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Serum Macrominerals, Trace Elements and Glutathione Peroxidase Activity from Late Gestation to Early Lactation in Holstein Cows, and Their Association with Indicators of Energy Balance

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Simple Summary

Around calving, dairy cows experience major changes in their energy and mineral needs. We followed 30 healthy Holstein cows from approximately three months before calving until approximately two months after calving and measured blood minerals, trace elements, antioxidant enzyme levels, and two markers for body fat mobilization. Phosphorus, magnesium, copper, iron, and zinc levels were lower during late pregnancy and the first week after calving. No significant change in blood calcium was detected during the times sampled. The fat mobilization markers and antioxidant enzyme levels were highest at the first sampling after calving (day 3). Average calcium, phosphorus, magnesium, and ketone values did not exceed the limits used to identify affected cows; however, some effects were seen in individual cows. Average fat mobilization markers were above the accepted limits shortly before and after calving, with potassium values higher than normal, probably resulting from methodological causes. Only a few links were found between the minerals and markers of energy balance. Late pregnancy and the first week after calving may be useful times for checking a small group of cows in the herd, although the benefit of such checks was not tested here.

Abstract

The transition from late gestation to lactation places considerable demands on the energy and mineral metabolism of dairy cows. However, the relationship between these metabolic processes in clinically healthy cows remains poorly understood. Blood was sampled from 30 multiparous Holstein cows on days −100 to −90, −60, −30, and −8 to −3 relative to expected calving, and on days 3, 8, 21–28, and 50–60 postpartum. Serum Ca, P, Mg, Na, K, Cl, Cu, Fe and Zn, glutathione peroxidase (GPx) activity, non-esterified fatty acids (NEFAs) and β-hydroxybutyric acid (BHBA) were analysed using repeated-measures ANOVA and linear mixed models. Ca showed no significant change ($p > 0.05$). P was lowest before calving and on day 8, Cu and Zn were reduced from day −30 and Fe from day −5, with Cl declining after calving ($p < 0.001$). NEFA, BHBA, and GPx were highest on day 3; mean BHBA was below the subclinical ketosis threshold, but 3 of 30 cows exceeded it. NEFA was associated with Mg ($p = 0.012$), and GPx with K ($p = 0.036$) and Ca ($p = 0.006$); these

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associations are exploratory. Herd-level monitoring in late gestation and the first week of lactation merits evaluation in future studies.

Keywords: dairy cattle; β-hydroxybutyric acid; metabolic adaptation; non-esterified fatty acids; oxidative status; periparturient period

1. Introduction

The weeks around calving are the most demanding part of the production cycle for a dairy cow. During this transition period, conventionally defined as the three weeks before and the three weeks after parturition, cows must support colostrogenesis and the onset of milk synthesis at a time when dry matter intake (DMI) is falling [1]. The resulting energy shortfall drives cows into a negative energy balance (NEB). Independently of energy balance, trace mineral status may also become marginal for some herds, depending mainly on their dietary supply and absorption. Body fat is mobilized as non-esterified fatty acids (NEFAs), part of which is incompletely oxidized in the liver and converted to β-hydroxybutyric acid (BHBA). In high-yielding cows these changes belong to normal homeorhetic adaptation, but when cows fail to adapt to this metabolic load, the risk of metabolic and infectious disease increases. Additionally, both production and reproductive performance can be negatively affected well beyond the transition period [2].

An adequate and balanced supply of energy, protein, electrolytes, macrominerals, and trace elements around calving determines how well cows manage these demands [3,4]. Both deficiency and excess of these minerals can compromise reproductive outcomes, including resumption of ovarian cyclicity, conception rate, and embryo survival [5].

Macrominerals (Ca, P, Mg, Na, K, and Cl) and trace elements (Cu, Fe, and Zn) underpin a wide range of biochemical functions [6]. Ca, P, and Mg are required for skeletal integrity and neuromuscular function, while Cu, Fe, and Zn act as cofactors in numerous enzyme systems [7,8]. Imbalances during the transition period have been linked to delayed uterine involution, retained fetal membranes, and periparturient immunosuppression, with direct consequences for herd productivity [9,10]. The circulating concentrations of trace element cations are only partly determined by intake because intestinal absorption is regulated in response to changing metabolic demands [11]. Maintaining homeostasis through this period involves a shifting balance between mineral requirements for energy production, structural function, resumption of reproductive activity, and immune competence [12].

Deficiencies or imbalances in Ca, P, Mg, Se, and Zn during this period have been linked to metabolic disorders, impaired immune function and economic losses [13].

Quantitative mineral requirements at each stage of the production cycle remain incompletely defined [14]. Serum and milk concentrations of Cu, Mn, Fe, and Zn are not constant across lactation, and monthly monitoring has been recommended [15]. Muiño et al. reported that serum Mg, P, and Ca levels fall during the first week after calving and return to prepartum values within a month [16].

Glutathione peroxidase (GPx) has long been used as a functional indicator of selenium status [5,17], although some trace mineral supplementation trials have found no changes in GPx or superoxide dismutase activity [18].

Many earlier studies on periparturient mineral metabolism have covered only the weeks around calving, have measured either macrominerals or trace elements, or have related serum minerals to disease or reproductive outcomes rather than to the indicators of energy balance [1,15,16]. Less is known about how macrominerals, trace elements, and GPx activity change together in individual cows from late lactation through the dry period into early lactation, and whether serum mineral concentrations are associated with NEFA, BHBA,

or GPx activity throughout this period. Therefore, a group of clinically healthy multiparous Holstein cows were followed from before dry-off to 50–60 days in milk (DIM), with serum macrominerals, trace elements, and GPx profiles recorded at eight sampling points. The aim was to describe how these variables change across the dry period, late gestation, and early lactation, and to test whether serum mineral concentrations are associated with the two conventional indicators of energy balance, NEFA and BHBA, and GPx activity. We hypothesized that serum mineral concentrations would be associated with NEFA, BHBA, and GPx activity during this period.

2. Materials and Methods

2.1. Animals

This study was conducted between September 2021 and June 2022 in a commercial dairy herd in Morphou, Cyprus, using 30 clinically healthy multiparous Holstein cows aged between 5 and 6 years. The mean daily milk yield in the preceding lactation period ranged from 20 to 30 L, and the body condition score (BCS) at enrolment ranged from 2.75 to 3.00. All cows were clinically healthy on examination at enrolment and remained clinically healthy throughout the study; no cow was excluded, and all 30 cows completed the eight sampling points. Clinical examinations were repeated at each sampling visit as part of the herd health monitoring program, and no clinical disease requiring treatment was recorded during the study. Individual DMI and milk yield were not recorded during the study period.

All animals were housed under identical conditions and managed as a single group throughout the study. The animals were housed in a free-stall system and had ad libitum access to feed and water. Their diet consisted of a base ration given ad libitum as a daily total mixed ration (TMR). The feeding frequency of the TMR was twice per day. Group-level dry matter intake and the proportion of forage in the rations were as follows: during the early dry period, 11–13 kg (75–85%); for the end of the dry period, 9–11 kg (60–70%); for the early postpartum period, 15–19 kg (40–45%); and for the late postpartum period, 18–22 kg (50–60%). Body condition scores were determined to be between 2.75 and 3.50, dependent on the assessment period. Ration composition was adjusted by stage of production: the dry cow ration contained approximately 12% crude protein (CP), 3% crude fat, and 35% neutral detergent fiber (NDF), and the lactation ration approximately 18% CP, 4% crude fat, and 30% NDF. The postpartum diet contained corn silage, hay, green grass, TMR, and an admixture of minerals and vitamins, and was adjusted to contain 17–20% CP, 3–5% crude fat, and 27–34% NDF on a dry matter (DM) basis. The CP, crude fat, and NDF contents given above are formulated values from the ration plan and were not analysed. The mineral composition of the rations was not analysed, and formulation records with mineral concentrations were not available for the study period; dietary mineral concentrations and the dietary cation–anion difference (DCAD) therefore could not be reported.

2.2. Blood Sampling

Blood sampling was performed as part of the routine herd health monitoring program of the unit. No procedure was performed beyond those normally applied during herd health monitoring. Blood was collected at eight predefined time points covering the late dry period, late gestation, and early lactation (Table 1). Prepartum sampling days were scheduled relative to the expected calving date, which was determined from the date of artificial insemination, whereas postpartum samples were collected relative to the actual calving date. The interval between the D−5 sample and the actual calving date was verified retrospectively and ranged from 3 to 8 days before calving. In the analysis, each sample was assigned to its scheduled time point (Table 1). All animals came from the same herd;

therefore, housing, ration, and management were held constant across the study. Samples were obtained around 08:00 am, after the morning feeding, by jugular venipuncture into 9 mL evacuated tubes with clot activator (BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA), transported to the laboratory under refrigeration, and centrifuged within 1 h of collection at 2000× *g* for 10 min. Serum was harvested, visually inspected for haemolysis (no visible haemolysis in any of the samples), and stored at −20 °C until analysed.

Table 1. Blood sampling schedule and labels used for each time point, expressed relative to the expected calving date (prepartum) or the actual calving date (postpartum).

Sampling Window	Label	Stage
Day −100 to −90	D−100	4 to 6 weeks before dry-off
Day −60	D−60	At dry-off
Day −30	D−30	Early dry period (first month)
Day −8 to −3	D−5	Antepartum
Day +3	D+3	Postpartum, day 3
Day +8	D+8	Postpartum, day 8
Day +21 to +28	D+25	Postpartum, weeks 3 to 4
Day +50 to +60	D+50	Postpartum, days 50 to 60

2.3. Laboratory Analyses

All analyses were performed at the Diagnostic Laboratory of the Near East University Animal Hospital using an automated clinical chemistry analyser (BS240-VET, Mindray, Shenzhen, China) with commercial ready-to-use reagent kits (Mindray, Shenzhen, China; Randox Laboratories, Crumlin, UK), following the manufacturers' procedures and quality control protocols. Calibration was performed using Randox Calibration Serum Level 3 (CAL2351), and each run included Randox Bovine Chemistry Assayed Control (AN1026) and, for GPx, the Ransel control (SC692) (Randox Laboratories, Crumlin, UK). The coefficients of variation of the control materials were below 5% within each run and below 10% between runs. Samples were analysed in runs as they were collected at each sampling point during the study.

Macrominerals were determined as calcium (Ca; Mindray CA0102, arsenazo III), phosphorus (P; Mindray P0104, phosphomolybdate), magnesium (Mg; Mindray MG0102, xylidyl blue), sodium (Na; Randox NA7167, enzymatic colorimetric β-galactosidase method), potassium (K; Randox PT1600, colorimetric sodium tetraphenylborate method) and chloride (Cl; Randox CL1645, mercuric thiocyanate). Trace elements were determined as copper (Cu; Randox CU2340, 3,5-DiBr-PAESA), iron (Fe; Mindray FE0102, ferrozine) and zinc (Zn; Randox ZN2341, colorimetric method after deproteinization). Na and K were therefore measured in serum by colorimetric methods rather than by ion-selective electrodes. All samples were collected, processed and analysed with the same protocols.

Serum BHBA was measured using a 3-hydroxybutyrate dehydrogenase method (RANBUT RB1007) and NEFA by an acyl-CoA synthetase/acyl-CoA oxidase enzymatic colorimetric method (FA115), both with commercial kits (Randox Laboratories, Crumlin, UK), on the same analyser.

GPx activity was measured in serum using a commercial kit (RANSEL RS504, Randox Laboratories, Crumlin, UK) based on the method described by Paglia and Valentine [19], with tert-butyl hydroperoxide used as the substrate instead of cumene hydroperoxide [20], and expressed per gram of total serum protein (activity in U/L divided by total protein in g/L) to adjust for changes in serum protein concentration around calving. The manufacturer describes this kit for use with whole blood; in the present study, it was applied to serum. Total serum protein was determined using the biuret method (TP245, Randox Laboratories, Crumlin, UK).

2.4. Statistical Analysis

Data were analysed in IBM SPSS Statistics 26.0 (IBM Corp., Armonk, NY, USA). Changes across the eight sampling points were tested by repeated-measures ANOVA. The assumption of sphericity was evaluated using Mauchly's test, which confirmed that sphericity was not violated ($p > 0.05$); therefore, no corrections were applied. Residual normality and homogeneity of variance were assessed by visual inspection of residual diagnostic plots. Additionally, the dataset was balanced with equal sample sizes and no missing observations across any of the repeated measurements. Pairwise differences between time points were identified by post hoc testing with Bonferroni correction. Relationships between serum minerals and the metabolic indicators were examined with a separate model for each mineral–indicator pair (27 models), with the serum mineral concentration as the response variable. Linear mixed-effects models (LMMs) were fitted using Restricted Maximum Likelihood (REML) estimation to examine the associations between serum mineral concentrations and metabolic indicators (NEFA, BHBA, and GPx). In each model, individual cow (Cow) was included as a random intercept to account for within-subject clustering across repeated measurements. Sampling time point (Period, 8 levels) and the respective metabolic indicator were included as fixed effects. A first-order autoregressive (AR(1)) covariance structure was specified for the repeated sampling periods to model serial correlation over time. Model results are reported as fixed-effect regression coefficients (β) with standard errors (SEs) and 95% confidence intervals (95% CIs), alongside Type III *F*-tests and variance components. Nominally significant associations were further described using regression coefficients (β) and their 95% confidence intervals. To account for multiple testing, Bonferroni correction was applied across all 27 metabolite–mineral comparisons; adjusted *p* values were calculated by multiplying the nominal *p* values by 27. Results are presented as mean ± standard deviation, and significance was set at $p < 0.05$. In addition, the number of cows beyond clinically relevant cut-points was counted at each time point: Ca < 8.0 mg/dL (2.0 mmol/L) [21], P < 4.5 mg/dL [22], Mg < 1.8 mg/dL [23], K > 5.8 mmol/L and Na < 132 mmol/L [24], NEFA ≥ 0.30 mmol/L before calving [25] and ≥0.70 mmol/L after calving [26], and BHBA ≥ 1.2 mmol/L [27,28]. No fixed cut-points were applied to Cu, Fe and Zn, because reference limits for these elements differ between laboratories and serum concentrations are insensitive indicators of body stores [29].

3. Results

3.1. Macrominerals

Serum macromineral concentrations varied significantly during the sampling period (Table 2). Serum phosphorus (P) concentration was 7.77 mg/dL before dry-off (D−100) and decreased significantly to 6.25 mg/dL antepartum at D−5 ($p < 0.001$). The most pronounced reduction was recorded at D+8, where the mean concentration decreased to 5.76 mg/dL ($p < 0.001$). The overall time effect was significant ($F(7, 112.972) = 14.911$, $p < 0.001$).

Magnesium (Mg) concentrations were significantly reduced at D−60 (1.98 mg/dL) and D−30 (1.86 mg/dL) compared with D−100 (2.47 mg/dL; $p < 0.001$). Although a slight recovery was observed at D−5 (2.05 mg/dL), concentrations decreased significantly again in early lactation, to 1.98 mg/dL at D+3 and 1.87 mg/dL at D+8 ($p < 0.001$). The overall time effect was significant ($F(7, 143.346) = 17.139$, $p < 0.001$).

Potassium (K) levels increased significantly from 6.08 mmol/L at D−100 to 7.83 mmol/L at D−60 ($p < 0.001$) and remained elevated at D−30, where the highest mean of the study was recorded (8.49 mmol/L). The most marked reductions occurred at D−5 (4.41 mmol/L) and D+3 (4.64 mmol/L; $p < 0.001$), after which concentrations increased progressively towards baseline from D+8 onwards. Mean K exceeded the upper reference limit of 5.8 mmol/L [24] at all time points except D−5 and D+3, and individual values ranged

from 2.45 to 14.06 mmol/L. The overall time effect was significant ($F(7, 116.772) = 40.814$, $p < 0.001$).

Sodium (Na) decreased significantly from 141.38 mmol/L at D−100 to 130.45 mmol/L at D−30 ($p < 0.001$) and reached its lowest concentration at D−5 (129.00 mmol/L; $p < 0.001$). Concentrations remained low at D+3 and D+25 (133.70 and 131.07 mmol/L), but were significantly elevated at D+8 and D+50 (141.79 and 143.44 mmol/L; $p < 0.001$). The overall time effect was significant ($F(7, 104.843) = 61.894$, $p < 0.001$).

Chloride (Cl) levels were significantly reduced at D+3 compared to the preceding time points (106.65 mmol/L; $p < 0.001$) and declined progressively thereafter, reaching 100.88 mmol/L at D+50 ($p < 0.001$). The overall time effect was significant ($F(7, 133.549) = 5.427$, $p < 0.001$).

No statistically significant differences in serum calcium (Ca) concentration were detected between time points ($p > 0.05$). The lowest mean value was recorded at D+3 (8.99 mg/dL), when 4 of 30 cows (13%) had Ca < 8.0 mg/dL. The overall time effect was not significant ($F(7, 115.848) = 1.549$, $p = 0.158$).

Table 2. Serum macromineral concentrations at each time point (mean ± SD; $n = 30$).

Time Point	P (mg/dL)	Mg (mg/dL)	K (mmol/L)	Na (mmol/L)	Cl (mmol/L)	Ca (mg/dL)
D−100	7.77 ± 1.18 ^{a,c}	2.47 ± 0.30 ^{c}	6.08 ± 0.77 ^{c}	141.38 ± 7.04 ^{c}	111.49 ± 11.05 ^{c}	9.41 ± 0.57 $^{n.s.}$
D−60	7.96 ± 1.15 ^{a,c}	1.98 ± 0.22 ^{d,e}	7.83 ± 1.05 ^{d,e}	143.79 ± 3.68 ^{c}	108.38 ± 1.92 ^{c,g}	9.46 ± 0.43 $^{n.s.}$
D−30	7.13 ± 1.27 ^{d}	1.86 ± 0.18 ^{d,e}	8.49 ± 2.38 ^{d,g}	130.45 ± 3.51 ^{d,g}	108.20 ± 2.49 ^{c,g}	9.35 ± 0.50 $^{n.s.}$
D−5	6.25 ± 0.83 ^{d,h}	2.05 ± 0.52 ^{d}	4.41 ± 0.73 ^{d,f,h,i}	129.00 ± 5.35 ^{d,g}	106.74 ± 1.24 ^{c,g}	9.26 ± 0.50 $^{n.s.}$
D+3	7.47 ± 1.19 ^{g}	1.98 ± 0.23 ^{d,g}	4.64 ± 1.00 ^{d,f,i}	133.70 ± 3.79 ^{d,f,h}	106.65 ± 1.99 ^{d,e}	8.99 ± 0.69 $^{n.s.}$
D+8	5.76 ± 1.33 ^{d,h}	1.87 ± 0.24 ^{d,f,h}	6.45 ± 0.90 ^{c,f,h,j}	141.79 ± 4.40 ^{d,e,h}	105.10 ± 2.95 ^{f,h,k}	9.32 ± 1.29 $^{n.s.}$
D+25	6.94 ± 1.39 ^{b,g}	2.26 ± 0.21 ^{c,g}	6.45 ± 1.64 ^{h,j}	131.07 ± 5.86 ^{d,f}	103.37 ± 2.62 ^{d,h}	9.50 ± 0.70 $^{n.s.}$
D+50	7.62 ± 1.25 ^{g}	1.98 ± 0.28 ^{d,h}	6.31 ± 1.05 ^{c,f,h,j}	143.44 ± 4.62 ^{d,e,h}	100.88 ± 2.63 ^{d,f,h,l}	9.29 ± 0.39 $^{n.s.}$
Overall time effect (p-value)	<0.001	<0.001	<0.001	<0.001	<0.001	0.158

Superscript letters in the same column indicate a statistically significant difference between time points. P: D−100, D−60 vs. D+25 = a:b $p < 0.05$; D−100, D−60 vs. D−30, D−5, D+8 = c:d $p < 0.001$; D+3, D+25, D+50 vs. D−5, D+8 = g:h $p < 0.001$. Mg: D−100, D+25 vs. D−60, D−30, D−5, D+3, D+8, D+50 = c:d $p < 0.001$; D−60, D−30 vs. D+8 = e:f $p < 0.001$; D+3, D+25 vs. D+8, D+50 = g:h $p < 0.001$. K: D−100, D+8, D+50 vs. D−60, D−30, D−5, D+3 = c:d $p < 0.001$; D−60 vs. D−5, D+3, D+8, D+50 = e:f $p < 0.001$; D−30 vs. D−5, D+8, D+25, D+50 = g:h $p < 0.001$; D−5, D+3 vs. D+8, D+25, D+50 = i:j $p < 0.001$. Na: D−100, D−60 vs. D−30, D−5, D+3, D+8, D+25, D+50 = c:d $p < 0.001$; D+8, D+50 vs. D+3, D+25 = e:f $p < 0.001$; D−30, D−5 vs. D+3, D+8, D+50 = g:h $p < 0.001$. Cl: D−100, D−60, D−30, D−5 vs. D+3, D+25, D+50 = c:d $p < 0.001$; D+3 vs. D+8, D+50 = e:f $p < 0.001$; D−60, D−30, D−5 vs. D+8, D+25, D+50 = g:h $p < 0.001$; D+8 vs. D+50 = k:l $p < 0.001$. Ca: $^{n.s.}$, not significant.

3.2. Trace Elements

Copper (Cu) decreased significantly from 15.52 µmol/L at D−100 and 16.01 µmol/L at D−60 to 8.03 µmol/L at D−30 and 6.57 µmol/L at D−5 ($p < 0.05$ and $p < 0.001$, respectively; Table 3). Variation among cows at D−100 was pronounced (median 14.22 µmol/L; range 1.84 to 44.63 µmol/L). Concentrations remained significantly reduced relative to the dry period at D+3, D+8, and D+25 (10.13, 9.98, and 9.88 µmol/L; $p < 0.001$) and increased significantly again at D+50 (12.80 µmol/L; $p < 0.001$). The overall time effect was significant ($F(7, 92.891) = 14.440$, $p < 0.001$).

Iron (Fe) concentrations were significantly reduced at D+25 (11.60 µmol/L), D+3 (13.76 µmol/L), D+8 (14.32 µmol/L), and D−5 (14.36 µmol/L; $p < 0.001$) compared with the dry period. Values increased significantly at D+50 and returned to baseline (16.65 µmol/L; $p < 0.001$). The overall time effect was significant ($F(7, 106.997) = 5.019$, $p < 0.001$).

Table 3. Serum trace element concentrations at each time point (mean ± SD; $n = 30$).

Time Point	Cu (µmol/L)	Fe (µmol/L)	Zn (µmol/L)
D−100	15.52 ± 11.10 [a]	16.55 ± 8.82	19.34 ± 5.12 [d]
D−60	16.01± 8.74 [a,f]	17.46 ± 6.44 [d]	15.75 ± 6.62 [d]
D−30	8.03 ± 4.09 [b,g,h]	18.81 ± 6.12 [d]	12.22 ± 5.14 [e]
D−5	6.57 ± 1.66 [e,g,j]	14.36 ± 4.04 [e]	12.01 ± 3.48 [e]
D+3	10.13 ± 3.42 [c,k]	13.76 ± 4.84 [e]	11.78 ± 4.33 [e]
D+8	9.98 ± 3.96 [c,k]	14.32 ± 4.78 [e]	12.36 ± 2.96 [e]
D+25	9.88 ± 4.89 [c,k]	11.60 ± 5.55 [e,h]	13.48 ± 5.06 [e]
D+50	12.80 ± 4.53 [c,i]	16.65 ± 4.99 [j]	11.60 ± 3.15 [e]
Overall time effect (p-value)	<0.001	<0.001	<0.001

Superscript letters in the same column indicate statistically significant differences between time points. Cu: D−100, D−60 vs. D−30 = a:b $p < 0.05$; D−100, D−60 vs. D−5 = a:e $p < 0.001$; D+3, D+8, D+25, D+50 vs. D−30, D−5 = c:g $p < 0.001$; D−60 vs. D−30, D−5 = f:g $p < 0.001$; D−30 vs. D+50 = h:i $p < 0.001$; D−30 vs. D−5 = h:j $p < 0.001$; D−5 vs. D+3, D+8, D+25 = j:k $p < 0.001$. Fe: D−60, D−30 vs. D−5, D+3, D+8, D+25 = d:e $p < 0.001$; D+25 vs. D+50 = h:j $p < 0.001$. Zn: D−100, D−60 vs. D−30, D−5, D+3, D+8, D+25, D+50 = d:e $p < 0.001$.

Zinc (Zn) concentrations at D−100 and D−60 were significantly higher than those at all subsequent time points ($p < 0.001$). From D−30 onwards, Zn remained significantly below the pre-dry-off and dry-off values, whereas differences between the subsequent time points were not statistically significant ($p > 0.05$). The overall time effect was significant ($F(7, 107.403) = 15.920$, $p < 0.001$).

3.3. Metabolic Indicators and Glutathione Peroxidase

BHBA increased significantly at D+3 (0.615 mmol/L) and D+8 (0.410 mmol/L) with significant decreases at later time points ($p < 0.001$; Table 4). NEFA was significantly elevated antepartum at D−5 (0.560 mmol/L) and was highest at D+3 (0.983 mmol/L; $p < 0.001$), remaining significantly above baseline levels at D+8 (0.689 mmol/L). Concentrations were significantly reduced at D+25 and D+50 (0.247 and 0.161 mmol/L; $p < 0.01$). At D−5, 21 of 30 cows (70%) had NEFA ≥ 0.30 mmol/L; at D+3 and D+8, 19 (63%) and 13 (43%) cows had NEFA ≥ 0.70 mmol/L. Three cows (10%) had BHBA ≥ 1.2 mmol/L at D+3 (1.98 to 2.17 mmol/L), and no cow exceeded this threshold at the later time points (Table 5).

GPx activity significantly increased antepartum at D−5 (12.31 U/g protein; $p < 0.001$) and reached its highest value at D+3 (19.96 U/g protein). Activity remained significantly elevated at D+8 (15.89 U/g protein) and decreased at later postpartum time points. The overall time effect was significant for BHBA, NEFA, and GPx activity (all $p < 0.001$; Table 4).

3.4. Relationships Between Minerals and Metabolic Indicators

The associations between mineral concentrations and metabolic indicators are presented in Table 6. Of the 27 comparisons, three associations were nominally significant. NEFA was negatively associated with Mg ($\beta = -0.160$; 95% CI: −0.283 to −0.036; $p = 0.012$), whereas GPx was negatively associated with Ca ($\beta = -0.024$; 95% CI: −0.040 to −0.007; $p = 0.006$) and K ($\beta = -0.032$; 95% CI: −0.062 to −0.002; $p = 0.036$). No statistically significant associations were detected between NEFA and the other measured minerals or between BHBA and any of the minerals. Similarly, GPx was not significantly associated with P, Mg, Na, Cl, Cu, Fe, or Zn. None of the three nominally significant associations remained statistically significant after Bonferroni correction (adjusted $p = 0.324$, 0.162, and 0.972 for NEFA–Mg, GPx–Ca, and GPx–K, respectively).

Table 4. Serum glutathione peroxidase activity, BHBA, and NEFA at each time point (mean ± SD; $n = 30$).

Time Point	GPx (U/g Protein)	BHBA (mmol/L)	NEFA (mmol/L)
D−100	7.50 ± 1.78 ^{e}	0.339 ± 0.189 ^{a}	0.438 ± 0.252 ^{a,e}
D−60	8.02 ± 3.08 ^{i}	0.375 ± 0.137 ^{a}	0.225 ± 0.106 ^{c,f,i}
D−30	10.03 ± 5.61 ^{f,g}	0.241 ± 0.103 ^{a}	0.206 ± 0.162 ^{f,g}
D−5	12.31 ± 6.63 ^{f,g}	0.271 ± 0.107 ^{a,e}	0.560 ± 0.443 ^{d,h}
D+3	19.96 ± 8.17 ^{f,h,j,k}	0.615 ± 0.525 ^{b}	0.983 ± 0.534 ^{f,h,j}
D+8	15.89 ± 8.23 ^{f,j}	0.410 ± 0.183 ^{f,i}	0.689 ± 0.247 ^{b,h,j,k}
D+25	9.28 ± 1.95 ^{f,l}	0.324 ± 0.112 ^{a}	0.247 ± 0.156 ^{b,i,l}
D+50	8.55 ± 2.31 ^{l}	0.284 ± 0.104 ^{j}	0.161 ± 0.091 ^{f,i,l}
Overall time effect (p-value)	<0.001	<0.001	<0.001

Superscript letters in the same column indicate statistically significant differences between time points. GPx: D−100 vs. D−30, D−5, D+3, D+8, D+25 = e:f $p < 0.001$; D−30, D−5 vs. D+3 = g:h $p < 0.001$; D−60 vs. D+3, D+8 = i:j $p < 0.001$; D+3 vs. D+25, D+50 = k:l $p < 0.001$. BHBA: D−100, D−60, D−30, D−5, D+25 vs. D+3 = a:b $p < 0.05$; D−100, D−60, D−30, D−5, D+25 vs. D+8 = a:f $p < 0.001$; D−5 vs. D+8 = e:f $p < 0.001$; D+8 vs. D+50 = i:j $p < 0.001$. NEFA: D−100 vs. D+8, D+25 = a:b $p < 0.05$; D−100 vs. D−60, D−30, D+3, D+50 = a:f $p < 0.001$; D−60 vs. D−5 = c:d $p < 0.05$; D−60 vs. D−5, D+3, D+8 = c:h $p < 0.001$; D−100 vs. D−60, D−30, D+3, D+50 = e:f $p < 0.001$; D−30 vs. D−5, D+3, D+8 = g:h $p < 0.001$; D−60, D+25, D+50 vs. D+3, D+8 = i:j $p < 0.001$; D+8 vs. D+25, D+50 = k:l $p < 0.001$.

Table 5. Number (%) of cows with values beyond clinically relevant cut-off points at each time point ($n = 30$).

Variable	D−100	D−60	D−30	D−5	D+3	D+8	D+25	D+50
Ca < 8.0 mg/dL	0 (0%)	0 (0%)	1 (3%)	2 (7%)	4 (13%)	1 (3%)	0 (0%)	0 (0%)
P < 4.5 mg/dL	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	8 (27%)	0 (0%)	0 (0%)
Mg < 1.8 mg/dL	0 (0%)	6 (20%)	8 (27%)	12 (40%)	8 (27%)	9 (30%)	0 (0%)	9 (30%)
K > 5.8 mmol/L	19 (63%)	30 (100%)	26 (87%)	1 (3%)	6 (20%)	23 (77%)	21 (70%)	24 (80%)
Na < 132 mmol/L	1 (3%)	0 (0%)	21 (70%)	22 (73%)	10 (33%)	1 (3%)	15 (50%)	1 (3%)
NEFA ≥ 0.30 mmol/L (prepartum)	–	–	–	21 (70%)	–	–	–	–
NEFA ≥ 0.70 mmol/L (postpartum)	–	–	–	–	19 (63%)	13 (43%)	0 (0%)	0 (0%)
BHBA ≥ 1.2 mmol/L (postpartum)	–	–	–	–	3 (10%)	0 (0%)	0 (0%)	0 (0%)

Cut-off points: Ca < 8.0 mg/dL (2.0 mmol/L) [21]; P < 4.5 mg/dL [22]; Mg < 1.8 mg/dL [23]; K > 5.8 mmol/L and Na < 132 mmol/L [24]; NEFA ≥ 0.30 mmol/L before calving (applied at D−5 only) [25] and ≥0.70 mmol/L after calving [26]; BHBA ≥ 1.2 mmol/L after calving [27,28]. –, cut-point not applied at this time point.

Table 6. Linear mixed-effects model results for associations between serum minerals and metabolic indicators.

Mineral	Metabolic Indicator	F	p	β	95% CI	Bonferroni-Adjusted p
Ca	NEFA	0.025	0.875	—	—	1.000
Ca	BHBA	2.324	0.129	—	—	1.000
Ca	GPx	7.670	0.006	−0.024	−0.040 to −0.007	0.162
P	NEFA	0.484	0.487	—	—	1.000
P	BHBA	0.445	0.505	—	—	1.000
P	GPx	0.561	0.454	—	—	1.000
Mg	NEFA	6.470	0.012	−0.160	−0.283 to −0.036	0.324
Mg	BHBA	0.570	0.451	—	—	1.000
Mg	GPx	1.394	0.239	—	—	1.000
Na	NEFA	2.160	0.143	—	—	1.000
Na	BHBA	0.940	0.333	—	—	1.000
Na	GPx	1.567	0.212	—	—	1.000
K	NEFA	0.002	0.965	—	—	1.000
K	BHBA	0.051	0.822	—	—	1.000
K	GPx	4.461	0.036	−0.032	−0.062 to −0.002	0.972
Cl	NEFA	0.337	0.562	—	—	1.000
Cl	BHBA	1.282	0.259	—	—	1.000

Table 6. *Cont.*

Mineral	Metabolic Indicator	F	*p*	β	95% CI	Bonferroni-Adjusted *p*
Cl	GPx	0.779	0.379	—	—	1.000
Cu	NEFA	0.362	0.564	—	—	1.000
Cu	BHBA	0.209	0.648	—	—	1.000
Cu	GPx	0.384	0.536	—	—	1.000
Fe	NEFA	0.004	0.951	—	—	1.000
Fe	BHBA	0.817	0.367	—	—	1.000
Fe	GPx	0.092	0.762	—	—	1.000
Zn	NEFA	0.265	0.607	—	—	1.000
Zn	BHBA	1.105	0.294	—	—	1.000
Zn	GPx	0.164	0.686	—	—	1.000

Bonferroni-adjusted *p* values were calculated across all 27 comparisons (adjusted p = nominal $p \times 27$; values greater than 1.000 are shown as 1.000). β and 95% confidence intervals are presented for the three nominally significant associations. F, F value for fixed effects. Each model was based on 240 observations from 30 cows (eight sampling periods per animal).

Parameter estimates and variance components are presented in Table 7. The sampling period had a significant fixed effect in the models for Mg ($F = 17.671$, $p < 0.001$), Na ($F = 61.276$, $p < 0.001$), and K ($F = 32.364$, $p < 0.001$), but not for Ca ($F = 0.527$, $p = 0.813$).

Table 7. Parameter estimates, 95% confidence intervals, and covariance components from linear mixed-effects models evaluating the associations between serum mineral concentrations and metabolic indicators, adjusted for the sampling period.

Dependent Variable (Mineral)	Fixed Effect/Covariate	β (Estimate)	SE	95% Confidence Interval (Lower, Upper)	*F*-Value	*p*-Value	Random Intercept Variance (σ^2_{cow})	Residual Variance (σ^2_{res})	AR(1) ρ
Mg	**NEFA**	**−0.160**	0.063	**−0.283, −0.036**	**6.470**	**0.012**	<0.001 [a]	0.083	0.197
	Sampling period	— [b]	—	—	17.671	<0.001			
Na	**NEFA**	−1.532	1.042	−3.587, 0.523	2.160	0.143	3.848	20.276	0.116
	Sampling period	— [b]	—	—	61.276	<0.001			
K	**GPx**	**−0.032**	0.015	**−0.062, −0.002**	**4.461**	**0.036**	0.165	1.485	−0.083
	Sampling period	— [b]	—	—	32.364	<0.001			
Ca	**GPx**	**−0.024**	0.009	**−0.040, −0.007**	**7.670**	**0.006**	0.021	0.472	0.040
	Sampling period	— [b]	—	—	0.527	0.813			

All models were fitted using Restricted Maximum Likelihood (REML) with individual cow (Cow) as a random intercept and a first-order autoregressive (AR(1)) covariance structure across the eight repeated sampling periods. Bold values indicate nominally significant associations (unadjusted $p < 0.05$). Each model was based on 240 observations from 30 cows (eight sampling periods per cow). β is the change in serum mineral concentration (in the units of Tables 2 and 3) per unit increase in the metabolic indicator (mmol/L for NEFA and BHBA; U/g protein for GPx). Full parameter estimates and 95% confidence intervals for non-significant models (BHBA and remaining minerals; all $p > 0.05$) are available from the corresponding author upon reasonable request. [a] The random intercept variance converged to a boundary value (<0.001) as the within-subject correlation was captured by the AR(1) residual structure. [b] The sampling period was modeled as an 8-level categorical fixed factor (Period 8 as reference); overall Type III F- and *p*-values are reported for brevity.

4. Discussion

Maternal mineral and trace element status shapes both the health of the dam and, through placental transfer, the health of the calf [30]. Inadequate energy and nutrient intake during the transition period can produce metabolic imbalance, oxidative stress, and periparturient immunosuppression [31]. This study aimed to describe how macrominerals, trace elements, and the metabolic indicators of energy balance move together in cows showing no clinical abnormality across the dry period, transition period and early lactation. Early studies reported marked antepartum and early postpartum decreases in trace elements [32], macrominerals [16,33], and metabolites [34,35], followed by later recovery during lactation.

All three trace elements were lower around the calving time than during dry period: Cu and Zn were already reduced at D−30, Fe was lowest at D+25, with Cu and Fe, but not Zn,

recovering by D+50. Hansard and Mohammed [36] observed that fetal Zn concentrations rise steeply during gestation, suggesting that Zn metabolism shifts in favor of the fetus as its growth requirement increases [30]. The decreases documented in this study would be consistent with that interpretation, as they coincided with the period of greatest fetal demand; however, fetal transfer was not measured. Serum Cu and Zn are also affected by factors other than supply; most serum Cu is bound to ceruloplasmin, approximately two-thirds of serum Zn is bound to albumin, inflammation lowers serum Zn, and serum concentrations poorly reflect body stores [29]. Therefore, the results are related to changes in the circulating pool rather than as evidence of deficiency or adequacy.

Iron participates in erythropoiesis, oxygen transport, energy metabolism, and immune function [37]. In our study, Fe did not change significantly between D−100 and D−30, fell significantly from D−5 onwards, remained low until D+25, and recovered only at D+50. Serum Fe has been reported to reach its nadir about a week before calving and to return to earlier values a month later [16]; in our cows, the decrease also began before calving, but recovery was only seen at D+50. Copper followed a similar course but began to decline earlier, at D−30. Unnecessary Fe supplementation has been associated with mastitis, reduced milk yield, and reduced intestinal motility [38]. Therefore, it is important to determine whether such temporary decreases fall below the standard values before supplementation is considered. Although mean serum Fe concentrations decreased during the periparturient period, they remained within the reference interval used in this study [39]. In this situation, it is considered more appropriate to measure the values in a group of cows and act only if the values are found to be consistently low.

Copper and zinc supplementation during the transition period has been shown to improve dry matter digestibility relative to unsupplemented controls [40]. Cows given organic Cu, Mn, and Zn immediately after calving also had higher immunoglobulin A and M concentrations than unsupplemented cows [40], which points to both the functional relevance of the postpartum decrease and to the possibility of correcting it. Pregnant animals absorb Cu and Zn more efficiently than non-pregnant animals as gestation advances, and this continues after parturition [41]; adaptive changes in intestinal absorption may contribute to the transient nature of the decreases recorded here, although absorption was not measured. Because individual DMI, and therefore individual trace mineral intake, was not recorded, it cannot be determined whether the low prepartum Cu values (6.57 µmol/L at D−5) reflect supply, dietary antagonists, or redistribution.

The K and Na results require cautious interpretation. Serum K was highest in the dry period (D−60 and D−30), lowest around calving (D−5 and D+3) and intermediate from D+8 onwards. This pattern agrees with Muiño et al. [16], who found lower serum K at one week and one month after calving than in late pregnancy, and with earlier reports of lower K in the periparturient period [32]. The decrease around calving can be explained by lower feed intake before calving and the start of milk secretion, as milk is a major route of K loss in lactating cows [42]. Mean K at D−60 and D−30 (7.83 and 8.49 mmol/L) and at most postpartum time points exceeded the upper limit of the bovine reference interval (5.8 mmol/L [24]), whereas mean values at D−5 and D+3 were within it. Reference intervals for K depend on the sample type and the analytical method. Serum K is higher than plasma K because K is released from platelets during clotting [43], and serum mineral concentrations are sensitive to haemolysis and to delayed separation of serum from the clot [29], and K is particularly affected because it is released from blood cells [24]. K is also one of the less stable analytes in bovine blood before separation [44]. In addition, K and Na were measured in serum by colorimetric methods rather than by ion-selective electrodes. All samples were collected, processed and analysed with the same protocol; however, this does not ensure that preanalytical and analytical effects were constant across sampling

points, and their contribution to the temporal differences in K cannot be excluded. K results are therefore interpreted only as changes over time within the study, not against external reference intervals, and the temporal pattern of K, including the wider spread of individual values at D−30, should be interpreted with caution. Mean Na was lower from D−30 to D+3 (about 129–134 mmol/L) than at D−100 and D−60 (about 141–144 mmol/L), which agrees in direction with the decrease around calving reported by Muiño et al. [16]. The alternating values after calving, lower at D+25 than at D+8 and D+50, are more difficult to explain because serum Na is tightly regulated, and they may partly reflect the colorimetric method; the Na results are therefore interpreted with caution. Haemolysis was assessed only visually, and automated haemolysis indices were not recorded. Earlier studies have reported decreases in Cl, Na and K before calving with recovery afterwards [16,45], and mammary gland activity [46], changes in renin activity [47] and milk yield [16] have been proposed as explanations, but these mechanisms could not be evaluated here.

Serum Cl levels declined progressively after calving. High Cl levels have been described as a biomarker of diet-induced acidification that contributes to the regulation of acid–base balance through the stimulation of the renal compensatory mechanisms [48]. Whether the prepartum Cl values reflect the anionic properties of the prepartum ration cannot be determined from serum data alone because the DCAD of the ration was not available and urine pH and blood acid–base status were not measured.

Phosphorus levels fall significantly on days 3, 10, and 15 after calving [49], which is consistent with our findings. In our study, P decreased at D−5, transiently returned to baseline at D+3, reaching its lowest value at D+8, and recovering from D+25 onwards. Increased uptake by the mammary glands during this window [50,51], the high P content of colostrum [49,52], and skeletal mobilization at the onset of lactation [53] are plausible explanations, although none of these were measured here. Fadlalla et al. [54] similarly recorded their lowest *p* values postpartum. Mean P did not fall below 4.5 mg/dL [22], but 8 of 30 cows (27%) had values below this limit at D+8; no cow developed clinical signs attributable to hypophosphatemia. At all time points except D+8, mean P was above the upper normal limit of approximately 6 mg/dL given by Goff [55]; P above this limit before calving can reduce the renal production of 1,25-dihydroxyvitamin D and thereby increase the risk of hypocalcemia [55], but no clinical hypocalcemia was observed in these cows.

For herd monitoring, the results are best interpreted as the proportion of cows beyond a meaningful cut-point rather than as group means, because the group mean may remain within the reference interval even when a considerable proportion of cows are affected [12]. In the present study, the time points at which the mean P and Mg decreased were also those at which the largest proportions of cows fell below the cut-points: 27% of cows had P < 4.5 mg/dL at D+8, when the lowest mean P was recorded, and 20 to 40% of cows had Mg < 1.8 mg/dL from D−60 to D+8 and at D+50. These mean level decreases reflect a substantial number of affected cows rather than a small shift in all animals. An alarm threshold of 10% of sampled cows has been suggested for subclinical ketosis [12], and for Mg at least 9 of 10 cows sampled after calving should have serum Mg of 2.0 mg/dL or more [55]; the proportions observed here for Mg exceeded these levels, and that for P at D+8 was similarly high. In contrast, Ca was below 8.0 mg/dL in at most 4 cows (13%) at any time point, which is consistent with the absence of a significant change in mean Ca.

No significant change in serum calcium was detected across the sampling points, and the lowest mean value was recorded at D+3. A dairy cow carries 2 to 4 g of Ca in blood, half of it in the ionized form. On the first day of lactation, colostrum synthesis and secretion remove an amount of Ca equivalent to 7 to 10 times the circulating pool [56]. Homeostatic mechanisms that increase Ca influx into blood must therefore be recruited to maintain normocalcemia [21]. Feeding low-calcium rations during the transition period and

formulating the close-up ration with attention to DCAD (Dietary Cation–Anion Difference) are the standard means of supporting this adaptation [57]. Because individual Ca intake, urine pH and blood acid–base status were not measured, the present data do not show whether the transition diet met Ca requirements. Calcium levels reach their lowest point in the first three days after birth due to colostrum production and rise between the fourth and seventh days postpartum [58]. As the first postpartum sample was taken on day 3, the nadir may have been missed, and the proportion of cows with subclinical hypocalcemia (13% with Ca < 8.0 mg/dL at D+3) is probably underestimated. At D+3, Ca in these four cows ranged from 7.79 to 7.99 mg/dL, and the lowest value of the study was 7.69 mg/dL at D−5. None of these cows showed clinical signs of hypocalcemia. Because the samples were analysed in runs after collection, low Ca values were not known at the time of sampling; in the absence of clinical signs, these cases may be considered subclinical hypocalcemia [21]. This proportion is lower than the 41 to 54% reported for second- and later-lactation cows within 48 h after calving [59]. Milk yield is also relevant, since Ca demand rises with production [52,60]; the 20 to 30 L/day reported for these cows refers to the preceding lactation, and milk yield was not recorded during the study period. Fadlalla et al. found daily milk yield to be positively correlated with Ca concentration ($p < 0.05$) [54]. The transition period in cows is characterised by endocrine and homeostatic changes aimed at keeping fluctuations in calcium levels within a minimal range [61]. Serum Ca was therefore relatively stable at the sampling times used, but the data do not allow conclusions on Ca homeostasis in the first 48 h after calving.

Magnesium was lower at D−60 and D−30 than at D−100, remained low at D+3 and D+8, and rose transiently at D+25. No cases of clinical hypocalcemia or hypomagnesemic tetany were recorded. Reported normal Mg values lie between 1.8 and 2.4 mg/dL [23]; mean values in this study were close to the lower limit, and 20 to 40% of cows had values below 1.8 mg/dL at D−60, D−30, D−5, D+3, D+8 and D+50, although the values at D−5 were unusually variable. It has been found that Mg levels are at their highest during the prepartum period and decline during the postpartum period [54], and a decrease in Ca levels around parturition may be accompanied by an increase in Mg levels [62,63], because parathyroid hormone secreted in response to low plasma Ca also increases the renal threshold for Mg [60]. As no significant change in Ca was detected in this study, no such reciprocal Mg response was expected. Because serum Mg depends mainly on daily absorption from the diet, the proportion of cows with low Mg suggests that Mg supply or absorption may have been limiting; high dietary K, which reduces ruminal Mg absorption [9], may be relevant in this respect (dietary Mg and K concentrations were not available).

Thresholds for the metabolic indicators are usually given as 1.2 mmol/L or above for BHBA and 0.7 mmol/L or above for NEFA [26]. For ketosis specifically, serum or plasma BHBA above 1.2 to 1.4 mmol/L is used to diagnose subclinical ketosis, or hyperketonemia, in the absence of clinical signs, and values above 3.0 mmol/L indicate clinical ketosis [27,28]. NEFA had already begun to rise at D−5 (0.560 mmol/L), both NEFA and BHBA were highest at D+3 (0.983 and 0.615 mmol/L), and both metabolites fell again at the later time points. Mean BHBA remained below the threshold for hyperketonemia at all time points, but this does not exclude high values in individual cows: 3 of 30 cows (10%) had BHBA ≥ 1.2 mmol/L at D+3, which corresponds to the 10% alarm level suggested for herd monitoring [12]. Similarly, 57% of cows had NEFA ≥ 0.40 mmol/L at D−5, compared with the 18 to 35% typically reported for the last week before calving [12], and 63% had NEFA ≥ 0.70 mmol/L at D+3.

The transition period in dairy cows is usually defined as the last three weeks of gestation and the first three weeks of lactation. In healthy cows, plasma NEFA rises from below

0.2 mmol/L about two weeks before calving to roughly 0.3 mmol/L three days before calving, then increases sharply in the last day or two before parturition and typically peaks on the second or third day of lactation, usually between 0.8 and 1.0 mmol/L, before declining slowly over the following two to three weeks [12,64,65]. The rapid rise in NEFA levels on the day of calving is likely stress-related, although hormonal changes (such as an increase in growth hormone) may also be associated with this condition. In healthy cows, plasma NEFA concentrations fall rapidly after calving, but remain at levels higher than those before calving for several weeks [66–68].

The prepartum rise in NEFA is generally taken to reflect increased lipolysis in adipose tissue in response to the progressive NEB that develops as energy requirement rises and DMI falls. Mobilized NEFAs are transported to the liver, where they are either completely oxidized to meet energy demand or partially oxidized to ketone bodies, principally BHBA. When glucose availability is limiting and hepatic oxidative capacity is exceeded, incomplete oxidation of NEFA generates excess ketone bodies and subclinical or clinical ketosis develops [69]. In our cows both NEFA and BHBA increased in early lactation, but mean BHBA stayed below the diagnostic threshold for hyperketonemia and only three cows exceeded it. NEFA and BHBA are indirect indicators—they do not measure energy balance or hepatic oxidative capacity directly—and DMI and milk yield were not recorded. The data are therefore consistent with an adaptive response in most cows, but they do not show that hepatic fatty acid oxidation prevented excessive ketogenesis, and the high proportion of cows above the NEFA thresholds indicates marked lipid mobilization in part of the herd [2,69,70].

Increases in GPx activity have been reported 14 and 7 days before calving [71]. Activity that was high on days 1 and 3 antepartum was found to have fallen below prepartum values by days 25 to 35 postpartum [72]. Other studies have reported low GPx activity in both the antepartum and postpartum periods [73,74]. Conversely, higher GPx has been described in late gestation than at either stage of lactation [75], and higher GPx activity has been reported in mid-lactation than at the start of lactation, with a non-significant decrease from late pregnancy to early lactation [72,76]. It has been argued that the increase in GPx levels during the transition and postpartum periods is linked to the adaptive changes exhibited by cows in response to oxidative stress [73,76]. In the present study, GPx activity increased at D−5 and was highest at D+3 and D+8, which is consistent with these reports. In the serum of Holstein–Friesian cows, GPx activity measured with tert-butyl hydroperoxide as substrate was likewise lowest in dry cows and highest in early lactation [20]. However, an increase in GPx activity alone does not show improved antioxidant protection or an adaptive response to oxidative stress, because neither markers of oxidative damage nor Se status were measured. Serum GPx activity is considered to reflect the short-term Se supply; it was correlated with serum Se across cows at different stages of lactation, but not within the individual stages [20], so that changes related to the stage of lactation cannot be separated from changes in Se status without Se measurements. Serum GPx activity is also not equivalent to the erythrocyte GPx activity for which the method was originally described [19]. The kit used is described by the manufacturer for whole blood and was applied here to serum with tert-butyl hydroperoxide instead of cumene hydroperoxide as substrate, meaning the activities are not directly comparable with values obtained with the unmodified kit; expressing activity per gram of total protein makes the result partly dependent on changes in serum protein around calving. The GPx results are therefore interpreted only as changes in serum enzyme activity.

Three associations were nominally significant: NEFA–Mg, GPx–K, and GPx–Ca. The regression coefficients indicated negative associations for NEFA–Mg ($\beta = -0.160$, 95% CI: −0.283 to −0.036), GPx–K ($\beta = -0.032$, 95% CI: −0.062 to −0.002), and GPx–Ca ($\beta = -0.024$, 95% CI: −0.040 to −0.007). However, none of these associations remained statistically

significant after Bonferroni correction for the 27 comparisons. Therefore, these findings should be interpreted as exploratory and do not provide conclusive evidence of associations between these metabolic indicators and mineral concentrations. No statistically significant associations were detected between BHBA and the measured minerals. However, this lack of statistical significance should not be interpreted as evidence that BHBA has no biological effect on mineral concentrations.

In the linear mixed models, GPx activity was associated with K ($p = 0.036$) and Ca ($p = 0.006$) but not with the other minerals. These two associations should be read cautiously and are regarded as exploratory. Because both serum Ca and antioxidant enzyme activity change with the inflammatory and metabolic challenge around calving [31,68], an association between GPx and Ca may reflect a common response to that challenge rather than a direct link; this could not be tested here. The GPx–K association should be interpreted with particular caution, because mean K was above the reference interval at most time points (see above).

BHBA was not significantly associated with any of the measured minerals ($p > 0.05$), and NEFA was associated only with serum Mg ($p = 0.012$); like the GPx associations, this association is regarded as exploratory. As NEFA in circulation reflects the mobilisation of adipose tissue during a negative energy balance [25], this may indicate that lipid mobilization is accompanied by differences in specific minerals rather than by a general change in mineral concentrations. During the transition period, increases in NEFA and BHBA levels are metabolic indicators of the transition from pregnancy to lactation, and this is particularly true in high-yielding dairy cows [77]. However, the absence of significant associations for the remaining minerals cannot be taken as evidence that mineral homeostasis was preserved, or that the rise in BHBA had no effect on minerals, because nonsignificant results from 30 cows are also compatible with modest associations that the study could not detect.

This study has several limitations. It was observational and was carried out in a single commercial herd with 30 multiparous cows of similar age, BCS and previous milk yields, which limits generalization to other herds, parities, production levels and feeding systems, and therefore limits the power to detect modest associations. Individual DMI, and therefore individual mineral intake, and milk yield were not recorded during the study, and the rations were characterized only by their ingredients and main nutrient composition, and their mineral composition and DCAD were not available, so the serum changes cannot be related to individual mineral supply or to directly measured energy balance. Because ration changes coincided with the physiological stages, the effects of diet, intake and stage of production on serum concentrations cannot be separated in this design. Prepartum sampling was scheduled relative to the expected calving date, the first postpartum sample was taken on day 3, after the expected Ca nadir, and samples were not collected in trace element tubes. Because the time points were defined relative to calving, samples belonging to the same time point were collected on different calendar dates, so that calendar- or run-related variation may have added to the differences between cows within a time point. Finally, the mixed models did not separate within-cow from between-cow associations, so the coefficients in Table 7 combine both sources of variation.

NEFA reflects the mobilization of lipid from adipose tissue, BHBA reflects ketone body metabolism, GPx activity represents one component of the antioxidant system, and minerals reflect a wider range of regulatory and nutritional processes. Although the associations between these variables were limited, their distinct temporal patterns across the sampling period illustrate the stage-dependent nature of metabolic regulation in dairy cows [31,33].

5. Conclusions

In this herd, serum P, Mg, Cu, Fe and Zn were lowest in late gestation and the first weeks of lactation, Cl declined after calving, and no significant change in serum Ca was detected at the sampling times used, while NEFA, BHBA and GPx activity were highest at the day 3 postpartum sampling. Group means did not cross the cut-points used for Ca, P, Mg or BHBA, whereas mean NEFA exceeded the stage-specific cut-points at D−5 and D+3, and some cows had low Ca, P or Mg, or high BHBA, around calving; mean K was above the reference interval at most time points, which is probably related to the serum sample and the colorimetric method, mean Na was below it at three time points, and K and Na results should be interpreted with caution. Associations between serum minerals and NEFA, BHBA and GPx were few and are regarded as exploratory. Because the study was observational and conducted in a single herd without individual intake data, these results describe serum changes rather than mineral utilization or antioxidant adaptation. Monitoring a subset of cows in late gestation and the first week of lactation may help to identify such changes at herd level, but the benefit of this approach was not tested here and should be evaluated in future studies.

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Abbreviations

The following abbreviations are used in this manuscript:

BCS	Body condition score
BHBA	β-hydroxybutyric acid
CP	Crude protein
DCAD	Dietary cation–anion difference
DIM	Days in milk
DMI	Dry matter intake
GPx	Glutathione peroxidase
NDF	Neutral detergent fiber
NEB	Negative energy balance
NEFA	Non-esterified fatty acid
TMR	Total mixed ration

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