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# Time profiles of energy balance in dairy cows in association with metabolic status, inflammatory status, and disease

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## ABSTRACT

The early lactation period in dairy cows is characterized by complex interactions among energy balance (EB), disease, and alterations in metabolic and inflammatory status. The objective of this study was to cluster cows based on EB time profiles in early lactation and investigate the association between EB clusters and inflammatory status, metabolic status, oxidative stress, and disease. Holstein-Friesian dairy cows (n = 153) were selected and monitored for disease treatments during wk 1 to 6 in lactation. Weekly EB was calculated based on energy intake and energy requirements for maintenance and milk yield in wk 1 to 6 in lactation. Weekly plasma samples were analyzed for metabolic variables in wk 1 to 6, and inflammatory and oxidative stress variables in wk 1, 2, and 4 in lactation. Liver activity index (LAI) was computed from plasma albumin, cholesterol, and retinol-binding protein concentration. First, cows were clustered based on time profiles of EB, resulting in 4 clusters (SP: stable positive; MN: mild negative; IN: intermediate negative; SN: severe negative). Cows in the SN cluster had higher plasma nonesterified fatty acids and BHB concentrations, compared with cows in the SP cluster, with the MN and IN clusters being intermediate. Cows in the SN cluster had a higher milk yield, lower DMI in wk 1, lower insulin concentration compared with cows in the SP cluster, and lower glucose and IGF-1 concentration compared with cows in the SP and MN clusters. Energy balance clusters were not related to plasma haptoglobin, cholesterol, albumin, paraoxonase, and LAI. Second, cows were grouped based on health status: IHP, cows with treatment for inflammatory health problem (endometritis, fever, clinical mastitis, vaginal discharge or retained placenta); OHP, cows with no IHP but treatment for other health problem (milk fever, cystic ovaries, claw and leg problems, rumen and intestine problems, or other diseases); and NHP, cows with no treatments, in the first 6 wk after calving. Energy balance was not different among health status groups. The IHP cows had lower nonesterified fatty acids and greater insulin concentration in plasma compared with OHP cows. The IHP cows had lower plasma albumin concentration, lower LAI, and higher haptoglobin concentration compared with OHP and NHP. Overall, EB time profiles were associated with the metabolic status of dairy cows in early lactation, but were only limitedly related to markers of inflammation and oxidative stress status. Inflammatory and metabolic status were related to disease events in early lactation and caused prolonged effects on liver metabolism.
**Key words:** negative energy balance, health, clustering, inflammation

## INTRODUCTION

During the first 3 wk of lactation, around one-third of dairy cows experienced at least one clinical disease, including metritis, mastitis, lameness, or respiratory issues (Koeck et al., 2012; Ribeiro and Carvalho, 2017). The high incidence of health problems at the start of lactation has been attributed to both the calving process and the energy deficiency to support the start of a new lactation (Collard et al., 2000), and the diseases and disorders in early lactation have a negative effect on milk yield, energy balance (**EB**), and reproductive performance of dairy cows (LeBlanc., 2010; Vergara et al., 2014).

Recently, cows were characterized using cluster analysis, for example, orthogonal components, milk, or plasma variables, respectively (Tremblay et al., 2018; De Koster et al., 2019; Foldager et al., 2020), exploiting the

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clustering approach to combine information from a collection of diverse variable types to identify cows at risk for a compromised metabolic status, severe negative EB, and health problems. Earlier studies predicted metabolic status based on blood variables (Tremblay et al., 2018; De Koster et al., 2019; Cattaneo et al., 2021) or predicted metabolic status and EB using milk variables (Grelet et al., 2019; Foldager et al., 2020; Giannuzzi et al., 2023). In these studies, metabolic status was defined with a set of metabolites and metabolic hormones in plasma, with some variation in plasma metabolic variables included, but in most studies plasma glucose was included and plasma nonesterified fatty acids (**NEFA**) and BHB concentration were always included. Furthermore, these traditional studies typically analyzed variables at subsequent time points in early lactation separately (De Koster et al., 2019; Foldager et al., 2020; Xu et al., 2020) or had changing clusters of cows from week to week (Tremblay et al., 2018), which would potentially complicate management decisions based on these predictions. More recently, we clustered cows based on time profiles of EB, including EB information during 10 wk peripartum (wk −3 to +7) and not allowing cows to change cluster from week to week (Vossebeld et al., 2022). The clusters of EB time profiles were clearly mirrored by peripartum profiles for metabolites including plasma NEFA concentration, glucose, IGF-1, but less clearly by plasma BHB and insulin concentration (Vossebeld et al., 2022).

In addition to alterations in metabolic status and EB, inflammation also plays an important role in the etiology of health disorders in early lactation and the adaptation of cows to a new lactation (Bertoni et al., 2008; Huzzey et al., 2009; Dubuc et al., 2010). Acute-phase proteins (**APP**), such as albumin and haptoglobin, are involved in the acute and systemic response to inflammation (Ceciliani et al., 2012). Moreover, mobilization of body fat during negative EB (**NEB**) and the associated increase in plasma NEFA concentration may contribute to the inflammatory response in dairy cows in early lactation (as reviewed by Sordillo and Raphael, 2013). Inflammation during NEB was associated with proinflammatory cytokines (Grimble, 1990), increased synthesis of positive APP (e.g., haptoglobin and ceruloplasmin), and reduced synthesis of negative APP (e.g., albumin; Bionaz et al., 2007). Earlier studies indicated that increased blood haptoglobin concentration was related to uterine diseases such as clinical metritis (Huzzey et al., 2009; Dubuc et al., 2010). Cows with fatty liver had increased concentrations of haptoglobin and ceruloplasmin and decreased concentrations of albumin and paraoxonase in serum (Katoh, 2002; Ametaj et al., 2005; Janovick et al., 2023). Cows affected with ketosis after calving had higher concentrations of ceruloplasmin and haptoglobin in plasma than healthy cows (El-Deeb and El-Bahr, 2017; Mezzetti et al., 2019). Concentrations of oxidative stress biomarkers, for example, reactive oxygen metabolites (**ROM**) and ferric-reducing antioxidant power (**FRAP**), were also found to be related to the occurrence of ketosis in dairy cows (Mezzetti et al., 2019).

None of the previously mentioned studies characterizing dairy cows in early lactation using clustering included information on inflammatory status or oxidative stress. It can be hypothesized that the characterization of the physiological response of dairy cows to the start of a new lactation can be further fine-tuned by adding information on inflammatory status and oxidative stress. The objective of this study was to cluster cows based on EB time profiles in early lactation and investigate the association between EB clusters and inflammatory status, metabolic status, oxidative stress, and disease.

## MATERIALS AND METHODS

### Experimental Design and Animals

The Institutional Animal Care and Use Committee of Wageningen University & Research (Wageningen, the Netherlands) approved the experimental protocol, which complies with the Dutch law on Animal Experimentation (protocol number 2016.D-0038.005). The experiment was conducted at the Dairy Campus research farm (Leeuwarden, the Netherlands) between December 2017 and January 2020.

***Animals.*** From the Dairy Campus research herd of 500 lactating cows 154 Holstein-Friesian dairy cows (41 primiparous and 113 multiparous cows) were selected based on the following criteria: no twin pregnancy, no clinical mastitis or SCC > 250,000 cells/mL at the final 2 milk test days before dry-off, and expected to finish a complete lactation. The original study was designed to evaluate consequences of voluntary waiting period (**VWP**) for lactation performance (Burgers et al., 2021) and cows were assigned to a VWP of 50 d, 125 d, or 200 d in wk 6 after calving. For the current study, data and samples were used from the first 6 wk of the first lactation during the experiment when the cows were not yet allocated to VWP treatments. Cows were milked twice daily around 0600 h and 1800 h in a 40-cow rotary milking parlor (GEA, Dusseldorf, Germany). Cows were treated for health problems according to standard protocols by trained technicians at Dairy Campus. For the current study, retrospectively, cows were (1) clustered based on time profiles of EB; and separately, (2) grouped based on health status, which are both explained in more detail below.

***Rations.*** Diet composition and feeding strategy were described earlier (Burgers et al., 2021). Cows were fed a lactation ration with a partial mixed ration (**PMR**) that

consisted of grass silage, corn silage, soybean meal, and wheat meal, supporting a milk production of 22 kg/d. Concentrate supply started at 1 kg/d from calving and increased stepwise to 9 kg/d (for primiparous cows) or 10 kg/d (for multiparous cows) from d 21 onward. Additionally, 1 kg of extra concentrate was provided to each cow daily during milking. The ration administered during the dry period comprised grass silage and corn silage, supplemented with wheat straw and concentrate. In the final 10 d before the anticipated calving date, cows were given 1 kg of concentrate daily.

### Measurements and Sampling

***Milk, DMI, and BW.*** Milk yield was recorded at every milking. Milk samples were collected from each cow 4 times per week (Tuesday afternoon, Wednesday morning, Wednesday afternoon, and Thursday morning) in 10-mL tubes containing bronopol as a preservative. Dry matter intake, including concentrate and PMR intake, was recorded daily (RIC Insentec bins, Marknesse, the Netherlands) and reported weekly. Body weight was recorded twice daily after each milking, by a scale (GEA, Dusseldorf, Germany) that the cows walked over when returning from the milking rotary to the freestall, and averaged per week.

***Blood Sampling.*** Blood samples were collected weekly on Thursday (Burgers et al., 2023). After the morning milking and 3h before feeding, blood samples were collected from the tail vein. Blood was collected in evacuated tubes (Vacuette, Greiner BioOne, Kremsmunster, Austria) containing NaF for glucose; EDTA for insulin, NEFA, and BHB; lithium-heparin for IGF-I, albumin, cholesterol, creatinine, total protein, urea, globulin, ceruloplasmin, calcium, glutamic oxaloacetic transaminase (**GOT**), haptoglobin, paraoxonase, myeloperoxidase (**MPO**), FRAP, ROM, vitamin A, vitamin E, and β-carotene. Samples were kept cold on ice for a maximum of 2 h until they were centrifuged at 3,000 × *g* for 15 min at 4°C. Plasma was decanted, aliquoted, and frozen at −20°C until analysis.

### Laboratory Analysis

***Milk Composition.*** Milk samples were collected from each individual cow 4 times weekly (Tuesday afternoon, Wednesday morning, Wednesday afternoon, Thursday morning) using 10-mL tubes containing bronopol as a preservative. These samples were pooled and analyzed for fat, protein, and lactose percentage (standard 9622, ISO, 2013; Qlip, Zutphen, the Netherlands).

***Metabolite and Hormone Concentrations.*** The weekly plasma samples from cows in wk 1 until 6 in lactation were analyzed at the Veterinary Physiology group of the Vetsuisse Faculty, University of Bern (Bern, Switzerland). The concentration of glucose was measured using commercial kit no. 61269 and no. 61974 from BioMérieux (Marcy l'Étoile, France). Concentrations of NEFA and BHB were measured using kit no. 994–75409 from Wako Chemicals (Neuss, Germany) and kit no. RB1007 from Randox Laboratories (Ibach, Switzerland). The concentration of IGF-1 was measured using kit no. A15729 from Beckman Coulter (Fullerton, CA), and insulin was measured using kit no. PI-12K from EMD Millipore Corporation (Billerica, MA). The inter- and intra-assay CV for NEFA, BHB, and glucose was <1%. The inter- and intra-assay CV for insulin and IGF-1 was <15%.

***Inflammatory Biomarkers and Oxidative Stress Variables.*** The plasma samples from cows in wk 1, 2, and 4 in lactation were analyzed at the Department of Animal Sciences, Food and Nutrition (DIANA) the Istituto di Zootecnica of the Università Cattolica del Sacro Cuore (Piacenza, Italy), following the procedures previously described by Calamari et al. (2016) using a clinical auto-analyzer (ILAB 650, Instrumentation Laboratory, Lexington, MA). In short, albumin, cholesterol, total protein, urea, calcium, and creatinine concentration were measured using the IL Test purchased from Instrumentation Laboratory Spa (Werfen Co., Milan, Italy). Globulin concentration was calculated as the difference between total protein and albumin concentration. Haptoglobin concentration was determined with the method described by Skinner et al. (1991). This method is based on the peroxidase activity of the methemoglobin-haptoglobin complex measured by the rate of oxidation of guaiacol (hydrogen donor) in the presence of hydrogen peroxide (oxidizing substrate). Ceruloplasmin concentration was determined with the method described by Sunderman and Nomoto (1970). The test is based on the measurement of color, which originates from the oxidation of p-phenylenediamine dihydrochloride induced by ceruloplasmin. Concentrations of ROM were measured using commercial kits (kit d-ROMs-test MC003; Diacron International s.r.l., Grosseto, Italy). Antioxidant potential was assessed as FRAP using the colorimetric method of Benzie and Strain (1996). Plasma paraoxonase activity was measured by adapting the method of Ferré et al. (2002) to the ILAB 650 conditions. Plasma vitamins A and E and β-carotene were extracted with hexane and analyzed by reverse-phase HPLC using a Spherisorb ODS-2, 3 m, in a 150 × 4.6 mm column (Alltech, Deerfield, IL); a UV detector set at 325 nm (for vitamin A), 290 nm (for vitamin E), and 460 nm (for β-carotene) using 80:20 methanol: tetrahydrofurane as the mobile phase. The inter- and intra-assay CV for albumin, cholesterol, total protein, urea, calcium, creatinine, ceruloplasmin, ROM, vitamin A, and vitamin E was <5%. The inter- and intra-assay

CV for FRAP, paraoxonase, haptoglobin, and β-carotene was <10%.

The liver activity index (**LAI**) was calculated according to Trevisi et al. (2010). This aggregate index includes the average plasma concentration (in wk 1, 2, and 4 of lactation) of proteins synthesized by the liver: albumin, lipoproteins (indirectly measured as total cholesterol; Bruss, 1997), and retinol-binding protein because the retinol released from the liver depends mainly on the synthesis of apo-B. Data of these 3 blood variables were transformed into units of SD obtained for each cow as follows: the mean value of the herd population of each plasma variable (albumin, total cholesterol, and retinol-binding protein) was subtracted from each cow value in wk 1, 2, and 4 of lactation and divided by the corresponding SD. Thus, the final LAI of each cow is the result of the arithmetical mean of the 3 partial values obtained from the 3 selected blood indices at the 3 sampling moments.

## Data Handling and Calculations

***Energy Balance and Fat- and Protein-Corrected Milk.*** Energy balance was calculated according to the Dutch NE system for lactation (Van Es, 1975; CVB, 2012). Weekly EB was calculated in the first 6 wk postcalving. It was determined by calculating the difference between the intake of net energy (**NE**) and the requirements of NE for maintenance and milk production (CVB, 2012). Net energy intake was computed based on the individual daily intake of PMR and intake of concentrate. The NE requirement for maintenance was assumed to be 291.18 kJ/$BW^{0.75}$, and the NE requirement for milk production was assumed to be 3,049.8 kJ/kg of fat- and protein-corrected milk (**FPCM**). Energy intake requirements and EB are expressed in kJ/$BW^{0.75}$ per day. Milk production was converted to FPCM using the following formula (CVB, 2012):

$$\text{FPCM (kg)} = \text{milk (kg)} \times (0.337 + 0.116 \times \text{fat (\%)} + 0.06 \times \text{protein (\%)}$$

***Handling of Missing Data and Data Imputation.*** Using a conservative approach of discarding animals with 3 or more missing time points, 1 animal was omitted because of 4 missing values. For 36 out of 153 cows (corresponding to 44 of 918 weekly EB measures), EB values were imputed to obtain complete EB times series. Using the MissForest method based on Random Forest (Breiman, 2001; Stekhoven and Bühlmann, 2012), a multiple imputation framework was considered when performing clustering of the imputed time series (Van Buuren, 2018).

## Clustering and Regrouping of Cows

***Clustering Based on EB Time Profiles.*** The clustering approach was based on a global alignment kernel (Cuturi et al., 2007). After the algorithms were applied to the 51 complete (nonimputed) time series, consensus clustering among the 1,000 solutions was obtained using the Hungarian method (Kuhn, 1955), in combination with majority voting (Wang et al., 2013) as described in Vossebeld et al. (2022). The optimal number of clusters was selected using 2 different criteria: the elbow approach (Thorndike, 1953) and the silhouette method (Rousseeuw, 1987). The elbow method is a heuristic approach entailing plotting the sum of the squared distances between data points and their cluster center as function of the number of clusters: the optimal number of clusters $K$ correspond to the minimum K after which the within-cluster sums of squares do not decrease any more. The silhouette method allows to characterize the separation between the resulting clusters: for each cluster the silhouette value ranges between −1 and 1, with 1 indicating a compact cluster solution. Given $K$, the closer the average of the silhouette values taken over the K cluster the better the cluster solution. Both methods indicated that $K = 4$ clusters was the optimal solution; all subsequent analyses were performed by considering 4 clusters of EB time series. Descriptive names to each cluster were assigned based on the characteristics of the EB time profiles in postpartum weeks according to Vossebeld et al. (2022): **SP** = stable positive cow cluster; **MN** = mild negative cow cluster; **IN** = intermediate negative cow cluster, **SN** = severe negative cow cluster.

***Grouping Cows Based on Health Status.*** Cows were grouped into 3 groups based on treatments in the first 6 wk in lactation (**IHP**: cows with treatment for inflammatory health problem; **OHP**: cows with no treatment for IHP but with treatment for other health problem **NHP**: cows with no treatment for a health problem, in the first 6 wk of lactation), where IHP was defined as treatment for acute endometritis, fever, clinical mastitis, vaginal discharge or retained placenta; OHP was defined as treatment for milk fever, cystic ovaries, claw and leg problems, rumen and intestine problems, or other diseases. Disease categories were adapted from Mayasari et al. (2017) by renaming the clinical health problem group IHP, adding vaginal discharge to the IHP group, and splitting the group with no clinical health problem into 2 groups: OHP and NHP.

## Statistical Analysis

Statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC) and R (The R Project; www.r-project.org/).

***Descriptive Statistics for the EB Clusters.*** A linear repeated measurements model in SAS (PROC MIXED, SAS version 9.4, SAS Institute Inc.) with cow as the repeated subject was used to analyze the descriptive statistics for the EB clusters (Model 1). Dependent variables in Model 1 were milk yield, DMI, and EB. Independent variables in Model 1 were EB clusters (SP, MN, IN, or SN), parity (1 or ≥2), and week (1, 2, 3, 4, 5, and 6 postpartum), and their 2-way interactions. The purpose of this analysis was to describe the characteristics of the EB cluster and its relationship with the underlying variables EB, DMI, and milk yield. The residuals of the model were checked and found to be normally distributed. Values are presented as LSM ± SEM. All *P*-values of pair-wise comparisons of LSM were corrected with a Bonferroni adjustment.

***Relationships Between EB Clusters and Inflammatory Biomarkers, Metabolic Variables.*** A linear repeated measurements model in SAS (PROC MIXED, SAS version 9.4, SAS Institute Inc.) with cow as the repeated subject was used to analyze the relationships between EB clusters and metabolic variables in plasma (Model 2) and EB clusters and inflammatory and oxidative stress variables in plasma (Model 3). Dependent variables in Model 2 were NEFA, BHB, glucose, insulin, and IGF-1. Independent variables in Model 2 were EB clusters (SP, MN, IN, or SN), parity (1 or ≥2), and week (1, 2, 3, 4, 5, and 6 postpartum), and their 2-way interactions. Dependent variables in Model 3 were albumin, cholesterol, creatinine, total protein, globulin, urea, calcium, ceruloplasmin, GOT, haptoglobin, FRAP, paraoxonase, MPO, ROM, vitamin A, vitamin E, β-carotene, and LAI. Independent variables in Model 3 were EB cluster (SP, MN, IN, or SN), parity (1 or ≥2), week (1, 2, and 4 postpartum), and their 2-way interactions. Residuals of the models were checked and found to be normally distributed. Values are presented as LSM ± SEM. All *P*-values of pair-wise comparisons of LSM were corrected with a Bonferroni adjustment.

***Relationships Between Health Status and Metabolic, Inflammatory, and Oxidative Stress Status.*** Whether an association existed between probability of health problem treatments and EB clusters was analyzed with a chi-squared test (PROC FREQ, SAS version 9.4, SAS Institute Inc.). A linear repeated measurements model in SAS (PROC MIXED, SAS version 9.4, SAS Institute Inc.) with cow as the repeated subject was used to analyze the relationship between health status (IHP, OHP, or NHP) and metabolic status (Model 4) and health status and inflammatory and oxidative stress status (Model 5). Cow was the repeated subject in these models. Dependent variables in Model 4 were milk yield, DMI, NEFA, BHB, glucose, insulin, IGF-1, and EB. Independent variables in Model 4 were health status (IHP, OHP, or NHP), parity (1 or ≥2), and week (1, 2, 3, 4, 5, and 6 postpartum), and their 2-way interactions. Dependent variables in Model 5 were albumin, cholesterol, creatinine, total protein, globulin, urea, calcium, ceruloplasmin, GOT, haptoglobin, FRAP, paraoxonase, MPO, ROM, vitamin A, vitamin E, β-carotene, and LAI. Independent variables in Model 5 were health status (IHP, OHP, or NHP), parity (1 or ≥2), week (1, 2, and 4 postpartum), and their 2-way interactions. Residuals of the models were checked and found to be normally distributed. Values are presented as LSM ± SEM. All *P*-values of pair-wise comparisons of LSM were corrected with a Bonferroni adjustment.

In a preliminary analysis, both compound symmetry and first-order autoregressive covariance matrixes were assessed; the first-order autoregressive covariance matrix had the best fit according to the Akaike information criterion and was used to account for within-cow variation in models 1 through 4. Values are presented as LSM and were regarded as significant if *P*-values were <0.05.

## RESULTS

### Clustering Based on EB Time Profiles

In the current study, 153 cows were grouped into 4 clusters based on EB time profile in the first 6 wk after calving (Figure 1). Primiparous cows were mostly present in the SP and MN clusters, whereas multiparous cows were present in all clusters (Table 1). Differences among EB clusters in milk yield and DMI depended on the week in lactation (Table 2). Milk yield was increasing through the first 6 wk (Figure 2). Milk yield of cows in the SP cluster was in all weeks ($P < 0.01$) lower than in the other 3 clusters. In wk 4, 5, and 6, milk yield of cows in the MN cluster was lower than milk yield of cows in the IN or SN clusters ($P < 0.05$; Appendix Table A1). In wk 1, cows in the SN cluster had a lower DMI compared with cows in the SP cluster ($P = 0.01$), no evidence for differences was found for DMI in other weeks ($P > 0.05$).

### Relationships Between EB Clusters and Metabolic Status

During the first 6 wk after calving, cows in the SP cluster had a higher concentration of glucose, insulin, and IGF-1 in plasma compared with cows in other EB clusters (Table 3). In addition, cows in the MN cluster had a higher concentration of glucose and IGF-1 in plasma compared with cows in the SN cluster. Differences among EB clusters in concentration of NEFA and BHB depended on the week in lactation. Especially in the first 3 wk, cows in the SP cluster had a lower level of NEFA in plasma compared with the other 3 clusters (all $P < 0.01$) and lower levels of BHB compared with cows in the SN cluster ($P < 0.01$).

### Relationships Between EB Clusters, Inflammatory Biomarkers, and Oxidative Stress Markers

Plasma urea concentration was greater for cows in the MN cluster than for cows in the IN cluster ($P = 0.045$), but did not differ from the SP or SN clusters (Table 4). No evidence for difference among EB clusters was found for other inflammatory biomarkers or oxidative stress variables ($P > 0.05$).

### Health Status Groups

Of the 153 cows in the experiment, 66 (43%) cows had an overall total of 100 treatments for a health problem in the first 6 wk postpartum. Of these treatments, 69 treatments occurred in the IHP group (49 cows) and 31 treatments in the OHP group (16 cows; Table 5). Of the health problem treatments, 16 disease treatments occurred in the first week after calving. Table 6 presents the distribu-

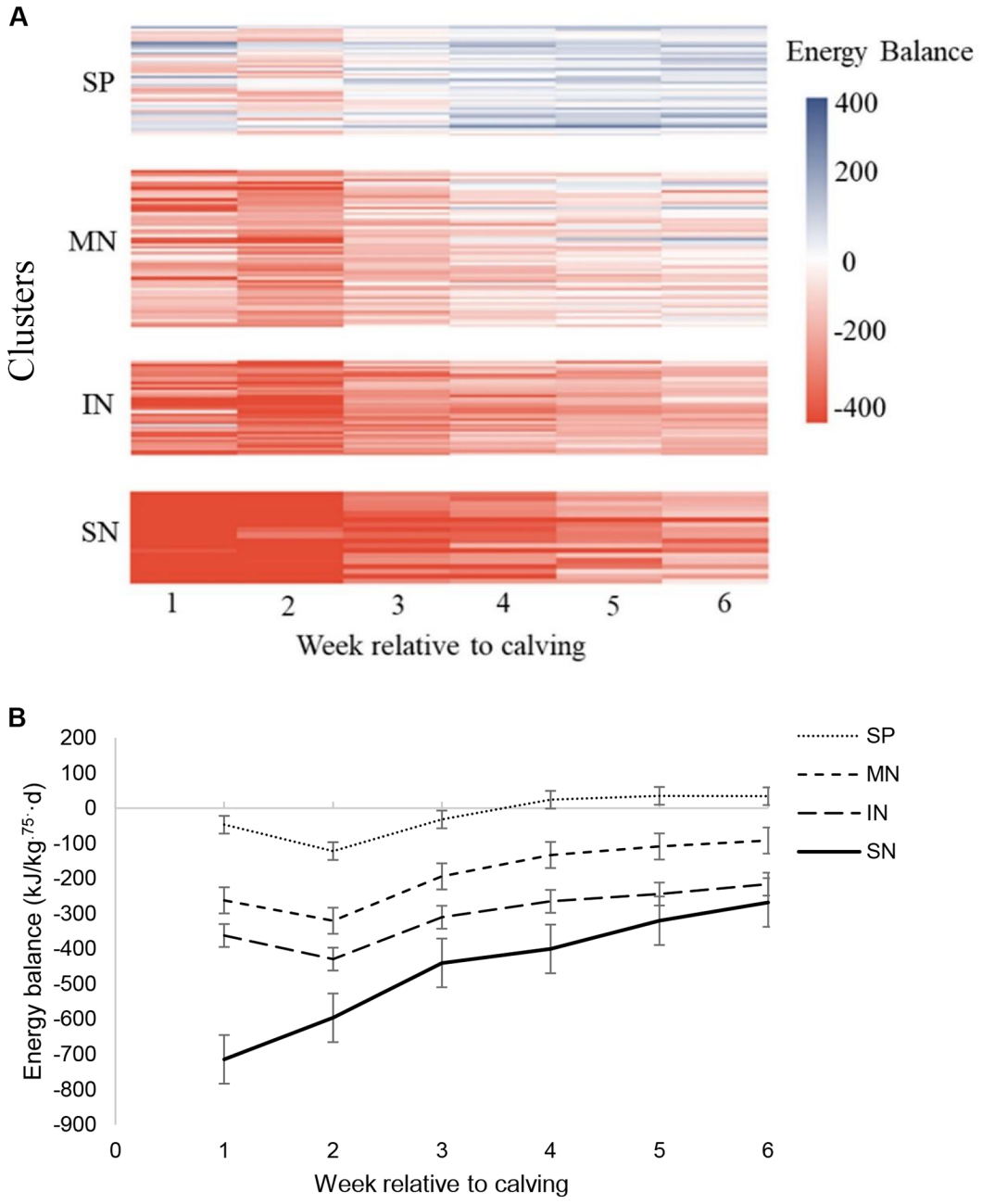


**Figure 1.** (A) Clustering of EB time profiles. Each line represents the time series of the EB of an individual cow. (B) Energy balance of cows in different clusters of time series of EB. Values represent LSM ± SEM. For both A and B, EB is expressed in kJ/kg$^{0.75}$ per day. SP = stable positive cow cluster; MN = mild negative cow cluster; IN = intermediate negative cow cluster, SN = severe negative cow cluster.

tion of cows among disease treatment groups and clusters for EB time profiles in the first 6 wk postpartum. Health problem treatments for cows in the various clusters were 30% (13/43) for SP, 44% (25/56) for MN, 53% (19/36) for IN, and 44% (8/18) for SN. There was no evidence for an association between probability of health problem treatments and EB clusters ($P = 0.31$, chi-squared).

### Relationships Between Health Status and Milk Yield, DMI, Metabolic Status and EB

During the first 6 wk after calving, plasma NEFA concentration was lower for cows with IHP ($P = 0.01$) or NHP ($P < 0.01$), compared with OHP cows (Table 7). Plasma insulin concentration was higher for cows with IHP ($P = 0.01$) compared with OHP cows, but both did not differ from NHP cows ($P > 0.05$). The effect of health status on plasma glucose concentration depended on week after calving, with specifically in wk 1 after calving higher plasma glucose concentration for NHP cows ($P = 0.02$), compared with OHP cows, but both did not differ from IHP cows ($P > 0.05$; Figure 3; Appendix Table A2). No relationships were found between the health status of cows during the first 6 wk of lactation and DMI, EB, and BHB and IGF-1 concentration in plasma ($P > 0.05$).

### Relationships Between Health Status, Inflammatory Biomarkers, and Oxidative Stress Markers

Cows with IHP had a lower concentration of albumin ($P < 0.01$) and a lower LAI ($P = 0.03$) compared with NHP or OHP cows (Table 8). Cows with IHP had a higher concentration of haptoglobin ($P < 0.01$), compared with NHP cows, but both did not differ from OHP cows. The effect of health status on albumin ($P = 0.03$) and creatinine ($P = 0.02$) depended on week in lactation, but differences between individual groups were not significant. No relationships were found between the health status of cows during the first 6 wk of lactation and cholesterol, total protein, globulin, calcium, and any of the oxidative stress variables in plasma ($P > 0.05$).

**Table 1.** Distribution of cows among clusters of time profiles of EB per parity class

| | Parity class | | |
|---|---|---|---|
| Energy balance cluster[1] | 1 | ≥2 | Total |
| SP | 20 | 23 | 43 |
| MN | 15 | 41 | 56 |
| IN | 4 | 32 | 36 |
| SN | 1 | 17 | 18 |
| Total | 40 | 113 | 153 |

[1]SP = stable positive EB; MN = mild negative EB; IN = intermediate negative EB; SN = severe negative EB.

**Table 2.** Descriptive statistics for milk yield, DMI, and EB of 153 dairy cows in wk 1 to 6 after calving where cows were categorized according to the cluster of time series of EB during the first 6 wk after calving

| | Energy balance cluster[1] | | | | | Parity | | | *P*-value | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Item | SP | MN | IN | SN | SEM | 1 | ≥2 | SEM | EB | Par[2] | Week | EB × parity | EB × week | Par × week |
| Cows, n | 43 | 56 | 36 | 18 | | | | | | | | | | |
| Week 1–6 | | | | | | | | | | | | | | |
| Milk yield (kg/d) | 26.5[a] | 31.8[b] | 36.1[c] | 38.0[c] | 1.96 | 28.2 | 38.0 | 1.11 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 |
| DMI (kg/d) | 17.5 | 17.3 | 17.4 | 16.1 | 0.96 | 14.5 | 19.7 | 0.54 | 0.57 | <0.01 | <0.01 | 0.24 | <0.01 | 0.02 |
| EB[3] (kJ/kg$^{0.75}$ per day) | −18.5[d] | −183[c] | −292[b] | −448[a] | 30.8 | −230 | −240 | 17.3 | <0.01 | 0.58 | <0.01 | 0.35 | <0.01 | 0.18 |

[a–d]For each variable, a significant difference between clusters of EB time profile is shown by letters; $P < 0.05$.
[1]Cluster of time series of EB: SP = stable positive cow cluster; MN = mild negative cow cluster; IN = intermediate negative cow cluster, SN = severe negative cow cluster.
[2]Par = parity.
[3]EB = energy balance.

## DISCUSSION

In the current study, clusters for EB time profiles were related to milk yield and metabolic status of cows in the first 6 wk of lactation. Milk yield, plasma NEFA, and BHB were greater and plasma glucose, insulin, and IGF-1 were lower for cows in the SN and IN clusters compared with cows in the MN and SP clusters. This is in agreement with our earlier study where we clustered cows based on EB time profiles in the peripartum period (Vossebeld et al., 2022), but also in agreement with an extensive list of experimental and observational studies evaluating EB and metabolic status of dairy cows in early lactation (e. g., Doepel et al., 2002; Hammon et al., 2009; Chen et al., 2015). Moreover, in earlier studies focusing on clustering cows based on peripartum metabolic status, plasma NEFA, BHB, glucose, and IGF-1 also played a prominent role in differentiating cow clusters (Grelet et al., 2019; Foldager et al., 2020; Xu, et al., 2020).

In the current study, cows were clustered based on time profiles of EB in the first 6 weeks of lactation, resulting in 4 clusters with 28% cows in the SP cluster, 37% in the MN cluster, 23% in the IN cluster, and 12% in the SN cluster. In our earlier study, clustering cows based on time profiles of EB in the peripartum period (wk −3 to wk +7) resulted in 17% of the cows in the SP cluster, 25% in the MN cluster, 32% in the IN cluster, and 26% in the SN cluster (Vossebeld et al., 2022). Although the overall clustering procedure was the same in both studies, the proportions of cows assigned to each cluster were not similar. Differences in the distribution of cows among EB clusters can be related to different peripartum weeks included in the clustering or different diets and dry period lengths, resulting in differences in EB between studies. Moreover, cow characteristics like milk yield level or parity, with no primiparous cows included in the earlier clustering study in contrast to the current study, could contribute to different distributions of cows among

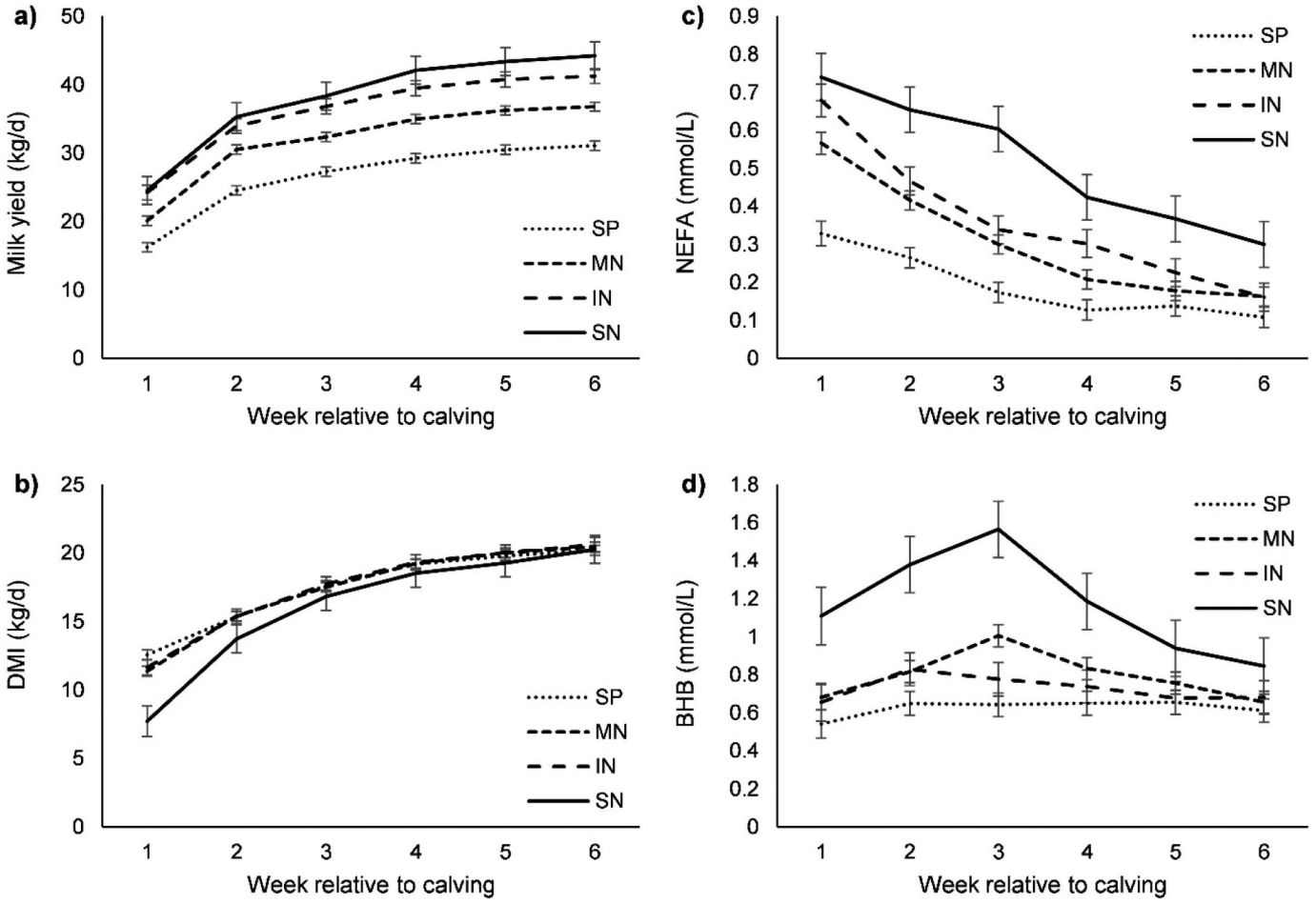


**Figure 2.** Milk yield (a), DMI (b), NEFA (c), and BHB (d) of cows in different clusters of time series of EB (SP = stable positive cow cluster; MN = mild negative cow cluster; IN = intermediate negative cow cluster, SN = severe negative cow cluster) in the first 6 wk after calving. Values represent LSM ± SEM.

**Table 3.** Plasma metabolites (NEFA, BHB, and glucose), and metabolic hormones (IGF-1 and insulin) of 153 dairy cows in wk 1 to 6 after calving where cows were categorized according to the cluster of time series of EB during the first 6 wk after calving

| | Energy balance cluster[1] | | | | | Parity | | | *P*-value | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Item | SP | MN | IN | SN | SEM | 1 | ≥2 | SEM | EB | Par[2] | Week | EB × parity | EB × week | Par × week |
| Cows, n | 43 | 56 | 36 | 18 | | | | | | | | | | |
| Week 1–6 | | | | | | | | | | | | | | |
| NEFA[3] (mmol/L) | 0.19[a] | 0.30[b] | 0.36[b] | 0.51[c] | 0.05 | 0.36 | 0.32 | 0.03 | <0.01 | 0.15 | <0.01 | 0.57 | <0.01 | 0.53 |
| BHB (mmol/L) | 0.62[a] | 0.79[b] | 0.73[ab] | 1.17[c] | 0.12 | 0.90 | 0.75 | 0.07 | <0.01 | 0.04 | <0.01 | 0.13 | <0.01 | 0.01 |
| Glucose (mmol/L) | 3.57[c] | 3.32[b] | 3.23[ab] | 2.96[a] | 0.12 | 3.33 | 3.20 | 0.07 | <0.01 | 0.08 | <0.01 | 0.27 | 0.94 | 0.28 |
| Insulin (µU/mL) | 15.1[b] | 12.2[a] | 11.0[a] | 9.39[a] | 1.78 | 11.4 | 12.5 | 1.02 | <0.01 | 0.30 | <0.01 | 0.73 | 0.94 | 0.42 |
| IGF-1 (ng/mL)[5] | 139[b] | 109[a] | 85.7[a] | 75.7[a] | 17.2 | 110 | 95.3 | 9.87 | <0.01 | 0.17 | <0.01 | 0.59 | 0.29 | 0.04 |

[a–c]For each variable, a significant difference between clusters of EB time profile is shown by letters; $P < 0.05$.

[1]Cluster of time series of EB: SP = stable positive cow cluster; MN = mild negative cow cluster; IN = intermediate negative cow cluster, SN = severe negative cow cluster.

[2]Par = parity.

[3]NEFA = nonesterified fatty acids.

EB clusters. This illustrates that it seems not valuable to compare the cow clusters of both studies, but to restrict ourselves to a comparison of clusters within data sets or studies, but not directly across studies. Ultimately, a baseline correction per study or dataset could be evaluated to correct for differences among studies and enable comparison of clusters based on EB time profiles across studies. Moreover, both in our earlier study (Vossebeld et al., 2022) and in the current study the clustering approach resulted in an unbalanced distribution of cows among clusters, with for example, primiparous cows being present mostly in the 2 more positive EB clusters. Although this makes sense from a biological perspective because primiparous cows mostly have a lower priority for milk production and therefore a better EB, these unbalanced cluster sizes complicate later statistical analysis.

In contrast to expectations, a major proportion of the set of inflammatory and oxidative stress variables in the current study were not different among EB clusters. This could be related to the possibly limited contrast in actual EB among clusters, cows treated for health problems present in all EB clusters, or to the sampling pattern of inflammatory and oxidative stress variables in wk 1, 2, and 4 after calving. In our earlier study, we used the same sampling timing as in the current study, but the variation in EB was much greater due to an experimental contrast in dry period length (0, 30, or 60 d), which possibly explains the reported consequences for the inflammatory and oxidative stress status in the first weeks of lactation (Mayasari et al., 2017). In addition, according to Trevisi et al. (2010), the inflammation (measured with positive APP: haptoglobin and ceruloplasmin) showed only temporary differences in cows in response to NEB. In contrast, the effects of NEB or clinical health problems (e.g., ketosis) on liver metabolism remained for a longer time and consequently caused problems in the recovery from diseases and lower milk yield.

Cows in the SP cluster were characterized by the most positive EB during wk 1 to 6 in lactation. This positive EB was due to a lower milk yield for cows in the SP cluster, whereas their DMI was not different from that of cows in the MN or IN cluster.s The lower milk yield could be related to lower genetic merit for milk yield in this group, but in the SP cluster 30% of the cows were treated for health problems compared with 44%, 53%, and 44% for cows in the MN, IN, and SN clusters, respectively. This indicates that also in the more positive EB clusters, there were still cows treated for health problems. It can be hypothesized that the lower milk yield in the SP cluster was related to a health problem. Also in an earlier study, severe metabolic disease or occurrence of multiple disease events for the same cows in early lactation affected milk yield (Hostens et al., 2012). Therefore, the SP cluster may not be the best-performing cluster

**Table 4.** Inflammatory biomarkers (albumin, cholesterol, total protein, globulin, ceruloplasmin, and haptoglobin), index of liver function (paraoxonase, LAI), and oxidative stress markers (GOT, MPO, ROM, FRAP, creatinine, urea, calcium, vitamin A, vitamin E, and β-carotene) in plasma of 153 dairy cows[1]

| | Energy balance cluster[2] | | | | | Parity | | | *P*-value | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Item[3] | SP | MN | IN | SN | SEM | 1 | ≥2 | SEM | EB | Par[4] | Week | EB × parity | EB × week | Par × week |
| Cows, n | 43 | 56 | 36 | 18 | | 40 | 113 | | | | | | | |
| Weeks 1, 2, and 4 | | | | | | | | | | | | | | |
| Albumin (g/L) | 33.6 | 34.2 | 34.0 | 35.1 | 1.06 | 33.8 | 34.7 | 0.60 | 0.37 | 0.15 | 0.04 | 0.98 | 0.89 | 0.92 |
| Cholesterol (mmol/L) | 2.96 | 2.98 | 3.04 | 2.86 | 0.33 | 2.82 | 3.10 | 0.19 | 0.97 | 0.18 | <0.01 | 0.95 | 0.41 | 0.62 |
| Creatinine (μmol/L) | 82.2 | 84.6 | 83.6 | 87.3 | 2.63 | 84.0 | 84.8 | 1.49 | 0.08 | 0.62 | <0.01 | 0.90 | 0.98 | 0.89 |
| Total protein (g/L) | 73.1 | 72.6 | 72.7 | 74.7 | 2.22 | 72.0 | 74.6 | 1.27 | 0.80 | 0.05 | <0.01 | 0.95 | 0.29 | 0.18 |
| Globulin (g/L) | 39.5 | 38.3 | 38.7 | 39.5 | 2.48 | 38.1 | 39.9 | 1.41 | 0.72 | 0.24 | <0.01 | 0.99 | 0.09 | 0.04 |
| Urea (mmol/L) | 3.50[ab] | 3.72[b] | 3.29[a] | 3.70[ab] | 0.26 | 3.54 | 3.57 | 0.15 | 0.03 | 0.86 | 0.05 | 0.23 | 0.58 | 0.42 |
| Calcium (mmol/L) | 2.43 | 2.45 | 2.44 | 2.41 | 0.06 | 2.46 | 2.41 | 0.03 | 0.87 | 0.17 | 0.08 | 0.97 | 0.54 | 0.54 |
| Ceruloplasmin (μmol/L) | 1.73 | 1.86 | 1.80 | 2.13 | 0.17 | 1.89 | 1.87 | 0.10 | 0.09 | 0.80 | 0.10 | 0.27 | 0.17 | 0.15 |
| GOT (U/L) | 78.2 | 84.7 | 85.0 | 90.1 | 8.56 | 78.5 | 90.5 | 4.87 | 0.21 | 0.02 | <0.01 | 0.07 | 0.20 | 0.03 |
| Haptoglobin (g/L) | 0.48 | 0.48 | 0.44 | 0.59 | 0.12 | 0.54 | 0.45 | 0.07 | 0.75 | 0.23 | <0.01 | 0.90 | 0.15 | 0.53 |
| FRAP (μmol/L) | 161 | 166 | 148 | 128 | 23.4 | 148 | 154 | 13.3 | 0.30 | 0.66 | 0.03 | 0.26 | 0.83 | 0.11 |
| Paraoxonase (U/mL) | 69.5 | 75.3 | 67.2 | 67.8 | 8.19 | 72.6 | 67.3 | 4.67 | 0.23 | 0.28 | <0.01 | 0.54 | 0.56 | 0.37 |
| MPO (U/L) | 442 | 434 | 413 | 458 | 27.3 | 441 | 434 | 15.6 | 0.30 | 0.68 | <0.01 | 0.10 | 0.25 | 0.11 |
| ROM (mg $H_2O_2$/100 mL) | 13.6 | 13.7 | 13.4 | 14.8 | 1.30 | 13.9 | 13.8 | 0.74 | 0.81 | 0.97 | 0.03 | 0.23 | 0.25 | 0.22 |
| Vitamin A (mg/100 mL) | 33.2 | 34.6 | 31.6 | 29.6 | 4.10 | 31.4 | 33.1 | 2.33 | 0.47 | 0.51 | <0.01 | 0.80 | 0.57 | 0.36 |
| Vitamin E (mg/mL) | 2.42 | 2.48 | 2.60 | 1.74 | 0.45 | 2.07 | 2.55 | 0.26 | 0.40 | 0.08 | <0.01 | 0.75 | 0.65 | 0.68 |
| β-carotene (mg/100 mL) | 0.57 | 0.55 | 0.56 | 0.46 | 0.10 | 0.52 | 0.55 | 0.06 | 0.77 | 0.56 | <0.01 | 0.08 | 0.68 | 0.72 |
| LAI all | −0.11 | 0.02 | −0.08 | −0.10 | 0.29 | −0.20 | 0.06 | 0.16 | 0.75 | 0.13 | | 0.93 | | |

[a,b]For each variable, a significant difference between clusters of EB time profile is shown by letters; $P < 0.05$.

[1]Samples were obtained in wk 1, 2, and 4 after calving and categorized according to the cluster of time series of EB in the first 6 wk after calving.

[2]Cluster of time series of EB: SP = stable positive cow cluster; MN = mild negative cow cluster; IN = intermediate negative cow cluster, SN = severe negative cow cluster.

[3]GOT = glutamic oxaloacetic transaminase, FRAP = ferric-reducing antioxidant power, MPO = myeloperoxidase, ROM = reactive oxygen metabolites, LAI = liver activity index.

[4]Par = parity.

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**Table 5.** The number of disease treatments by week for cows during the first 6 wk after calving

| Group and disease[1] | Wk 1 | Wk 2 | Wk 3 | Wk 4 | Wk 5 | Wk 6 | Total |
|---|---|---|---|---|---|---|---|
| IHP | | | | | | | |
| Fever | 0 | 1 | 0 | 0 | 1 | 0 | 2 |
| Clinical mastitis | 1 | 1 | 0 | 0 | 1 | 0 | 3 |
| Retained placenta | 9 | 1 | 0 | 0 | 0 | 0 | 10 |
| Vaginal discharge | 1 | 1 | 8 | 20 | 7 | 1 | 38 |
| Acute endometritis | 5 | 1 | 2 | 3 | 2 | 3 | 16 |
| OHP | | | | | | | |
| Milk fever | 9 | 0 | 0 | 0 | 0 | 0 | 9 |
| Cystic ovaries | 0 | 0 | 0 | 0 | 0 | 5 | 5 |
| Claw and leg problems | 0 | 0 | 0 | 0 | 1 | 2 | 3 |
| Rumen and intestine problems[2] | 0 | 0 | 3 | 0 | 0 | 0 | 3 |
| Other | 1 | 2 | 2 | 4 | 2 | 0 | 11 |
| Total | 26 | 7 | 15 | 27 | 14 | 11 | 100 |

[1]IHP = inflammatory health problem, OHP = other health problem.
[2]Treated rumen and intestine problems: rotavirus, diarrhea, peritonitis.

because the better EB in this group was due to a lower milk yield, which could be due to lower genetic merit of animals or health problems that reduced the milk yield in this cluster or both.

Within cows with IHP, 23% (16/69) of the health problem treatments happened in wk 1 after calving. Cows with IHP had lower NEFA and higher insulin concentrations in plasma in the first 6 wk after calving, compared with OHP cows. These alterations in metabolic status for IHP cows might be related to the lower milk yield ($P = 0.08$) in this group. Also, earlier studies reported that an inflammatory status, indicated by a low LAI in the first month after calving, resulted in milk yield losses (Bertoni et al., 2008). Indeed cows with IHP had a lower LAI ($P < 0.01$) compared with OHP or NHP cows and a greater plasma haptoglobin and lower plasma albumin concentration compared with NHP cows. Herewith, it can be hypothesized that the inflammatory disease in the IHP group resulted in a lower milk yield for this group.

**Table 6.** Distribution of cows among health status groups and clusters of time profiles of EB

| | Health status[2] | | | |
|---|---|---|---|---|
| Energy balance cluster[1] | IHP | OHP | NHP | Total |
| SP | 12 | 1 | 30 | 43 |
| MN | 19 | 6 | 31 | 56 |
| IN | 13 | 6 | 17 | 36 |
| SN | 5 | 3 | 10 | 18 |
| Total | 49 | 16 | 88 | 153 |

[1]SP = stable positive EB, MN = mild negative EB, IN = intermediate negative EB, and SN = severe negative EB.
[2]IHP = cows with treatment for inflammatory health problem, OHP = cows with no treatment for IHP, but treatment for other health problem; NHP = cows with no treatment for a health problem, in the first 6 wk after calving.

In the current study, cows with IHP had a lower concentration of albumin in plasma compared with OHP and NHP cows. Our findings are in line with those of Tóthová et al. (2017), who reported that cows with postpartum metritis, as well as those with mastitis, had lower plasma albumin concentrations compared with healthy cows. Cows with retained placenta and metritis had lower concentrations of plasma albumin compared with healthy cows (Green et al., 2009; Burke et al., 2010). This general decrease in albumin concentrations in sick animals may be attributed to the role of albumin as a negative APP (Gruys et al., 1994).

In contrast to albumin, cows with IHP had higher concentrations of haptoglobin in plasma compared with OHP and NHP cows. Serum haptoglobin has been stated as a positive APP that can distinguish diseased animals from healthy animals (Eckersall and Bell, 2010) and is especially effective in the diagnosis and prognosis of mastitis, enteritis, and endometritis (Petersen et al., 2004). Additionally, it has been reported that healthy cows have plasma haptoglobin concentrations <20 mg/L, which can increase to >2 g/L within 2 d after the occurrence of an infection (Eckersall and Bell, 2010). One recent study (Qu et al., 2014), in which 161 transition cows were monitored, found that the haptoglobin was elevated in serum around parturition, even in cows that appeared to be healthy, but cows that experienced diseases or calving difficulties had significantly greater concentrations compared with healthy animals. The concentration of haptoglobin in healthy cows in the current study, however, was much higher than 20 mg/L. In contrast to the cows in the study of Eckersall and Bell (2010) that were not undergoing any known inflammatory processes, the early lactation stage may explain the higher concentrations found in our current study. Also, the differences in the laboratory analysis methods or statistical methods may

**Table 7.** Milk yield, DMI, plasma metabolites (NEFA, BHB, and glucose), metabolic hormones (insulin and IGF-1), and EB of 153 dairy cows in wk 1 to 6 after calving categorized according to the health status in the first 6 wk after calving

| | Health status[1] | | | | Parity | | | *P*-value[2] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Item[3] | IHP | OHP | NHP | SEM | 1 | ≥2 | SEM | HS | Par | Week | HS × Par | HS × week | Par × week |
| Cows, n | 49 | 16 | 88 | | 40 | 113 | | | | | | | |
| Week 1–6 | | | | | | | | | | | | | |
| Milk yield (kg/d) | 29.9 | 32.2 | 34.1 | 1.00 | 25.8 | 38.4 | 0.63 | 0.08 | <0.01 | <0.01 | 0.42 | 0.31 | <0.01 |
| DIM (kg/d) | 17.0 | 17.4 | 17.2 | 0.38 | 14.4 | 20.1 | 0.22 | 0.61 | <0.01 | <0.01 | 0.25 | 0.82 | <0.01 |
| NEFA (mmol/L) | 0.29[b] | 0.41[a] | 0.28[b] | 0.02 | 0.32 | 0.34 | 0.01 | 0.03 | 0.62 | <0.01 | 0.15 | 0.08 | 0.87 |
| BHB (mmol/L) | 0.70 | 0.92 | 0.74 | 0.05 | 0.80 | 0.77 | 0.07 | 0.15 | 0.65 | <0.01 | 0.31 | 0.20 | 0.03 |
| Glucose (mmol/L) | 3.40 | 3.16 | 3.38 | 0.05 | 3.44 | 3.18 | 0.03 | 0.11 | <0.01 | <0.01 | 0.86 | 0.01 | 0.45 |
| Insulin (μU/mL) | 13.3[a] | 9.34[b] | 12.7[ab] | 1.66 | 11.8 | 11.8 | 0.42 | 0.04 | 0.96 | <0.01 | 0.49 | 0.11 | 0.24 |
| IGF-1 (ng/mL) | 113 | 98 | 117 | 6.65 | 130 | 88.5 | 4.25 | 0.40 | <0.01 | <0.01 | 0.22 | 0.19 | 0.06 |
| EB (kJ/kg$^{0.75}$ per day) | −154 | −256 | −171 | 25.3 | −149 | −238 | 16.1 | 0.24 | 0.03 | <0.01 | 0.49 | 0.82 | 0.79 |

[a,b]For each variable, a significant difference between groups of health status is shown by letters; $P < 0.05$.
[1]IHP = cows with treatment for inflammatory health problem, OHP = cows with no treatment for IHP, but treatment for other health problem, NHP = cows with no treatment for a health problem, in the first 6 weeks after calving.
[2]HS = health status; Par = parity.
[3]NEFA = nonesterified fatty acids, IGF-1 = insulin-like growth factor 1, EB = energy balance.

lead to the different concentrations of the haptoglobin in different studies.

The substantially reduced LAI in cows with IHP in the current study was possibly due to the lower plasma concentration of albumin and higher concentration of haptoglobin compared with OHP and NHP cows. A low LAI in plasma was found to be related to a high frequency of inflammatory conditions and serious clinical health problems (Trevisi et al., 2008). Low levels of LAI are related to metabolic and infectious diseases, and cows with low LAI should be closely monitored so that diseases can be identified at an early stage (Trevisi et al., 2010). No

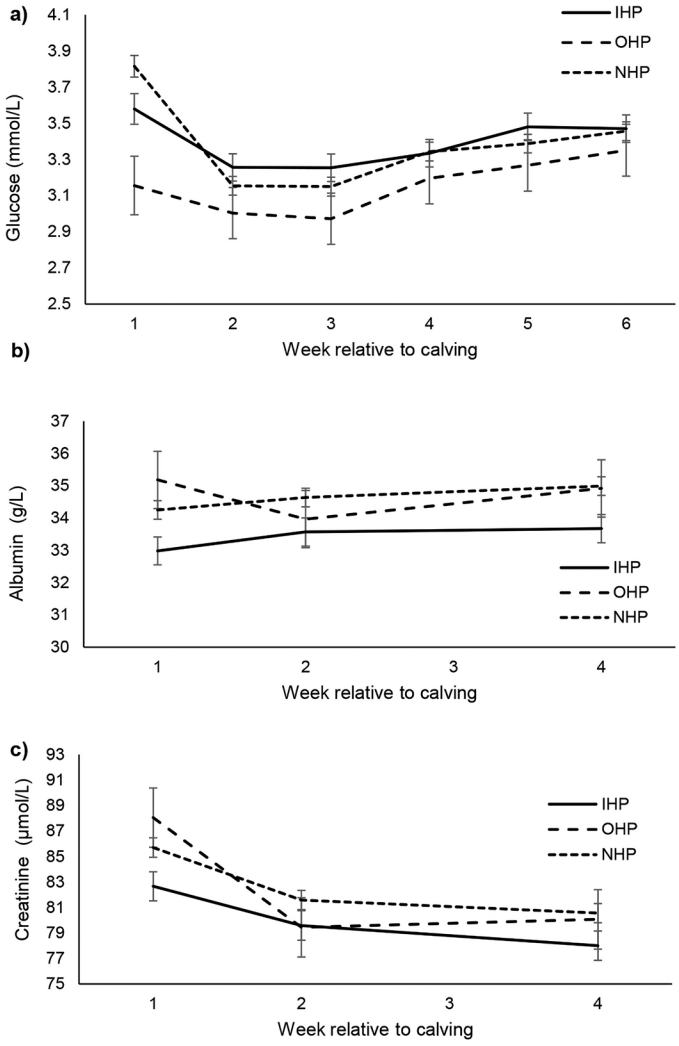


**Figure 3.** Plasma glucose (a), albumin (b), and creatinine (c) concentration of cows in different health status groups (IHP, OHP, and NHP) within the first 6 wk after calving. IHP = cows with treatment for inflammatory health problem; OHP = cows with no treatment for IHP, but treatment for other health problem; NHP = cows with no treatment for a health problem, in the first 6 weeks after calving. Values represent LSM ± SEM.

**Table 8.** Inflammatory biomarkers (albumin, cholesterol, total protein, globulin, ceruloplasmin, and haptoglobin), index of liver function (paraoxonase) and oxidative stress markers (GOT, MPO, ROM, FRAP, creatinine, urea, calcium, vitamin A, vitamin E, β-carotene and LAI) in plasma of 153 dairy cows in wk 1, 2, and 4 after calving categorized according to the health status in first 6 wk after calving

| | Health status[1] | | | | Parity | | | *P*-value[2] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Item[3] | IHP | OHP | NHP | SEM | 1 | ≥2 | SEM | HS | Par | Week | HS × Par | HS × week | Par × week |
| Cows, n | 49 | 16 | 88 | | 40 | 113 | | | | | | | |
| Weeks 1, 2, and 4 | | | | | | | | | | | | | |
| Albumin (g/L) | 33.4[a] | 34.7[b] | 34.6[b] | 0.60 | 32.7 | 33.5 | 0.45 | 0.03 | <0.01 | 0.14 | 0.69 | 0.03 | 0.96 |
| Cholesterol (mmol/L) | 3.23 | 3.06 | 3.48 | 0.27 | 3.80 | 2.74 | 0.09 | 0.17 | 0.07 | <0.01 | 0.78 | 0.19 | 0.12 |
| Creatinine (μmol/L) | 80.1 | 82.5 | 82.6 | 0.64 | 81.1 | 82.3 | 1.00 | 0.10 | 0.49 | <0.01 | 0.35 | 0.02 | 0.49 |
| Total protein (g/L) | 74.2 | 72.1 | 73.9 | 0.88 | 70.8 | 76.0 | 0.98 | 0.62 | <0.01 | <0.01 | 0.14 | 0.29 | 0.23 |
| Globulin (g/L) | 40.8 | 37.5 | 39.3 | 0.91 | 37.3 | 41.1 | 0.58 | 0.21 | 0.01 | <0.01 | 0.13 | 0.57 | 0.09 |
| Urea (mmol/L) | 3.40 | 3.90 | 3.64 | 0.10 | 3.71 | 3.58 | 0.07 | 0.06 | 0.44 | 0.56 | 0.12 | 0.14 | 0.97 |
| Calcium (mmol/L) | 2.43 | 2.44 | 2.46 | 0.02 | 2.46 | 2.43 | 0.03 | 0.61 | 0.36 | 0.64 | 0.78 | 0.39 | 0.70 |
| Ceruloplasmin (μmol/L) | 1.89 | 1.82 | 1.77 | 0.07 | 1.81 | 1.85 | 0.07 | 0.31 | 0.71 | 0.80 | 0.77 | 0.51 | 0.12 |
| GOT (U/L) | 78.0 | 85.5 | 82.2 | 3.27 | 82.2 | 82.9 | 2.08 | 0.73 | 0.89 | <0.01 | 0.45 | 0.36 | 0.01 |
| Haptoglobin (g/L) | 0.52[b] | 0.34[ab] | 0.35[a] | 0.04 | 0.41 | 0.40 | 0.04 | <0.01 | 0.91 | <0.01 | 0.73 | 0.73 | 0.14 |
| FRAP (μmol/L) | 155 | 154 | 154 | 3.13 | 155 | 154 | 1.98 | 0.99 | 0.95 | 0.18 | 0.76 | 0.47 | 0.44 |
| Paraoxonase (U/mL) | 71.5 | 70.8 | 76.3 | 3.21 | 77.2 | 68.5 | 2.02 | 0.39 | 0.09 | 0.43 | 0.97 | 0.39 | 0.02 |
| MPO (U/L) | 434 | 401 | 424 | 10.9 | 421 | 418 | 6.84 | 0.42 | 0.87 | 0.12 | 0.78 | 0.16 | 0.01 |
| ROM (mg $H_2O_2$/100mL) | 14.2 | 12.6 | 13.4 | 0.35 | 13.4 | 13.4 | 0.35 | 0.34 | 0.94 | 0.50 | 0.76 | 0.13 | 0.07 |
| Vitamin A (mg/100mL) | 34.8 | 37.0 | 36.1 | 1.59 | 37.0 | 35.0 | 1.01 | 0.74 | 0.43 | <0.01 | 0.51 | 0.92 | 0.95 |
| Vitamin E (mg/mL) | 2.57 | 2.83 | 2.80 | 0.19 | 2.46 | 3.01 | 0.12 | 0.58 | 0.08 | <0.01 | 0.63 | 0.73 | 0.58 |
| β-carotene (mg/100mL) | 0.58 | 0.64 | 0.60 | 0.04 | 0.58 | 0.63 | 0.02 | 0.80 | 0.37 | <0.01 | 0.71 | 0.35 | 0.35 |
| LAI all | −0.36[a] | −0.05[b] | 0.04[b] | 0.30 | −0.44 | −0.06 | 0.19 | <0.01 | <0.01 | | 0.71 | | |

[a,b]For each variable, a significant difference between groups of health status is shown by letters; $P < 0.05$.

[1]IHP = cows with treatment for inflammatory health problem, OHP = cows with no treatment for IHP, but treatment for other health problem, NHP = cows with no treatment for a health problem, in the first 6 weeks after calving.

[2]HS = health status; Par = parity.

[3]GOT = glutamic oxaloacetic transaminase, FRAP = ferric-reducing antioxidant power, MPO = myeloperoxidase, ROM = reactive oxygen metabolites, LAI = liver activity index.

effects of health status on oxidative stress variables were found in this study.

The definition of health status categories in this study was based on our earlier study (Mayasari et al., 2017) and are grounded in several earlier studies (e.g., Trevisi et al., 2008; 2010). All these studies specifically focus on diseases with an inflammatory character, and the category named "inflammatory health problems" includes specifically inflammation-related diseases. This makes sense from the perspective of the focus of these studies on inflammatory status. It can be argued, however, that also diseases with a noninflammatory character can be clinical, for example milk fever, and can be also supported by inflammatory status (Horst et al., 2021). Therefore, alternative definitions of health status categories could be considered, possibly resulting in different associations between health status and EB or inflammatory or oxidative stress variables.

The current study presents a clustering approach based on time profiles of EB in relation to metabolic, inflammatory, and oxidative stress variables. The relationships of EB clusters with disease events found in our current study were limited. Partly, this may have been due to the limited animal numbers in some of the parity class × EB clusters. Moreover, timing of disease in relation to start of lactation and dynamics of the EB profile might be key in the presence or absence of a relationship between EB cluster and metabolic, inflammatory and oxidative stress status. Also the timing of disease might determine a possible predictive value of EB time profiles for disease. In the current study, the relationship between EB time profiles during the first 6 wk of lactation and disease treatments within the same relatively limited period was explored. This limitation may affect the practical applicability of the findings. Further exploration of time profiles of EB or plasma variables pre- and postdisease could be an interesting focus to explore the value of time profile clustering. Adding data, including plasma or milk metabolomics or sensor data, to the cluster definition could increase the value of (noninvasive) measures to characterize the dynamics of energy or metabolic status of cows in the peripartum period.

## CONCLUSIONS

In the current study, we confirmed the approach that dairy cows can be clustered based on time profiles of EB in the first 6 wk of lactation. Moreover, EB clustering was related to milk yield level and metabolic status, but was limitedly related to inflammatory and oxidative stress status. Health status groups were related to metabolic and inflammatory variables, including NEFA, insulin, albumin, creatinine, haptoglobin, and LAI, but not to oxidative stress variables. To characterize the physiological functioning and health of cows in early lactation, it can be concluded that EB alone is not a sufficient marker. Health problems in early lactation are also associated with metabolic and inflammatory variables.

## NOTES

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**Nonstandard abbreviations used:** APP = acute phase proteins; EB = energy balance; FPCM = fat- and protein-corrected milk; FRAP = ferric-reducing antioxidant power; GOT = glutamic oxaloacetic transaminase; IHP = cows with treatment for an inflammatory health problem; IN = intermediate negative cow cluster; LAI = liver activity index; MN = mild negative cow cluster; MPO = myeloperoxidase; NE = net energy; NEB = negative energy balance; NEFA = nonesterified fatty acids; NHP = cows with no treatment for a health problem; OHP = cows with no treatment for IHO but with treatment for other health problem(s); Par = parity; PMR = partial mixed ration; ROM = reactive oxygen metabolites; SN = severe negative cow cluster; SP = stable positive cow cluster; VWP = voluntary waiting period.

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## APPENDIX

**Table A1.** Milk yield, DMI, and plasma metabolites (NEFA and BHB) of 153 dairy cows in wk 1 to 6 after calving where cows were categorized according to the cluster of time series of EB during the first 6 wk after calving[1]

| | Energy balance cluster[2] | | | |
|---|---|---|---|---|
| Item[3] | SP | MN | IN | SN |
| Cows, n | 43 | 56 | 36 | 18 |
| Milk yield (kg/d) | | | | |
| wk 1 | 16.2 ± 0.7[a] | 20.1 ± 0.7[b] | 24.2 ± 1.1[c] | 24.5 ± 2.0[bc] |
| wk 2 | 24.5 ± 0.7[a] | 30.5 ± 0.7[b] | 34.0 ± 1.1[b] | 35.3 ± 2.0[b] |
| wk 3 | 27.3 ± 0.7[a] | 32.4 ± 0.7[b] | 36.8 ± 1.1[c] | 38.3 ± 2.0[bc] |
| wk 4 | 29.3 ± 0.7[a] | 35.0 ± 0.7[b] | 39.5 ± 1.1[c] | 42.1 ± 2.0[c] |
| wk 5 | 30.5 ± 0.7[a] | 36.2 ± 0.7[b] | 40.7 ± 1.1[c] | 43.4 ± 2.0[c] |
| wk 6 | 31.1 ± 0.7[a] | 36.8 ± 0.7[b] | 41.2 ± 1.1[c] | 44.2 ± 2.0[c] |
| DMI (kg/d) | | | | |
| wk 1 | 12.6 ± 0.4[a] | 11.4 ± 0.4[ab] | 11.6 ± 0.6[ab] | 7.7 ± 1.1[b] |
| wk 2 | 15.4 ± 0.4 | 15.4 ± 0.4 | 15.4 ± 0.6 | 13.7 ± 1.0 |
| wk 3 | 17.6 ± 0.4 | 17.5 ± 0.4 | 17.7 ± 0.6 | 16.8 ± 1.0 |
| wk 4 | 19.2 ± 0.4 | 19.2 ± 0.4 | 19.3 ± 0.6 | 18.5 ± 1.0 |
| wk 5 | 19.8 ± 0.4 | 20.0 ± 0.4 | 20.0 ± 0.6 | 19.3 ± 1.0 |
| wk 6 | 20.2 ± 0.4 | 20.5 ± 0.4 | 20.6 ± 0.6 | 20.3 ± 1.0 |
| NEFA (mmol/L) | | | | |
| wk 1 | 0.33 ± 0.03[a] | 0.57 ± 0.03[b] | 0.68 ± 0.04[b] | 0.74 ± 0.06[b] |
| wk 2 | 0.26 ± 0.03[a] | 0.42 ± 0.03[b] | 0.47 ± 0.04[bc] | 0.65 ± 0.06[c] |
| wk 3 | 0.17 ± 0.03[a] | 0.30 ± 0.03[b] | 0.34 ± 0.04[b] | 0.60 ± 0.06[c] |
| wk 4 | 0.13 ± 0.03[a] | 0.21 ± 0.03[ab] | 0.30 ± 0.04[bc] | 0.42 ± 0.06[c] |
| wk 5 | 0.14 ± 0.03[a] | 0.18 ± 0.03[ab] | 0.23 ± 0.04[ab] | 0.37 ± 0.06[b] |
| wk 6 | 0.11 ± 0.03 | 0.16 ± 0.03 | 0.16 ± 0.04 | 0.30 ± 0.06 |
| BHB (mmol/L) | | | | |
| wk 1 | 0.54 ± 0.07[a] | 0.68 ± 0.07[ab] | 0.66 ± 0.10[ab] | 1.11 ± 0.15[b] |
| wk 2 | 0.65 ± 0.06[a] | 0.82 ± 0.06[a] | 0.83 ± 0.09[a] | 1.38 ± 0.15[b] |
| wk 3 | 0.64 ± 0.06[a] | 1.01 ± 0.06[a] | 0.78 ± 0.09[ab] | 1.57 ± 0.15[b] |
| wk 4 | 0.65 ± 0.06[a] | 0.83 ± 0.06[ab] | 0.74 ± 0.09[ab] | 1.19 ± 0.15[b] |
| wk 5 | 0.66 ± 0.06 | 0.76 ± 0.06 | 0.68 ± 0.09 | 0.94 ± 0.15[b] |
| wk 6 | 0.61 ± 0.06 | 0.66 ± 0.06 | 0.68 ± 0.09 | 0.85 ± 0.15[b] |

[a–c]For each variable per week, a significant difference ($P < 0.05$) between clusters of EB time profile is shown by letters.

[1]Presented variables have an interaction between EB cluster and week after calving. Values are LSM ± SEM

[2]Cluster of time series of EB: SP = stable positive cow cluster; MN = mild negative cow cluster; IN = intermediate negative cow cluster, SN = severe negative cow cluster.

[3]NEFA = nonesterified fatty acids.

**Table A2.** Glucose, albumin, and creatinine in plasma of 153 dairy cows in wk 1 to 6 after calving categorized according to the HS in the first 6 wk after calving[1]

| | Health status[2] | | |
|---|---|---|---|
| Item | IHP | OHP | NHP |
| Cows, n | 49 | 16 | 88 |
| Glucose (mmol/L) | | | |
| wk 1 | 3.58 ± 0.08[ab] | 3.16 ± 0.16[a] | 3.82 ± 0.06[b] |
| wk 2 | 3.26 ± 0.08 | 3.00 ± 0.14 | 3.15 ± 0.05 |
| wk 3 | 3.25 ± 0.08 | 2.97 ± 0.14 | 3.15 ± 0.05 |
| wk 4 | 3.33 ± 0.08 | 3.20 ± 0.14 | 3.34 ± 0.05 |
| wk 5 | 3.48 ± 0.08 | 3.27 ± 0.14 | 3.39 ± 0.05 |
| wk 6 | 3.47 ± 0.08 | 3.35 ± 0.14 | 3.46 ± 0.05 |
| Albumin (g/L) | | | |
| wk 1 | 33.0 ± 0.4 | 35.2 ± 0.9 | 34.2 ± 0.3 |
| wk 2 | 33.6 ± 0.4 | 34.0 ± 0.9 | 34.6 ± 0.3 |
| wk 4 | 33.7 ± 0.4 | 34.9 ± 0.9 | 35.0 ± 0.3 |
| Creatinine (μmol/L) | | | |
| wk 1 | 82.7 ± 1.1 | 88.1 ± 2.3 | 85.7 ± 0.8 |
| wk 2 | 79.6 ± 1.2 | 79.4 ± 2.3 | 81.6 ± 0.8 |
| wk 4 | 78.0 ± 1.1 | 80.1 ± 2.3 | 80.6 ± 0.8 |

[a,b]For each variable per week, a significant difference ($P < 0.05$) between groups of health status is shown by letters.

[1]Presented variables have an interaction between HS group and week after calving. Values are LSM ± SEM.

[2]IHP = inflammatory health problem; OHP = other health problem; NHP = no health problem in the first 6 wk after calving.