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ARTICLE

NMR-Based Metabonomic Investigation of Heat Stress in Myotubes Reveals a Time-Dependent Change in the Metabolites

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NMR-based metabonomics was applied to elucidate the time-dependent stress responses in mouse myotubes after heat exposure of either 42 or 45 °C for 1 h. Principal component analysis (PCA) revealed that the gradual time-dependent changes in metabolites contributing to the clustering and separation of the control samples from the different time points after heat stress primarily are in the metabolites glucose, leucine, lysine, phenylalanine, creatine, glutamine, and acetate. In addition, PC scores revealed a maximum change in metabolite composition 4 h after the stress exposure; thereafter, samples returned toward control samples, however, without reaching the control samples even 10 h after stress. The results also indicate that the myotubes efficiently regulate the pH level by release of lactate to the culture medium at a heat stress level of 42 °C, which is a temperature level reached in muscles of pigs during exposure to slaughter stress.

KEYWORDS: Metabonomics; proton nuclear magnetic resonance; Hsp70; heat shock protein; slaughter stress; overshoot; lactate; acetate; metabolites; muscle cells; multivariate data analysis

INTRODUCTION

When pigs are handled in relation to slaughter, they are exposed to stressors which influence subsequent meat quality development. The pigs may experience stress from the transportation to the abattoir, mixing with unfamiliar pigs, the lairage and handling at the abattoir, and the actual slaughter procedure itself (*1*−*4*). These stressors cause a release of stress hormones, which results in a faster muscle metabolism and causes breakdown of high-energy metabolites, increased muscle temperature, a faster decline in pH in the muscle, and an increase in the drip loss, resulting in inferior meat quality (*1*, *2*, *5*−*9*).

Several experimental setups have been conducted in order to mimic preslaughter stress, including running, nose snare, or electrical goad, and the effect on meat quality traits has been investigated (*9*−*11*). Some studies find no relation between the stress exposure and meat quality (*10*, *11*), whereas others find effects of stress on, for example, temperature, pH, and drip loss (*1*, *2*, *9*, *12*). This apparent discrepancy may be explained by different experimental setups, including the stress resistance of the breed and the type and duration of the stressor, but Young et al. (*9*) also observed an overshooting effect on several parameters when sampling/slaughtering 1−3 h after stress, indicating that the time of sampling/slaughter after stress is critical for the findings and conclusions. Hence, elucidating factors that influence meat quality traits is rather complex in whole animal systems, and for studying basic mechanisms at the cellular level the application of myotube cultures as a model for muscles represents a valuable controlled experimental system. Differentiated muscle cell cultures have previously been used for studying basic cellular mechanisms (*13*−*16*) and also for studying the effect of stress exposures of interest in relation to slaughter (*17*, *18*). Explorative "omics" techniques have the advantage of measuring multiple variables simultaneously. Nuclear magnetic resonance (NMR) based metabonomics has been successfully applied to investigate both short-term and long-term effects of stress on the metabolic profile (*19*−*22*). Furthermore, multivariate data analysis has proven to be a very useful tool to assess the complex changes in the numerous metabolites identified with metabonomics, and principal component analysis (PCA) has been applied to distinguish control samples and samples from different time points after stress exposure (*19*−*22*). Proton (^{1}H) NMR spectroscopy has been used to investigate the influence of preslaughter treadmill stress on metabolite levels by acquiring plasma from pigs (*21*). ^{1}H NMR spectroscopy has also proven to be a valuable tool to investigate the slaughter-related stressors heat and anoxia in mouse C2C12 myotubes (*17*).

An increase in muscle temperature is often experienced under stressful conditions in connection with slaughter (*1*, *2*, *9*, *12*), and in the study by Young et al. (*9*) temperatures of up to 42 °C have

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been found in muscles of pigs exposed to treadmill stress. To achieve a physiological response to stress in myotube cultures, a stress hormone could be applied, but in a myotube monolayer it would not be possible to achieve heat stress, for example, by exposure to a hormone. Exposing pig muscle fiber strips to heat (*23*) or exercising of humans in the heat (*24*) results in changes in the muscles similar to those experienced at slaughter stress: that is, release of stress hormones, faster decline in the breakdown of high-energy metabolites, and faster decline in the pH. Hence, in the present study a stress level of 42 °C was used to imitate a heat level which has been demonstrated in relation to slaughter (*9*). However, to possibly get more distinct changes in the metabolites, a more extreme stress level of 45 °C was also applied. Hence, the aim of the present study was to investigate changes in metabolites by use of NMR-based metabonomics in myotubes after exposure to heat stress for 1 h at 42 or 45 °C. The suitability of the porcine primary cells and the mouse C2C12 cell line was established, and the changes in metabolites were followed before stress, immediately after heat stress, and up to 10 h after stress exposure in order to elucidate the time-dependent effects of stress in the myotubes. The suitability of the two different muscle model systems was assessed by monitoring the transcription level of the heat shock protein Hsp70 mRNA at different temperatures.

MATERIALS AND METHODS

Porcine Primary Muscle Cell Cultures. Porcine primary satellite cells isolated from *M. semimembranosus* of female pigs at the age of 6 weeks (8−12 kg) were grown to establish myotube cultures. The original method of Bischoff with some modifications was applied (*25*, *26*). Muscle tissue was excised, stripped for visible fat and connective tissue, placed in ice-cold transport medium consisting of 1% glucose, 500 IU/ml of penicillin, 500 μg/mL of streptomycin, 15 μg/mL of amphotericin B, and 100 μg/mL of gentamycin in Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS), and transferred to a laminar flow bench. The muscle tissue was finely chopped with a pair of scissors and digested for 20 min in 20 mL of PBS (Ca^{2+} free) containing 1% glucose, 1.5 mg/mL of collagenase II, 0.25% trypsin, and 0.01% DNase (digestion medium). The digestion medium was aspirated, another 20 mL of digestion medium was added, and the muscle tissue was left to digest for another 20 min. The procedure was repeated to give a total of 3 × 20 min digestion and a total volume of approximately 60 mL of digest. Following digestion the cells were transferred to a primary growth medium (PGM) consisting of Dulbecco's Modified Eagle's Medium (DMEM), 10% (v/v) fetal calf serum (FCS), 10% (v/v) horse serum (HS), 100 IU/mL of penicillin, 100 μg/mL of streptomycin, 3 μg/mL of amphotericin B, and 20 μg/mL of gentamycin and triturated 10 times. The cell suspension was centrifuged at 630*g* for 8 min at 4 °C, resuspended, and filtered through a 200 μm and then a 50 μm Nytex filter. Percoll gradients of 20% were used to enrich the relative proportion of satellite cells in the cell suspension (*16*). Cells were kept in liquid nitrogen until use, where cells were thawed at 37 °C and seeded in 24-well plates coated with matrigel (1/50 v/v) at a density of 40 000 cells/cm^2. The cells were grown in PGM under an atmosphere of 95% air and 5% CO_2 at 37 °C. Cells were made to fuse after 6 days of proliferation by switching to DMEM with 10% FCS, 1 μM insulin, and antibiotics for 24 h and then to DMEM containing 5% FCS, 1 μM insulin, antibiotics, and 1 μM cytosine arabinosid (differentiation medium) for 24 h. After 24 h in differentiation medium the cultures contained multinuclear myotubes and were ready for experimental use.

Mouse C2C12 Muscle Cell Line Cultures. The mouse myoblast cell line C2C12, originally derived from a mouse thigh muscle (*27*) (American Type Culture Collection, Manassas, VA), was grown to establish myotube cultures. Briefly, a clone that effectively fuses and forms myotubes was grown in a 75 cm^2 culture flask in 10 mL of growth medium consisting DMEM, 10% (v/v) FCS, 100 IU/mL of penicillin, 100 μg/mL of streptomycin, 3 μg/mL of amphotericin B, and 20 μg/mL of gentamycin. Cells were maintained under an atmosphere of 95% air and 5% CO_2 at 37 °C. Prior to confluence, cells were harvested in 0.25% trypsin and seeded in 6- or 24-well plates at a density of 10 000 cells/cm^2. Cells were grown to confluence in growth medium and left to fuse in differentiation medium containing DMEM, 4% (v/v) FCS, and antibiotics. The latter medium was changed after 48 h of incubation. After ∼4 days in differentiation medium the cultures contained multinuclear myotubes and were ready for experimental use.

Experimental Setup for Real-Time RT-PCR and ^{1}H NMR Spectroscopy. For the reverse transcription polymerase chain reaction (RT-PCR) the porcine and mouse myotube cultures were grown in 24-well plates, whereas for ^{1}H NMR spectroscopy the mouse myotube cultures were grown in 6-well plates. All the myotubes were harvested in 0.25% trypsin. For RT-PCR the porcine and mouse myotubes were harvested immediately before the stress exposure of 42 or 45 °C for 1 h (controls), immediately after stress (t = 0 h), and 0.5, 1, 2, 4, 6, 8, 10, 14, 18, and 24 h after the heat exposure. For the ^{1}H NMR spectroscopic experiments the culture medium was collected and myotubes were harvested immediately before stress exposure to 42 °C (a) or 45 °C (b) for 1 h (controls), and subsequently the medium was collected and myotubes were harvested immediately after stress (t = 0 h), and 1, 4, 8, and 10 h after the heat exposure. The harvested myotubes and the culture medium were stored in Eppendorf tubes at −80 °C until extraction of RNA or NMR analysis.

RNA Extraction and Real-Time RT-PCR. The RNA was purified using the AllPrep RNA/protein kit (Qiagen, Albertslund, Denmark), and the RNA concentration and purity were determined after dilution by application of a NanoDrop ND-1000 spectrophotometer (Nano-Drop Technologies, Inc., Wilmington, DE). After the RNA concentrations of all the samples were adjusted to the same level (for porcine myotubes to 9 ng/μL and for mouse myotubes to 90 ng/μL), which is one of the normalization procedures recommended by Bustin et al. (*28*), the RNA was reverse transcribed with oligo-dT primers and Superscript II RNase H reverse transcriptase kit (Invitrogen, Taastrup, Denmark). Reverse transcribed material (1 μL) was amplified with TaqMan Universal PCR Master Mix (Applied Biosystems, Stockholm, Sweden).

For porcine and mouse myotubes the quantity of the inducible heat shock protein 70 (Hsp70) mRNA was detected by primers and probe designed specifically for the Hsp70 gene in pig (accession number: M69100) and mouse (accession number: NM_010479.2), respectively, by use of Primer Express 2.0 software (Applied Biosystems, Stockholm, Sweden). The sequences of the forward primer and the TAMRA probe were identical for pig and mouse (5′-GGCAAGGCCAACAAGATCAC-3′, 5′-ACAAGGGCCGCCTGAGCAAGG-3′, respectively), whereas the sequence for the reverse primer was 5′-TTCTCAGCCTCCTGCACCAT-3′ for pig (*29*) and 5′-GCACCATGCGCTCGATCT-3′ for mouse. The amplicon lengths were tested after real time RT-PCR analysis on 2% agarose gels. Only one PCR product was amplified for the Hsp70 gene in each species, and the length agreed with the predicted length of 86 bp in pig and 73 bp in mouse based on the nucleotide sequences (data not shown). Quantities of the Hsp70 mRNA from the porcine and the mouse myotubes were detected by a gene-specific TAMRA probe labeled with FAM fluorophore in the 5′-end and a nonfluorescent quencher in the 3′-end.

For PCR, 40 cycles at 95 °C for 15 s and 60 °C for 60 s were applied to amplify the PCR products. A selected sample was diluted serially and analyzed in triplicate to test the linearity and efficiency of the PCR amplifications. Furthermore, control wells with water, genomic pig, or genomic mouse DNA were used as negative controls. The samples were analyzed using an ABI 7900HT detection system (Applied Biosystems, Stockholm, Sweden). To evaluate mRNA quantities, data were obtained as Ct values (the cycle number at which logarithmic plots cross a calculated threshold line). The relative mRNA quantity was calculated using the formula relative quantity = $2^{-\Delta Ct}$, where the mRNA level is expressed relative to cells without stress. At each time point myotubes were harvested from two wells (n = 2) and analyzed for Hsp70 mRNA in duplicate. For the porcine myotubes the two wells represent isolation of cells from two different pigs.

^{1}H NMR Spectroscopy and Multivariate Data Analysis. Myotubes from two wells were pooled in 600 μL of D_2O containing 0.000 05% (w/v) sodium trimethylsilyl[2,2,3,3-D_4]-1-propionate (TSP) and centrifuged at 10 000*g* for 10 min, and the supernatant was transferred to a 5 mm NMR tube. For the medium samples 250 μL of medium from each of two wells was mixed with 100 μL of D_2O containing 0.025% (w/v) TSP in a 5 mm NMR tube. ^{1}H NMR spectra were recorded at 25 °C on a

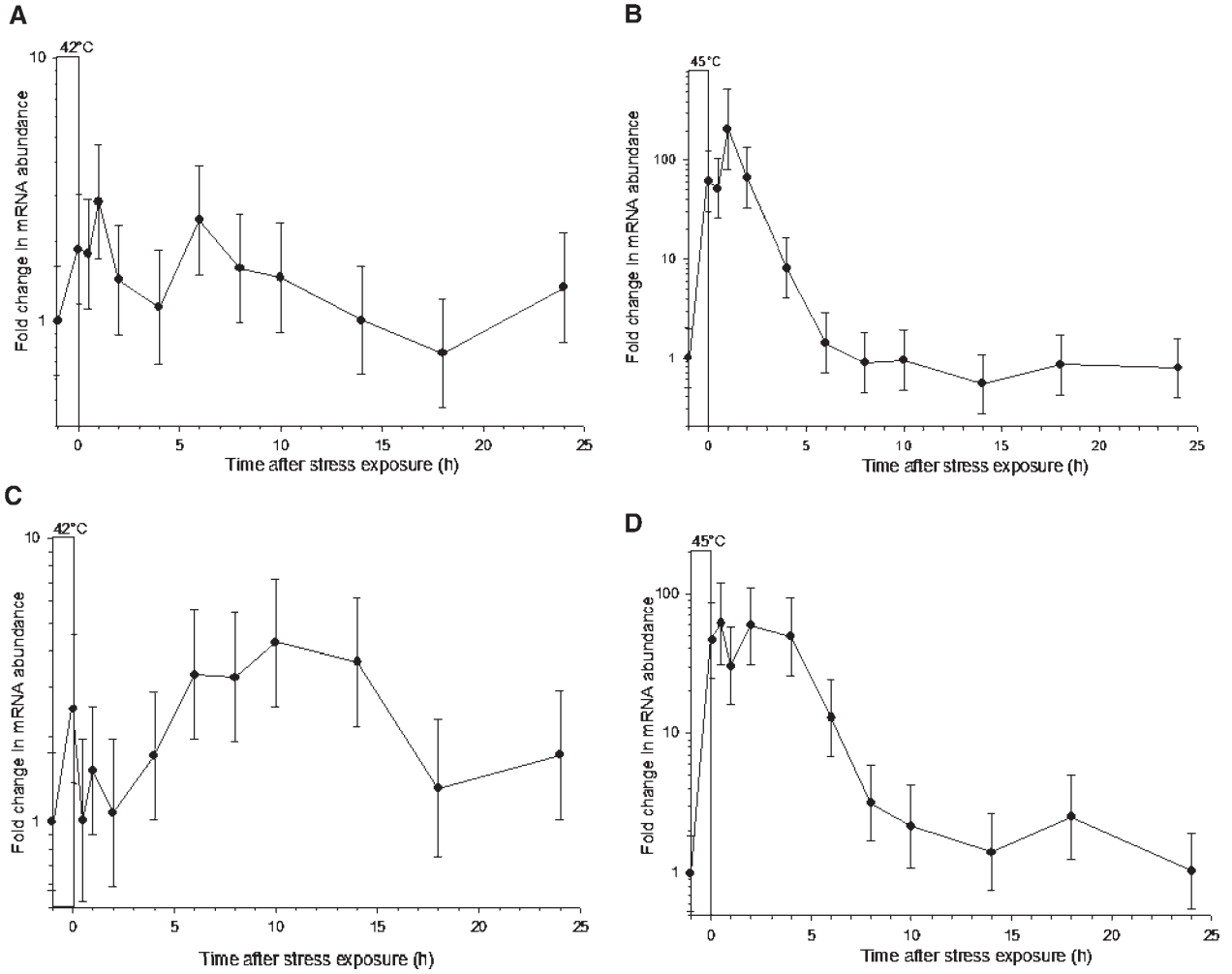

Figure 1. Fold changes in Hsp70 mRNA level with 95% CI in mouse (**A** and **B**) and porcine (**C** and **D**) myotubes after heat exposure at 42 °C (**A** and **C**) or 45 °C (**B** and **D**), respectively, for 1 h. The time point $t = 0$ h is immediately after the 1 h heat exposure. The mRNA level is expressed relative to the control cells.

Bruker Avance 600 spectrometer, operating at a ^{1}H frequency of 600.13 MHz, equipped with a 5 mm ^{1}H TXI probe (Bruker BioSpin, Rheinstetten, Germany). Standard one-dimensional (1D) ^{1}H NMR spectra were acquired using a single 90° pulse experiment, and each spectrum was the sum of 256 FIDs. Water suppression was achieved by irradiating the water peak during the relaxation delay of 2 s, and 32K data points spanning a spectral width of 12.02 ppm were collected. All spectra were referenced to TSP at 0 ppm. In addition, to aid spectral assignment, two-dimensional (2D) ^{1}H−^{1}H COSY and 2D ^{1}H−^{13}C HSQC spectra were recorded on selected cell and medium samples. ^{1}H NMR spectra were obtained on five replicates ($n = 5$) for each of the six time points by pooling myotubes or medium from two wells for each of the treatment groups: (a) 42 °C for 1 h and (b) 45 °C for 1 h mentioned above. A few of the medium samples were removed from the data sets because of problems with the baseline or because of poor spectral quality (see below).

Quantification of the various metabolites was carried out by integration of peak areas using Topspin 2.1 software (Bruker BioSpin, Rheinstetten, Germany). The ^{1}H NMR spectra were integrated in the spectral range 0.9−8.5 ppm, excluding the intervals 4.5−5.0 and 4.7−5.0 ppm containing the residual water signal in the myotubes and the media, respectively, as well as signals in the intervals at 2.40−3.92 and 2.00−4.20 ppm containing the HEPES in the myotubes and the media, respectively. The integrals of the ^{1}H NMR spectra were normalized to a total intensity of 1000 to reduce the effect of concentration differences between samples. Multivariate data analysis was carried out using mean-centered data and Pareto scaling, in which each variable is weighted by the square root of its standard deviation. The spectral data were analyzed by PCA with SIMCA-P 12.0.1 software (Umetrics, Umeå, Sweden). Cross-validation was performed by iterating seven times with every seventh sample removed from the analysis to evaluate how well each of the variables could be predicted.

Statistics. All the data were analyzed by using the mixed procedure of SAS (SAS Institute Inc., Cary, NC). The level of Hsp70 mRNA and the integral values of the ^{1}H NMR spectra were analyzed in a model with the time point as a fixed effect and replicates as a random effect. For quantification of mRNA, normalization was achieved by adjusting the RNA level for all samples to the same concentration, as described above. The mRNA level of Hsp70 is presented as least-squares means (LSMeans) ± 95% confidence interval (CI), whereas the integral values of the NMR spectra are presented as LSMeans ± standard errors of LSMeans (SEM).

RESULTS

Effects of Heat Stress on Hsp70 mRNA Level in the Mouse and Porcine Myotubes. Changes in the heat shock protein Hsp70 mRNA level over time, before heat stress and after exposing the mouse (**Figure 1A**,**B**) or porcine (**Figure 1C**,**D**) myotubes to 42 or 45 °C, respectively, for 1 h, was investigated with real-time RT-PCR.

From **Figure 1** it is apparent that the Hsp70 mRNA level peaked initially 1 h after stress in the mouse myotubes (**Figure 1A**) and immediately after stress ($t = 0$ h) in the porcine myotubes (**Figure 1C**) after exposure to 42 °C for 1 h. The increase in the transcription of Hsp70 for both mouse and porcine myotubes was approximately 2-fold in comparison to control cells significant in both the mouse and the porcine myotubes.

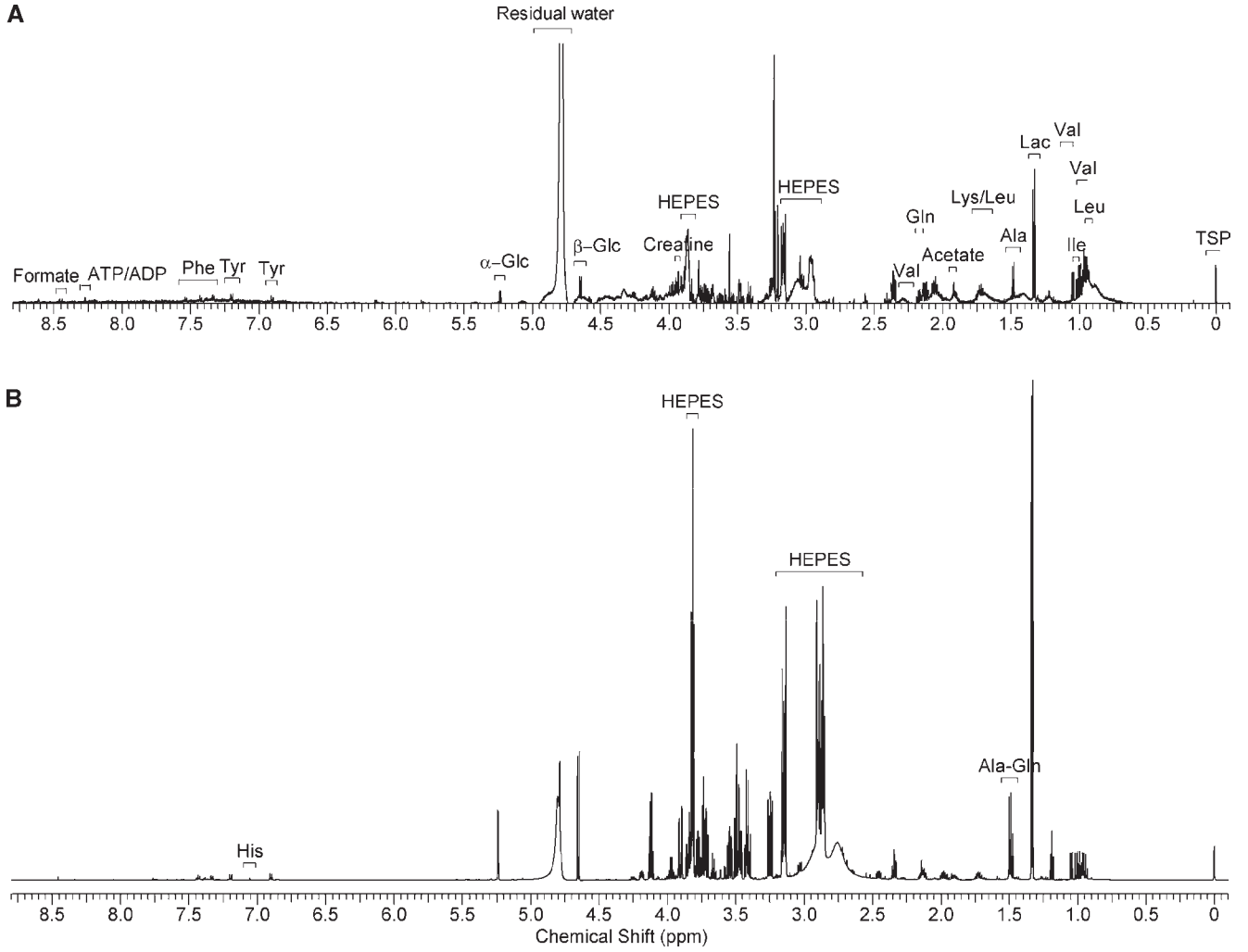

Figure 2. Representative ^{1}H NMR spectra of mouse myotubes (**A**) and culture medium (**B**). Legend: Leu, leucine; Val, valine; Ile, isoleucine; Lac, lactate; Ala, alanine; Lys, lysine; Gln, glutamine; Glc, glucose; Tyr, tyrosine; Phe, phenylalanine; Aln-Gln, alanyl-glutamine; His, histidine. TSP is an internal reference. The spectra were acquired under identical conditions, but scaling factors differ between the plots for the myotubes and the medium.

When the myotubes were exposed to 45 °C for 1 h, the Hsp70 mRNA level peaked 1 h after stress for the mouse myotubes (**Figure 1B**) and 0.5 h after stress for the porcine myotubes (**Figure 1D**). The very significant increase in the transcription of Hsp70 was approximately 200-fold in the mouse myotubes and 60-fold in the porcine myotubes in comparison to control cells.

^{1}H NMR Spectra of Mouse Myotubes and Culture Medium following Heat Stress. The mouse myotubes were exposed to 42 or 45 °C for 1 h, and cells were harvested and the culture medium was collected before the stress exposure (control) and 0, 1, 4, and 10 h after stress. **Figure 2** shows representative ^{1}H NMR spectra of the myotubes and the culture medium. The relative levels of the metabolites for the controls and at different time points after exposure to 42 or 45 °C for 1 h are given for the myotubes with statistics in **Tables 1** and **2** and for clarity changes in selected metabolites are illustrated in **Figure 3**. For the culture medium the data with statistics are given in **Tables 3** and **4**.

Mouse Myotubes. Exposure to 42 °C for 1 h resulted in an immediate significant decrease in lactate, followed by an increase reaching significance after 10 h, without reaching the level of the control (**Figure 3A** and **Table 1**). For the myotubes exposed to 45 °C for 1 h, an increase in lactate was observed after stress, significant after 1 h, and reaching a plateau after 4 h (**Figure 3C** and **Table 2**). Both 42 and 45 °C heat stress resulted in increases in the glucose levels, followed by decreases after 4 and 1−4 h, respectively, although the changes in the glucose levels were not significant (**Figure 3A**,**C** and **Tables 1** and **2**). Increases in creatine were observed after 42 and 45 °C heat stress, being significant after 8 and 0 h, respectively (Tablea **1** and **2**). The changes in acetate at 42 and 45 °C heat stress also followed similar patterns, reaching a minimum after 4 h and 1−4 h, respectively, after which the acetate levels returned to control levels after 10 h, but with no overall significance at 42 °C (**Figure 3A**,**C** and **Tables 1** and **2**). For the amino acids leucine, lysine, and phenylalanine very similar changes were observed over time at 42 and 45 °C heat stress (**Figure 3B**,**D** and **Tables 1** and **2**). The level of these amino acids decreased significantly after stress, reaching a minimum after 4 h, followed by an increase but not returning to the control level 10 h after stress.

Mouse Myotube Culture Medium. For the culture medium from myotubes exposed to 42 or 45 °C significant decreases in glucose and all amino acids were observed (**Tables 3** and **4**). For all metabolites the decreases were larger after 45 °C heat exposure, in comparison to 42 °C heat exposure. Significant increases were observed for lactate, acetate, and formate, and the increases were larger after 45 °C heat exposure, compared to 42 °C heat exposure.

PCA of ^{1}H NMR Spectra from Mouse Myotubes and Culture Medium. PCA was performed on the integral values for the myotubes and the culture medium of each of the two experiments

Table 1. ^{1}H NMR Chemical Shift Ranges and LSMeans of the Relative Integral Intensities, with Calculated Standard Errors of the LSMeans (SEM) for Myotubes Exposed to 42 °C for 1 h[a]

metabolite	shift range (ppm)	control (n = 5)	0 h (n = 5)	1 h (n = 5)	4 h (n = 5)	8 h (n = 5)	10 h (n = 5)	SEM	P
Leu (δ-CH_3)	0.923−0.981	196.5^{a}	147.8^{b}	153.9^{b}	123.1^{b}	133.8^{b}	152.3^{b}	11.4	**0.0045**
Val (CH_3)	0.982−1.004	34.1^{b}	33.1^{b}	34.6^{b}	36.9^{ab}	39.6^{a}	35.1^{b}	1.4	0.0540
Ile (β-CH_3)	1.004−1.027	21.7^{c}	23.0c	25.0^{bc}	30.3^{ab}	32.8^{a}	26.5^{bc}	2.1	**0.0081**
Val (CH_3)	1.033−1.059	19.4^{d}	20.6^{cd}	23.5^{bcd}	29.1^{ab}	30.3^{a}	25.8^{abc}	2.1	**0.0049**
Lac (CH_3)	1.314−1.346	131.5^{a}	92.0^{c}	109.7^{bc}	99.9^{bc}	110.9^{abc}	120.6^{ab}	7.2	**0.0127**
Ala (Ala-Gln) (CH_3)	1.460−1.520	31.4^{c}	33.2^{c}	47.7^{b}	37.8^{c}	48.4^{ab}	56.4^{a}	2.8	**<0.0001**
Lys/Leu (δ-CH_2)/(CH_2)	1.600−1.780	98.3^{ab}	69.9^{c}	102.8^{a}	77.8^{c}	79.4^{bc}	98.2^{ab}	6.6	**0.0079**
Acetate (CH_3)	1.914−1.929	12.0^{ab}	10.5^{ab}	12.3^{a}	8.9^{b}	9.1^{b}	12.5^{a}	1.2	0.0810
Gln (Ala-Gln) (β-CH_2)	2.099−2.206	20.6^{d}	27.6^{d}	64.4^{b}	42.9^{c}	54.7^{bc}	83.6^{a}	5.3	**<0.0001**
Val (β-CH)	2.238−2.307	5.8^{ab}	3.2^{ab}	9.0^{a}	8.8^{a}	1.2^{b}	8.0^{a}	2.3	0.0896
creatine (CH_2)	3.929−3.940	34.1^{c}	33.3^{c}	37.0^{bc}	31.4^{c}	46.0^{ab}	49.7^{a}	3.8	**0.0071**
α-Glc (H1)	5.220−5.261	25.7^{bc}	45.8^{ab}	41.0^{abc}	58.9^{a}	38.6^{abc}	19.6^{c}	8.7	0.0543
Tyr (H3/H5)	6.880−6.931	9.2	8.0	7.0	8.9	10.0	9.5	1.4	0.7216
Tyr (H2/H6)	7.180−7.221	15.1	12.6	13.9	13.7	14.9	13.5	1.1	0.5943
Phe (H2/H6/H4/H3/H5)	7.300−7.561	81.9^{a}	63.9^{b}	62.1^{b}	54.5^{b}	63.3^{b}	64.7^{b}	4.0	**0.0034**
ATP/ADP (ring H2)	8.250−8.296	6.0^{a}	4.6^{ab}	2.9^{bc}	1.8c	3.5^{abc}	3.2^{bc}	0.9	0.0527
formate (CH)	8.454−8.470	2.7^{a}	2.3^{ab}	1.4^{bc}	1.0^{c}	1.1^{c}	1.1^{c}	0.4	**0.0143**

[a] The time point t = 0 h is immediately after the 1 h heat exposure. Abbreviations are as defined in the caption for **Figure 2**. LSMeans within a row with different superscript letters (a−d) differ significantly (P < 0.05).

Table 2. ^{1}H NMR Chemical Shift Ranges and LSMeans of the Relative Integral Intensities, with Calculated Standard Errors of the LSMeans (SEM) for Myotubes Exposed to 45 °C for 1 h[a]

metabolite	shift range (ppm)	control (n = 5)	0 h (n = 5)	1 h (n = 5)	4 h (n = 5)	8 h (n = 5)	10 h (n = 5)	SEM	P
Leu (δ-CH_3)	0.923−0.981	179.6^{a}	164.3^{ab}	145.9^{bc}	143.4^{c}	158.0^{bc}	159.4^{abc}	7.4	**0.0159**
Val (CH_3)	0.982−1.004	39.9^{ab}	41.5^{a}	35.8^{cd}	33.6^{d}	38.5^{b}	37.7^{bc}	0.8	**<0.0001**
Ile (δ-CH_3) (β-CH_3)	1.004−1.027	28.4^{ab}	30.5^{a}	27.8^{abc}	25.1^{c}	29.4^{ab}	27.1^{bc}	0.9	**0.0116**
Val (CH_3)	1.033−1.059	27.7^{b}	31.1^{a}	25.8^{bc}	23.2^{c}	27.5^{b}	27.2^{b}	1.0	**0.0007**
Lac (CH_3)	1.314−1.346	127.2^{d}	134.1^{cd}	156.7^{bc}	187.1^{a}	163.2^{ab}	177.1^{ab}	9.8	**0.0020**
Ala (Ala_Gln) (CH_3)	1.460−1.520	59.1^{a}	57.7^{a}	47.8^{b}	49.8^{b}	53.5^{ab}	53.2^{ab}	2.6	**0.0457**
Lys/Leu (δ-CH_2)/(CH_2)	1.600−1.780	149.2^{a}	131.3^{b}	107.1^{cd}	104.0^{d}	124.3^{bc}	124.5^{bc}	6.4	**0.0003**
Acetate (CH_3)	1.914−1.929	18.2^{a}	16.8^{ab}	12.5^{c}	12.6^{c}	14.6^{bc}	16.6^{ab}	0.8	**<0.0001**
Gln (Ala-Gln) (β-CH_2)	2.099−2.206	59.5^{b}	81.1^{a}	77.7^{a}	71.0^{ab}	66.0^{ab}	71.4^{ab}	5.8	0.0980
Val (β-CH)	2.238−2.307	14.9^{a}	11.8^{abc}	8.2^{c}	7.3^{c}	10.0^{bc}	12.8^{ab}	1.9	**0.0225**
creatine (CH_2)	3.929−3.940	21.5^{c}	25.0^{b}	29.0^{a}	32.0^{a}	28.7^{a}	29.8^{a}	1.2	**0.0001**
α-Glc (H1)	5.220−5.261	13.4	16.5	22.7	22.4	14.4	9.8	6.4	0.5965
Tyr (H3/H5)	6.880−6.931	9.5^{bc}	12.7^{a}	10.4^{abc}	8.6^{c}	10.6^{abc}	11.3^{ab}	0.8	**0.0307**
Tyr (H2/H6)	7.180−7.221	16.5^{a}	16.7^{a}	14.8^{a}	12.7^{b}	16.6^{a}	15.8^{a}	0.7	**0.0026**
Phe (H2/H6/H4/H3/H5)	7.300−7.561	76.0^{a}	71.7^{ab}	66.0^{bc}	59.1^{c}	68.3^{b}	66.4^{bc}	2.6	**0.0033**
ATP/ADP (ring H2)	8.250−8.296	3.9^{ab}	3.1^{b}	4.8^{ab}	5.0^{ab}	5.5^{a}	3.6^{ab}	0.6	0.0988
formate (CH)	8.454−8.470	1.0	0.8	1.0	1.2	0.9	1.1	0.2	0.4500

[a] The time point t = 0 h is immediately after the 1 h heat exposure. Abbreviations are as defined in the caption for **Figure 2**. LSMeans within a row with different superscript letters (a−d) differ significantly (P < 0.05).

where the mouse myotubes were exposed to 42 or 45 °C for 1 h, respectively. For the mouse myotubes the two first principal components, PC1 and PC2, account for 74% (42 °C) and 77% (45 °C) of the total variance, respectively. In the medium PC1 and PC2 account for 97% (42 °C) and 95% (45 °C) of the total variance, respectively. In the following primarily metabolites with an explained variance above 50% will be included in the description of the data.

Mouse Myotubes. When the myotubes were exposed to 42 °C, clear tendencies for clustering of the control samples and the samples from the different time points after stress was observed in the PCA score plot (**Figure 4A**). The stress exposure resulted in a time-dependent gradual change in the levels of the metabolites apparent in both PC1 and PC2. Hence, the stress exposure resulted in a shift to the left-hand side of the plot (PC1) with a maximum shift reached after 4 h and then returning toward control, without reaching the control 10 h after stress. From the corresponding loading plot (**Figure 4C**; **Figure 3A**,**B** and **Table 1**) it is apparent that the metabolic changes mainly contributing to this shift were an increase followed by a decrease in glucose and decreases followed by increases in leucine, lysine, lactate, phenylalanine, and acetate. In the 10 h time period after stress a progressive shift upward in the PCA score plot (PC2) was observed (**Figure 4A**). From the corresponding loading plot (**Figure 4C**; **Table 1** and **Figure 3A**,**B**) it is apparent that the metabolites exerting the greatest influence on this shift with time after stress were increases in glutamine, alanine, and creatine and to a lesser degree decreases in leucine, lysine, and phenylalanine.

Overall similar patterns, in comparison to 42 °C heat stress, were observed in the score and loading plots when the myotubes were exposed to 45 °C (**Figure 4B**,**D**). The stress exposure of 45 °C also resulted in a time-dependent gradual change in the levels of the metabolites apparent in both PC1 and PC2 (**Figure 4B**), however, with a shift in the opposite direction along PC1 in comparison to 42 °C. The shift to the right-hand side of the plot (PC1) with time after stress also reached a maximum after 4 h and then returned toward control, without reaching the control 10 h after stress. From the corresponding loading plot (**Figure 4D**; **Figure 3C**,**D** and **Table 2**)

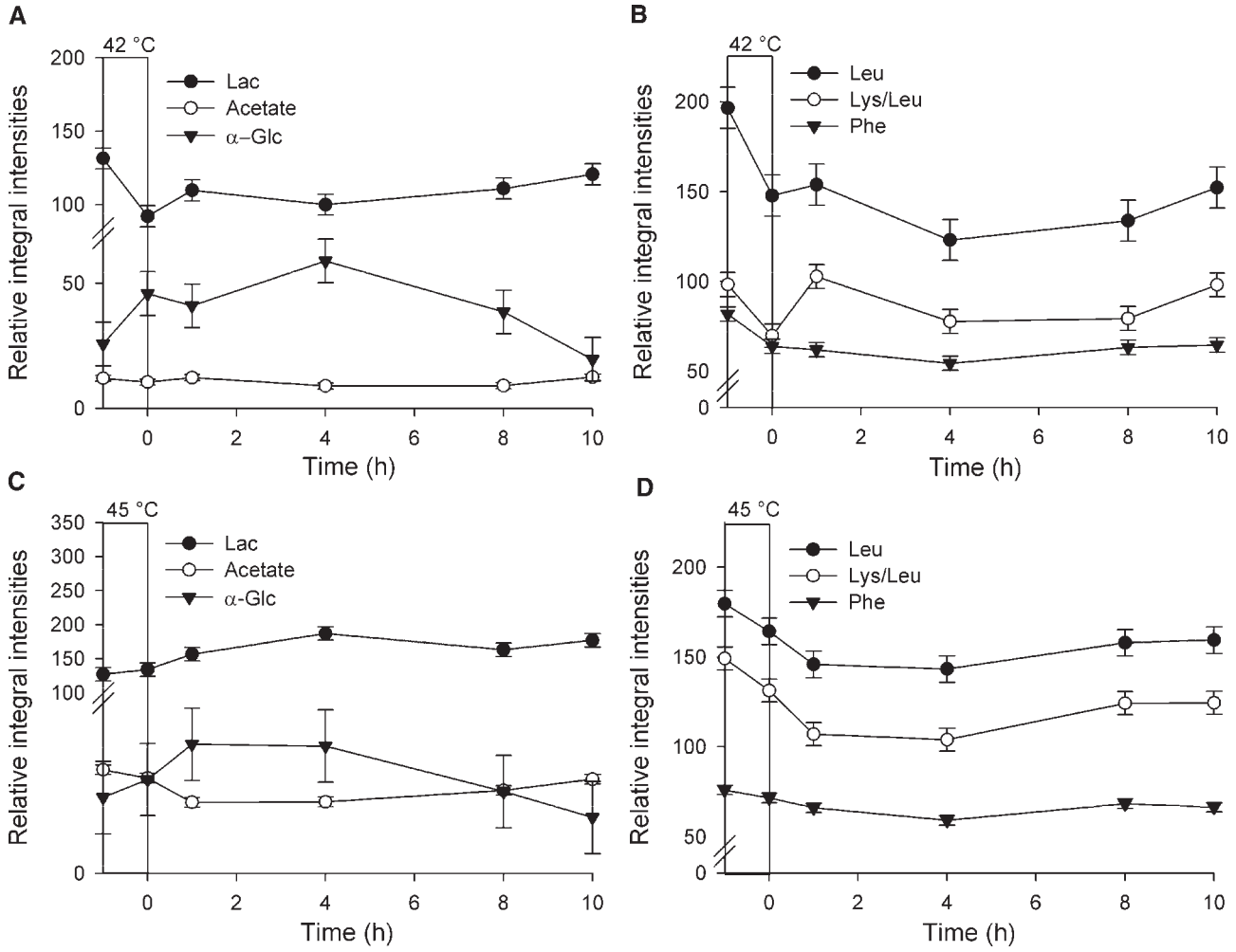

Figure 3. Intensities (LSMeans ± SEM, $n = 5$) of the relative integrals of the metabolites lactate, acetate, glucose, leucine, lysine, and phenylalanine from the ^{1}H NMR spectra for myotubes exposed to 42 °C (**A** and **B**) or to 45 °C (**C** and **D**) for 1 h. The time point $t = 0$ h is immediately after the 1 h heat exposure. Abbreviations are as defined in the caption for **Figure 2**.

Table 3. ^{1}H NMR Chemical Shift Ranges and LSMeans of the Relative Integral Intensities, with Calculated Standard Error of the LSMeans (SEM) for the Medium from Myotubes Exposed to 42 °C for 1 h[a]

metabolite	shift range (ppm)	control ($n = 5$)	0 h ($n = 4$)	1 h ($n = 5$)	4 h ($n = 4$)	8 h ($n = 3$)	10 h ($n = 5$)	SEM	P	decrease (%)	increase (%)
Leu (δ-CH_3)	0.918−0.978	50.7^a	50.7^a	48.7^{bc}	49.2^{ab}	47.8^{bc}	47.4^c	0.7	**0.0014**	6.5	
Val (CH_3)	0.979−1.001	25.4^a	25.0^b	24.6^c	24.5^c	23.9^d	23.4^e	0.2	**<0.0001**	7.7	
Ile (δ-CH_3) (CH_3)	1.001−1.023	23.7^a	23.1^b	22.9^{bc}	22.8^c	22.2^d	21.8^e	0.2	**<0.0001**	8.3	
Val (CH_3)	1.029−1.060	25.6^a	25.6^a	24.8^b	24.7^b	24.2^c	23.9^d	0.3	**<0.0001**	6.8	
Lac (CH_3)	1.300−1.358	290.0^{cd}	280.1^d	300.1^c	325.3^b	349.1^a	363.6^a	12.5	**<0.0001**		25.4
Ala-Gln (CH_3)	1.372−1.537	141.5^a	142.8^a	136.3^b	135.3^b	132.4^c	129.4^d	1.7	**<0.0001**	8.5	
Lys/Leu (δ-CH_2)/(CH_2)	1.600−1.781	51.8^b	55.0^a	48.8^c	48.7^c	47.6^d	47.2^d	0.4	**<0.0001**	8.7	
acetate (CH_3)	1.917−1.930	4.2^b	2.7^c	4.2^b	4.9^a	4.5^{ab}	4.7^a	0.2	**<0.0001**		10.8
β-Glc (H1)	4.635−4.669	93.3^a	95.7^a	89.5^b	87.3^b	82.6^c	81.0^c	2.4	**<0.0001**	13.2	
α-Glc (H1)	5.220−5.259	74.0^a	74.7^a	71.4^b	69.5^b	65.8^c	63.8^c	1.9	**<0.0001**	13.8	
Tyr (H3/H5)	6.870−6.926	7.8^a	7.4^b	7.2^{cd}	7.2^c	7.2^{cd}	7.1^d	0.1	**<0.0001**	9.6	
His (H4)	7.020−7.070	2.2^a	2.3^a	1.9^b	1.9^b	1.9^b	1.8^b	0.1	**<0.0001**	15.2	
Tyr (H2/H6)	7.169−7.230	8.3^a	8.3^a	7.7^b	7.6^{bc}	7.6^{bc}	7.4^c	0.1	**<0.0001**	10.8	
Phe (H2/H6/H4/H3/H5)	7.300−7.470	27.1^a	27.1^a	25.9^b	25.7^b	25.5^{bc}	25.1^c	0.3	**<0.0001**	7.5	
formate (CH)	8.454−8.462	0.8^{bc}	0.8^c	0.9^{ab}	0.9^{abc}	0.9^a	0.9^a		**0.0134**		15.5

[a] The time point t = 0 h is for medium collected immediately after the heat exposure. The decreases or increases in the relative integral intensities are the 10 h levels relative to the control levels. Abbreviations are as defined in the caption for **Figure 2**. LSMeans within a row with different superscript letters (a−e) differ significantly ($P < 0.05$).

it is apparent that the metabolic changes mainly contributing to this shift were an increase followed by a decrease in glucose, an increase followed by plateau level in creatine, and decreases followed by increases in leucine, lysine, phenylalanine, alanine, and acetate. At 45 °C stress exposure, a progressive shift upward in the PCA score plot (PC2) after stress was observed (**Figure 4B**), as for the myotubes exposed to 42 °C, but the maximum shift was reached after 4 h and then returned toward control, without reaching the control 10 h after stress. The corresponding loading plot (**Figure 4D**; **Figure 3C**,**D** and **Table 2**) shows that the metabolic changes exerting the greatest

Table 4. ^{1}H NMR Chemical Shift Ranges and LSMeans of the Relative Integral Intensities, with Calculated Standard Error of the LSMeans (SEM) for the Medium from Myotubes Exposed to 45 °C for 1 h[a]

metabolite	shift range (ppm)	control (n = 4)	0 h (n = 4)	1 h (n = 5)	4 h (n = 4)	8 h (n = 5)	10 h (n = 5)	SEM	P	decrease (%)	increase (%)
Leu (δ-CH_3)	0.918−0.978	48.3^a	47.7^{ab}	46.8^{bc}	45.8^{cd}	46.5^{bc}	44.7^d	0.5	**0.0002**	7.5	
Val (CH_3)	0.979−1.001	24.1^a	23.3^b	23.1^b	22.6^c	22.3^d	21.8^e	0.1	**<0.0001**	9.7	
Ile (δ-CH_3) (CH_3)	1.001−1.023	22.7^a	21.2^b	21.1^b	20.6^c	20.3^d	20.0^e	0.1	**<0.0001**	11.7	
Val (CH_3)	1.029−1.060	24.6^a	23.8^b	23.5^c	23.1^d	22.5^e	22.2^f	0.1	**<0.0001**	9.7	
Lac (CH_3)	1.300−1.358	333.8^f	350.1^e	373.1^d	394.3^c	422.4^b	435.9^a	2.8	**<0.0001**		30.6
Ala-Gln (CH_3)	1.372−1.537	137.7^a	136.3^a	132.2^b	130.0^b	124.3^c	122.4^c	1.1	**<0.0001**	11.1	
Lys/Leu (δ-CH_2)/(CH_2)	1.600−1.781	49.1^b	51.2^a	48.8^{bc}	47.2^c	44.1^d	43.3^d	0.7	**<0.0001**	11.7	
acetate (CH_3)	1.917−1.930	4.5^b	2.9^d	3.1^d	3.9^c	5.5^a	5.6^a	0.2	**<0.0001**		24.1
β-Glc (H1)	4.635−4.669	84.8^a	80.4^{ab}	80.0^b	73.6^c	66.6^d	64.9^d	1.6	**<0.0001**	23.4	
α-Glc (H1)	5.220−5.259	67.7^a	64.0^b	61.5^c	58.4^d	54.4^e	52.4^f	0.6	**<0.0001**	22.6	
Tyr (H3/H5)	6.870−6.926	7.6^a	6.9^b	6.9^b	6.8^{bc}	6.8^{bc}	6.6^c	0.1	**<0.0001**	12.8	
His (H4)	7.020−7.070	2.1^a	2.1^a	2.0^a	1.9^a	1.6^b	1.6^b	0.1	**0.0005**	22.9	
Tyr (H2/H6)	7.169−7.230	7.9^a	7.7^a	7.6^{ab}	7.3^b	6.8^c	6.7^c	0.1	**<0.0001**	15.4	
Phe (H2/H6/H4/H3/H5)	7.300−7.470	25.4^a	24.8^b	24.6^b	24.0^c	23.0^d	22.6^d	0.2	**<0.0001**	11.0	
formate (CH)	8.454−8.462	0.8^{bc}	0.8^c	0.8^{bc}	0.8^{bc}	0.9^{ab}	0.9^a		**0.0112**		17.4

[a] The time point t = 0 h is for medium collected immediately after the heat exposure. The decreases or increases in the relative integral intensities are the 10 h levels relative to the control levels. Abbreviations are as defined in the caption for **Figure 2**. LSMeans within a row with different superscript letters (a−f) differ significantly (P < 0.05).

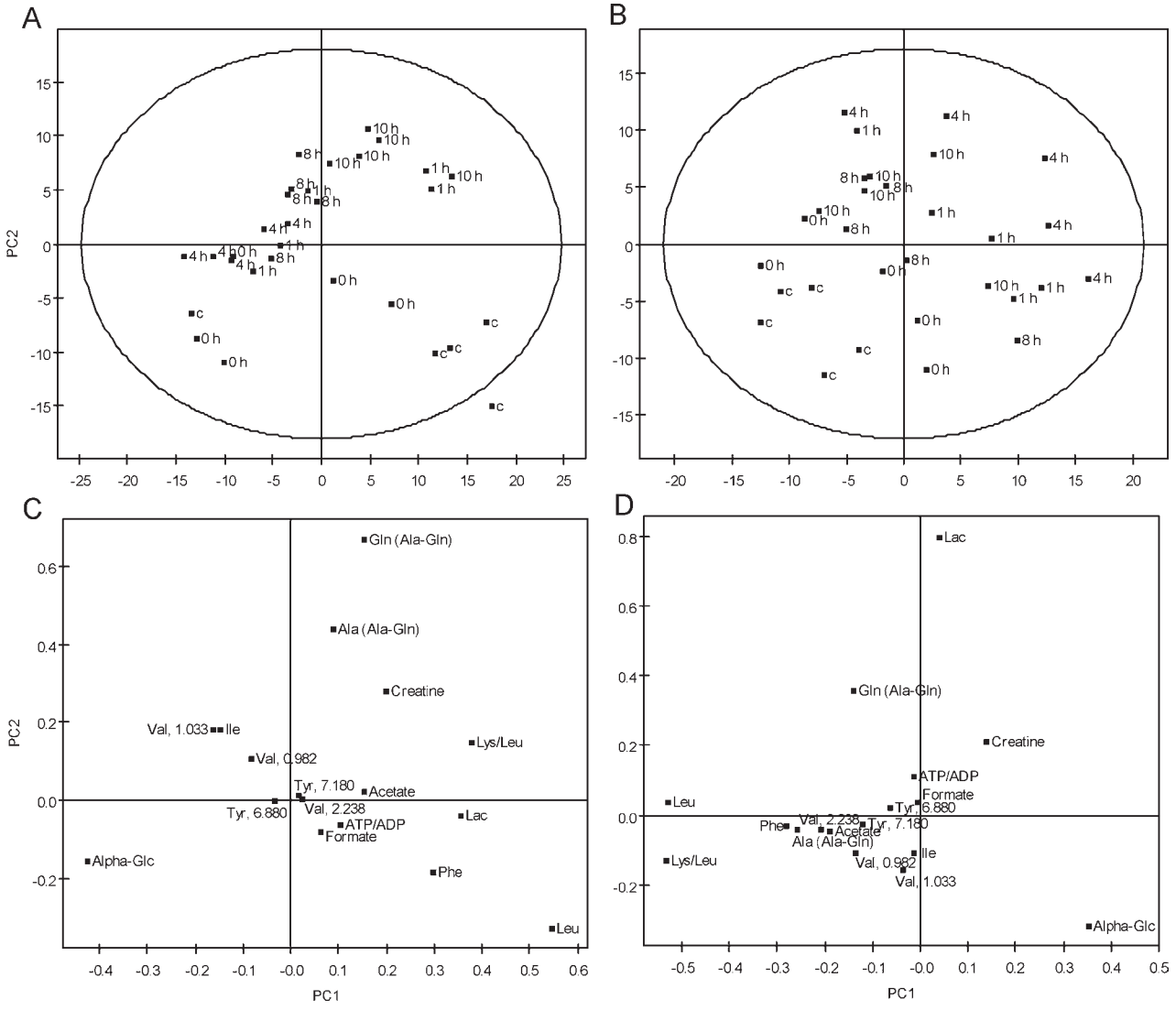

Figure 4. PCA score plots (**A** and **B**) mapping the LSMeans for control samples and each of the time points after stress for PC1 and PC2, and the corresponding loading plots (**C** and **D**) for mouse myotubes exposed to 42 °C (**A** and **C**) or 45 °C (**B** and **D**), respectively, for 1 h. The time point t = 0 h is immediately after the 1 h heat exposure (c denotes the control). Abbreviations are as defined in the caption for **Figure 2**.

influence on this shift with time after stress were increases followed by plateau levels in lactate, glutamine, and creatine and an increase followed by a decrease in glucose.

Mouse Myotube Culture Medium. For the culture medium seven samples were classified as outliers and removed from the analyses. Four samples were removed from the medium from

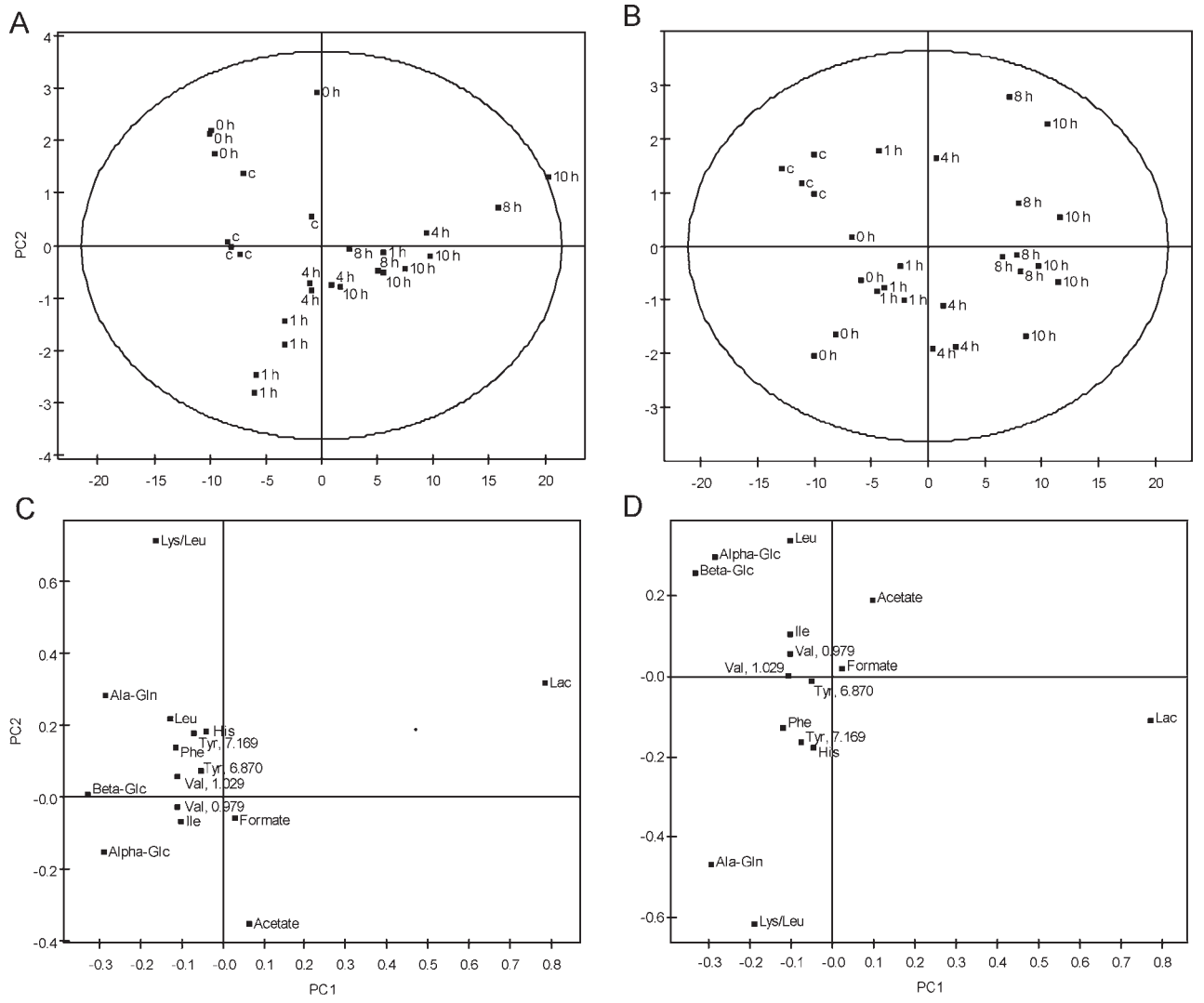

Figure 5. PCA score plots (**A** and **B**) mapping the LSMeans for control samples and each of the time points after stress for PC1 and PC2 and the corresponding loading plots (**C** and **D**) for the culture media from mouse myotubes exposed to 42 °C (**A** and **C**) or 45 °C (**B** and **D**), respectively, for 1 h. The time point t = 0 h is immediately after the 1 h heat exposure (c denotes the control). Abbreviations are as defined in the caption for **Figure 2**.

myotubes exposed to 42 °C and two samples from the medium from myotubes exposed to 45 °C due to spectral baseline problems because of suboptimal suppression of the water resonance. One sample was removed from the medium from myotubes exposed to 45 °C because of poor spectral quality.

When the medium from the myotubes exposed to 42 °C was analyzed with PCA, clustering of the control samples and the samples from the different time points after stress was observed in the PCA score plot (**Figure 5A**). The most apparent changes after stress were decreases or increases in metabolites over time, resulting in a shift to the right-hand side (PC1), with PC1 explaining 95% of the variance (**Figure 5A**). The corresponding loading plot (**Figure 5C**; **Table 3**) shows that the metabolic changes primarily contributing to this shift were a significant decrease in glucose and a significant increase in lactate over time and, to a minor degree, significant decreases in amino acids and significant increases in acetate and formate.

Similar but more clear patterns, in comparison to 42 °C heat stress, were apparent when the culture medium from myotubes exposed to 45 °C was analyzed (**Figure 5B**,**D**). Clustering of both the control samples and the samples from the different time points after stress was very obvious, and the control samples and the samples from the different time points were almost completely separated in the PCA score plot (**Figure 5B**). As for myotubes exposed to 42 °C, the changes after 45 °C stress were decreases or increases in metabolites over time also resulting in a shift to the right-hand side (PC1), with PC1 explaining 93% of the variance. This is reflected in a very similar pattern for the corresponding loading plot (**Figure 5D**), as for the myotubes exposed to 42 °C. Hence, the metabolites primarily contributing to this shift were also a significant decrease in glucose and a significant increase in lactate over time and, to a minor degree, significant decreases in amino acids and significant increases in acetate and formate (**Table 4**).

DISCUSSION

In relation to slaughter, the magnitude and duration of stressful conditions can vary and hence result in varying stress exposures, consequently varying meat quality development. Also, the time of rest after stress greatly influences the meat quality. Hence, the use of a muscle model system could aid in estimating the optimal time period to monitor stress markers and assist in estimating the adequate resting time after stress before the pigs are slaughtered.

At a temperature stress of 42 °C in the present experiments an increase in the transcription level of Hsp70 was found, significant

in both the mouse and the porcine myotubes (**Figure 1**). It was also found that porcine and mouse myotubes respond in a similar way regarding the transcription level of Hsp70 mRNA on exposure to heat stress. Thus, both cell models were evaluated as suitable, but the mouse cell line has the advantage of being easy to cultivate due to its continuous growth for several passages without changing characteristics. Furthermore, cultivation of the mouse cell line results in a higher yield in comparison to the porcine primary cells. Hence, in the present study the mouse cell line was used as a model system to investigate responses to heat stress, a stressor that is frequently observed when pigs are slaughtered.

PCA of the metabolites detected by ^{1}H NMR metabonomics revealed that it is possible to distinguish stressed mouse myotubes from nonstressed myotubes after heat exposure of either 42 or 45 °C (**Figure 4A**,**B**). Also, a gradual time-dependent change in metabolites after stress was evident after both 42 and 45 °C heat exposure. From the two corresponding loading plots (**Figure 4C**, **D**) it is apparent that the metabolic changes resulting in the clustering of the control samples and samples from the same time point and the time-dependent changes are very similar for both temperatures. In both cases similar changes with time after stress in primarily the metabolites glucose, leucine, lysine, phenylalanine, creatine, glutamine, and acetate are contributing to the clustering and separation of the control samples from the different time points after stress. These results are consistent with a study where rats were exposed to psychological stress, and multivariate analysis revealed alterations in the levels of glucose, glutamine, lactate, acetate, alanine, isoleucine, leucine, valine, and lysine in the plasma within the 6 h after stress by use of NMR-based metabonomics (*19*). This suggests that these very different types of stress cause similar reactions. ^{1}H NMR spectroscopy on mouse hearts revealed that stress introduced during ischemia/reperfusion injury resulted in higher levels of the metabolites glucose, lactate, glutamine, and creatine (*30*), which is in agreement with higher levels of these metabolites in the heat-stressed myotubes in the present experiments (**Figure 3A**,**C** and **Tables 1** and **2**). Despite the apparent overall changes in glucose in the present study, the glucose levels varied considerably between the control samples and between the samples from different time points after stress. Thus, even though the changes in the glucose levels were pronounced, they were not significant.

A few differences in the myotubes' response to 42 and 45 °C were apparent, most noticeably the changes in the lactate levels. For the myotubes exposed to 42 °C a decrease in lactate was observed (**Figure 3A** and **Table 1**), whereas for the myotubes exposed to 45 °C an increase in lactate was observed after stress (**Figure 3C** and **Table 2**). The analyses of the media showed that lactate is released to the media, the increase in release after stress being highest after exposure to 45 °C (30.6%), in comparison to the release after exposure to 42 °C (25.4%). In cultured human myotubes increased extracellular acidification resulting from lactate and carbon dioxide was used to identify malignant hyperthermia (*31*). After heat stress in the present study an increase in lactate was only observed for myotubes exposed to 45 °C, whereas at 42 °C a decrease in lactate was observed after stress, not even reaching the control level 10 h after stress. In pigs exposed to exercise stress a relation was found between the pH measured 1 h post mortem in the muscles and the lactate level in the plasma (*21*). This indicates that the myotubes regulate the pH level by release of lactate to the medium. For myotubes exposed to 45 °C stress the lactate level does not return to the control level 10 h after stress but reaches a plateau after 4 h. In contrast, the myotubes exposed to 42 °C apparently regulate the lactate level more efficiently by release to the medium. The decrease in lactate immediately after stress does not return to the control level within 10 h, indicating an overshooting effect. Overshoot effects have previously been observed in relation to stress exposure in vivo (*9*). For pigs slaughtered immediately after treadmill stress, a decrease in pH in the muscles measured 45 min after slaughter was found; however, in pigs resting before slaughter the pH was in some cases found to be higher in comparison to that in control pigs.

The findings in the present study are also in agreement with our previous ^{13}C and ^{1}H NMR experiments where myotubes incubated with [$^{13}C_1$]glucose showed increases in newly synthesized ^{13}C-labeled lactate 3 h after exposure to 42 or 45 °C for 1 h that was not significant, however, in comparison to control, whereas a decrease in the unlabeled lactate was observed to be significant at 45 °C (*17*). Hence, one needs to be careful when planning experiments and assessing results because of complex regulation mechanisms after stress, and also the choice of methods has to be considered carefully.

The increased lactate production after stress in the medium from myotubes exposed to 45 °C in comparison to the medium from the myotubes exposed to 42 °C indicates an increase in anaerobic metabolism with an increase in temperature. The accumulation of lactate inside the myotubes observed in the present experiments at 45 °C, which was significant 1 h after stress, also indicates an increase in anaerobic metabolism. Furthermore, as mentioned above, an increase in lactate production, though not significant, has also been observed by application of ^{13}C NMR spectroscopy in our previous heat stress experiments at 42 and 45 °C (*17*). However, in general higher metabolic turnover was observed with an increase in temperature. Higher consumption of glucose and amino acids and a higher production of acetate and formate was observed in the media when myotubes were exposed to 45 °C in comparison to myotubes exposed to 42 °C (**Tables 3** and **4**).

In the myotubes a decrease in acetate was observed after heat stress, which returned to the control level within the 10 h recovery period after stress. However, increased acetate production was apparent as an increase in the release of acetate to the medium from the myotubes exposed to 45 °C (24.1%) in comparison to the myotubes exposed to 42 °C (10.8%). By use of NMR-based metabonomics it has been found that exercising pigs on a treadmill causes an increase in acetate in the plasma (*21*). These data support our findings that acetate is released from the myotubes to the medium, increasing with an increase in heat exposure.

Together with acetate, it has been found that lactate is the metabolite mainly increasing in the plasma after treadmill stress of pigs (*21*). Hence, lactate and acetate were the metabolites primarily contributing to the separation of control pigs from stressed pigs. Partial least-squares discriminate analysis revealed a strong correlation between the plasma metabolite profile and muscle temperature (*21*). Also, Jensen-Waern and Nyberg (*32*) found that lactate in the plasma is a good marker for stress after exercising pigs on a treadmill. These previous results are in good agreement with the results from the media in the present study, and the metabolites lactate and acetate may be good markers of stress. However, the results also show that the time point after stress is very crucial when monitoring the presence of these stress metabolites. In the myotubes only at 45 °C and not until 1 h after stress and onward was a significant increase in lactate observed in comparison to control myotubes. The alternations of the metabolites in the myotubes are complex and highly dependent on the stress level and the time after stress exposure.

The finding of increased lactate production with increased stress exposure in the present study is in agreement with known responses in relation to slaughter stress (*1*, *2*, *5*−*9*, *21*, *32*). However, in the present study the application of the explorative metabonomic approach also resulted in identifying alternation

in the levels of numerous amino acids in response to heat stress. The relation between changes in the levels of these amino acids and the influence on meat quality is not known, and further studies are needed to elucidate if alternations in these metabolites influence meat quality.

From the present experiments it is apparent that it is possible to distinguish myotube control samples from myotube samples exposed to heat stress of 42 or 45 °C for 1 h. To some extent the different time points after stress were also separated. Score and loading plots for 42 and 45 °C heat stress were very similar. Evidently, the 42 °C stress level, which is a temperature level that has been reached in the muscles of treadmill exercised pigs (*9*), resulting in a significant increase in the expression of Hsp70 in the present experiment is adequate to make a separation of control samples and the samples exposed to stress. A stress exposure of 45 °C did not result in a better separation of the samples.

In conclusion, by use of multivariate data analysis changes in metabolite levels can be used to distinguish control myotubes from different time points after stress, at a heat stress level experienced in pig muscles under stressful conditions. In general the metabolites identified in the present study vary significantly in comparison to control samples and samples immediately after stress, and for some metabolites significant changes in the metabolite levels in the recovery period after stress are also apparent. Hence, metabolites identified in the present study could possibly be used as markers for stress experienced in relation to slaughter, but further whole animal studies are needed to establish the stress-induced time-dependent cellular metabolic fluctuations in whole animals under stress.

ABBREVIATIONS USED

1D, one-dimensional; 2D, two-dimensional; Ala, alanine; Aln-Gln, alanyl-glutamine; CI, confidence interval; COSY, correlation spectroscopy; DMEM, Dulbecco's Modified Eagle's Medium; FCS, fetal calf serum; FID, free induction decays; Glc, glucose; Gln, glutamine; His, histidine; Hsp, heat shock protein; HSQC, heteronuclear singe-quantum coherence spectroscopy; Ile, isoleucine; Lac, lactate; Leu, leucine; LSMeans, least-squares means; Lys, lysine; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; PCA, principal component analysis; PGM, primary growth medium; Phe, phenylalanine; RT-PCR, reverse transcription polymerase chain reaction; SEM, standard errors of LSMeans; TSP, sodium trimethylsilyl[2,2,3,3-D_4]-1-propionate; Tyr, tyrosine; Val, valine.

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