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Phosphorus content of muscle tissue and muscle function in dairy cows fed a phosphorus-deficient diet during the transition period

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ABSTRACT

Phosphorus (P) deficiency and hypophosphatemia are believed to be associated with muscle function disturbances in dairy cows, particularly around parturition. The objective of this study was to determine the effect of dietary P deprivation during late gestation and early lactation on muscle P homeostasis and muscle function in periparturient dairy cows. Thirty-six multiparous dairy cows in late gestation were randomly assigned either to undergo dietary P depletion or to be offered a diet with adequate P content from 4 wk before to 4 wk after parturition. Phosphorus-deficient rations for dry and lactating cows contained 0.15 and 0.20% P on a dry matter basis, respectively. Blood and muscle tissue for biopsy were obtained and electromyographic examinations were conducted on biceps femoris and intercostal muscles in regular intervals throughout the study. Muscle tissue was analyzed for the total P, adenosine triphosphate, adenosine diphosphate, adenosine monophosphate, creatine phosphate, and tissue water content. Dietary P deprivation resulted in a pronounced and sustained decline of the plasma phosphate concentration, reaching a nadir at calving with mean values below 1.5 mg/dL and remaining below 2.0 mg/dL during the first 4 wk of lactation. Hypophosphatemia was not associated with signs of clinically apparent muscle weakness or disturbed muscle function and was not associated with a decline in the content of any of the studied P-containing compounds in muscle tissue. Accordingly, no association between plasma phosphate concentration and muscle tissue P content was found. Electromyographic examination identified subclinical effects on motor unit action potentials that are indicative of disturbed neuromuscular functionality. Increasing occurrence of pathologic spontaneous activity possibly resulting from membrane instability of nerve or muscle cells and suggestive of myopathy was also recorded as P deprivation progressed. These effects were predominantly observed in intercostal and to a lesser degree biceps femoris muscles. Electromyographic parameters affected by P deprivation were found to be associated primarily with the plasma phosphate and to a lesser extent with the amounts of energy storing P-containing compounds contained in muscle tissue. These results indicate that prolonged and pronounced dietary P deprivation in transition dairy cows leads to marked sustained hypophosphatemia without altering the muscle tissue P homeostasis or causing clinically apparent muscle function disturbances.
Key words: hypophosphatemia, electromyography, muscle biopsy, muscle weakness, neuromuscular disorder

INTRODUCTION

Hypophosphatemia is commonly encountered in dairy cows during the periparturient period and in early lactation (Macrae et al., 2006, 2012). Although subnormal plasma inorganic phosphorus (P) concentrations (**[Pi]**) have been reported in at least 50% of clinically healthy dairy cows around parturition, hypophosphatemia is also diagnosed in the majority of recumbent fresh cows, frequently in association with subnormal plasma calcium concentrations (**[Ca]**; Grünberg, 2014). Furthermore, hypophosphatemia was found to be more pronounced in recumbent dairy cattle not responding to intravenous calcium administration compared with recumbent cows making a full recovery after calcium supplementation (Ménard and Thompson, 2007). However, the proposed association between hypophosphatemia and recumbency in cattle is solely based on empirical evidence and has been discussed controversially for decades. Attempts to experimentally induce hypophosphatemic recumbency in cattle have been unsuccessful thus far, and reports about treatment efficacy of oral or parenteral P supplementation in cattle with presumed

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hypophosphatemic recumbency are inconsistent at best (Rodehutscord et al., 1994; Grünberg, 2014; Puggaard et al., 2014; Grünberg et al., 2015b). A potential mechanism through which P depletion or hypophosphatemia may result in disturbed muscle function has not been unraveled, but depletion of energy stores in muscle tissue in states of P deficiency has been proposed in combination with membrane instability (Grünberg, 2014; Grünberg et al., 2015b). Muscle fibers depend on the availability of creatine phosphate (**CPh**) and adenosine phosphates as a source of energy, and it is thought that hypophosphatemia is indicative of P deficiency and thus is accompanied by depletion of intracellular P stores.

Hypophosphatemia in early-lactating dairy cows is receiving increased attention because of recent incentives in many parts of the world aiming for the reduction of the P content in dairy cow rations, with the objective of containing environmental pollution with P of fecal origin (Goff, 2018). Producers and veterinarians raised concerns that this practice may exacerbate disturbances of the P balance occurring in periparturient cows and thereby jeopardize health and productivity of the fresh dairy cow.

The objective of this study was to determine the effects of pronounced dietary P deprivation during the transition period on muscle tissue P homeostasis and muscle function at a clinical and subclinical level. Based on the results of earlier studies conducted on nonperiparturient cattle, we hypothesized that dietary P deprivation would result in pronounced hypophosphatemia that is not associated with clinically apparent muscle function disturbances or alterations of the muscle tissue P balance.

MATERIALS AND METHODS

Ethics Statement

The national and institutional guidelines for the care and use of experimental animals were followed and all experimental procedures were approved by the Utrecht University Institutional Animal Care and Use Committee (permit no. AVD108002016616). This study is part of a multi-institutional project evaluating the effect of dietary P deprivation in dairy cows. To further understand the molecular mechanisms through which P depletion may affect various organ systems of the dairy cow at different stages of the lactation cycle, other laboratories are currently conducting studies on liver and immune cell function from these transition cows. The present study focuses on the effect of dietary P deprivation on muscle function in the periparturient period.

Animals and Housing

A total of 36 healthy, multiparous, pregnant Holstein-Friesian or Holstein-Friesian crossbreed dairy cows in late gestation were used for this study. Cows were purchased from commercial dairy farms in the Netherlands at least 1 mo before enrollment. For logistical reasons, the study was conducted in 2 consecutive replicates consisting of 18 cows each. Cows of each replicate were required to have been artificially inseminated within a range of 5 d to narrow the calving period of each replicate as much as possible. Only cows found to be healthy based on physical, hematological, and blood biochemical examination were included in the study.

Cows were housed in individual tiestalls with rubber bedding covered with sawdust in a temperature-controlled facility. Study animals were dried off at least 8 wk before the expected calving date (i.e., were dry for at least 2 wk before enrollment).

Study Design and Experimental Rations

The study was conceived as a randomized block design. Study animals of each replicate were blocked by lactation number and 305-d milk yield of the previous lactation and were then, within block, randomly assigned to a treatment with either deficient (**LP**) or adequate (**AP**) dietary P supply by drawing cards. The study period covered a minimum of 11 wk starting 6 wk before the expected calving date (Figure 1). The study was initiated with an acclimation period of 2 wk for both treatments that was followed by a period of deficient dietary P supply for LP cows and adequate dietary P supply for AP cows, extending from 4 wk before the expected week of calving to 4 wk after the expected week of calving (Figure 1). The LP cows of the first replicate underwent an additional 2-wk P repletion period immediately following the P-depletion period, whereas the AP cows of the first replicate were kept on the diet with adequate P supply. During acclimation both treatments received the same dry cow ration with adequate P content. During the P-repletion period of the first replicate, LP cows were switched to the AP diet and thus both groups were fed the same lactating cow ration. The objective of the repletion period was to determine whether effects that may have resulted from dietary P depletion in LP animals were reversed by dietary P supplementation over 2 wk.

Feed was offered as a balanced TMR for dry or lactating cows depending on the stage of the study. The same base ration formulated to meet current recommendations for dairy cows, with exception of the dietary P content, was prepared for both groups (NRC, 2001;

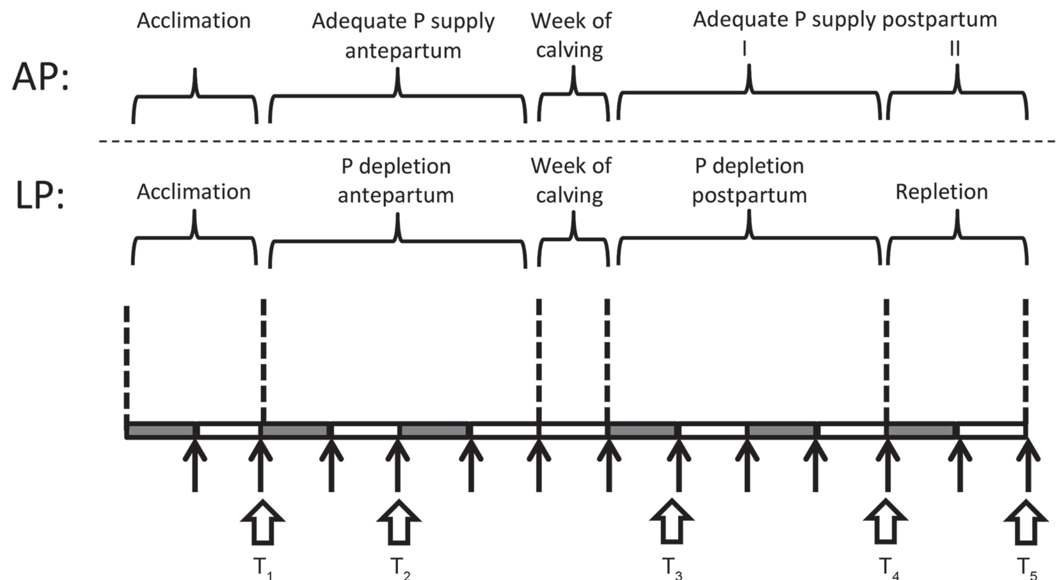

Figure 1. Experimental timeline for adequate-phosphorus (AP) and low-phosphorus (LP) treatments. Each shaded and open rectangle represents 1 wk of time. Open arrows represent the sampling times T_1 (end of acclimation period), T_2 (2 wk after onset of P depletion), T_3 (within 1 wk of parturition), T_4 (end of P depletion), and T_5 (end of P repletion), at which electromyograms were conducted and muscle biopsies were obtained. Small arrows represent the blood sampling time points. Only cows of replicate 1 underwent the repletion period following phosphorus depletion. During acclimation, cows in both treatments received the same dry cow ration with adequate P supply. During repletion and adequate P supply postpartum, both groups received the same lactating cow ration with adequate P supply; repletion and adequate P supply postpartum II applied only to the first replicate of the study.

Table 1). This P-deficient base ration was fed to LP cows, whereas AP cows were offered this ration supplemented with NaH_2PO_4 to obtain a dietary P content meeting or slightly exceeding current recommendations for dairy cows (NRC, 2001). The P content of dry and lactating cow LP rations was 0.15 and 0.20% P on a DM basis, respectively. Dry cow and lactating cow rations of the AP treatment had a dietary P content of 0.28 and 0.44% P in DM, respectively (Table 1). The dietary P content of the LP rations fed during the depletion period was the lowest of an otherwise balanced ration we were able to achieve using standard ration ingredients. The dietary P content of the lactating cow AP ration slightly exceeded current recommendations for dietary P supply but met the P content commonly fed to high-yielding dairy cows in practice (NRC, 2001). Orts were weighed back, and fresh feed was offered twice daily between 0600 and 0700 h and between 1800 and 1900 h. Access to feed was restricted to 12.5 kg of DM/d for dry cows and was ad libitum for lactating cows, and cows had ad libitum access to water. Lactating cows were milked twice daily between 0600 and 0700 h and between 1800 and 1900 h.

Sample Collection and Experimental Procedures

Animal Health, Feed Intake, Milk Production, and BW. Attitude and behavior of the study animals were monitored daily, and a complete physical examination was conducted once a week. Cows were weighed on an electronic scale at the end of the acclimation period ($\mathbf{T_1}$), after 2 wk of dietary P depletion ($\mathbf{T_2}$), during the first week of lactation ($\mathbf{T_3}$), at the end of the depletion period ($\mathbf{T_4}$), and, for cows of replicate 1, at the end of the repletion phase ($\mathbf{T_5}$; Figure 1). Milk yield and DMI were recorded daily, and the average daily DMI and milk yield of the 7 d before each of the 5 sampling times were calculated for every cow.

Blood and Feed Sample Collection. Blood samples were collected by venipuncture of a jugular vein every Friday between 0700 and 0800 h. Blood was collected into blood tubes containing lithium–heparin as anticoagulant (Vacuette, Greiner Bio-One, Kremsmünster, Austria) and was centrifuged within 30 min of collection at 1,000 × g for 15 min at 6°C. Harvested plasma was stored at −21°C until analyzed as described below.

Feed samples were collected once a week and were analyzed for their DM and P content to confirm the adequate P content of the experimental rations. Dry matter was determined by drying at 100°C to constant weight at atmospheric pressure; dietary P content was measured by inductively coupled plasma MS. The results of the P analysis of the experimental diets confirmed that the dietary P content of the dry cow LP ration was on average 0.15% P in DM (range: 0.14–0.18%).

Table 1. Ingredients (% of DM) and composition (g/kg of DM unless noted) of experimental dry cow (antepartum) and lactating cow (postpartum) rations of low-phosphorus (LP) and adequate-phosphorus (AP) treatments[1]

	Antepartum		Postpartum	
Item	AP	LP	AP	LP
Ingredient				
Corn silage	29.2	29.4	39.1	39.6
Grass seed straw[2]	25.8	26.0	12.4	12.6
Beet pulp	35.2	35.5	36.1	36.6
Soybean meal	0	0	8.7	8.8
Palatinose[3]	4.8	4.8	0	0
Urea	0.68	0.68	0.34	0.35
Coated urea	2.03	2.05	1.00	1.01
Mineral mix	1.56[4]	1.57[4]	1.10[5]	1.11[5]
NaH_2PO_4	0.67	0	1.23	0
Chemical analysis				
DM (% as fed)	60.0	59.3	58.3	58.2
NE_L (MJ/kg of DM)	5.90	5.88	6.48	6.54
DVE[6]	61.0	60.7	79.4	80.4
OEB[7] (g/d)	18.1	17.9	11.8	12.0
Starch	111	110	153	154
Ca	4.35	4.38	5.8	5.7
K	13.5	13.4	14.1	14.5
P	2.83	1.52	4.41	2.03
Mg	3.47	3.51	1.83	1.8
S	1.24	1.25	1.36	1.41
Na	4.3	1.82	3.62	1.5
DCAD (mEq/kg of DM)	109.9	105.0	294.6	290.8

[1]Cows assigned to the LP treatment received the antepartum ration of AP during acclimation and the postpartum ration of AP during the repletion period.

[2]Grass seed straw from smooth meadow grass (*Poa pratensis*) known for its quality and palatability, similar to common grass hay.

[3]Palatinose was used as a substitute for molasses to stimulate rumen fermentation but without increasing the dietary K content.

[4]Mineral mix contained Ca (0 g/kg), Mg (180 g/kg), Na (100 g/kg), Cu (1.655 g/kg), Co (0.022 g/kg), Mn (0.9 g/kg), Zn (0.65 g/kg), I (0.01 g/kg), Se (0.015 g/kg), vitamin A (340,000 IU/kg), β-carotene (0.6 g/kg), vitamin D_3 (140,000 IU/kg), and vitamin E (8,000 IU/kg).

[5]Mineral mix contained Ca (170 g/kg), Mg (38 g/kg), Na (120 g/kg), Cu (0.73 g/kg), Co (0.009 g/kg), Mn (1.2 g/kg), Zn (1.67 g/kg), I (0.042 g/kg), Se (0.017 g/kg), vitamin A (280,000 IU/kg), β-carotene (2.27 g/kg), vitamin D_3 (90,000 IU/kg), and vitamin E (900 IU/kg).

[6]Intestinal digestible protein (Tamminga et al., 1994).

[7]Degraded protein balance (Tamminga et al., 1994).

The mean dietary P content of the LP ration for lactating cows was 0.20% P in DM (range: 0.19–0.20%). The mean P content of AP diets was 0.28% (range: 0.26–0.32%) and 0.44% (range: 0.42–0.47%) for the dry cow and lactating cow rations, respectively.

Electromyographic Examination. Cows on study underwent repeated electromyographic examination to detect pathologic spontaneous activity as an indicator of muscular dysfunction and to conduct motor unit action potential (**MUAP**) and interference pattern analyses (Finsterer et al., 1997; Rubin, 2012). Electromyographic examinations were conducted at the time points T_1, T_2, T_3, T_4, and, for cows of replicate 1, T_5. Electromyographies were always conducted and analyzed by the same investigator as described in earlier studies (Wijnberg et al., 2004; Grünberg et al., 2015b). Briefly, the electrical activity of intercostal (**IC**) and biceps femoris (**BF**) muscles was determined to evaluate pathologic spontaneous activity and to assess the properties of isolated MUAP and interference patterns (Liguori et al., 1997). Interference patterns of the IC muscle were recorded during breathing at rest and those of the BF muscle were recorded during weight bearing with an elevated contralateral limb (Nirkko et al., 1995). Electromyographies were recorded using portable equipment (Viking Quest EMG system, Nicolet Biomedical Inc., Madison, WI; Kimura, 2001; Daube and Rubin, 2009) and were made using disposable concentric 26-gauge, 50-mm and 21-gauge, 100-mm electromyography needle electrodes (MEDcat, Emmen, the Netherlands). Cows were placed in stocks in a calm environment and remained without sedation for the examination. Motor unit action po-

tentials of the IC muscle were recorded during normal breathing with the cow standing calmly in the stocks. No extra manipulation was required to induce MUAP in this muscle. Motor unit action potentials of the BF muscle were generated by inducing voluntary muscle contractions as described earlier by lifting the contralateral limb (Grünberg et al., 2015b).

Muscle Tissue Collection. Muscle tissue for biopsy of the BF muscle was obtained from all animals on the days of electromyographic examination (T_1–T_5) immediately following electromyography and from the limb opposed to the one used for the foregoing electromyography. Tissue samples were obtained under local anesthesia (procaine hydrochloride 4%, V.M.D. n.v., Arendonk, Belgium) and following administration of a nonsteroidal anti-inflammatory drug (meloxicam 0.5 mg/kg s.c.; Metacam, Boehringer Ingelheim) using a suction-modified Bergstrom biopsy needle (6-mm diameter; Walter Veterinär-Instrumente, Baruth, Germany) as described earlier in cattle (Grünberg et al., 2015b). Two specimens, each approximately 0.2 to 0.3 g, were obtained at each sampling time. Collected tissue was submerged in liquid nitrogen within 10 s of collection and stored in liquid nitrogen until processed for further analysis as described below.

Analyses

Plasma Biochemical Analysis. Plasma was analyzed for [Pi] (ammonium molybdate method); total [Ca] (Arsenazo III method); the concentrations of creatinine (**[Crea]**; enzymatic method), nonesterified fatty acids (**[NEFA]**; acetyl-CoA-synthetase–acetyl-CoA-oxydase method), BHB (**[BHB]**; UV method), and total bilirubin (dichloraniline method); and the enzyme activity of aspartate aminotransferase (**AST**; UV method without pyridoxal phosphate) and creatine kinase (**CK**; *N*-acetylcysteine-creatine phosphate method). Plasma biochemical analyses were performed by a commercial veterinary diagnostic laboratory and conducted on an automated analyzer (ABX Pentra 400; Horiba, Europe GmbH, Langenhagen, Germany).

Electromyographic Examination. Insertional activity, pathologic spontaneous activity, MUAP, satellite potentials, and interference pattern analysis were recorded and studied as described in detail for an earlier study conducted in cattle (Grünberg et al., 2015b). Briefly, the occurrence of pathologic spontaneous activity was determined outside the endplate region at the same site from where MUAP were obtained. Four types of pathologic spontaneous activity were differentiated: fibrillation potentials, positive sharp waves, complex repetitive discharges, and neuromyotonia. Pathologic spontaneous activity was counted as such when occurring in at least 2 locations within the same muscle; the incidence of pathologic spontaneous activity was categorized semiquantitatively into 4 groups from 0 (no pathologic spontaneous activity) to +++ (very frequent pathologic spontaneous activity; Georgesco and Salerno, 2000). For quantitative MUAP analysis, the amplitude, duration, number of phases, and number of turns, area under the action potential curve (referred to as "area"), and size index were calculated from at least 20 recorded MUAP per muscle (Wijnberg et al., 2002a, 2004). For interference pattern analysis of the IC muscle, a minimum of 20 contractions were evaluated by determining the maximum voluntary activity expressed as the ratio turns/second and amplitude/turns during spontaneous breathing patterns.

Muscle Tissue Biochemical Analysis. The content in muscle tissue wet weight ($_{ww}$) of adenosine triphosphate (**ATP**), adenosine diphosphate (**ADP**), adenosine monophosphate (**AMP**), creatine, and CPh were determined as described earlier (Teerlink et al., 1993; Jiang et al., 2012; Grünberg et al., 2015b). Briefly, frozen muscle tissue was homogenized in 2 mL of ice-cold 0.66 *M* perchloric acid. After centrifugation at 3,500 × *g* and 4°C for 10 min, the supernatant was transferred into a 5-mL glass tube. The residue was extracted and centrifuged again as described above. Both supernatants were mixed and the volume was topped up with perchloric acid to 5 mL. Samples were stored at −20°C until analyzed as described below. Before analysis, 0.5 mL of extract was neutralized by mixing with 0.3 mL of 2 *M* KOH, 0.1 mL of 1 *M* $KHCO_3$, and 0.1 mL of water. The sample was refrigerated for 20 min, centrifuged as described above, and finally diluted 5 times with water. The analysis of AMP_{ww}, $creatine_{ww}$, and CPh_{ww} was conducted on an HPLC-MS system consisting of 2 HPLC pumps, an autosampler with tray cooling set at 4°C (PE200 series, PESciex, Norwalk, CT), and a triple quadrupole MS detector (ABSciex, Framingham, MA) with a turbo ion spray source. The HPLC column was a hypercarb (5-μm porous graphitic carbon, 150 × 2.1 mm; Thermo Scientific, Breda, the Netherlands). Eluent 1 for pump A was 8 m*M* ammonium acetate, adjusted to pH 9.8 with 25% ammonia solution, and eluent 2 for pump B was 8 m*M* ammonium acetate, pH 9.8/acetonitrile (40/60; vol/vol). The flow rate was 200 μL/min, and a gradient run was made. The first 3 min after injection was discarded by a divert valve. The MS was operated in multiple reaction monitoring mode. Analysis of ADP_{ww} and ATP_{ww} was also done by gradient HPLC with UV detection at 259 nm (Shimadzu, 's-Hertogenbosch, the Netherlands). Eluent A was 50 m*M* KH_2PO_4 and 10

m*M* tetrabuyl ammonium hydrogen sulfate (pH 6.5), and eluent B was methanol. The column was a Luna C18 (3 μL, 150 × 2 mm; Phenomenex, Torrance, CA). The HPLC was controlled by Lab Solutions version 5.63 software (Shimadzu, Duisburg, Germany).

The P and potassium (K) concentrations in the solution containing the tissue homogenate were determined by inductively coupled optical emission spectrometry. Potassium, like P, is a predominantly intracellular electrolyte and was analyzed to assess whether changes in muscle tissue P content over time are specific to P or whether they also occur with other intracellular electrolytes. The total amounts of P, K, ATP, ADP, AMP, and CPh per gram of wet weight muscle tissue were then calculated from the concentration of each compound in the homogenate and the mass of the wet weight of each tissue specimen. The DM content of each muscle tissue specimen was determined by drying to constant weight at 85°C at atmospheric pressure.

Statistical Analysis

Data are expressed as arithmetic mean ± standard deviation or as median and interquartile range. The significance level was set at $P < 0.05$. Values were log transformed when necessary to achieve normal distribution. Repeated-measures ANOVA with an autoregressive(1) covariance matrix with animal ID as subject was used to determine effects of replicate number, block, treatment, time (sampling time or, in the case of blood biochemical parameters, week relative to calving), and the interaction between treatment and time using PROC MIXED (SAS 9.4, SAS Institute Inc., Cary, NC). Repeated-measures ANOVA on ranks was conducted with the model as described above with severity of pathologic spontaneous activity as the dependent variable. For this purpose, categories of 0 to +++ were transformed to numerical values 0 to 3. If a significant replicate number effect was determined for one parameter, it was analyzed for each replicate separately. An autoregressive(1) covariance structure was chosen based on the lowest Akaike information criterion. Bonferroni-adjusted *P*-values were used to assess differences between treatments at specific sampling times whenever the *F*-test was significant.

Forward stepwise regression analyses were conducted for each of the following parameters of the muscle tissue analysis as dependent variables for each of the 5 sampling times T_1 to T_5: P_{ww}, K_{ww}, ATP_{ww}, ADP_{ww}, AMP_{ww}, CPh_{ww}, and DM. The parameters included as independent variables with $P = 0.2$ as entry and $P = 0.05$ as exit, with exception of treatment and animal ID that were forced into the model, were chosen to identify possible factors affecting the intracellular P balance. These were lactation number, DMI, BW, age, and previous 305-d milk yield as cow-specific parameters; [Pi] as the parameter reflecting the extracellular P balance; [Ca] as the parameter associated with disturbed muscle function in periparturient cows (periparturient hypocalcemia); [Crea] as the parameter associated with muscle tissue metabolism; CK and AST as indicators for muscle cell injury; [NEFA] and [BHB] as indicators for disturbed energy balance; total bilirubin as an indicator for liver function; DM as an indicator for changes of muscle tissue water content; and K_{ww} as an indicator for changes of intracellular electrolyte balance in muscle tissue that is not specific for P. The final model was checked for variance inflation by determining correlations between independent variables remaining in the model and screening tolerance and variance inflation factors of each variable in the final model. In case of significant associations between independent variables in the final model, the variable with the lowest coefficient of determination was removed and the analysis was rerun. Pearson correlation analyses were conducted to compare the associations of P_{ww}, K_{ww}, [Pi], and DM with other parameters of the intracellular P homeostasis (ATP_{ww}, ADP_{ww}, AMP_{ww}, CPh_{ww}) at the different sampling times.

Forward multiple stepwise regression analyses were also conducted for each of the following parameters of the MUAP analysis of IC and BF muscles stratified by sampling time: MUAP duration, amplitude, number of phases, number of turns, area under the action potential curve, and size index. For the interference pattern analysis, dependent variables included in the analysis were turns per second (M), the amplitude per turn (T), and the turns per second:amplitude per turn ratio (**M/T**). Independent variables included in these analyses were treatment, animal ID, and, for IC muscles, the replicate number (all forced into the model) as well as lactation number, DMI, BW, previous 305-d milk yield, age, [Pi], [Ca], [Crea], [NEFA], [BHBA], total bilirubin, CK, AST, P_{ww}, K_{ww}, ATP_{ww}, ADP_{ww}, AMP_{ww}, creatine ww, and CPh_{ww}. Possible variance inflation was determined as described above. Pearson correlation analyses were again conducted to compare the associations of MUAP duration, amplitude, number of phases, number of turns, area under the action potential curve, and size index and IPA turns per second, amplitude per turn, and M/T with parameters of the intra- and extracellular P homeostasis such as ATP_{ww}, ADP_{ww}, AMP_{ww}, CPh_{ww}, and [Pi] as well as with [Ca] at the different sampling times.

The required sample size for this study was estimated on the basis of results obtained from an earlier study

investigating the effects of dietary P deprivation in mid-lactating dairy cows (Grünberg et al., 2015b). All analyses were conducted with SAS software (SAS 9.4, SAS Institute Inc.).

RESULTS

Study Animals

The LP and AP cows were 4.7 ± 1.2 and 4.5 ± 1.2 yr old, respectively, and in lactation 3 ± 1. The 305-d milk yield of the previous lactation was 8,935 ± 2,534 and 9,127 ± 1,731 kg for LP and AP cows, respectively. Because cows were blocked by lactation number and 305-d milk yield, differences of these parameters between groups were not significant. The mean BW for LP and AP cows at the time of enrollment was 692 ± 79 and 662 ± 69 kg, respectively, and did not differ between treatments. Cow-related parameters mentioned above were not different between first and second replicates.

All 36 cows delivered healthy calves either spontaneously or with mild to moderate assistance. The cows calved within a range of 13 d and 24 calved within the predicted week of calving and thus were on the experimental dry cow ration and the experimental lactating cow ration for at least 4 wk each. Four AP cows calved between 1 and 3 d before the predicted week of calving, shortening the prepartum period on the experimental ration accordingly. Five LP and 3 AP cows calved between 1 and 3 d following the end of the predicted calving week.

Clinical periparturient hypocalcemia, confirmed by blood biochemical analysis, occurred in 4 AP cows (2 in each replicate). In all cases cows were successfully treated with oral and parenteral administration of calcium salts. Four cows, all assigned to the LP treatment, were excluded from the study 2 to 4 wk after parturition, thus between 1 and 2 wk after T_3. Three of these cows developed postparturient hemoglobinuria during the second week of lactation, which was characterized by severe intravascular hemolysis and ensuing profound anemia and hemoglobinuria. One cow that delivered twins had retained fetal membranes and later developed metritis and clinical ketosis and was excluded due to ongoing deterioration. Results from these cows obtained up to the first week of lactation (T_3) were included in the data analysis. Two cows (1 from each group) with abomasal displacement in their second week of lactation underwent surgical correction of the condition immediately after diagnosis, recovered uneventfully, and completed the study. Other clinically apparent health events that occurred during the study period include 4 animals with febrile metritis (including the animal mentioned above; 3 LP and 1 AP) as well 3 cows with clinical ketosis (defined as depressed demeanor and marked ketonuria without other clinically apparent causes of disease; 2 LP and 1 AP). Treatment of metritis consisted of 3 consecutive days of parenteral administration of amoxicillin (10 mg/kg; Norobrittin, Norbrook Laboratories Ltd. Newry, Northern Ireland) and single or repeated administration of a nonsteroidal anti-inflammatory drug (meloxicam 0.5 mg/kg; Metacam, Boehringer Ingelheim, Ingelheim am Rhein, Germany). Treatment for ketosis consisted of a single or repeated (up to 3 times) intravenous administration of 500 mL of 30% dextrose solution (Glucose 30%, B. Braun, Melsungen, Germany) and oral administration of 250 mL of feed-grade propylene glycol twice daily until ketosis resolved. Dextrose treatments were never administered within 48 h before T_3 or T_4. Blood samples obtained within 48 h after dextrose administration were retrospectively excluded from the data analysis.

Blood Biochemical Analysis

The concentration–time curves for plasma [Pi] and [Ca] stratified by treatment are presented in Figure 2. Significant treatment, time, and treatment × time interaction effects (all $P < 0.0001$) for plasma [Pi] were identified. Mean concentrations determined in LP were below AP values throughout the entire depletion period (Figure 2), and mean plasma [Pi] of LP determined during the depletion period were below values measured during the acclimation period and below the reference for [Pi] in cattle (4–8 mg/dL; Figure 2; Goff, 1999). Values did not differ between treatments during the acclimation and repletion periods of the study (Figure 2). Significant treatment and time effects ($P = 0.0154$ and $P < 0.0001$, respectively) were identified for plasma [Ca], with higher concentrations in LP cows than AP cows (Figure 2).

Muscle Tissue Biochemical Analysis

The muscle tissue P_{ww}, ATP_{ww}, ADP_{ww}, AMP_{ww}, and CPh_{ww} at the different sampling times are presented in Figure 3. A replicate number effect was not determined for any of these parameters so that results from both replicates of cows were pooled per treatment for the statistical analysis.

The P_{ww} revealed neither a treatment nor time effect, whereas the treatment × time interaction effect was significant ($P < 0.0001$). The mean P_{ww} of LP obtained at T_5 was lower than values obtained at T_1, T_3, and T_4 (Figure 3). At T_5 the mean P_{ww} of LP was below values determined in AP cows. The P_{ww} showed a strong posi-

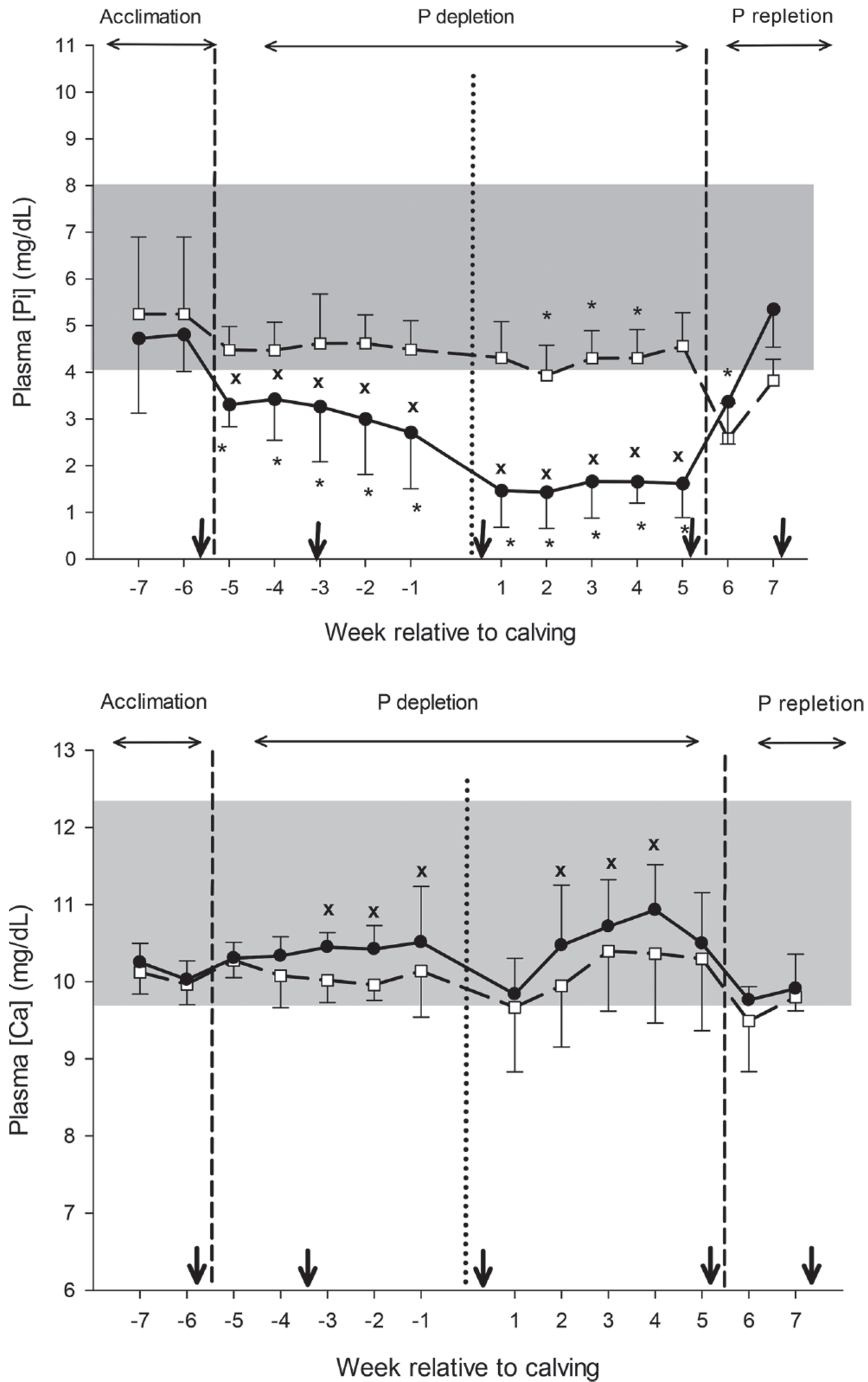

Figure 2. Mean ± SD plasma phosphorus concentrations ([Pi]; upper panel) and plasma calcium concentrations ([Ca]; lower panel) over time in cows assigned to the low-phosphorus (LP; closed circles/solid line) and adequate-phosphorus (AP; open squares/dashed line) diets. Vertical dashed lines mark the start and end of the P-depletion phase, and the vertical dotted line marks the time of parturition. The shaded area represents the reference range for plasma [Pi] and [Ca] in cattle. Time points labeled with an × differ significantly between groups ($P < 0.05$); time points labeled with an asterisk in the upper panel differ significantly from values measured during the acclimation period (i.e., wk −7 and wk −6) within each group ($P < 0.05$, Bonferroni corrected). Arrows mark the sampling times T_1 to T_5 (T_1 = end of acclimation period, T_2 = 2 wk after onset of P depletion, T_3 = within 1 wk of parturition, T_4 = end of P depletion, and T_5 = end of P repletion).

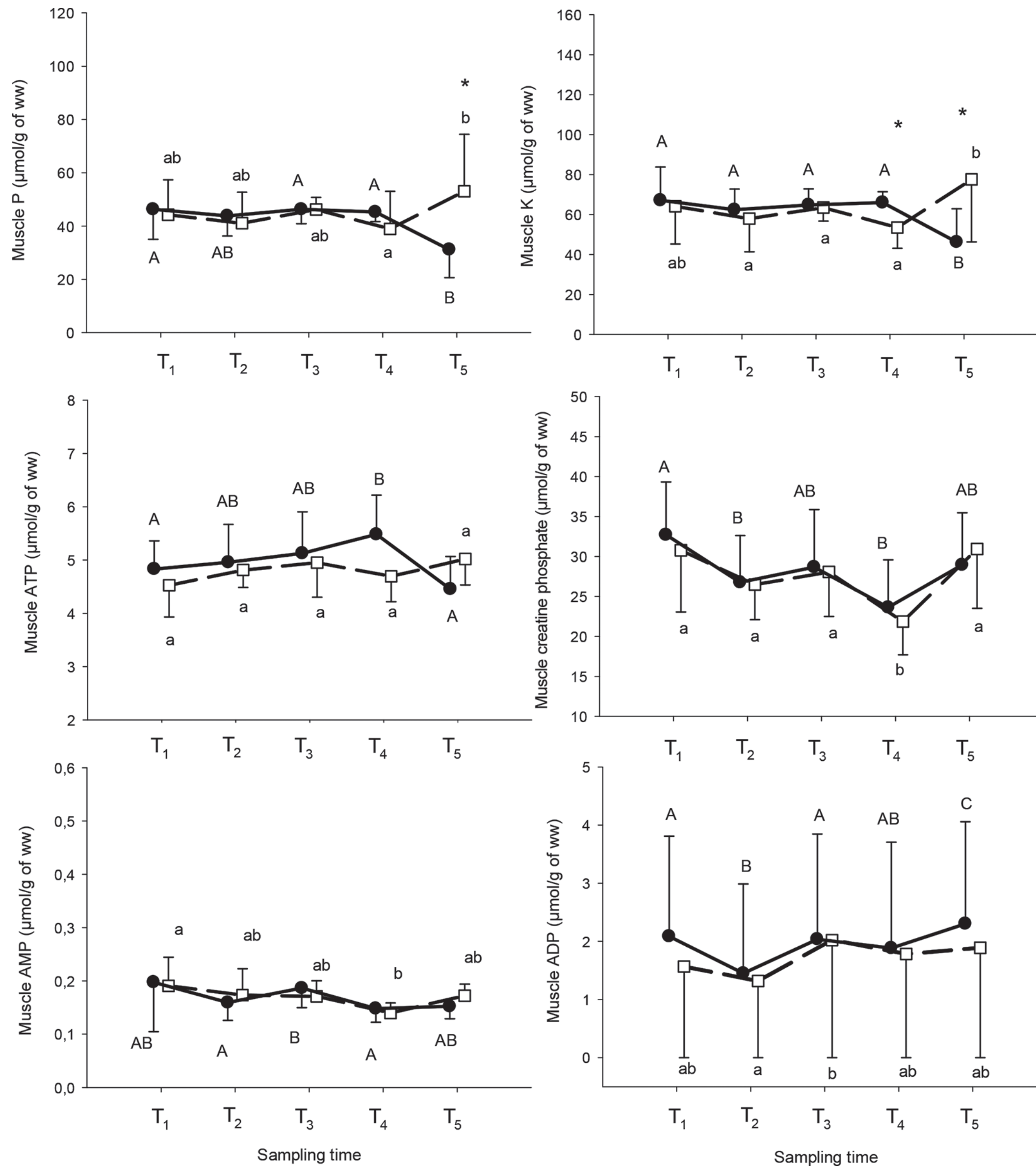

Figure 3. Mean ± SD muscle tissue total phosphorus (P; upper left panel), total potassium (K; upper right panel), adenosine triphosphate (ATP; middle left panel), creatine phosphate (middle right panel), adenosine monophosphate (AMP; lower left panel), and adenosine diphosphate (ADP; lower right panel) in μmol/g of wet weight (ww) for cows on low-phosphorus (LP; closed circles/solid line) and adequate-phosphorus (AP; open squares/dashed line) diets for each of the 5 sampling times (T_1 = end of acclimation period, T_2 = 2 wk after onset of P depletion, T_3 = within 1 wk of parturition, T_4 = end of P depletion, and T_5 = end of P repletion). Time points with different uppercase (LP) or lowercase (AP) letters (A–C and a,b, respectively) differ between sampling times within treatment ($P < 0.05$, Bonferroni corrected). Time points marked with an asterisk differ significantly between groups ($P < 0.05$).

tive association with K_{ww} for both treatments and at all sampling times. Accordingly, a treatment × time interaction effect ($P = 0.0002$) but no treatment or time effects were also identified for K_{ww}. As for P_{ww} values, the K_{ww} measured at T_5 were lower than at all other sampling times in group LP and were lower than AP values at T_4 and T_5 (Figure 3).

The CPh_{ww} showed a significant time effect ($P < 0.0001$) but neither a treatment nor a treatment × time interaction effect. The mean CPh_{ww} of both groups combined declined significantly from 31.72 ± 7.16 μmol/g at T_1 to 22.66 ± 5.04 μmol/g at T_4 and increased again to 30.24 ± 6.94 μmol/g at T_5 (Figure 3).

For ATP_{ww}, a time effect ($P = 0.0301$) and a treatment × time interaction effect ($P = 0.0089$) were determined, but no treatment effect was determined. The ATP_{ww} content of both groups combined was higher at T_3 (5.04 ± 0.71 μmol/g) and T_4 (5.05 ± 0.72 μmol/g)

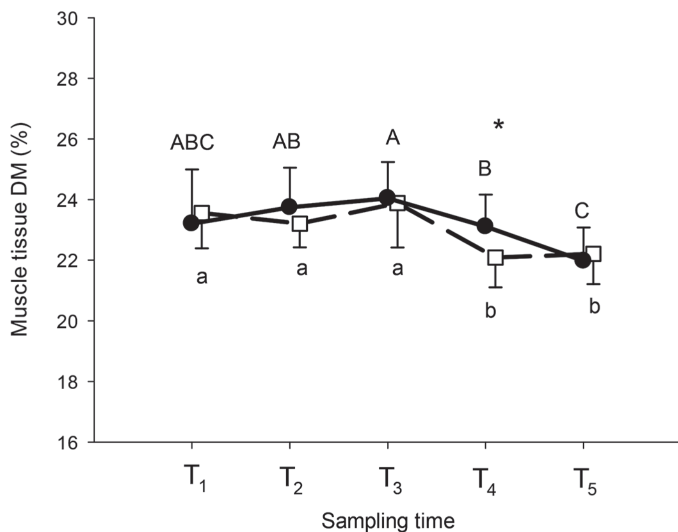

Figure 4. Mean ± SD of muscle tissue DM content (% of wet weight) for cows on low-phosphorus (LP; closed circles/solid line) and adequate-phosphorus (AP; open squares/dashed line) diets for each of the 5 sampling times (T_1 = end of acclimation period, T_2 = 2 wk after onset of P depletion, T_3 = within 1 wk of parturition, T_4 = end of P depletion, and T_5 = end of P repletion). Time points with different uppercase (LP) or lowercase (AP) letters (A–C and a,b, respectively) differ between sampling times within treatment ($P < 0.05$, Bonferroni corrected). Time points marked with an asterisk differ significantly between treatments ($P < 0.05$).

compared with T_1 (4.67 ± 0.58 μmol/g). In LP cows, the highest ATP_{ww} values were determined at T_4 and were different from values measured at T_1 and T_5. Differences between treatments were not observed at any time.

Significant time effects ($P < 0.0001$) and treatment × time interaction effects ($P = 0.0097$) were identified for ADP_{ww}. The most remarkable finding was a significant decline of ADP_{ww} in LP at T_5 relative to all other sampling times (Figure 3). Muscle tissue AMP_{ww} showed a significant time effect ($P = 0.0009$) but neither a treatment nor a treatment × time interaction effect (Figure 3).

Muscle tissue DM was subject to a significant time effect ($P < 0.0001$) but neither a treatment nor a treatment × time interaction effect. Values determined between T_1 and T_3 ranged between 23.4 ± 1.5% and 24.0 ± 1.3% and declined by more than 5% to 22.5 ± 1.1 and 22.1 ± 1.0% at the sampling times T_4 and T_5, respectively (Figure 4).

The results of the multiple stepwise regression analysis conducted with P_{ww}, CPh_{ww}, ATP_{ww}, ADP_{ww}, and AMP_{ww} as dependent variables for each sampling time are presented in Table 2. The most prominent finding was again a tight positive association between P_{ww} and K_{ww} at all sampling times. Positive associations with total bilirubin in plasma were identified for ATP_{ww}

Table 2. Results of multiple stepwise regression analyses with phosphorus (P_{ww}), creatine phosphate (CPh_{ww}), adenosine triphosphate (ATP_{ww}), adenosine diphosphate (ADP_{ww}), and adenosine monophosphate (AMP_{ww}) in wet muscle tissue ($_{ww}$) as well as muscle tissue DM as dependent variables[1,2]

Item	T_1	T_2	T_3	T_4	T_5
P_{ww}	K_{ww}: $+pr^2 = 0.968$; $P < 0.0001$	K_{ww}: $+pr^2 = 0.896$; $P < 0.0001$	K_{ww}: $+pr^2 = 0.816$; $P < 0.0001$	K_{ww}: $+pr^2 = 0.264$; $P < 0.0001$	K_{ww}: $+pr^2 = 0.719$; $P \geq 0.0001$
CPh_{ww}	BW: $-pr^2 = 0.205$; $P = 0.008$	K_{ww}: $+pr^2 = 0.304$; $P = 0.001$	K_{ww}: $+pr^2 = 0.540$; $P < 0.0001$	NA[3]	NA
ATP_{ww}	[Ca]: $+pr^2 = 0.134$; $P = 0.03$	K_{ww}: $+pr^2 = 0.466$; $P < 0.0001$ [TBil]: $+pr^2 = 0.112$; $P = 0.01$	K_{ww}: $+pr^2 = 0.674$; $P < 0.0001$ [TBil]: $+pr^2 = 0.154$; $P < 0.0001$	[CK]: $-pr^2 = 0.131$; $P = 0.02$	[TBil]: $+pr^2 = 0.454$; $P = 0.001$
ADP_{ww}	NA	[Ca]: $+pr^2 = 0.1276$; $P = 0.03$ K_{ww}: $-pr^2 = 0.126$; $P = 0.02$	K_{ww}: $-pr^2 = 0.354$; $P < 0.0001$	NA	[TBil]: $+pr^2 = 0.325$; $P = 0.006$
AMP_{ww}	[BHB]: $-pr^2 = 0.276$; $P = 0.003$	[BHB]: $-pr^2 = 0.273$; $P = 0.005$ K_{ww}: $-pr^2 = 0.203$; $P = 0.005$	[BHB]: $-pr^2 = 0.144$; $P = 0.0225$	DM: $+pr^2 = 0.141$; $P = 0.04$	[CK]: $-pr^2 = 0.353$; $P = 0.02$
DM	NA	[TBil]: $+pr^2 = 0.225$; $P = 0.002$	[NEFA]: $+pr^2 = 0.197$; $P = 0.01$	ATP_{ww}: $+pr^2 = 0.151$; $P = 0.02$	NA

[1]Parameters included as independent variables were treatment and cow ID (both forced into the model) as well as age, lactation number, BW, 305-d milk yield of the previous lactation, and DMI; plasma concentrations of inorganic phosphate ([Pi]), calcium ([Ca]), potassium ([K]), total bilirubin ([TBil]), creatinine, nonesterified fatty acids ([NEFA]), and BHB ([BHB]); and the activity in plasma of creatinine kinase ([CK]) and aspartate aminotransferase. For the analysis of DM, the variables P_{ww}, CPh_{ww}, ATP_{ww}, ADP_{ww}, and AMP_{ww} were also included as independent variables.

[2]Results are stratified by sampling times—T_1 (end of acclimation period), T_2 (2 wk after onset of P depletion), T_3 (within 1 wk of parturition), T_4 (end of P depletion), and T_5 (end of P repletion for the second replicate)—and given as partial coefficient of determination (pr^2) and P-value. Associations are listed only when significant ($P < 0.05$) and (pr^2) > = 0.1. The direction of the association is indicated by the sign preceding pr^2 (+ = positive; − = negative).

[3]No significant association.

at T_2, T_3, and T_5 as well as for ADP_{ww} at T_5 (Table 2). Plasma [Pi] was not associated with any of the P-containing compounds at any of the sampling times.

The results of the Pearson correlation analysis are summarized in Table 3. The contents ATP, ADP, AMP, and CPh showed strong positive associations with P_{ww}, particularly at T_2 and T_3, whereas no associations were identified during acclimation and repletion. Association of these concentrations with K_{ww} were weaker than with P_{ww}. Positive associations with muscle DM were identified for P_{ww} and to a lesser extent with K_{ww} as well as with ATP_{ww}, ADP_{ww}, and AMP_{ww} at T_4 and for ATP at T_2 (Table 3). The plasma [Pi] was strongly negatively associated with ATP_{ww}, ADP_{ww}, and muscle DM at T_4 (Table 3).

Electromyographic Examination

Pathologic Spontaneous Activity. Occurrence of pathological spontaneous activity stratified by muscles and sampling times is summarized in Table 4. Minor pathological spontaneous activity of IC muscles was recorded in more than 20% of study animals of either treatment at T_1 but occurred with lower frequency in BF muscles (Table 4). The frequency of minor pathologic spontaneous activity increased over time in IC muscles and to a lesser degree in BF muscles for both treatments. A marked increase in the occurrence of moderate (++) and pronounced (+++) pathologic spontaneous activity of IC muscles at T_4 and T_5 compared with earlier sampling times was observed in LP animals. Treatment and time effects were significant ($P = 0.005$ and $P < 0.0001$, respectively) for IC muscles with higher frequency in LP cow than in AP cows and higher frequency at T_5 than at T_1, T_2, and T_3 as well as higher frequency at T_4 than at T_1. Significant treatment and time effects were also identified in BF muscles ($P = 0.005$ and $P < 0.0001$, respectively) with higher frequency in LP cows compared with AP cows and higher frequency at T_5 than at all other sampling times. Moderate or pronounced pathologic spontaneous activity was observed incidentally only in BF muscles of LP cows (Table 4). The most common types of pathologic spontaneous activity were fibrillation potentials followed by complex repetitive discharges and positive sharp waves.

MUAP Analysis. The MUAP parameters of IC muscles were analyzed for each replicate separately as a significant replicate number effect was determined on several parameters. Results are presented in Tables 5 and 6 for replicates 1 and 2, respectively. Time effects were identified on the MUAP amplitude ($P = 0.0008$ and $P < 0.0001$), the number of phases ($P = 0.0004$ and $P = 0.0002$), the number of turns ($P = 0.0007$ and $P = 0.0492$), and the size index ($P = 0.0029$ and $P < 0.0001$) for the first and second replicates, respectively (Tables 5 and 6). Treatment effects were only identified in the second replicate on MUAP amplitude (473 ± 153 μV in AP vs. 376 ± 145 μV in LP, $P = 0.0002$), the number of phases (3.13 ± 0.33 in AP vs. 2.92 ± 0.31 in LP, $P = 0.0013$), the number of turns (3.34 ± 0.62 in AP vs. 2.97 ± 0.50 in LP, $P = 0.0029$), and the size index (0.37 ± 0.23 in AP vs. 0.20 ± 0.21 in LP, $P = 0.0003$). Treatment × time interaction effects were also only apparent in the second replicate for the MUAP amplitude ($P = 0.0010$), area ($P = 0.0002$), and size index ($P = 0.0084$, Table 6). Specifically, the MUAP number of turns, MUAP area, and size index of IC muscles were higher at T_1 compared with T_2 to T_4 in both replicates and tended to increase again between T_4 and T_5 in replicate 1 (Tables 5 and 6).

The results of the MUAP analysis of BF muscles of both replicates of cows were pooled for statistical analysis as no replicate number effect was identified; the results are summarized in Table 7. A significant treatment effect was only observed for the MUAP size index (0.53 ± 0.20 in AP vs. 0.38 ± 0.21 in LP, $P < 0.0001$); the time effect was also significant ($P = 0.0011$).

The results of the multiple stepwise regression analysis with parameters of the MUAP analysis of IC and BF as dependent variables are summarized in Table 8. Plasma [Pi] was found to be positively associated with MUAP amplitude at T_3 in BF muscles and with MUAP duration at T_1 and T_5 as well as with the number of turns at T_5 in IC muscles (Table 8). Other positive associations were identified for plasma creatinine that was associated with MUAP duration, amplitude, and number of phases at various time points in BF muscles and plasma [Ca] at T_5 that was associated with the MUAP area and size index of IC muscles (Table 8). In contrast, weak negative associations with plasma [Ca] were identified for MUAP duration and the number of turns in IC muscles at T_2.

Results of the Pearson correlation analysis for IC muscles are summarized in Table 9. Strong positive associations with plasma [Pi] were identified for the MUAP amplitude, number of phases, number of turns, size index, and area at T_4 and with MUAP duration and size index at T_3 (Table 9). Positive associations were furthermore observed between CPh_{ww} and MUAP amplitude, number of phases, size index, and MUAP area at different sampling times (Table 9). Negative associations were identified between plasma [Ca] and the MUAP duration, the number of phases, and number of turns at T_2 as well as with MUAP amplitude at T_4

Table 3. Results of the Pearson correlation analysis (r; *P*-values in parentheses) of muscle wet weight ($_{ww}$) adenosine triphosphate (ATP_{ww}), adenosine diphosphate (ADP_{ww}), adenosine monophosphate (AMP_{ww}), and creatine phosphate (CPh_{ww}) with muscle phosphorus (P_{ww}), muscle potassium (K_{ww}), plasma inorganic phosphorus concentration [Pi], total bilirubin (TBil), and muscle tissue DM stratified by sampling time[1,2]

Item	Time	P_{ww}	K_{ww}	[Pi]	TBil	DM	ATP_{ww}	ADP_{ww}	AMP_{ww}
P_{ww}	T_1		0.99 ($P < 0.0001$)	NA[3]	NA	NA			
	T_2		0.97 ($P < 0.0001$)	NA	0.41 ($P = 0.0205$)	NA			
	T_3		0.93 ($P < 0.0001$)	NA	NA	NA			
	T_4		0.82 ($P < 0.0001$)	−0.61 ($P < 0.0001$)	NA	0.42 ($P = 0.02$)			
	T_5		0.99 ($P < 0.0001$)	NA	NA	NA			
ATP_{ww}	T_1	NA	NA	NA	NA	NA			
	T_2	0.68 ($P < 0.0001$)	0.66 ($P < 0.0001$)	NA	0.59 ($P = 0.0002$)	0.35 ($P = 0.04$)			
	T_3	0.88 ($P < 0.0001$)	0.84 ($P < 0.0001$)	NA	0.56 ($P = 0.0004$)	NA			
	T_4	NA	NA	−0.52 ($P = 0.002$)	NA	0.53 ($P = 0.002$)			
	T_5	NA	NA	NA	0.81 ($P = 0.0001$)	NA			
ADP_{ww}	T_1	0.37 ($P = 0.03$)	0.35 ($P = 0.0275$)	NA	NA	NA	NA		
	T_2	0.54 ($P = 0.002$)	0.47 ($P = 0.007$)	NA	NA	NA	0.56 ($P = 0.0004$)		
	T_3	0.70 ($P < 0.0001$)	0.62 ($P < 0.0001$)	NA	0.48 ($P = 0.003$)	NA	0.65 ($P < 0.0001$)		
	T_4	0.40 ($P = 0.03$)	0.42 ($P = 0.02$)	−0.59 ($P = 0.0007$)	NA	0.47 ($P = 0.005$)	0.84 ($P < 0.0001$)		
	T_5	NA	NA	NA	0.76 ($P = 0.0007$)	NA	0.89 ($P < 0.0001$)		
AMP_{ww}	T_1	NA	NA	NA	0.37 ($P = 0.03$)	NA	NA	0.48 ($P = 0.004$)	
	T_2	0.59 ($P = 0.0005$)	0.50 ($P = 0.004$)	NA	0.37 ($P = 0.03$)	NA	NA	NA	
	T_3	0.37 ($P = 0.03$)	NA	NA	NA	NA	NA	0.42 ($P = 0.002$)	
	T_4	NA	NA	NA	NA	0.35 ($P = 0.05$)	0.53 ($P < 0.0001$)	NA	
	T_5	NA	NA	NA	NA	NA	NA	NA	
CPh_{ww}	T_1	NA	NA	NA	−0.45 ($P = 0.007$)	NA	NA	NA	NA
	T_2	0.61 ($P = 0.0002$)	0.57 ($P = 0.0007$)	NA	0.41 ($P = 0.003$)	NA	0.36 ($P = 0.03$)	0.41 ($P = 0.01$)	0.58 ($P = 0.0003$)
	T_3	0.69 ($P < 0.0001$)	0.74 ($P < 0.0001$)	NA	NA	NA	0.62 ($P < 0.0001$)	0.34 ($P = 0.04$)	NA
	T_4	NA	NA	NA	NA	NA	0.45 ($P = 0.0091$)	0.58 ($P = 0.0005$)	0.45 ($P = 0.01$)
	T_5	NA	NA	NA	NA	NA		NA	NA
DM	T_1	NA	NA	NA	NA				
	T_2	NA	NA	NA	0.54 ($P = 0.001$)				
	T_3	NA	NA	NA	0.37 ($P = 0.03$)				
	T_4	0.42 ($P = 0.02$)	0.36 ($P = 0.04$)	−0.36 ($P = 0.04$)	NA				
	T_5	NA	NA	NA	NA				

[1]Sampling times were T_1 (end of acclimation period), T_2 (2 wk after onset of P depletion), T_3 (within 1 wk of parturition), T_4 (end of P depletion), and T_5 (end of P repletion).
[2]Empty cells are either cells with the same parameter in row and column or duplicates of cells of the table.
[3]NA = no significant association.

Table 4. Frequency of pathological spontaneous activity in intercostal and biceps femoris muscles for treatment with low (LP) and adequate (AP) dietary P supply[1]

			T_1			T_2			T_3			T_4			T_5		
Muscle	Group	Grade[2]	No.	Total	%	No.	Total	%	No.	Total	%	No.	Total	%	No.	Total	%
Intercostal	LP	0	14	18	78	8	18	44	9	18	50	2	14	14	0	7	0
		+	3	18	20	9	18	50	7	18	38	8	14	57	1	7	14
		++	1	18	2	1	18	6	1	18	6	3	14	22	1	7	14
		+++	0	18	0	0	18	0	1	18	6	1	14	7	5	7	72
	AP	0	12	18	66	11	18	61	11	18	61	9	18	50	3	9	33
		+	3	18	17	6	18	33	6	18	33	9	18	50	5	9	56
		++	3	18	17	1	18	6	1	18	6	0	18	0	0	9	0
		+++	0	18	0	0	18	0	0	18	0	0	18	0	1	9	11
Biceps femoris	LP	0	18	18	100	16	18	89	14	18	78	14	14	78	3	7	43
		+	0	18	0	2	18	11	4	18	22	4	14	22	2	7	29
		++	0	18	0	0	18	0	0	18	0	0	14	0	1	7	14
		+++	0	18	0	0	18	0	0	18	0	0	14	0	1	7	14
	AP	0	18	18	100	17	18	94	14	18	78	18	18	100	6	9	67
		+	0	18	0	1	18	6	4	18	22	0	18	0	3	9	33
		++	0	18	0	0	18	0	0	18	0	0	18	0	0	9	0
		+++	0	18	0	0	18	0	0	18	0	0	18	0	0	9	0

[1]Sampling times were T_1 (end of acclimation), T_2 (after 2 wk of dietary P deprivation antepartum), T_3 (within 1 wk postpartum), T_4 (at the end of dietary P deprivation), and T_5 (after 2 wk of dietary P repletion). At T_4, 4 LP cows were removed from the experiment because of health issues; muscle tissue analyses during T_4 excluded those cows. At T_5, only cows in the first replicate of the experiment were included.

[2]Occurrence of pathologic spontaneous activity was graded as absent (0), mild (+), moderate (++), or pronounced (+++).

Table 5. Results of motor unit action potential analysis of intercostal muscles of cows of the first replicate on adequate (AP) and low (LP) dietary P supply stratified by sampling time[1,2]

	T_1		T_2		T_3		T_4		T_5	
Parameter	AP (n = 9)	LP (n = 9)	AP (n = 9)	LP (n = 9)	AP (n = 9)	LP (n = 9)	AP (n = 9)	LP (n = 7)	AP (n = 9)	LP (n = 7)
Duration (ms)	5.2 ± 0.9	6.2 ± 1.0	5.3 ± 1.2	4.9 ± 0.9	5.0 ± 1.0	5.1 ± 1.0	4.9 ± 1.1	5.0 ± 0.9	6.3 ± 2.4	5.7 ± 1.6
Amplitude (μV)	572 ± 98	667 ± 203	548 ± 161	466 ± 142	453 ± 138	430 ± 145	506 ± 139	285 ± 80	387 ± 103	475 ± 303
No. of phases	3.4 ± 0.3^{A}	3.3 ± 0.5^{A}	3.1 ± 0.4^{AB}	3.0 ± 0.2^{AB}	3.2 ± 0.4^{AB}	3.0 ± 0.2^{AB}	$3.1 \pm 0.3^{AB,*}$	2.7 ± 0.3^{B}	2.9 ± 0.3^{B}	3.0 ± 0.4^{AB}
No. of turns	3.4 ± 0.5	3.7 ± 0.9^{A}	3.1 ± 0.6	3.0 ± 0.5^{B}	3.1 ± 0.7	2.8 ± 0.4^{B}	3.0 ± 0.4	2.4 ± 0.3^{B}	3.1 ± 0.5	3.0 ± 0.5^{B}
Size index	0.48 ± 0.07	0.67 ± 0.27^{A}	0.49 ± 0.30	0.35 ± 0.21^{AB}	0.39 ± 0.29	0.35 ± 0.27^{B}	0.26 ± 0.19	0.14 ± 0.08^{B}	0.27 ± 0.18	0.46 ± 0.45^{AB}
Area (ms/mV)	560 ± 151	757 ± 300^{A}	565 ± 243	456 ± 190^{BC}	458 ± 223	462 ± 220^{BC}	459 ± 199	265 ± 64^{B}	374 ± 156	546 ± 438^{AC}

[A–C]Values of the same treatment with different superscripts at different sampling times are significantly different ($P < 0.05$, Bonferroni corrected).

[1]Sampling times were T_1 (end of acclimation), T_2 (after 2 wk of dietary P deprivation antepartum), T_3 (within 1 wk postpartum), T_4 (at the end of dietary P deprivation), and T_5 (after 2 wk of dietary P repletion).

[2]Results are presented as mean ± SD.

*Values differ significantly between groups at 1 sampling time ($P < 0.05$).

Table 6. Results of motor unit action potential analysis of intercostal muscles of cows of the second replicate on adequate (AP) and low (LP) dietary P supply stratified by sampling time[1,2]

	T_1		T_2		T_3		T_4	
Parameter	AP (n = 9)	LP (n = 9)	AP (n = 9)	LP (n = 9)	AP (n = 9)	LP (n = 9)	AP (n = 9)	LP (n = 7)
Duration (ms)	5.2 ± 0.7	5.7 ± 0.9	5.5 ± 1.2	4.9 ± 0.7	6.0 ± 0.9	4.5 ± 0.4	7.3 ± 4.8	5.1 ± 1.4
Amplitude (μV)	476 ± 54^{A}	550 ± 85^{A}	$429 \pm 121^{A,*}$	280 ± 62^{BC}	$623 \pm 196^{B,*}$	400 ± 121^{B}	368 ± 87^{A}	247 ± 48^{C}
No. of phases	3.2 ± 0.3	3.1 ± 0.2^{A}	3.0 ± 0.2	2.8 ± 0.2^{BC}	3.3 ± 0.3	3.1 ± 0.2^{AB}	3.1 ± 0.5*	2.5 ± 0.3^{C}
No. of turns	3.2 ± 0.3	3.2 ± 0.3^{A}	3.1 ± 0.3	2.8 ± 0.5^{AB}	3.6 ± 0.4	3.2 ± 0.5^{AB}	3.4 ± 1.1	2.6 ± 0.3^{B}
Size index	0.36 ± 0.18^{AB}	0.45 ± 0.24^{A}	0.26 ± 0.21^{A}	0.08 ± 0.07^{B}	$0.55 \pm 0.20^{B,*}$	0.19 ± 0.15^{B}	$0.29 \pm 0.25^{A,*}$	0.06 ± 0.05^{B}
Area (ms/mV)	444 ± 101^{A}	552 ± 173^{A}	374 ± 140^{A}	240 ± 54^{BC}	590 ± 134^{B}	335 ± 108^{B}	397 ± 133^{A}	209 ± 45^{C}

[A–C]Values of the same treatment with different superscripts at different sampling times are significantly different ($P < 0.05$, Bonferroni corrected).

[1]Sampling times were T_1 (end of acclimation), T_2 (after 2 wk of dietary P deprivation antepartum), T_3 (within 1 wk postpartum), T_4 (at the end of dietary P deprivation), and T_5 (after 2 wk of dietary P repletion).

[2]Results are presented as mean ± SD.

*Values differ significantly between groups at 1 sampling time ($P < 0.05$).

Table 7. Results of motor unit action potential analysis of biceps femoris muscles of both replicates of cows on adequate (AP) and low (LP) dietary P supply stratified by sampling time[1,2]

	T_1		T_2		T_3		T_4		T_5	
Parameter	AP (n = 18)	LP (n = 18)	AP (n = 18)	LP (n = 18)	AP (n = 18)	LP (n = 18)	AP (n = 18)	LP (n = 14)	AP (n = 9)	LP (n = 7)
Duration (ms)	5.9 ± 1.1	6.1 ± 0.8	5.9 ± 1.0	6.0 ± 0.8	5.9 ± 1.1	5.9 ± 0.6	5.8 ± 1.0	6.2 ± 0.8	5.6 ± 09	6.2 ± 1.0
Amplitude (μV)	579 ± 147	507 ± 126	586 ± 152	503 ± 123	594 ± 160	501 ± 118	576 ± 154	493 ± 111	554 ± 147	437 ± 56
No. of phases	3.2 ± 0.2	3.1 ± 0.2	3.2 ± 0.2	3.1 ± 0.4	3.2 ± 0.2	3.1 ± 0.2	3.2 ± 0.2	3.1 ± 0.2	3.2 ± 0.1	3.1 ± 0.1
No. of turns	3.3 ± 0.4	3.2 ± 0.4	3.3 ± 0.4	3.2 ± 0.4	3.3 ± 0.5	3.1 ± 0.4	3.3 ± 0.5	3.2 ± 0.3	3.3 ± 0.4	3.2 ± 0.3
Size index	0.51 ± 0.18^{AB}	0.48 ± 0.23^{A}	0.48 ± 0.23^{A}	0.37 ± 0.16^{AB}	$0.59 \pm 0.21^{AB,*}$	0.38 ± 0.18^{AB}	$0.52 \pm 0.15^{AB,*}$	0.24 ± 0.18^{B}	0.66 ± 0.27^{B}	0.47 ± 0.24^{A}
Area (ms/mV)	577 ± 142	527 ± 146	580 ± 129	519 ± 138	580 ± 147	508 ± 135	556 ± 130	525 ± 141	560 ± 142	491 ± 85

[A,B]Values of the same treatment with different superscripts at different sampling times are significantly different ($P < 0.05$, Bonferroni corrected).

[1]Sampling times were T_1 (end of acclimation), T_2 (after 2 wk of dietary P deprivation antepartum), T_3 (within 1 wk postpartum), T_4 (at the end of dietary P deprivation), and T_5 (after 2 wk of dietary P repletion).

[2]Results are presented as mean ± SD.

*Values differ significantly between groups at 1 sampling time ($P < 0.05$).

Table 8. Results of multiple stepwise regression analysis for biceps femoris and intercostal muscles with parameters of the motor unit action potential (MUAP) analysis as dependent variables stratified by sampling time[1,2,3]

MUAP parameter	T_1	T_2	T_3	T_4	T_5
Biceps femoris					
Duration (ms)	[AST]: $+pr^2 = 0.24$; $P = 0.004$ [CK]: $-pr^2 = 0.18$; $P = 0.005$	AMP_{ww}: $-pr^2 = 0.22$; $P = 0.01$	[Crea]: $+pr^2 = 0.14$; $P = 0.04$	[TBil]: $+pr^2 = 0.30$; $P = 0.0009$ [Crea]: $+pr^2 = 0.12$; $P = 0.02$	NA[4]
Amplitude (μV)	NA	[Crea]: $-pr^2 = 0.17$; $P = 0.02$	[Pi]: $+pr^2 = 0.29$; $P = 0.0002$	NA	NA
No. of phases	NA	NA	AMP_{ww}: $-pr^2 = 0.12$; $P = 0.05$	[BHB]: $+pr^2 = 0.16$; $P = 0.03$	BW: $-pr^2 = 0.50$; $P = 0.004$ [Crea]: $-pr^2 = 0.15$; $P = 0.04$
No. of turns	NA	NA	NA	NA	ADP_{ww}: $-pr^2 = 0.23$; $P = 0.03$
Size index	NA	NA	[AST]: $+pr^2 = 0.14$; $P = 0.02$	NA	[AST]: $-pr^2 = 0.27$; $P = 0.04$ [CK]: $+pr^2 = 0.37$; $P = 0.0006$
Area (ms/mV)	NA	NA	DMI : $+pr^2 = 0.15$; $P = 0.02$ AMP_{ww}: $-pr^2 = 0.11$; $P = 0.04$	NA	NA
Intercostal					
Duration (ms)	[Pi]: $+pr^2 = 0.17$; $P = 0.006$ LN: $+pr^2 = 0.11$; $P = 0.02$	[Ca]: $-pr^2 = 0.14$; $P = 0.002$	NA	LN: $+pr^2 = 0.20$; $P = 0.01$ [BHB]: $+pr^2 = 0.12$; $P = 0.03$	CPh_{ww}: $+pr^2 = 0.31$; $P = 0.04$ [Pi]: $+pr^2 = 0.25$; $P = 0.03$
Amplitude (μV)	NA	NA	[BHB]: $+pr^2 = 0.16$; $P = 0.01$	NA	NA
No. of phases	LN: $+pr^2 = 0.29$; $P = 0.001$	DMI: $-pr^2 = 0.21$; $P = 0.006$	NA	NA	[NEFA]: $-pr^2 = 0.39$; $P = 0.02$ ATP_{ww}: $+pr^2 = 0.21$; $P = 0.03$
No. of turns	LN: $+pr^2 = 0.17$; $P = 0.02$	[Ca]: $-pr^2 = 0.18$; $P = 0.02$	NA	[NEFA]: $+pr^2 = 0.14$; $P = 0.02$	[Pi]: $-pr^2 = 0.36$; $P = 0.003$ CPh_{ww}: $+pr^2 = 0.29$; $P = 0.05$ [AST]: $-pr^2 = 0.16$; $P = 0.001$
Size index	NA	NA	[BHB]: $+pr^2 = 0.18$; $P = 0.007$	NA	[Ca]: $+pr^2 = 0.25$; $P = 0.04$ ATP_{ww}: $+pr^2 = 0.19$; $P = 0.02$
Area (ms/mV)	NA	NA	[BHB]: $+pr^2 = 0.14$; $P = 0.02$	NA	[Ca]: $+pr^2 = 0.23$; $P = 0.03$ [CK]: $+pr^2 = 0.14$; $P = 0.05$ [TBil]: $-pr^2 = 0.14$; $P = 0.009$

[1]Parameters included as independent variables were animal ID, treatment, and, for intercostal muscles, replicate number (all forced into the model) as well as age, lactation number (LN), BW, 305-d milk yield of the previous lactation, and DMI; plasma concentration of inorganic phosphate ([Pi]), calcium ([Ca]), creatinine ([Crea]), nonesterified fatty acids ([NEFA]), and BHB ([BHB]); total bilirubin concentration ([TBil]); the activity in plasma of creatinine kinase ([CK]) and aspartate aminotransferase ([AST]); and the total content of phosphorus (P_{ww}), creatine phosphate (CPh_{ww}), creatine (C_{ww}), ATP (ATP_{ww}), ADP (ADP_{ww}), and AMP (AMP_{ww}) in muscle tissue wet weight.

[2]Sampling times are T_1 (end of acclimation period), T_2 (2 wk after onset of P depletion), T_3 (within 1 wk of parturition), T_4 (end of P depletion), and T_5 (end of P repletion).

[3]Results are given as partial coefficient of determination (pr^2) and P-value. Associations are listed only when significant ($P < 0.05$) and partial coefficient of determination (pr^2) $\geq$ 0.1. The direction of the association is indicated by the sign preceding pr^2 (+ = positive; − = negative).

[4]No significant association.

(Table 9). The ATP_{ww} was negatively associated with the number of phases at T_2 and the MUAP area at T_4 and T_5 (Table 9). Neither P_{ww} nor AMP_{ww} were found to be associated with any of the studied MUAP parameters.

The Pearson correlation analysis between parameters of the MUAP analysis of BF muscles and parameters characterizing the extracellular and muscle tissue P homeostasis revealed strong positive associations of the plasma [Pi] with the MUAP size index at T_4 (r = 0.68, $P < 0.0001$). Negative associations were identified between ATP_{ww} and MUAP size index at T_4 (r = −0.37, $P = 0.0471$) as well as between AMP_{ww} and MUAP duration at T_1 (r = −0.41, $P = 0.0182$) and T_5 (r = −0.51, $P = 0.0357$).

The results of the interference pattern analysis conducted on IC muscles of cows of the second replicate are summarized in Table 10. Time effects ($P = 0.0031$) and treatment effects were identified for the amplitude per turn of the action potentials [240 (209–279) for treatment AP vs. 234 (195–232) for treatment LP, $P = 0.0055$; interquartile range in parentheses] but not for the number of turns per seconds or the M/T. The stepwise regression analysis with number of turns per second as the dependent variable yielded significant associations only at T_2, with the strongest associations with ADP_{ww} (partial $R^2 = 0.37$, $P = 0.04$). For the interference pattern ratio (M/T), significant associations were identified for sampling time T_1, with the strongest associations with ADP_{ww} (partial $R^2 = 0.38$, $P = 0.03$) and plasma [NEFA] (partial $R^2 = 0.20$, $P = 0.05$). The Pearson correlation analysis at T_1 revealed significant associations of the interference pattern ratio (M/T) with plasma [Ca] (r = 0.60, $P = 0.0403$), ADP_{ww} (r = −0.58, $P = 0.0484$), and ATP_{ww} (r = 0.65, $P = 0.0210$). At T_2, a significant correlation was determined between number of turns per second and ADP_{ww} (r = −0.60, $P = 0.0237$).

Table 9. Results of the Pearson correlation analysis (r; *P*-values in parentheses) of parameters of the motor unit action potential (MUAP) analysis of intercostal muscles[1,2]

Parameter	Time	[Pi]	[Ca]	ATP_{ww}	ADP_{ww}	CPh_{ww}
Duration	T_1	0.39 ($P = 0.02$)	NA[3]	NA	NA	NA
	T_2	NA	−0.51 ($P = 0.002$)	NA	NA	NA
	T_3	0.39 ($P = 0.02$)	NA	NA	NA	NA
	T_4	NA	NA	NA	NA	NA
	T_5	NA	NA	NA	NA	NA
Amplitude	T_1	NA	NA	NA	NA	0.36 ($P = 0.03$)
	T_2	NA	NA	NA	NA	NA
	T_3	NA	NA	NA	NA	NA
	T_4	0.54 ($P = 0.001$)	−0.48 ($P = 0.005$)	NA	NA	0.36 ($P = 0.05$)
	T_5	NA	NA	NA	NA	NA
No. of phases	T_1	NA	NA	NA	NA	NA
	T_2	NA	−0.41 ($P = 0.01$)	−0.50 ($P = 0.004$)	−0.57 ($P = 0.0008$)	0.35 ($P = 0.05$)
	T_3	NA	NA	NA	NA	NA
	T_4	0.48 ($P = 0.006$)	NA	NA	NA	NA
	T_5	NA	NA	NA	NA	NA
No. of turns	T_1	NA	NA	NA	NA	NA
	T_2	NA	−0.44 ($P = 0.007$)	NA	0.38 ($P = 0.03$)	NA
	T_3	NA	NA	NA	NA	NA
	T_4	0.36 ($P = 0.04$)	NA	NA	NA	NA
	T_5	NA	NA	NA	NA	NA
Size index	T_1	NA	NA	NA	NA	NA
	T_2	NA	NA	NA	NA	0.40 ($P = 0.02$)
	T_3	0.36 ($P = 0.03$)	NA	NA	NA	NA
	T_4	0.39 ($P = 0.03$)	NA	NA	0.38 ($P = 0.04$)	0.49 ($P = 0.006$)
	T_5	NA	NA	NA	NA	NA
Area	T_1	NA	NA	NA	NA	0.36 ($P = 0.04$)
	T_2	NA	NA	NA	NA	0.40 ($P = 0.02$)
	T_3	NA	NA	NA	NA	NA
	T_4	0.53 ($P = 0.002$)	NA	−0.39 ($P = 0.03$)	−0.40 ($P = 0.03$)	NA
	T_5	NA	NA	NA	NA	NA

[1]Parameters of the MUAP analysis were tested for associations with plasma inorganic phosphate ([Pi]), calcium ([Ca,]), and the content of adenosine triphosphate (ATP_{ww}), adenosine diphosphate (ADP_{ww}), and creatine phosphate (CPh_{ww}) in muscle tissue wet weight.

[2]Sampling times are T_1 (end of acclimation period), T_2 (2 wk after onset of P depletion), T_3 (within 1 wk of parturition), T_4 (end of P depletion), and T_5 (end of P repletion). The sample size at T_1 to T_3 was n = 36, at T_4 was n = 32, and at T_5 was n = 16.

[3]No association.

Table 10. Results of the interference pattern analysis of intercostal muscles of cows on adequate (AP) and low (LP) dietary P supply of the second replicate, stratified by sampling time[1]

Parameter	T_1		T_2		T_3		T_4	
	AP (n = 9)	LP (n = 9)	AP (n = 9)	LP (n = 9)	AP (n = 9)	LP (n = 9)	AP (n = 9)	LP (n = 7)
No. of turns[2]	96 ± 34	82 ± 24	91 ± 37	90 ± 27	99 ± 37	92 ± 38	99 ± 32	72 ± 13
Amplitude[3]	233 (205–293)[A]	233 (230–278)[a]	249 (206–255)[B,*]	223 (203–275)[b]	222 (213–259)[B]	177 (162–217)[ab]	238 (206–254)[AB]	226 (209–243)[ab]
Ratio[2]	0.37 ± 0.14	0.33 ± 0.13	0.41 ± 0.19	0.44 ± 0.12	0.40 ± 0.14	0.40 ± 0.14	0.46 ± 0.15	0.35 ± 0.07

[a,b]Values for LP with different lowercase superscripts at different sampling times differ significantly ($P < 0.05$, Bonferroni corrected).

[A,B]Values for AP with different uppercase superscripts at different sampling times differ significantly ($P < 0.05$, Bonferroni corrected).

[1]Sampling times were T_1 (acclimation), T_2 (2 wk of P deprivation antepartum), T_3 (first week of lactation), and T_4 (end of P deprivation).

[2]Values are mean ± SD.

[3]Values are median (interquartile range).

*Values at 1 sampling time differ significantly between treatments ($P < 0.05$).

DISCUSSION

The most remarkable finding of this study was that dietary P deprivation that was associated with pronounced and sustained hypophosphatemia over several weeks was not associated with clinical signs of muscle weakness or with recumbency in any of the cows undergoing dietary P depletion. Clinical periparturient hypocalcemia was observed in 4 instances, all affecting AP cows, and significantly higher plasma [Ca] were measured in LP cows in the weeks before and after calving. The effects of P deprivation on calcium homeostasis have been studied in more detail on a subset of study animals, and the results have been published elsewhere (Cohrs et al., 2018). Although dietary P deprivation did not result in a clinically apparent disruption of muscle function, the results reported here revealed obvious subclinical effects of dietary P deprivation on muscle function as determined by electromyography. These are reflected in increased frequency of pathologic spontaneous activity and the decrease of several MUAP parameters that are indicative of muscle fiber functionality loss.

Phosphorus deprivation in late gestation and early lactation was not associated with clinically apparent effects until after the first week of lactation, when feed intake depression and lower milk production compared with cows on a diet with adequate P supply were observed (data not shown). A similar outcome on feed intake and productivity has been reported in earlier studies (Valk and Sebek, 1999; Puggaard et al., 2014). In particular, feed intake depression can be considered as a well-accepted outcome of sustained P depletion in cattle and other species (Knochel, 1977; Valk and Sebek, 1999; Puggaard et al., 2014). All AP cows completed the study, whereas 4 LP cows had to be removed prematurely between the second and fourth weeks of lactation. Three animals developed a potentially life-threatening episode of postparturient hemoglobinuria, requiring blood transfusion. Postparturient hemoglobinuria in dairy cows is characterized by severe intravascular hemolysis and hemoglobinuria and has been empirically associated with hypophosphatemia in fresh dairy cows (Macwilliams et al., 1982).

The results of the plasma biochemical analysis confirmed that the experimental rations used in this study were effective in inducing and sustaining pronounced hypophosphatemia throughout the entire depletion period. In LP cows, plasma [Pi] declined continuously until parturition, when a nadir with mean plasma [Pi] below 1.5 mg/dL and with values below the detection limit (0.6 mg/dL) in several cows was reached. No further decline was observed after calving despite ongoing dietary P depletion. Dietary P supplementation during

the repletion period in LP cows of the first replicate was associated with a rapid increase in plasma [Pi], again confirming the efficacy of oral P supplementation to correct hypophosphatemia. Dietary P supplementation in LP cows was furthermore associated with an increase of MUAP parameters that apparently were affected by previous P deprivation. This observation suggests that the loss of contributing individual muscle fibers to the MUAP observed during P deprivation can be corrected at least partially with dietary P supplementation (Kimura, 2001; Daube and Rubin, 2009; Wijnberg and Franssen, 2016).

The biochemical analysis of muscle tissue revealed that P depletion and hypophosphatemia did not result in a remarkable disturbance of the muscle tissue P homeostasis. A treatment effect could not be identified for any of the biochemical parameters studied in muscle tissue. The P_{ww} that comprises all P present in muscle tissue remained unchanged from acclimation through the entire depletion phase. Interestingly, a decline in P_{ww} was observed at the end of the P supplementation phase in LP cows. This decline in P_{ww} between T_4 and T_5 was associated with a decline in ATP_{ww} and ADP_{ww}, 2 compounds forming part of the muscle tissue P_{ww} pool. Although there is no evident explanation for the changes observed during the P repletion phase, a similar decline in ATP_{ww} and ADP_{ww} during dietary P repletion following a depletion phase of 5 wk was observed in an earlier study (Grünberg et al., 2015b). This observed decline in P_{ww} during repletion was furthermore tightly associated with a concomitant decline of K_{ww} of a similar extent. Parallel changes in the content of K and P in muscle tissue, both predominantly intracellular electrolytes, imply an underlying mechanism that is not specific to P. Such a mechanism could, for example, be an increase in cytosolic volume of muscle cells that would increase the distribution volume of compounds dissolved in the cytosol to a similar degree. In this study, the muscle tissue DM content was used as an indicator for changes in tissue water content, giving a crude approximation for possible changes in cytosol volume. Muscle tissue DM decreased and thus water content in muscle tissue increased between T_3 and T_5 in both groups. This effect contributed to the observed decrease in P_{ww}, K_{ww}, ATP_{ww}, and ADP_{ww} at T_5 as implied by the results of the regression and correlation analyses. Changes in tissue water content in the form of an increase in empty body water (i.e., total body water excluding water contained in the digestive tract) in late gestation and early lactation have previously been reported in cattle and ewes (Degen and Young, 1980; Robinson, 1986). This effect has been attributed to increased water turnover at the onset of lactation as well as to the rapid loss of body fat around parturition that is tightly coupled with increased tissue water content.

Creatine phosphate was the only P-containing compound in muscle tissue with a time curve crudely reflecting the different phases of dietary P supply in P-deprived animals. However, similar changes were observed in AP cows, again suggesting that these effects are not directly attributable to P deprivation.

Associations between plasma [Pi] and intracellular P compounds were only identified at T_4. At this time, the Pearson correlation analysis revealed correlations of plasma [Pi] with P_{ww}, ATP_{ww}, and ADP_{ww} that were negative. These results are in agreement with earlier studies and indicate that plasma [Pi] is poorly suited to estimate muscle tissue P content. These findings specifically refute the concept that hypophosphatemia is indicative of intracellular P depletion of muscle tissue (Grünberg et al., 2015b). Maintenance of constant P content in tissue despite variable P supply and plasma [Pi] has also been reported for other tissues such as liver and erythrocytes (Grünberg et al., 2009, 2015a).

Plasma total bilirubin was found to be positively associated with all P compounds in muscle tissue at one or several sampling times. Animals with postparturient hemoglobinuria, a condition characterized by intravascular hemolysis and invariably associated with hyperbilirubinemia, are unlikely to have confounded these results because associations with total bilirubin were already observed antepartum. Furthermore, samples from animals with postparturient hemoglobinuria obtained at T_3 and later were not included in this analysis. Plasma total bilirubin at T_3 did not differ between groups but showed a transient mild increase in both groups during the second and third weeks of lactation. Mild hyperbilirubinemia may be indicative of impaired liver function frequently observed in early-lactating dairy cows. Because positive associations with total bilirubin were identified at T_1 and T_2, considerably before liver function is commonly hampered, hepatic hyperbilirubinemia also is deemed an improbable confounder. Muscle tissue contributes approximately 20% to plasma total bilirubin through its myoglobin turnover (Kamga et al., 2012). As the amount of muscle tissue myoglobin characterizes the capacity of aerobic glycolysis of myocytes, this may explain the positive association between compounds of adenosine phosphate metabolism and the metabolite of the myoglobin breakdown.

The amounts of P, K, ATP, ADP, AMP, and CPh in muscle tissue in this study are in good agreement with values determined in muscle tissue from bovines and other species in earlier studies (Fuller et al., 1976;

Angerås et al., 1991; Williams et al., 1991; Grünberg et al., 2015b; Schneider et al., 2016).

An important implication of the results reported here is that the degree and duration of P deprivation achieved in this study were not sufficient to negatively affect the muscle tissue P homeostasis or the adenosine phosphate metabolism in periparturient dairy cows. It is highly improbable that the degree of dietary P deprivation that was achieved in the present study would occur in dairy cattle under field conditions in Europe or North America. Studies conducted in other species and using P-deficient diets covering 10% of daily P requirements or less over several weeks reported a decline of the P content not only in blood but also in muscle tissue. Dietary P deprivation in these studies resulted in a reduction in muscle tissue P content in the range of 20% after 5 wk in dogs and of 45% after 4 wk in mice (Fuller et al., 1976; Hettleman et al., 1983). It has been proposed that the intracellular P homeostasis is resistant to changes of the plasma [Pi] up to a certain degree due to the properties of the Na-linked P transport ensuring efficient cellular P uptake from the extracellular space despite an unfavorable chemical gradient (Kemp, 1993). Dietary P deprivation of comparable severity in dairy cattle was associated with rapid induction of counterregulation in the form of bone mobilization releasing Pi together with calcium from bone in mid-lactating dairy cows (Grünberg et al., 2015b). In this previous study, plasma [Pi] was observed to increase after 10 d of dietary P depletion, which was attributed to the efficacy of counterregulation. In the present study, an increase in plasma [Pi] was not observed until P was supplemented orally. However, the lower incidence of clinical hypocalcemia and higher plasma [Ca] around parturition in LP cows compared with AP cows suggest that bone mobilization was indeed triggered by dietary P deprivation. It is conceivable that in the present study the combined challenge of P homeostasis through dietary P deprivation and the onset of lactation resulted in increased bone mobilization in combination with enhanced cellular P uptake to maintain intracellular P homeostasis. Such a scenario of increased P flux would preclude an increase of plasma [Pi] despite bone mobilization while intracellular P homeostasis is maintained.

In the present study, experimental animals were fed dry cow rations with moderately elevated DCAD, which is more common in Europe than in the United States. Moderately alcalogenic diets are associated with an increased risk for clinical and subclinical periparturient hypocalcemia, and hypocalcemia is thought to contribute to and exacerbate periparturient hypophosphatemia through increased parathyroid (**PTH**) secretion in hypocalcemic animals (Horst, 1986). Following this hypothesis, alcalogenic diets used in this study thus may have exacerbated periparturient hypophosphatemia. It should however be noted that results obtained in a subset of study animals and published elsewhere revealed that cows on LP diets had markedly lower plasma PTH concentrations and higher plasma [Ca] than AP cows around parturition, indicating that dietary P depletion in late gestation was associated with markedly hampered PTH release. This would at least have attenuated the hypothesized exacerbation of periparturient hypophosphatemia by high-DCAD diets in AP cows (Cohrs et al., 2018).

Results of the electromyographic examination indicate that overall, functionality of muscle fibers was decreased in P-deprived animals, as can be concluded from the decrease in size index and lower MUAP amplitudes over time in P-deprived animals. Furthermore, pathologic spontaneous activity that is indicative of instable muscle fiber or nerve membranes became more frequent in LP cows as P deprivation progressed, especially in IC muscles. Increasing occurrence of pathologic spontaneous activity was previously reported in mid-lactating dairy cows that were fed a P-deficient diet over a course of 5 wk (Grünberg et al., 2015b). Membrane instability developing in states of P deficiency may be attributable to disturbed synthesis of phospholipids that form an integral part of cell membranes that is well documented (Subramanian and Khardori, 2000; Chen et al., 2018). Dietary P supplementation over a course of 2 wk resulted in partial reversal of the observed effects of P deprivation on MUAP parameters, whereas the occurrence of pathologic spontaneous activity remained unchanged or even progressed during the repletion period of this study. These findings indicate that in contrast to disturbances of muscle fiber functionality that seem to respond to correction of P depletion, the effects of membrane instability reflecting in increased pathologic spontaneous activity are not easily reversed.

Parameters of the MUAP analysis seemed to be associated with the plasma [Pi] rather than with the content of energy-carrying compounds of muscle tissue. This observation suggests that altered muscle fiber function is indeed associated with hypophosphatemia per se and thus extracellular P homeostasis rather than with depletion of energy stores of muscle tissue or disturbances of the intracellular P balance of muscle fibers. The negative association of ATP metabolites with several MUAP parameters identified in this study suggests that more and larger muscle fibers are activated with lower ATP availability to maintain comparable muscle strength (Kimura, 2001; Wijnberg and Franssen, 2016). This interpretation is supported by the positive

association of the number of turns per second and the M/T of the interference pattern analysis and ATP_{ww}, AMP_{ww}, CPh_{ww}, and plasma [Ca] determined at T_1 and T_2 but not at later time points. This proposed hypothesis implies a loss of relation between muscle strength and available ATP, AMP, and calcium in muscle tissue in states of P deprivation. Calcium, ATP, and AMP are all required for muscle contraction; the logically ensuing positive association of these parameters with interference pattern parameters characterizing muscle strength could be identified early in the study but was lost or even inversed as P depletion progressed. These results thus partly support the concept of disturbed muscle function in states of P deprivation.

Several of the MUAP parameters were found to be positively associated with plasma [Pi], with the MUAP size index showing one of the most consistent responses to P depletion. It is this parameter that is considered one of the more robust in human electromyographic examination (Sonoo and Stalberg, 1993; Wijnberg et al., 2011; Wijnberg and Franssen, 2016). The observed correlations between plasma [Pi] and MUAP parameters at the end of the P-deprivation period indicate that the lower the plasma [Pi], the lower the number of functional muscle fibers. Indeed, several authors mention muscle cell membrane and structural integration disturbances as causes of myopathy noticed in hypophosphatemic patients (Bugg and Jones, 1998; Subramanian and Khardori, 2000; Chen et al., 2018). If muscle fibers do not contribute to the MUAP, the summation of the individual action potential parameters decreases, which is reflected in lower values of MUAP parameters such as MUAP amplitude or size index. The positive association of MUAP parameters with plasma [Pi] at the time alterations of the MUAP were most prominent, whereas association related to intracellular P homeostasis such as ATP_{ww} or CPh_{ww} were absent, suggesting that disturbances of extracellular P homeostasis may etiologically contribute to the observed effects even without measurable changes of the intracellular P homeostasis. In this context, it should be noted that most prominent effects were identified on IC muscles while muscle tissue specimens for biopsy were obtained from BF muscle exclusively. In view of the obvious difference in sensitivity to P depletion of these 2 muscles, it is conceivable that the time–concentration curves for intracellular P compounds in BF muscles presented in this study may not be representative of IC muscles. The results of the correlation and regression analysis between parameters of the MUAP analysis in IC muscles and P-containing compounds of BF muscles presented in Tables 8 and 9 should thus be interpreted cautiously. Hypocalcemia was also found to have an effect on MUAP parameters as a result of increased temporal dispersion leading to increased MUAP duration, number of turns, and number of phases (Wijnberg et al., 2002b). To complicate the interpretation of the results of the regression and correlation analysis, it must be kept in mind that plasma [Pi] and muscle P content are not associated with each other, a finding corroborated by the results presented here (Grünberg et al., 2015b). Furthermore, important diurnal variation in plasma [Pi] occurs in mammals and in cattle in particular (Bugg and Jones, 1998; Grünberg, 2014).

The differences between BF and IC muscles observed in this study might be explained by differences in fiber type composition of these muscles. Intercostal muscle that require aerobic activity at all times contain more oxidative type I fibers than BF muscles (Greer and Martin, 1990), and hypophosphatemia has been associated with impaired oxygen release and tissue hypoxia in monogastric species (Subramanian and Khardori, 2000). It is thus conceivable that aerobic muscle fibers are more susceptible to P depletion than more anaerobic muscle fibers. Also, IC muscles are activated consistently with constant strength during voluntary breathing, which is associated with a more consistent recruitment pattern of motor units than in the BF muscle. This more uniform recruitment pattern and the presumably smaller degree of variation associated with this constant recruitment pattern is likely to facilitate the disclosure of effects compared with BF muscles, where contraction had to be triggered by lifting the contralateral hind limb.

The reported study has several limitations. Most important, housing study animals in tiestalls, which strongly limited physical exercise, may have contributed to a certain degree to the observed subclinical effects on muscle function. Part of the increase in frequency of pathologic spontaneous activity observed in both treatments could have been associated with changes occurring around parturition but may as well have been the result of prolonged exercise restriction. Also, the chosen study design does not allow even remotely estimating a threshold for dietary P depletion required to obtain observed subclinical effects.

Marked and sustained P depletion in transition cows associated with severe hypophosphatemia did not cause clinically apparent signs of muscle weakness or recumbency despite electromyographic abnormalities suggestive of subclinical myopathic alterations. Furthermore, P depletion and hypophosphatemia did not measurably disturb the P balance of muscle tissue in this study, as determined in the example of the BF muscle. The increasing occurrence of pathologic spontaneous activity in P-deprived animals indicates disturbances of muscle

fiber function as P deficiency progressed, possibly resulting from membrane instability of muscle or nerve cells. The association of MUAP parameters with the plasma [Pi] indicates that disturbed muscle function and altered strength of muscle fiber contractility are associated with disturbances of P homeostasis. The absence of clinically apparent muscle weakness despite severe hypophosphatemia does not support the concept of a role of P deprivation in periparturient recumbency of dairy cows. It remains to be determined whether the observed subclinical effects on neuromuscular function are relevant for the health and well-being of dairy cows.

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