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Targeted assessment of the metabolome in skeletal muscle and in serum of dairy cows supplemented with conjugated linoleic acid during early lactation

Y. Yang,[1]* H. Sadri,[2]†‡ C. Prehn,[3] J. Adamski,[3,4,5,6] J. Rehage,[7] S. Dänicke,[8] M. H. Ghaffari,[1] and H. Sauerwein[1]
[1]Institute of Animal Science, Physiology Unit, University of Bonn, 53115 Bonn, Germany
[2]Department of Clinical Science, Faculty of Veterinary Medicine, University of Tabriz, 5166616471 Tabriz, Iran
[3]Research Unit Molecular Endocrinology and Metabolism, Genome Analysis Center, Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg 85764, Germany
[4]Lehrstuhl für Experimentelle Genetik, Technische Universität München, Freising-Weihenstephan 85350, Germany
[5]German Center for Diabetes Research (DZD), München-Neuherberg 85764, Germany
[6]Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, 8 Medical Drive, Singapore 117597, Singapore
[7]University for Veterinary Medicine, Foundation, Clinic for Cattle, 30173 Hannover, Germany
[8]Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI), 38116 Braunschweig, Germany

ABSTRACT

In the dairy cow, late gestation and early lactation are characterized by a complexity of metabolic processes required for the homeorhetic adaptation to the needs of fetal growth and milk production. Skeletal muscle plays an important role in this adaptation. The objective of this study was to characterize the metabolome in skeletal muscle (semitendinosus muscle) and in serum of dairy cows in the context of the physiological changes occurring in early lactation and to test the effects of dietary supplementation (from d 1 in milk onwards) with conjugated linoleic acids (sCLA; 100 g/d; supplying 7.6 g of *cis*-9,*trans*-11 CLA and 7.6 g of *trans*-10,*cis*-12 CLA per cow/d; n = 11) compared with control fat-supplemented cows (CTR; n = 10). The metabolome was characterized in skeletal muscle samples collected on d 21 and 70 after calving in conjunction with their serum counterpart using a targeted metabolomics approach (AbsoluteIDQ p180 kit; Biocrates Life Sciences AG, Innsbruck, Austria). Thereby 188 metabolites from 6 different compound classes (acylcarnitines, amino acids, biogenic amines, glycerophospholipids, sphingolipids, and hexoses) were quantified in both sample types. In both groups, dry matter intake increased after calving. It was lower in sCLA than in CTR on d 21, which resulted in reduced calculated net energy and metabolizable protein balances. On d 21, the concentrations of dopamine, Ala, and hexoses in the skeletal muscle were higher in sCLA than in CTR. On d 21, the changed metabolites in serum were mainly long-chain (>C24) diacyl phosphatidylcholine PC (PC-aa) and acyl-alkyl phosphatidylcholine (PC-ae), along with lysophosphatidylcholine acyl (lysoPC-a) C26:1 that were all lower in sCLA than in CTR. Supplementation with CLA affected the muscle concentrations of 22 metabolites on d 70 including 10 long-chain (>C22) sphingomyelin (SM), hydroxysphingomyelin [SM(OH)], PC-aa, and PC-ae along with 9 long-chain (>C16) lysoPC-a and 3 metabolites related to amino acids (spermine, citrulline, and Asp). On d 70, the concentrations of lysoPC-a C18:2 and C26:0 in serum were higher in the sCLA cows than in the CTR cows. Regardless of treatment, the concentrations of Ile, Leu, Phe, Lys, His, Met, Trp, and hydroxybutyrylcarnitine (C4-OH) decreased, whereas those of ornithine, Gln, and *trans*-4-hydroxyproline (t4-OH-Pro) increased from d 21 to 70 in muscle. The significantly changed metabolites in serum with time of lactation were 28 long-chain (>C30) PC-ae and PC-aa, 7 long-chain (>C16) SM and SM(OH), along with lysoPC-a C20:3 that were all increased. In conclusion, in addition to other significantly changed metabolites, CLA supplementation mainly led to changes in muscle and serum concentrations of glycerophospholipids and sphingolipids that might reflect the phospholipid compositional changes in muscle. The metabolome changes observed in sCLA on d 21 seem to be, at least in part, due to the lower DMI in these cows. The changes in the muscle concentrations of AA from d 21 to 70, which coincided with the steady energy and MP balances, might reflect a shift of protein synthesis/degradation balance toward synthesis.

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*Present address: College of Animal Science, Guizhou University, 550025 Guiyang, China.
†H. Sadri was a visiting scientist at the Institute of Animal Science, Physiology and Hygiene Unit, University of Bonn, 53115 Bonn, Germany, at the time the research was done.
‡Corresponding author: sadri@tabrizu.ac.ir

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INTRODUCTION

Reduced feed intake during the last days of gestation and increased nutrient demand for milk synthesis at the onset of lactation commonly result in a negative nutrient balance (Drackley, 1999), which might compromise the ability of dairy cows to adapt to the physiological needs of pregnancy and lactation. Adaptive failure during this crucial period might impair animal health and performance (Drackley, 1999; Sundrum, 2015). Most of the approaches to help cows through this critical phase focused on understanding the regulation of energy metabolism, and the main target tissues were liver and adipose tissue; consequently, less attention has been paid to the metabolic and regulatory role of skeletal muscle in the adaptation to early lactation. Skeletal muscle, the largest internal organ in mammals, plays an important role in regulating the systemic metabolic homeostasis (Baskin et al., 2015). Muscle protein mobilization seems to start ante partum, even before the onset of lipid mobilization (van der Drift et al., 2012), to overcome negative nutrient balance by providing AA for milk protein synthesis, direct oxidation, and hepatic gluconeogenesis, as well as for the immune system (Plaizier et al., 2000; Kuhla et al., 2011; Sadri et al., 2016). Moreover, the oxidative capacity of skeletal muscle for fatty acids (**FA**) released from adipose tissue lipolysis might also contribute to reducing the metabolic load of FA on the liver (Kuhla et al., 2011; Schäff et al., 2013), although the quantitative contribution of skeletal muscle to FA metabolism in dairy cows in early lactation is unknown.

In recent decades, dietary supplementation with CLA has gained special attention in human and animal nutrition due to the many bioactive health-related properties of CLA. Supplementation with *trans*-10,*cis*-12 CLA has evolved as a dietary strategy used in dairy cows, especially in early lactation for reducing milk fat content while improving energy balance to counteract the physiological negative energy balance (Sippel et al., 2009; Schlegel et al., 2012; Qin et al., 2018). In most animal feeding trials applying CLA, the consideration of skeletal muscle was limited to the assessment of the CLA tissue concentrations (e.g., Enser et al., 1999; De La Torre et al., 2006; Pinelli-Saavedra et al., 2019). However, in view of reports attributing CLA a role in modulating muscle metabolism in humans and in laboratory animals (Lehnen et al., 2015; Kim et al., 2016a,b), analogous effects in dairy cows are of interest. In human studies, characterizing the muscle metabolome is considered as a promising tool for understanding the metabolic processes related to the energetic status, glucose, and FA metabolism (Fazelzadeh et al., 2016; Xiang et al., 2017; Starnes et al., 2017); however, the studies performed in cattle to explore the metabolome in skeletal muscle are mostly limited to beef animals and meat (Goldansaz et al., 2017), whereas there has been only one paper dealing with dairy cows until now (Sadri et al., 2020). Using a targeted metabolomics approach, we tested the hypothesis that dietary supplementation with CLA would alter the lactation-related metabolomic signatures in skeletal muscle and in blood serum of dairy cows. Thus, the objective of this study was to characterize the skeletal muscle metabolome in conjunction with its serum counterpart in the context of the physiological changes occurring during early lactation in dairy cows as influenced by a rumen-protected CLA supplement.

MATERIALS AND METHODS

Animals, Treatment, and Experimental Design

The experimental procedures performed in this study were in accordance with the European Community regulations on the protection of experimental animals and the guidelines of the LAVES (Lower Saxony State Office for Consumer Protection and Food Safety, Germany, File Number 33.14.42502–04–071/07). The basic set-up of the study together with performance data has been described in detail previously (Pappritz et al., 2011). In the current study, a subset of animals and samples from this study were used. In brief, 1 d after calving, 21 multiparous Holstein cows were allocated to either a control (**CTR**; n = 10) group or a supplemental CLA (**sCLA**; n = 11) group. Cows were fed with a partial mixed ration (**PMR**) for ad libitum intake, according to the recommendations of the German Society of Nutrition Physiology (GfE, 2001). The PMR (6.8 MJ of NE_L/kg of DM) consisted of 37.8% corn silage, 25.2% grass silage, and 37% concentrate (on a DM basis). From DIM 1 throughout the observation period, the fat supplements were given with 4 kg of additional concentrate (8.8 MJ of NE_L/kg of DM) consisting of the same components as the concentrate used in the PMR. The animals in the sCLA group received a diet supplemented with rumen-protected CLA (Lutrell Pure, BASF SE, Ludwigshafen, Germany; 100 g per cow/d), providing 7.6 g of *cis*-9,*trans*-11 CLA and 7.6 g of *trans*-10,*cis*-12 CLA per day and cow (Pappritz et al., 2011). The CTR cows were fed a diet that included 100 g/d of rumen-protected control fat supplement (Silafat, BASF SE) in which CLA was substituted by stearic acid (C18:0) to form an isoenergetic control diet.

Blood and Muscle Tissue Sampling

Blood samples were collected via puncture of the jugular vein using evacuated tubes on d 1, 21, and 70 after parturition after the morning milking before cows had access to fresh feed and before collecting the muscle biopsies (see below). The centrifugation (at 4°C and 3,000 × *g* for 10 min) of plasma (heparin and EDTA) and serum was performed after sampling, and the samples were stored at −80°C for further analysis.

Muscle tissue samples were collected on d 21 and 70 after parturition under aseptic conditions from the semitendinosus muscle of the left or right hind limb about 10 cm ventrally of the tuber ischiadicum. At the biopsy site, the skin was shaved, washed, dried with paper towels, degreased with medical alcohol, and disinfected with iodine. Biopsies were taken by means of the Magnum Biopsy System (penetration depth 22 mm) using 12G Magnum Biopsy Needles, which were introduced through a 11G Bard TrueGuide coaxial needle (Bard GmbH, Karlsruhe, Germany). Thereafter, the skin wound was covered by aluminum spray (aniMedica GmbH, a Livisto company, Senden-Bösensell, Germany). For pain management, a sacral epidural anesthesia (7–8 mL Procain 2%; Pracasel 2%, Selectavet, Weyarn-Holzolling, Germany) was applied and the biopsy site infiltrated subcutaneously with 5 mL procaine 2%. For analgesia, ketoprofen (3 mg/kg BW IM; Romefen PR10%, CEVA Tiergesundheit GmbH, Düsseldorf, Germany) was administered for 2 consecutive days. Tissue samples were snap-frozen in liquid nitrogen and stored at −80°C until analysis. The muscle samples collected from one sCLA animal (DIM 21 and 70) and 2 CTR animals (DIM 70) had been expended for other analyses, and the amount of tissue remaining was insufficient for a reliable analysis.

Analysis of NEFA, Glucose, and Insulin in Blood Samples

Glucose was assayed in NaF-EDTA plasma; for quantification of NEFA and insulin, heparinized plasma was used. The plasma concentrations of glucose and NEFA were determined by an automatic analyzer (Cobas Mira Plus System, Roche Diagnostica Ltd., Basel, Switzerland) using commercial test kits (Glucose: Glucose Hexokinase Fluid 5+1, MTI Diagnostics GmbH, Idstein, Germany; NEFA: HR(2) R1+R2 Set, WAKO Chemicals GmbH, Neuss, Germany). The concentrations of insulin in plasma were measured using a commercially available antibody radioimmunoassay (DSL-1600, Diagnostic Systems Laboratories Inc., Webster, TX). The intra- and inter-assay coefficients of variation were 6.3% and 8.8%, respectively (Saremi et al., 2014).

Metabolite Profiling

The metabolome was characterized in muscle and serum samples by a targeted quantitative metabolomics approach employing a commercially available kit (AbsoluteIDQ p180 Kit; Biocrates Life Sciences AG, Innsbruck, Austria). The metabolite panel consists of 188 different metabolites including free carnitine, 40 acylcarnitines (Cx:y), 21 amino acids (19 proteinogenic amino acids, citrulline, and ornithine), 21 biogenic amines, hexose (H1; the sum of hexoses – about 90–95% glucose), 90 glycerophospholipids [14 lysophosphatidylcholines (lysoPC) and 76 phosphatidylcholines [PC diacyl (aa) and acyl-alkyl (ae)], and 15 sphingolipids (SMx:y). The complete list of the metabolites is given in Supplemental Table S1 (https://data.mendeley.com/datasets/nckrvjrjkf/1). The abbreviations, written as Cx:y, are used to describe the total number of carbons and double bonds of all chains, respectively. Sample preparation and all analyses were performed in the Helmholtz Zentrum München (GmbH), German Research Center for Environmental Health, Genome Analysis Center, Neuherberg, Germany. In the case of serum, 10 μL of the thawed sample was directly applied to the assay. In the case of muscle, 25 mg of frozen sample was homogenized and extracted using homogenization tubes containing ceramic beads with a diameter of 1.4 mm and a Precellys 24 homogenizer equipped with an integrated cooling unit (PEQLAB Biotechnology GmbH, Darmstadt, Germany). Three microliters of a dry ice-cooled mixture of ethanol/phosphate buffer (85/15, vol/vol) was added to each milligram of frozen muscle tissue. After centrifugation, 10 μL of the homogenate supernatant was applied to the well plate of the p180 kit. The detailed description of the tissue preparation, the assay procedures of the AbsoluteIDQ p180 Kit, as well as the metabolite nomenclature have been described in detail elsewhere (Zukunft et al., 2013, 2018). Sample handling was performed using a Hamilton Microlab STAR robot (Hamilton Bonaduz AG, Bonaduz, Switzerland) and an Ultravap nitrogen evaporator (Porvair Sciences, Leatherhead, UK) along with standard laboratory equipment. Mass spectrometric analyses were conducted using an API 4000 triple quadrupole instrument (Sciex Deutschland GmbH, Darmstadt, Germany) equipped with a 1200 Series HPLC (Agilent Technologies Deutschland GmbH, Böblingen, Germany) and an HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland) controlled by the software Analyst 1.6.1 (Sciex Deutschland GmbH). Data evaluation for quantification of muscle and serum metabolite concentrations and quality assessment was performed with the software MultiQuant 3.0.1 (Sciex Deutschland GmbH) and the Met*IDQ* software pack-

age (Biocrates Life Sciences AG), which is an integral part of the AbsoluteIDQ p180 kit. Internal standards were used as a reference to calculate the metabolite concentrations. The concentrations in muscle tissue samples are given in picomoles per milligram of tissue, and the concentrations of the serum samples are given in micromoles per liter.

Calculations and Statistical Analyses

The diet used for the experimental cows was evaluated by the NittanyCow Dairy Ration Evaluator (Mifflinburg, PA) to calculate postpartum net energy (NE_L) and MP balances for each cow according to the National Research Council (NRC) model (NRC, 2001). The NE_L balance was calculated with the following formula: NEB = (DMI × NE_L diet) − [(0.08 × $BW^{0.75}$) + (0.0929 × fat + 0.0563 × protein + 0.0395 × lactose) × milk yield]. In the case of MP balance, the supply of the different protein fractions and AA balances was estimated using actual DMI, milk yield, milk composition, BCS, and BW of the cows as explained in detail elsewhere (Yang et al., 2020). Feed efficiency was calculated as the ratio between 4% FCM (kg) and DMI, where FCM (4%) = 0.4 × kg of milk + 15 × kg of milk × fat content. Statistical analysis of the data for DMI, NE_L, and MP balances, feed efficiency, and blood parameters were analyzed using repeated measures in the MIXED procedure of SAS software (version 9.3; SAS Institute Inc., Cary, NC). The model included treatment, time, and interaction of treatment × time as fixed effects and cow as random effect. The first-order autoregressive covariance structure was determined as the most appropriate covariance structure for all repeated statements according to the information criteria of Akaike and Bayes. The Tukey-Kramer adjustment was applied to account for multiple comparisons. The data were tested for normality before analysis using the UNIVARIATE procedure and were log-transformed (base 10) when data were not normally distributed. The threshold of significance was set at $P \leq 0.05$, and trends were declared at $0.05 < P \leq 0.10$.

The statistical analysis of the muscle and serum metabolite data was carried out using the web-based metabolomics data processing tool MetaboAnalyst 4.0 (Chong et al., 2018). According to the "80% rule" proposed by Bijlsma et al. (2006), metabolites with more than 20% of missing values (i.e., values lower than the limit of detection; Supplemental Table S2, https://data.mendeley.com/datasets/nckrvjrjkf/1) were not considered for the statistical analysis. A total of 178 (94.7%) and 185 (98.4%) metabolites yielded useful measurements for the statistical evaluation in serum and muscle samples, respectively. Thus, only 10 and 3 metabolites, respectively, in serum and muscle were not considered for the statistical analysis. In case of missing values within those metabolites considered for the statistical analysis, the Bayesian Principal Component Analysis (BPCA) method offered by MetaboAnalyst (http://www.metaboanalyst.ca) was used to estimate the values of missing data. The metabolite data were transformed using generalized log transformation and then Pareto scaled to correct for heteroskedasticity, to reduce the skewness of the data and to reduce mask effects (van den Berg et al., 2006). Volcano plots were created to provide an overview of the significantly affected metabolites. Volcano plots display the size of the biological effect (fold-change) versus the statistical significance of the result. Important metabolites were identified by the volcano plot based on the fold-change threshold of 1.5 on the x-axis and *t*-tests threshold (*P*-value) of 0.05 on the y-axis. The metabolites determined through the use of a volcano plot were then analyzed using estimation methods and are presented as mean difference estimation plots (Claridge-Chang and Assam, 2016). Effect size was measured using Hedges' g (Greenland et al., 2016) and defined as "trivial" ($g < 0.2$), "small" ($0.2 < g < 0.5$), "moderate" ($0.5 < g < 0.8$), or "large" ($g > 0.8$) as per standard practice. To estimate precision, 95% confidence intervals for mean differences were calculated by bootstrap methods (resampled 5,000 times, bias-corrected, and accelerated). The Mann-Whitney U-test was used to calculate *P*-values for pro forma reporting exclusively (Crichton, 2000).

RESULTS

Blood Parameters, DMI, Calculated NE_L and MP Balances, and Feed Efficiency

The time courses of the plasma NEFA, glucose, and insulin concentrations are presented in Figure 1. Plasma NEFA concentrations did not differ between the groups but decreased in both groups postpartum ($P < 0.01$). The plasma concentrations of glucose were affected by time ($P < 0.01$), and the overall treatment effect was significant ($P = 0.04$). The mean concentrations of glucose across all time points in sCLA were greater than in CTR. Plasma insulin concentrations were affected by CLA supplementation ($P = 0.02$) and decreased ($P < 0.01$) with time of lactation in both groups but to a greater extent in CTR than in sCLA, resulting in lower ($P < 0.05$) insulin concentrations on d 21 and 70 in CTR than in sCLA.

Dry matter intake, and calculated NE_L balance and feed efficiency are presented in Figure 1. Postpartum DMI was 21.2 ± 0.24 kg/d (mean ± SEM) and 22.3 ± 0.24 kg/d in the sCLA and CTR groups, respectively.

The DMI increased ($P < 0.01$) in both groups postpartum, and an interaction of treatment × time was observed ($P < 0.01$). The DMI on d 21 was greater in CTR than in sCLA ($P < 0.01$). The effect of CLA on calculated energy balance depended on time in lactation ($P = 0.02$). The NE_L balance was estimated to be

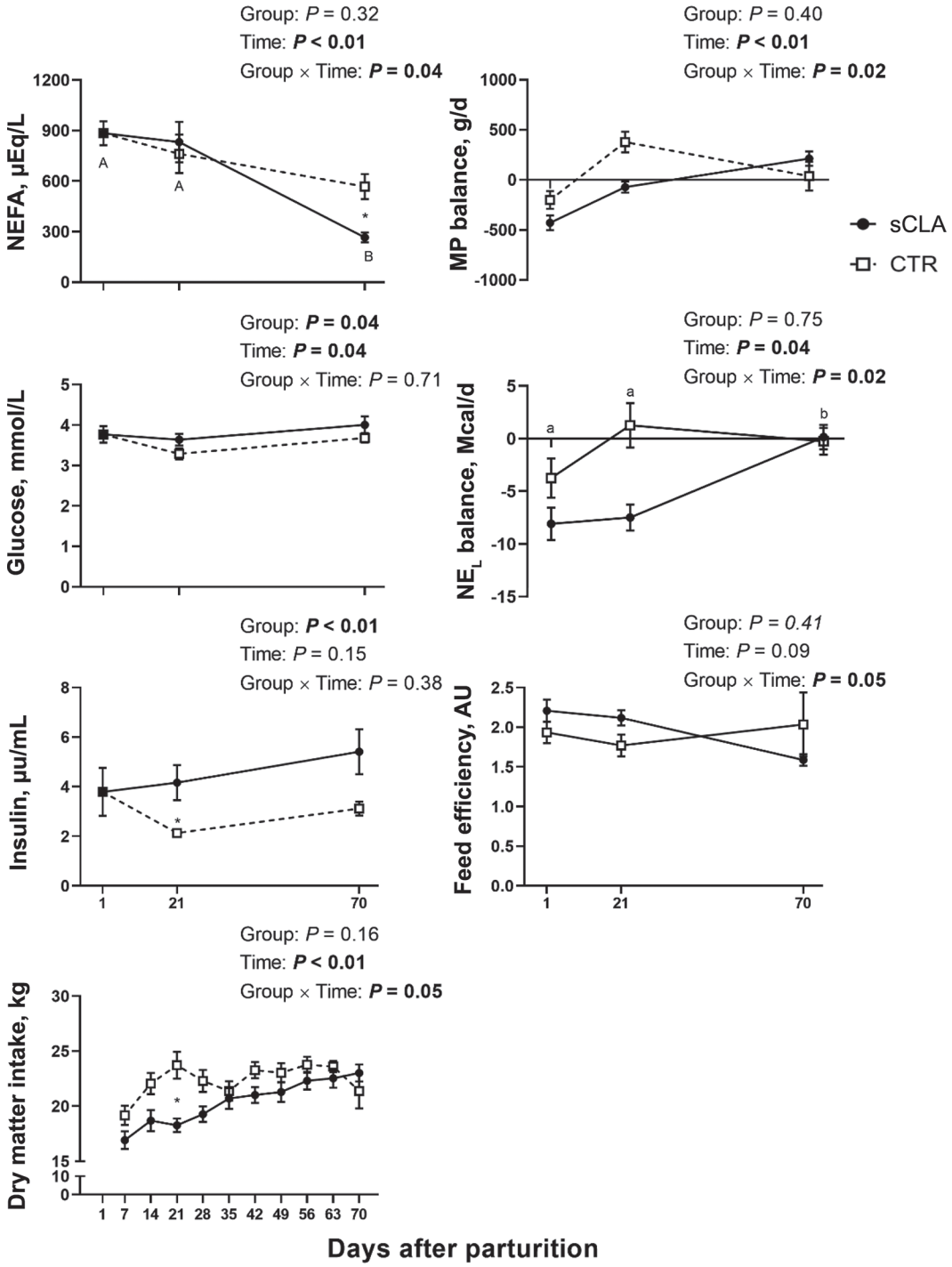

Figure 1. Time course of the circulating concentrations of nonesterified fatty acids (NEFA), glucose, and insulin, of DMI, MP balance, NE_L balance, and feed efficiency (4% FCM/DMI) in dairy cows during early lactation. Supplementation with CLA (sCLA) or control fat (CTR) was initiated at d 1 of lactation. Different letters indicate differences ($P < 0.05$) between the time points in the control (CTR; a, b) and in the sCLA (A, B) group. Asterisks indicate differences ($P < 0.01$) between sCLA and CTR at a given time point. Data are presented as means ± SEM. Data for NEFA, glucose, insulin, and DMI are from Saremi et al. (2014) and Yang et al. (2020).

about −8.09 ± 1.54 MCal/d (mean ± SEM) and −3.74 ± 1.86 MCal/d on d 1 in sCLA and CTR, respectively. The sCLA group was estimated to be still deficient in NE_L intake on d 21 (−7.45 ± 1.24 MCal/d), whereas the animals of the CTR group reached a steady NE_L balance. Both sCLA and CTR groups were in a steady energy balance on d 70. The effect of CLA on MP balance was dependent on time in lactation ($P = 0.02$). The MP balance was estimated to be about −201 ± 89 g/d (mean ± SEM) and −429 ± 74 g/d on d 1 in CTR and sCLA, respectively. The animals of the sCLA groups were estimated to be still deficient in MP intake on d 21 (−72 ± 56 g/d), whereas the CTR group had a positive calculated MP intake balance on d 21 (377 ± 104 g/d). Both groups had a positive calculated MP intake balance on d 70 (212 ± 72.9 and 173 ± 51 g/d, respectively). The calculated feed efficiency was affected by neither treatment nor time, but a treatment × time interaction was observed ($P = 0.05$). The calculated feed efficiency on d 21 was slightly greater in sCLA than in CTR, but it did not reach the level of significance after correction for multiple comparisons.

Muscle and Serum Metabolome

The univariate analysis with volcano plot was carried out to obtain a preliminary overview of the muscle and serum metabolites that are contributing most to discriminating sCLA and CTR cows on d 21 and 70 (Figures 2, 3, 4, and 5).

The muscle concentrations of dopamine, Ala, and hexoses (H1) were greater (effect size, $g \geq 0.99$; $P \leq 0.04$) in sCLA than in CTR on d 21 (Figure 2). On d 21, the serum concentrations of carnosine ($g = 1.68$; $P < 0.01$) were greater in the sCLA cows as compared

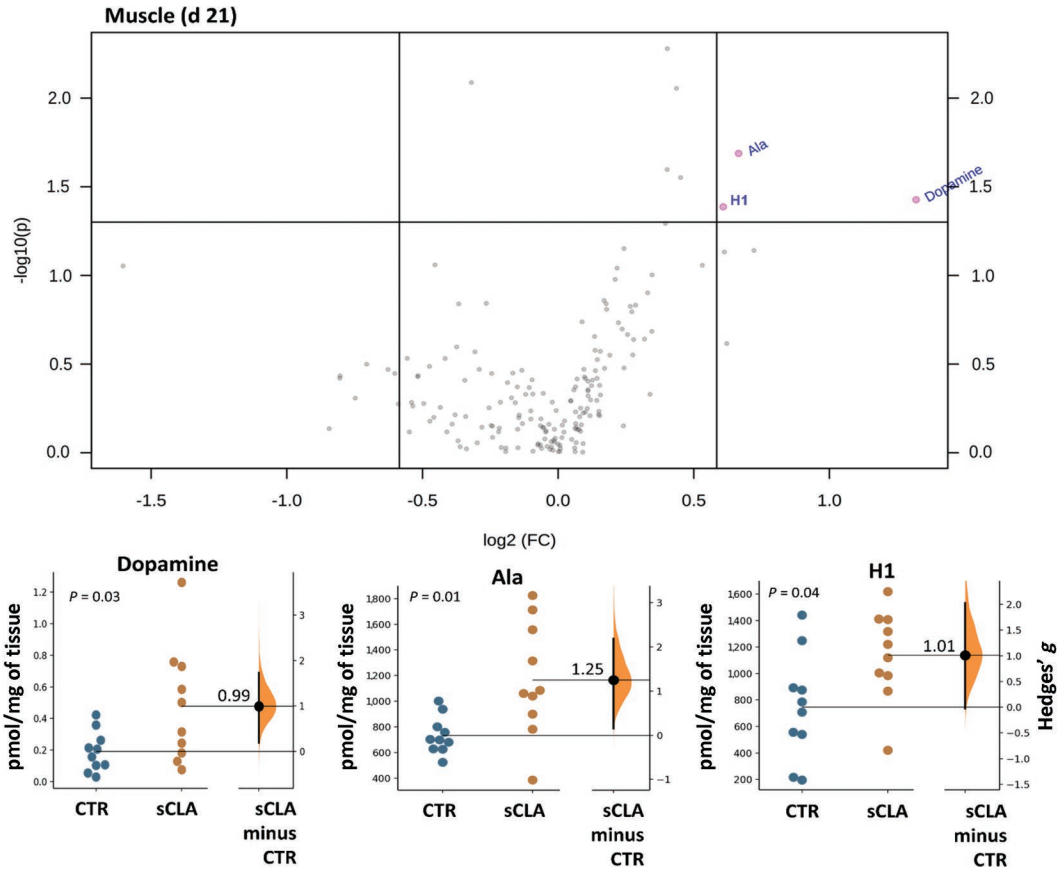

Figure 2. Volcano plot visualizing muscle metabolites in dairy cows on d 21 after calving. Supplementation with CLA (sCLA; n = 10) or control fat (CTR; n = 10) was initiated at d 1 of lactation. The x-axis represents the mean of $\log_2$ fold-change (FC) value, and the y-axis corresponds to the negative logarithm of the *P*-values. Each circle represents a single metabolite. The horizontal line represents the level of significance for the *t*-tests performed (0.05), and the vertical lines display the threshold set for fold-change (1.5). The pink circles show metabolites that were significantly changed by the CLA supplementation, and the gray circles show metabolites that were not significantly changed. Effect size is shown as Hedges' *g* between sCLA and CTR cows in the Gardner–Altman estimation plots of muscle metabolites. Both groups are plotted on the left axes; the mean difference is plotted on floating axes on the right as a bootstrap sampling distribution. The mean difference is depicted as a dot. H1 = hexose (sum of hexoses, about 90–95% glucose).

with the CTR cows, whereas those of methionine sulfoxide (Met-SO; $g = -1.24$; $P < 0.01$), PC-aa C24:0, C40:1, C40:2, C42:0, C42:1, C42:2, C42:4 ($g \leq -1.04$; $P \leq 0.02$), and PC-ae C38:1, C38:2, C40:1, C40:3, C40:4, C42:0, C44:6, C42:1, C42:2, C42:3, C42:4, C42:5, C44:3, C44:4, C44:5 ($g \geq -0.96$; $P \leq 0.02$), as well as LysoPC-a C26:1 showed a reverse change ($g = -1.01$; $P = 0.02$; Figure 3).

On d 70, the muscle concentrations of spermine, citrulline, Asp ($g \geq 0.88$; $P \leq 0.02$), SM C24:0, C26:1 ($g \geq 0.88$; $P \leq 0.05$), hydroxysphingomyelin [**SM(OH)**] C22:1, C24:1 ($g \geq 2.06$; $P < 0.01$), PC-aa C26:0, C32:0, C30:0, C28:1 ($g \geq 0.88$; $P \leq 0.05$), PC-ae C34:0, C30:1 ($g \geq 0.84$; $P \leq 0.03$), and lysoPC-a C18:2, C20:3, C26:1, C28:0, C28:1, C20:4, C16:1, C18:1, C26:0 ($g \geq 0.96$; $P \leq 0.05$, except for C20:4: $g = 0.80$; $P = 0.09$) were greater in the sCLA cows compared with the CTR cows (Figure 4). The serum concentrations of lysoPC-a C18:2 and C26:0 ($g \geq 1.11$; $P \leq 0.02$) were greater in sCLA than in CTR on d 70 (Figure 5).

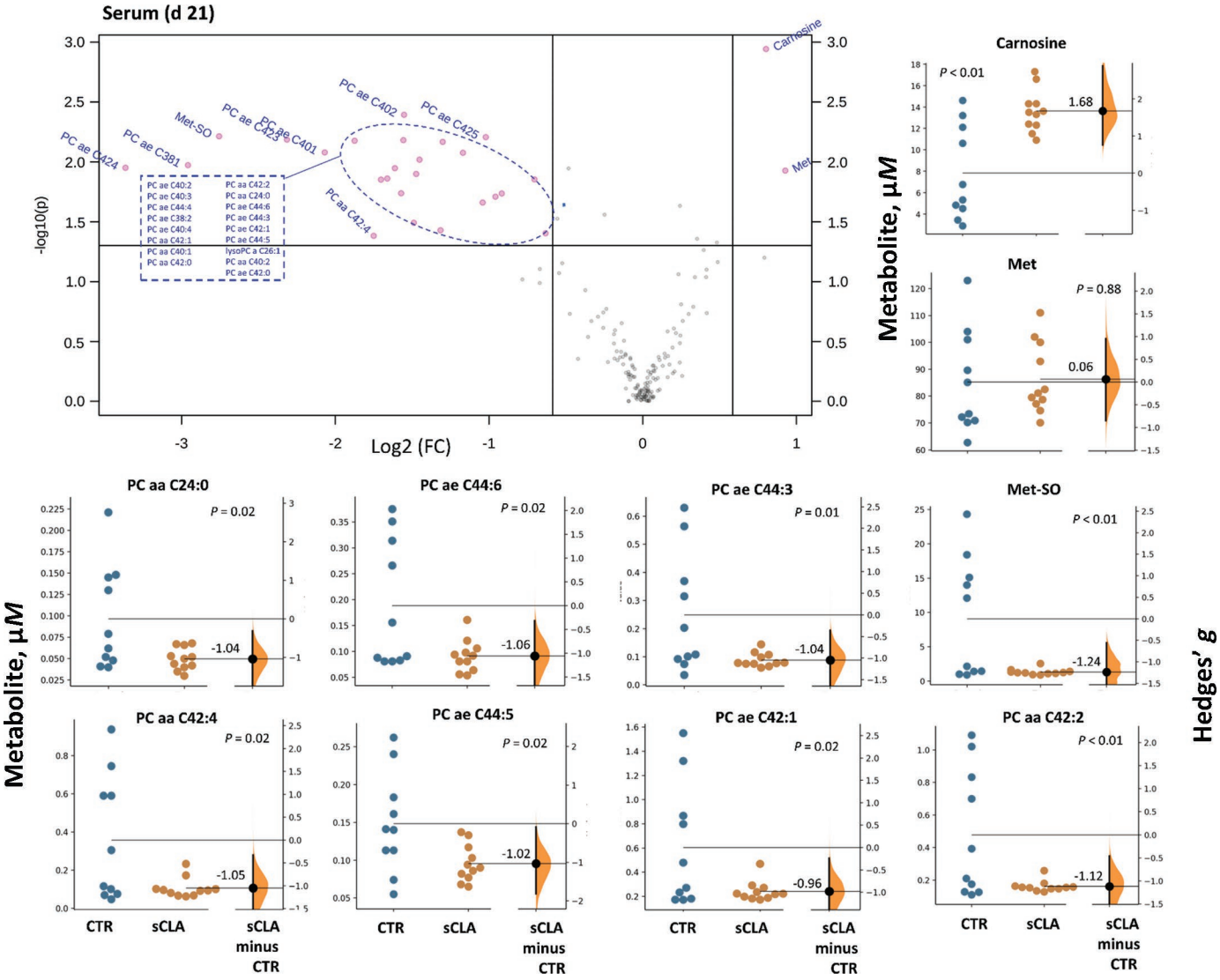

Figure 3. Volcano plot visualizing serum metabolites in dairy cows on d 21 after calving. Supplementation with CLA (sCLA; n = 11) or control fat (CTR; n = 10) was initiated at d 1 of lactation. The x-axis represents the mean of $\log_2$ fold-change (FC) value, and the y-axis corresponds to the negative logarithm of the *P*-values. Each circle represents a single metabolite. The horizontal line represents the level of significance for the *t*-tests performed (0.05), and the vertical lines display the threshold set for fold-change (1.5). The pink circles show metabolites that were significantly changed by the CLA supplementation, and the gray circles show metabolites that were not significantly changed. Effect size is shown as Hedges' *g* between sCLA and CTR cows in the Gardner–Altman estimation plots of muscle metabolites. Both groups are plotted on the left axes; the mean difference is plotted on floating axes on the right as a bootstrap sampling distribution. The mean difference is depicted as a dot. Met-SO = methionine sulfoxide; PC-aa = diacyl phosphatidylcholine; PC-ae = acyl-alkyl phosphatidylcholine; lysoPC-a = lysophosphatidylcholine acyl.

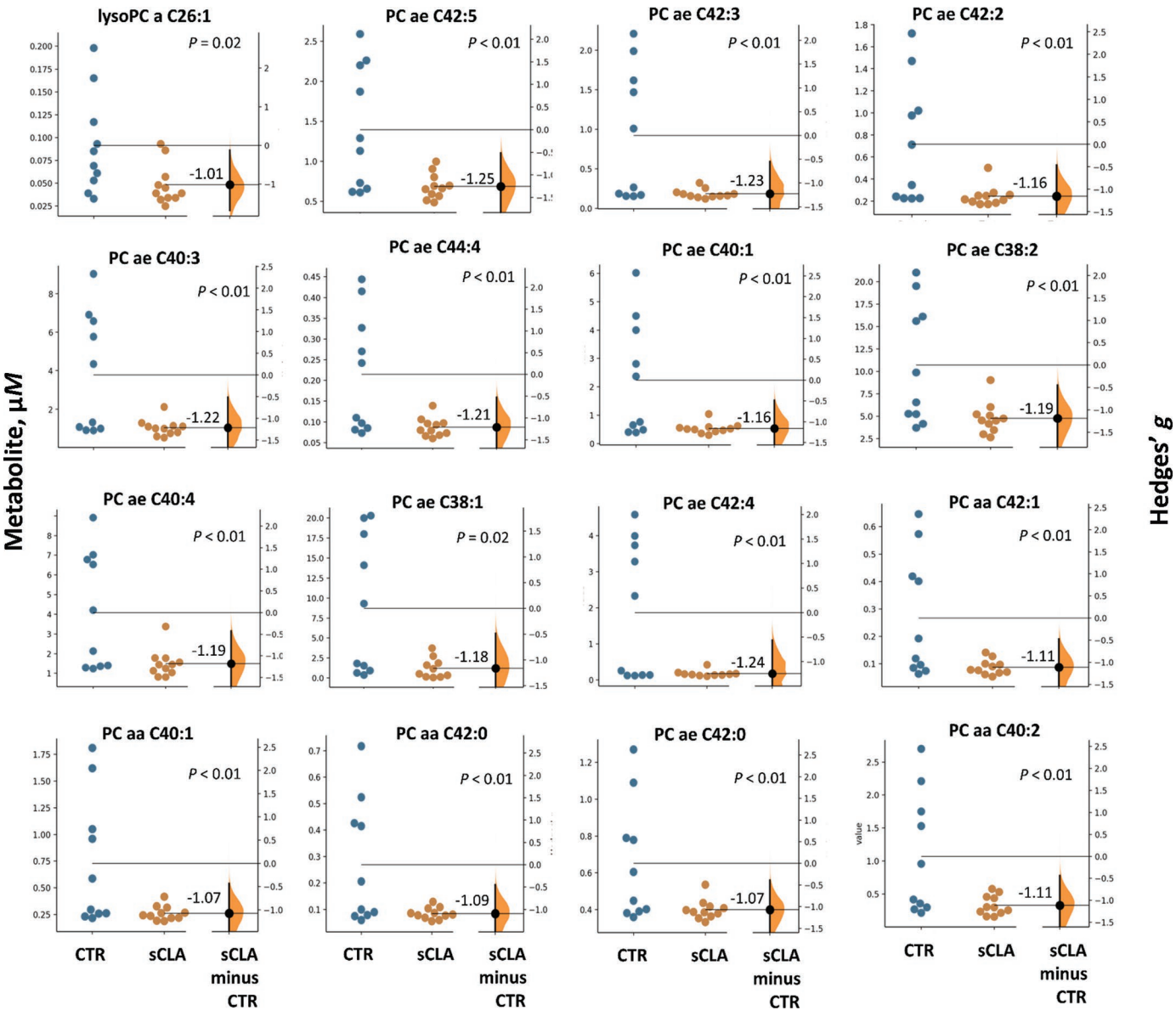

Figure 3 (Continued). Volcano plot visualizing serum metabolites in dairy cows on d 21 after calving. Supplementation with CLA (sCLA; n = 11) or control fat (CTR; n = 10) was initiated at d 1 of lactation. The x-axis represents the mean of $\log_2$ fold-change (FC) value, and the y-axis corresponds to the negative logarithm of the *P*-values. Each circle represents a single metabolite. The horizontal line represents the level of significance for the *t*-tests performed (0.05), and the vertical lines display the threshold set for fold-change (1.5). The pink circles show metabolites that were significantly changed by the CLA supplementation, and the gray circles show metabolites that were not significantly changed. Effect size is shown as Hedges' *g* between sCLA and CTR cows in the Gardner–Altman estimation plots of muscle metabolites. Both groups are plotted on the left axes; the mean difference is plotted on floating axes on the right as a bootstrap sampling distribution. The mean difference is depicted as a dot. Met-SO = methionine sulfoxide; PC-aa = diacyl phosphatidylcholine; PC-ae = acyl-alkyl phosphatidylcholine; lysoPC-a = lysophosphatidylcholine acyl.

The univariate analysis with volcano plot was also carried out to identify those metabolites that differed (1.5-fold-change and $P < 0.05$) in muscle (Supplemental Figure S1, https://data.mendeley.com/datasets/nckrvjrjkf/1) and serum (Supplemental Figure S2, https://data.mendeley.com/datasets/nckrvjrjkf/1) across the time points (regardless of treatment). The muscle concentrations of Ile, Leu, Phe, Lys, His, Met, Trp, and hydroxybutyrylcarnitine (C4-OH) decreased from d 21 to 70, whereas those of Orn, Gln, and *trans*-4-hydroxyproline (t4-OH-Pro) increased. The serum concentrations of PC-ae C30:0, C30:1, C32:2, C34:0,

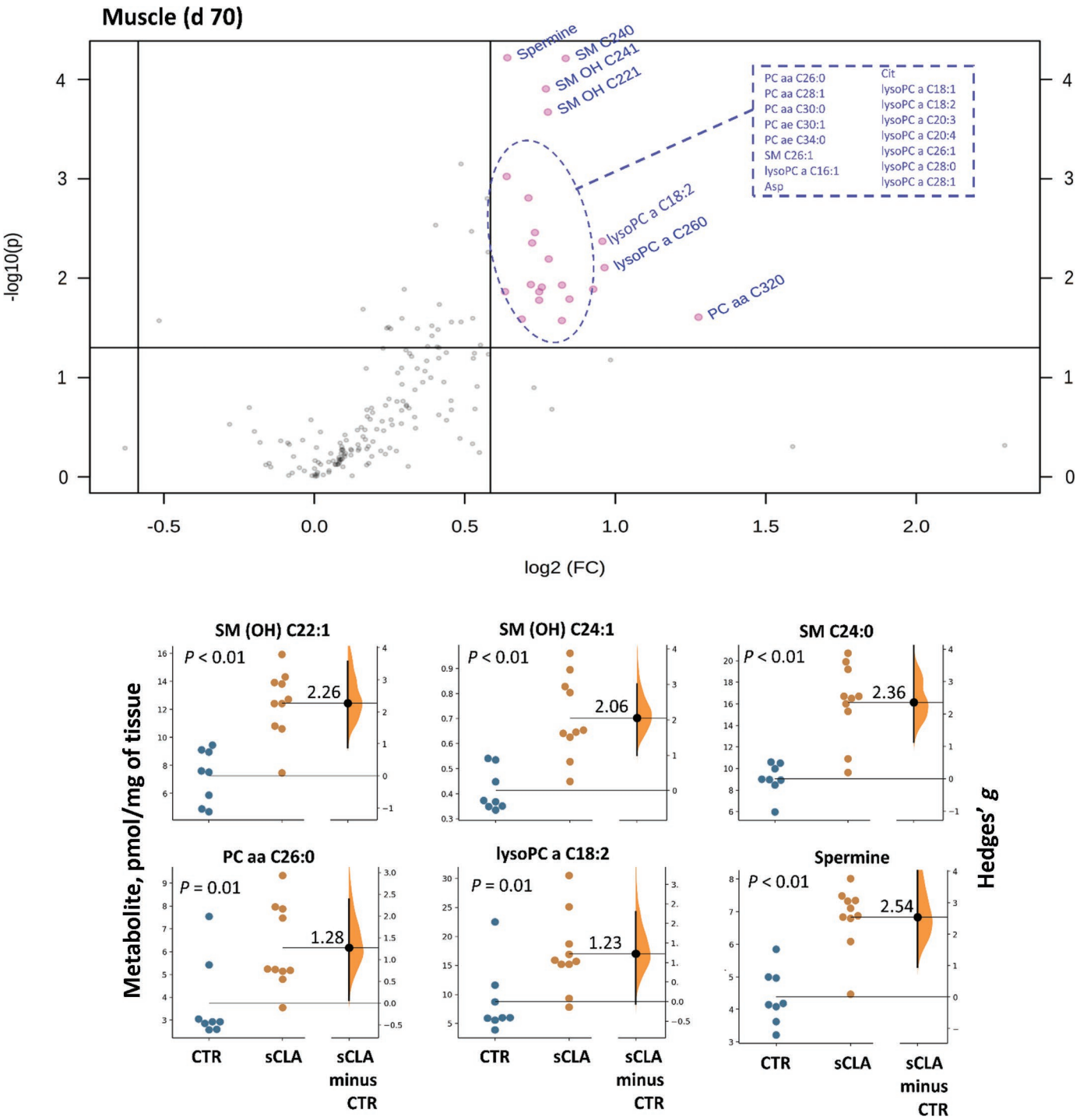

Figure 4. Volcano plot visualizing muscle metabolites in dairy cows on d 70 after calving. Supplementation with CLA (sCLA; n = 10) or control fat (CTR; n = 8) was initiated at d 1 of lactation. The x-axis represents the mean of $\log_2$ fold-change (FC) value, and the y-axis corresponds to the negative logarithm of the *P*-values. Each circle represents a single metabolite. The horizontal line represents the level of significance for the *t*-tests performed (0.05), and the vertical lines display the threshold set for fold-change (1.5). The pink circles show metabolites that were significantly changed by the CLA supplementation, and the gray circles show metabolites that were not significantly changed. Effect size is shown as Hedges' *g* between sCLA and CTR cows in the Gardner–Altman estimation plots of muscle metabolites. Both groups are plotted on the left axes; the mean difference is plotted on floating axes on the right as a bootstrap sampling distribution. The mean difference is depicted as a dot. LysoPC-a = lysophosphatidylcholine acyl; PC-aa = diacyl phosphatidylcholine; PC-ae = acyl-alkyl phosphatidylcholine; SM = sphingomyelin; SM(OH) = hydroxysphingomyelin.

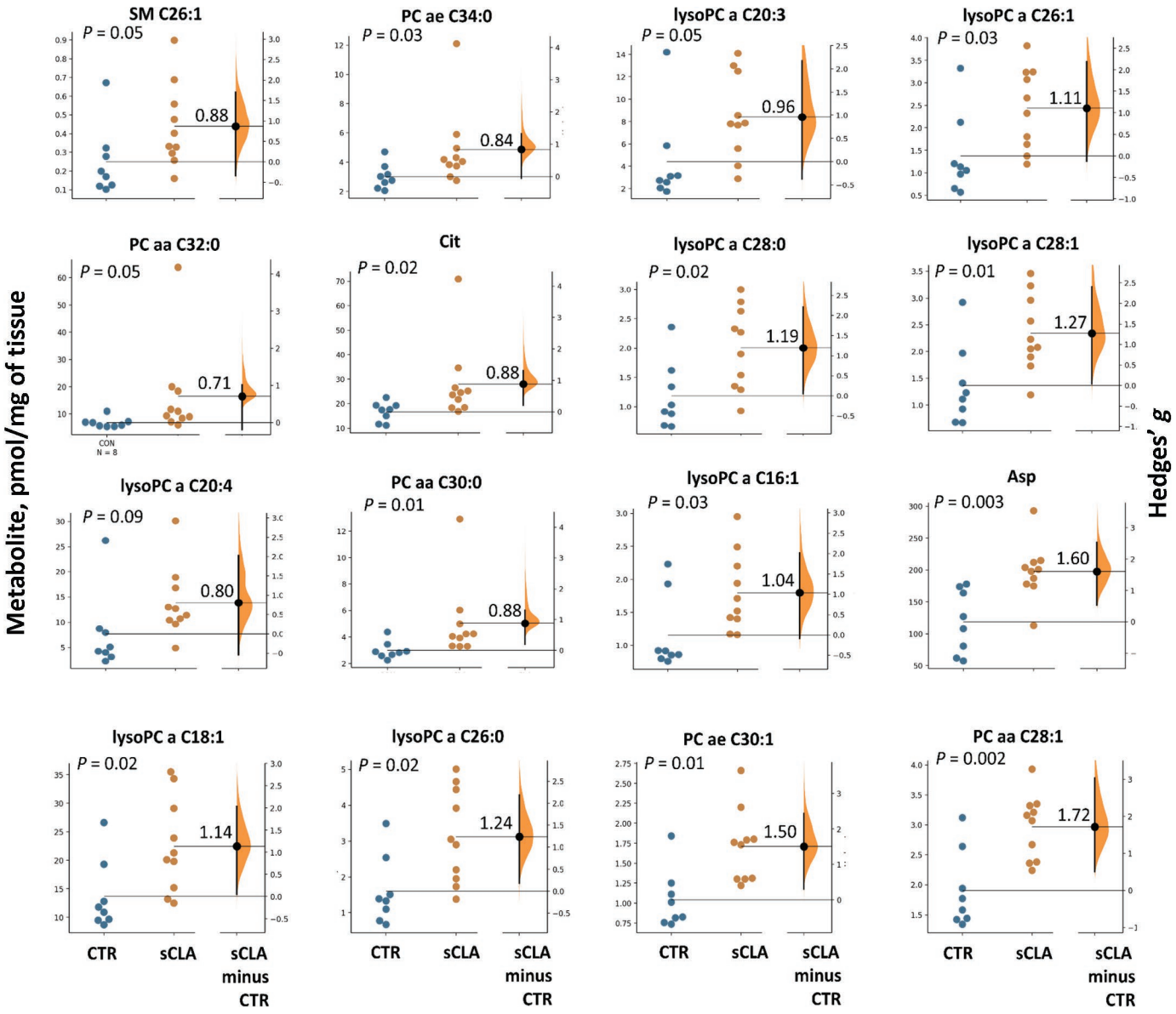

Figure 4 (Continued). Volcano plot visualizing muscle metabolites in dairy cows on d 70 after calving. Supplementation with CLA (sCLA; n = 10) or control fat (CTR; n = 8) was initiated at d 1 of lactation. The x-axis represents the mean of $\log_2$ fold-change (FC) value, and the y-axis corresponds to the negative logarithm of the *P*-values. Each circle represents a single metabolite. The horizontal line represents the level of significance for the *t*-tests performed (0.05), and the vertical lines display the threshold set for fold-change (1.5). The pink circles show metabolites that were significantly changed by the CLA supplementation, and the gray circles show metabolites that were not significantly changed. Effect size is shown as Hedges' *g* between sCLA and CTR cows in the Gardner–Altman estimation plots of muscle metabolites. Both groups are plotted on the left axes; the mean difference is plotted on floating axes on the right as a bootstrap sampling distribution. The mean difference is depicted as a dot. LysoPC-a = lysophosphatidylcholine acyl; PC-aa = diacyl phosphatidylcholine; PC-ae = acyl-alkyl phosphatidylcholine; SM = sphingomyelin; SM(OH) = hydroxysphingomyelin.

C34:2, C34:3, C36:0, C36:2, C36:3, C36:4, C36:5, C38:0, C38:3, C38:4, C38:5, PC-aa C32:2, C32:3, C34:4, C36:0, C36:2, C36:3, C38:0, C38:1, C38:3, C40:3, C40:4, C42:4, C42:5, SM C16:1, C18:1, C20:2, C24:0, C26:0, SM(OH) C16:1, C24:1, lysoPC-a C20:3, and serotonin increased from d 21 to 70, whereas those of acetylcarnitine (C2) showed the reverse change.

DISCUSSION

In the current study, sample preparation and all analyses were performed almost 4 yr after collecting the samples (with no previous thawing and freezing cycle). Thus, the absolute concentrations in these samples might vary due to the storage period, though the rela-

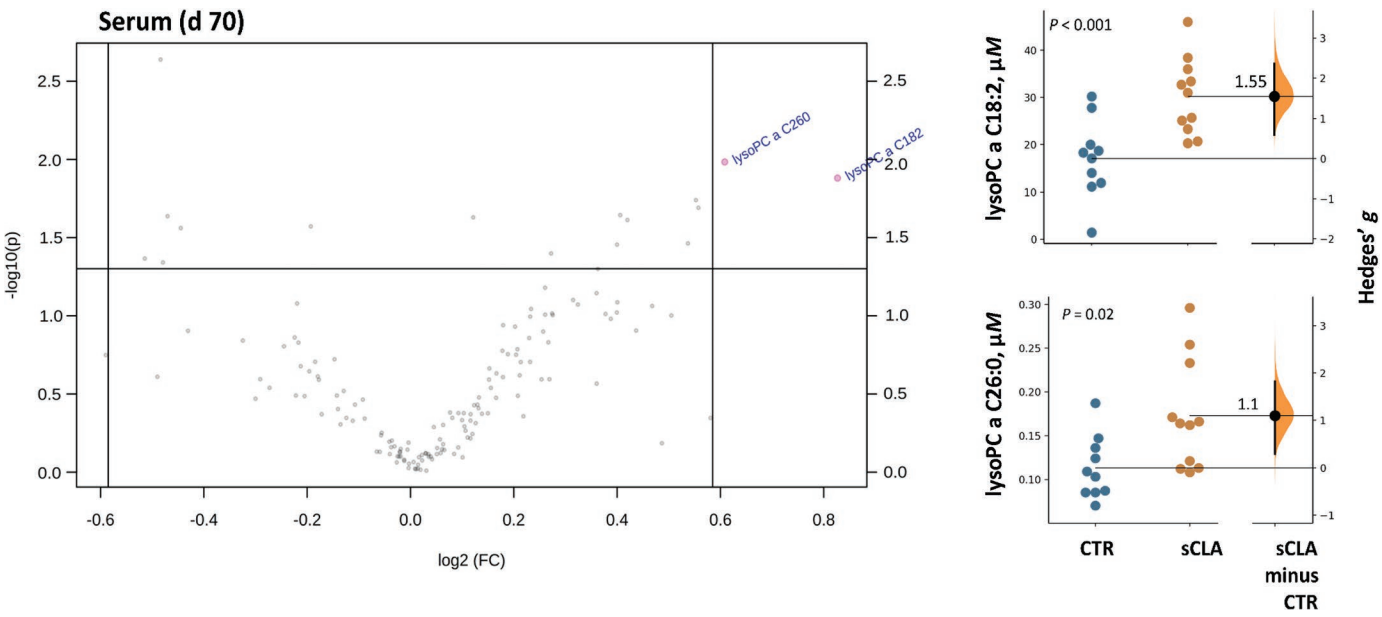

Figure 5. Volcano plot visualizing serum metabolites in dairy cows on d 70 after calving. Supplementation with CLA (sCLA; n = 11) or control fat (CTR; n = 10) was initiated at d 1 of lactation. The x-axis represents the mean of $\log_2$ fold-change (FC) value, and the y-axis corresponds to the negative logarithm of the *P*-values. Each circle represents a single metabolite. The horizontal line represents the level of significance for the *t*-tests performed (0.05), and the vertical lines display the threshold set for fold-change (1.5). The pink circles show metabolites that were significantly changed by the CLA supplementation, and the gray circles show metabolites that were not significantly changed. Effect size is shown as Hedges' *g* between sCLA and CTR cows in the Gardner–Altman estimation plots of muscle metabolites. Both groups are plotted on the left axes; the mean difference is plotted on floating axes on the right as a bootstrap sampling distribution. The mean difference is depicted as a dot. LysoPC-a = lysophosphatidylcholine acyl.

tive concentrations and the differences between groups would remain largely unchanged. The current data demonstrate that glycerophospholipids, including PC (a principal phospholipid circulating in the blood) in particular, underwent a shift in their serum and muscle concentrations of the sCLA cows when compared with those of the CTR cows in early lactation. In serum of the sCLA cows, 23 unequivocally identified phospholipids (22 PC and 1 lysPC) on d 21 after calving were less abundant than in the CTR cows. Phosphatidylcholines can be synthesized in the body through phosphorylation of choline, obtained from dietary sources or by the degradation of existing choline-containing lipids, mediated by phosphatidylethanolamine N-methyltransferase (PEMT) in the liver (Reo et al., 2002) as well as in the mammary gland (Robinson et al., 1987). In ruminants, dietary choline is extensively degraded in the rumen (Neill et al., 1979; Sharma and Erdman, 1989) and thus endogenous synthesis via the PEMT pathway is regarded as a critical source of choline (Baldi and Pinotti, 2006). It is known that the de novo synthesis of choline is carried out from ethanolamine when S-adenosylmethionine as a methyl-group donor is adequately supplied (Zeisel and Blusztajn, 1994). In the sCLA cows, the concentrations of serum PC on d 21 were decreased, without change in the levels of other phospholipids (except lysoPC-a C26:1). Due to the reduced DMI in the sCLA cows over the first days of lactation and possibly the related lower supply of dietary precursors such as methionine, we, therefore, reasoned that choline biosynthesis could be decreased or the use of PC as a substrate for energy metabolism could be increased during this period, or a combination of both. Interestingly, the relationship reversed on d 70: the concentrations of the 19 identified phospholipids [6 PC, 9 lysPC, 2 SM, and 2 SM(OH)] in muscle and of serum lysoPC-a C18:2 and C26:0 were elevated in the sCLA cows as compared with the CTR cows. The PC derived from hepatic PEMT activity might have a small contribution to total PC in ruminants (Robinson et al., 1987; Artegoitia et al., 2014). Unlike rodents and humans, where almost all PEMT activity occurs in the liver (Reo et al., 2002), the majority (about 82%) of endogenous choline synthesis via the PEMT activity appears to be supplied by extrahepatic tissues in sheep, with skeletal muscle contributing around 60% of this activity (Robinson et al., 1987). The mechanism yielding increased muscle and serum phospholipids in the sCLA cows on d 70 remains to be clarified; however, CLA have been demonstrated to influence several proteins involved in diverse metabolic processes linked to skeletal muscle anabolism, inflammation, and carbohy-

drate and lipid metabolism (Lehnen et al., 2015; Kim et al., 2016a,b). Thus, though speculative, CLA might have affected key proteins involved in phospholipid synthesis, augmenting the content of muscle PC, lysoPC, and SM. In addition to PC and SM, 9 lysoPC-a (C16:1, C18:1, C18:2, C20:0, C20:4, C26:1, C28:0, and C28:1) in the muscle and 2 lysoPC-a (C18:2, C26:0) in the serum of the sCLA cows were increased on d 70. Lysophosphatidylcholines, with structural and signaling roles, are a class of biomolecule that is primarily originating from cleaving of cell membrane-derived PC via the action of phospholipase A2 (van der Veen et al., 2017).

An interesting finding of the present study was the increased muscle dopamine concentrations on d 21 in cows supplemented with CLA. Dopamine, with multiple central and peripheral physiological effects, mediates its action via β-adrenergic receptors in skeletal muscle (Schubert et al., 1976; Smith et al., 2004). It has been shown that muscle cells respond to dopamine through increases in cAMP concentrations, which could be blocked by a β-adrenergic antagonist (Schubert et al., 1976). Thus, it seems possible that the greater dopamine content in the muscle of the sCLA cows would augment the cAMP concentrations in muscle via β-adrenergic receptors. The β-adrenergic action and cAMP have been implicated as negative regulators of insulin action, which consequently decrease glycogen synthase activity and insulin-stimulated glucose uptake in skeletal muscle (Smith et al., 2004). Lowering the activity of glycogen synthase activity could cause an intracellular accumulation of glucose-6-phosphate, which appears to decrease insulin-stimulated glucose transport into the muscle (Lee et al., 1997; Laurent et al., 2000). In the current study, the greater muscle dopamine coincided with the greater muscle hexoses (H1, about 90–95% glucose) concentrations, proposing the presence of insulin resistance in the muscle of the sCLA cows. As reported previously from this animal experiment, CLA supplementation was associated with a mitigation of the postpartum increase of circulating adiponectin (an adipokine that promotes insulin sensitivity) in the cows of the present study (Singh et al., 2014), further indicating decreased insulin sensitivity. In support of this, there was also a slight and transient increase of both glucose and insulin in the circulation of sCLA cows, and as shown in the companion study by Saremi et al. (2014), resulting in decreased values of a surrogate index for insulin sensitivity that is calculated from glucose, insulin, and NEFA concentrations.

In the current study, the concentrations of Ala in muscle were higher in the sCLA cows as compared with the CTR cows on d 21. In addition to Gln, Ala predominates among the AA released from muscle proteolysis in transferring both amino groups and carbon atoms (in particular from branched-chain amino acids) to the liver where the nitrogen enters the urea cycle and the pyruvate is used for gluconeogenesis (Frayn, 2010). The reduced DMI in the sCLA cows over the first days of lactation and the related lower calculated NE_L and MP balances were associated with the 1.7-fold greater abundance of *FBXO32* on d 21. *FBXO32* is one of the specific markers of muscle wasting (Foletta et al., 2011), and its upregulation might be related to an upregulation of the ubiquitin-proteasome system (the main proteolytic pathway in muscle) and consequent stimulation of protein degradation in the muscle. In addition, the mRNA for the α-polypeptide subunit of the branched-chain α-keto acid dehydrogenase E1 component was elevated in sCLA (Yang et al., 2020), likely suggesting a relatively high oxidative potential of the muscle in the sCLA cows on d 21. Taken together, the greater muscle concentrations of Ala in the sCLA cows on d 21 might be due to stimulation of muscle proteolysis and catabolism of released AA, in particular branched-chain amino acids, in response to the CLA supplementation or to the decreased DMI and the related lower calculated NE_L and MP balances during early lactation, or to a combination of both.

In the current study, we cannot determine whether or not the observations were independent of lower feed intake in the sCLA cows over the first 21 d of lactation. In a study reported by Hötger et al. (2013), cows supplemented with CLA tended to lower DMI as compared with control cows. Nevertheless, other studies about the effects of dietary supplemented CLA on the feed intake of dairy cows during the first weeks of lactation have yielded mostly consistent results, and in contrast to our findings, no influence on DMI was observed (Moore et al., 2004; Castañeda-Gutiérrez et al., 2005; Piamphon et al., 2009). The mechanisms behind the CLA-related transitory depression in DMI in the current study is not clear, but was apparently not related to compromised palatability of the CLA-containing concentrate because all cows consumed the concentrate feed as allocated.

The concentrations of free AA in muscle reflect the balance between various processes: the inward transport from the circulation and the outward release into the bloodstream, the incorporation into protein (synthesis), the release from protein degradation, and the loss by transamination and degradation (Frayn, 2010). In addition, in muscle wasting states, increased muscle protein breakdown is associated with raising intracellular concentrations of essential AA, such as Leu, Ile, Val, Phe, and Met (Holecek and Micuda, 2017; de Vasconcelos et al., 2019). Thus, in the current study, the decrease in muscle concentrations of Ile, Leu, Phe, Lys, His, Met, Trp, and C4-OH (mainly derived from the ketone body

β-hydroxybutyrate) from d 21 to 70, which coincided with the steady energy and MP balance on d 70 might reflect that the balance of protein synthesis/degradation was shifted toward synthesis. This is supported by the increased muscle Gln on d 70, which has been positively associated with muscle protein synthesis (Millward et al., 1989; Wu et al., 2011).

In the current study, phosphatidylcholines were the main metabolites in serum increasing in the course of lactation, which is in agreement with a previous report in dairy cows (Artegoitia et al., 2014). Phosphatidylcholine is a main component of all cell membranes and lipoprotein classes, reaching up to 60 to 80% of total phospholipids (Cole et al., 2012). The concentrations of lipoproteins, in particular of high-density lipoprotein, in the circulation increase with time of lactation in dairy cows (Raphael et al., 1973). Thus, the increased concentrations of PC from d 21 to 70 after calving might have resulted from changes in type and level of lipoproteins in the circulation that occur during lactation (Artegoitia et al., 2014).

CONCLUSIONS

This study presents the analysis of the metabolomes in muscle and serum of dairy cows receiving a diet supplemented with CLA during early lactation. For unknown reasons, supplementation of the diet with CLA was associated with a reduction in DMI over the first days of lactation and consequently a reduced calculated NE_L and MP balance. The CLA supplementation mainly led to changes in muscle and serum concentrations of glycerophospholipids and sphingolipids, reflecting the phospholipid compositional changes in muscle. The decreased long-chain PC in sCLA cows on d 21 might be due to the reduced DMI in these cows over the first days of lactation and the related lower supply of dietary precursors for choline biosynthesis or to the use of PC as a substrate for energy metabolism during this period. The changes observed in the muscle AA concentrations with time of lactation (regardless of treatment), which coincided with the steady energy and MP balance, might reflect a shift of protein synthesis/degradation balance toward synthesis.

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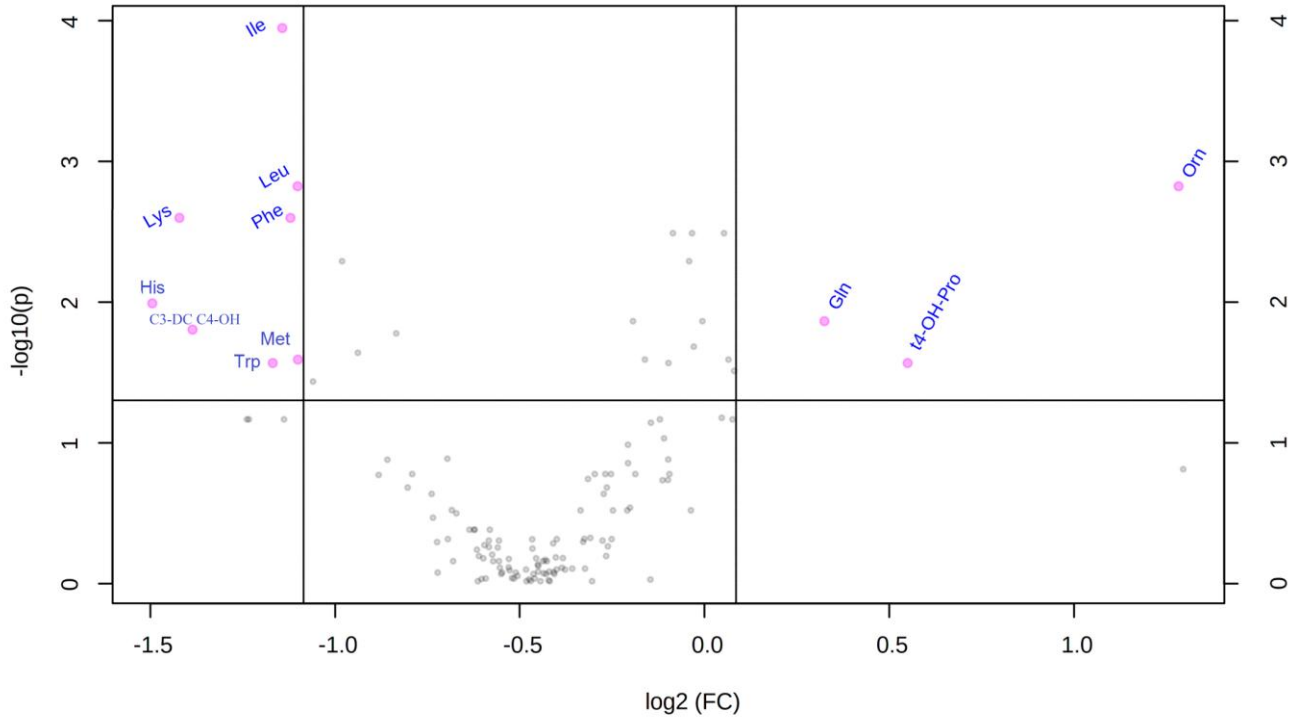
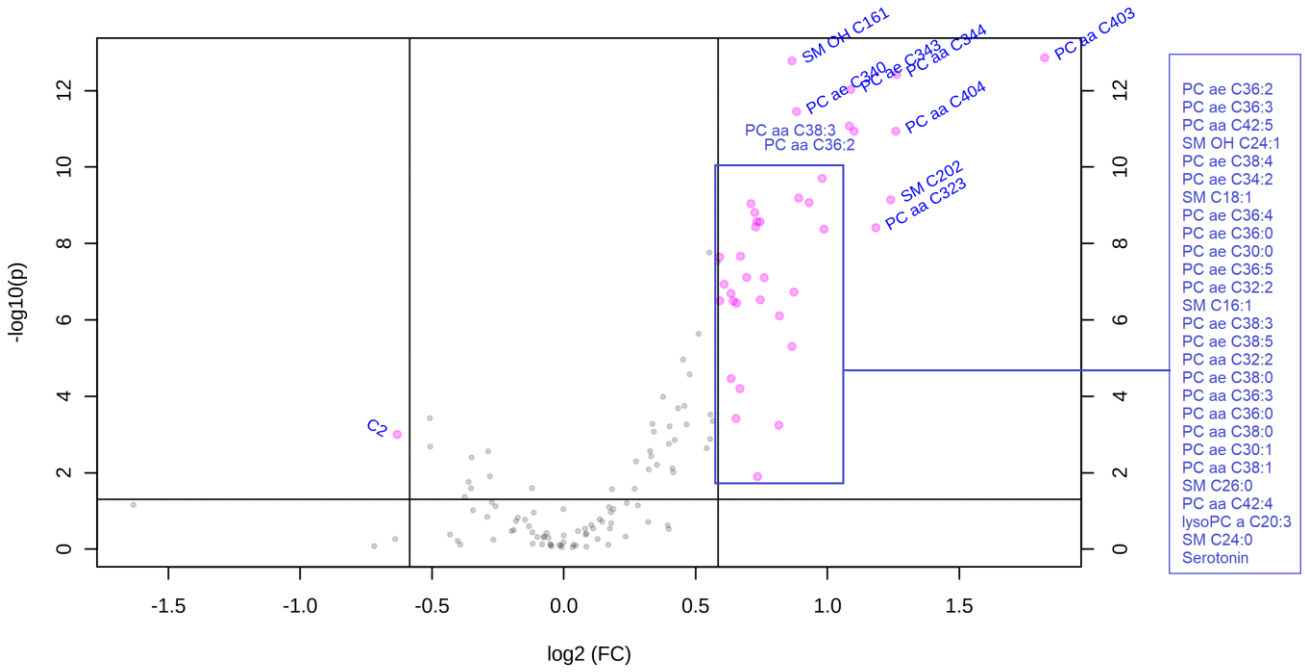


Supplemental Figure S1. Volcano plot visualizing muscle metabolites that were affected by time of lactation (d 21 versus d 70; regardless of treatment) in dairy cows. The x-axis represents the mean of $\log_2$ fold-change (FC) value, and the y-axis corresponds to the negative logarithm of the *P*-values. Each circle represents a single metabolite. The horizontal line represents the level of significance for the t-tests performed (0.05), and the vertical lines display the threshold set for fold change (1.5). The pink circles show metabolites that were significantly changed between two-time points and the gray circles show metabolites that were not significantly changed. C4- OH (C3-DC) = hydroxybutyrylcarnitine; Gln = gluthamine; His = histidine; Ile= isoleucine; Leu = leucine; Lys =lysine; Met = methionine; Orn = ornithine; Phe = phenylalanine; t4-OH-Pro = trans-4-hydroxyproline; Trp = tryptophan.



Supplemental Figure S2. Volcano plot visualizing serum metabolites that were affected by time of lactation (d 21 versus d 70; regardless of treatment) in dairy cows. The x-axis represents the mean of $\log_2$ fold-change (FC) value, and the y-axis corresponds to the negative logarithm of the *P*-values. Each circle represents a single metabolite. The horizontal line represents the level of significance for the t-tests performed (0.05), and the vertical lines display the threshold set for fold change (1.5). The pink circles show metabolites that were significantly changed between two-time points and the gray circles show metabolites that were not significantly changed. C2 = acetylcarnitine; lysoPC = lysophosphatidcholine; PC aa = di-acyl phosphatidylcholine; PC ae = acyl alkyl phosphatidylcholine; SM (OH) = hydroxysphingomyeline; SM = sphingomyeline.

Supplemental Table S1. List of metabolites measured with the Absolute-IDQ p180 Kit.

Compound class	Metabolites
Acylcarnitines	Carnitine (C0), Acetylcarnitine (C2), Propionylcarnitine (C3), Propenoylcarnitine (C3:1), Hydroxypropionylcarnitine (C3-OH), Butyrylcarnitine (C4), Butenylcarnitine (C4:1), Hydroxybutyrylcarnitine (C4- OH (C3-DC)), Valerylcarnitine (C5), Tiglylcarnitine (C5:1), Glutaconylcarnitine (C5:1-DC), Glutarylcarnitine (Hydroxyhexanoylcarnitine) (C5-DC (C6-OH)), Methylglutarylcarnitine (C5-M-DC), Hydroxyvalerylcarnitine (Methylmalonylcarnitine) (C5-OH (C3-DC-M)), Hexanoylcarnitine (Fumarylcarnitine) (C6 (C4:1-DC)), Hexenoylcarnitine (C6:1), Pimelylcarnitine (C7-DC), Octanoylcarnitine (C8), Nonaylcarnitine (C9), Decanoylcarnitine (C10), Decenoylcarnitine (C10:1), Decadienylcarnitine (C10:2), Dodecanoylcarnitine (C12), Dodecenoylcarnitine (C12:1), Dodecanedioylcarnitine (C12-DC), Tetradecanoylcarnitine (C14), Tetradecenoylcarnitine (C14:1), Hydroxytetradecenoylcarnitine (C14:1-OH), Tetradecadienylcarnitine (C14:2), Hydroxytetradecadienylcarnitine (C14:2- OH), Hexadecanoylcarnitine (C16), Hexadecenoylcarnitine (C16:1), Hydroxyhexadecenoylcarnitine (C16:1-OH), Hexadecadienylcarnitine (C16:2), Hydroxyhexadecadienylcarnitine (C16:2-OH), Hydroxyhexadecanoylcarnitine (C16-OH), Octadecanoylcarnitine (C18), Octadecenoylcarnitine (C18:1), Hydroxyoctadecenoylcarnitine (C18:1-OH), Octadecadienylcarnitine (C18:2)
Amino acids	Alanine (Ala), Arginine (Arg), Asparagine (Asn), Aspartate (Asp), Citrulline (Cit), Glutamine (Gln), Glutamate (Glu), Glycine (Gly), Histidine (His), Isoleucine (Ile), Leucine (Leu), Lysine (Lys), Methionine (Met), Ornithine (Orn), Phenylalanine (Phe), Proline (Pro), Serine (Ser), Threonine (Thr), Tryptophan (Trp), Tyrosine (Tyr), Valine (Val)
Biogenic amines	Acetylornithine (Ac-Orn), Asymmetric dimethylarginine (ADMA), Symmetric dimethylarginine (SDMA), alpha-Aminoadipic acid (alpha-AAA), Carnosine (Carnosine), Creatinine (Creatinine), Histamine (Histamine), Kynurenine (Kynurenine), Methioninesulfoxide (Met-SO), Nitrotyrosine (Nitro-Tyr), cis-4-Hydroxyproline (cis-OH-Pro), trans-4-Hydroxyproline (trans-OH-Pro), Phenylethylamine (PEA), Putrescine, Sarcosine, Serotonin, Spermidine, Spermine, Taurine, Dopamine, DOPA
Glycerophospholipids	Lyso-PC lysoPC a C14:0, lysoPC a C16:0, lysoPC a C16:1, lysoPC a C17:0, lysoPC a C18:0, lysoPC a C18:1, lysoPC a C18:2, lysoPC a C20:3, lysoPC a C20:4, lysoPC a C24:0, lysoPC a C26:0, lysoPC a C26:1, lysoPC a C28:0, lysoPC a C28:1 Diacyl-PC PC aa C24:0, PC aa C26:0, PC aa C28:1, PC aa C30:0, PC aa C30:2, PC aa C32:0, PC aa C32:1, PC aa C32:2, PC aa C32:3, PC aa C34:1, PC aa C34:2, PC aa C34:3, PC aa C34:4, PC aa C36:0, PC aa C36:1, PC aa C36:2, PC aa C36:3, PC aa C36:4, PC aa C36:5, PC aa C36:6, PC aa C38:0, PC aa C38:1, PC aa C38:3, PC aa C38:4, PC aa C38:5, PC aa C38:6, PC aa C40:1, PC aa C40:2, PC aa C40:3, PC aa C40:4, PC aa C40:5, PC aa C40:6, PC aa C42:0, PC aa C42:1, PC aa C42:2, PC aa C42:4, PC aa C42:5, PC aa C42:6 Acyl-alkyl-PC PC ae C30:0, PC ae C30:1, PC ae C30:2, PC ae C32:1, PC ae C32:2, PC ae C34:0, PC ae C34:1, PC ae C34:2, PC ae C34:3, PC ae C36:0, PC ae C36:1, PC ae C36:2, PC ae C36:3, PC ae C36:4, PC ae C36:5, PC ae C38:0, PC ae C38:1, PC ae C38:2, PC ae C38:3, PC ae C38:4, PC ae C38:5, PC ae C38:6, PC ae C40:1, PC ae C40:2, PC ae C40:3, PC ae C40:4, PC ae C40:5, PC ae C40:6, PC ae C42:0, PC ae C42:1, PC ae C42:2, PC ae C42:3, PC ae C42:4, PC ae C42:5, PC ae C44:3, PC ae C44:4, PC ae C44:5, PC ae C44:6
Sphingolipids	SM (OH) C14:1, SM (OH) C16:1, SM (OH) C22:1, SM (OH) C22:2, SM (OH) C24:1, SM C16:0, SM C16:1, SM C18:0, SM C18:1, SM C20:2, SM C22:3, SM C24:0, SM C24:1, SM C26:0, SM C26:1
Hexoses	Sum of hexoses (H1)

Supplemental Table S2. List of metabolites with more than 20% of missing values (i.e., values lower than the limit of detection) that were not considered for the statistical analysis.

Serum	Muscle
Decenoylcarnitine (C10:1)	c4-OH-Pro (*cis*-4-Hydroxyproline)
Dodecenoylcarnitine (C12:1)	Nitrotyrosine (Nitro-Tyr)
Dodecanedioylcarnitine (C12-DC)	Sarcosine
Dihydroxyphenylalanine (DOPA)	
Dopamine	
Histamine	
Phenylethylamine (PEA)	
c4-OH-Pro (*cis*-4-Hydroxyproline)	
Nitrotyrosine (Nitro-Tyr)	
Sarcosine	