Open Access
Asian-Australas J Anim Sci
Vol. 30, No. 11:1633-1642 November 2017
https://doi.org/10.5713/ajas.17.0167
pISSN 1011-2367 eISSN 1976-5517

AJAS
Asian-Australasian Journal of Animal Sciences

Analysis of metabolomic patterns in thoroughbreds before and after exercise

Hyun-Jun Jang[1,2,a], Duk-Moon Kim[3,a], Kyu-Bong Kim[1], Jeong-Woong Park[4], Jae-Young Choi[4], Jin Hyeog Oh[4], Ki-Duk Song[2], Suhkmann Kim[5,*], and Byung-Wook Cho[4,*]

* **Corresponding Authors:** Suhkmann Kim
Tel: +82-51-510-2240, **Fax:** +82-51-516-7421,
E-mail: suhkmann@pusan.ac.kr
Byung-Wook Cho
Tel: +82-55-350-5515, **Fax:** +82-55-350-5519,
E-mail: bwcho@pusan.ac.kr

[1] College of Pharmacy, Dankook University, Cheonan 31116, Korea
[2] Department of Animal Biotechnology, Chonbuk National University, Jeonju 54896, Korea
[3] Department of Animal Biotechnology, College of Applied Life Sciences, Jeju National University, Jeju 63243, Korea
[4] Department of Animal Science, College of Natural Resources and Life Sciences, Pusan National University, Miryang 50463, Korea
[5] Department of Chemistry and Chemistry Institute for Functional Materials, Pusan National University, Busan 46241, Korea

[a] These authors contributed equally to this work.
Submitted Feb 3, 2017; Revised May 3, 2017; Accepted Jun 2, 2017

Objective: Evaluation of exercise effects in racehorses is important in horseracing industry and animal health care. In this study, we compared metabolic patterns between before and after exercise to screen metabolic biomarkers for exercise effects in thoroughbreds.
Methods: The concentration of metabolites in muscle, plasma, and urine was measured by ^{1}H nuclear magnetic resonance (NMR) spectroscopy analysis and the relative metabolite levels in the three samples were compared between before and after exercise. Subsequently, multivariate data analysis based on the metabolic profiles was performed using orthogonal partial least square discriminant analysis (OPLS-DA) and variable important plots and t-test was used for basic statistical analysis.
Results: From ^{1}H NMR spectroscopy analysis, 35, 25, and 34 metabolites were detected in the muscle, plasma, and urine. Aspartate, betaine, choline, cysteine, ethanol, and threonine were increased over 2-fold in the muscle; propionate and trimethylamine were increased over 2-fold in the plasma; and alanine, glycerol, inosine, lactate, and pyruvate were increased over 2-fold whereas acetoacetate, arginine, citrulline, creatine, glutamine, glutarate, hippurate, lysine, methionine, phenylacetylglycine, taurine, trigonelline, trimethylamine, and trimethylamine *N*-oxide were decreased below 0.5-fold in the urine. The OPLS-DA showed clear separation of the metabolic patterns before and after exercise in the muscle, plasma, and urine. Statistical analysis showed that after exercise, acetoacetate, arginine, glutamine, hippurate, phenylacetylglycine trimethylamine, trimethylamine N-oxide, and trigonelline were significantly decreased and alanine, glycerol, inosine, lactate, and pyruvate were significantly increased in the urine ($p<0.05$).
Conclusion: In conclusion, we analyzed integrated metabolic patterns in the muscle, plasma, and urine before and after exercise in racehorses. We found changed patterns of metabolites in the muscle, plasma, and urine of racehorses before and after exercise.

Keywords: Racehorse; Thoroughbred; Metabolic Analysis; Exercise

INTRODUCTION

Exercise affects metabolic responses throughout the body [1]. During exercise, muscles generate ATP by using various intramuscular and extramuscular substrates such as creatine phosphate, muscle glycogen, blood-borne glucose, lactate, and free fatty acids. The various substrates for exercise metabolism are dependently determined by exercise intensity and duration as well as training status, dietary manipulation, and other environmental factors [2]. Exercise of maximal intensity increases the amount of lactate derived from the degradation of muscle glycogen, products of adenine nucleotide catabolism, and tricarboxylic acid cycle intermediates related to aerobic energy production [3]; it also promotes glycogenolysis, lipolysis, and ammonia metabolism [4]. Prolonged submaximal intensity exercise improves insulin sensitivity, arterial compliance, and endothelial function [5]; increases lipid catabolism [6]; decreases the catecholamine response

Copyright © 2017 by Asian-Australasian Journal of Animal Sciences
This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/4.0/), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

[7]; and maintains bone density, skeletal muscle mass, and muscle metabolic capacity during ageing [8].

The equine skeletal muscle displays intrinsic metabolic adaptations based on myofiber structure and function, substrate and by-product transport across the sarcolemma, and coordinated integration of metabolic pathways to produce ATP in response to exercise [9]. Equine muscles store a large amount of glycogen (300 to 650 mol/g dry weight) in fast fibers. The stored glycogen is used as the most important source of energy for muscle contraction during both submaximal (<85% VO_{2max}) and maximal exercise (>85% VO_{2max}) [9]. During prolonged submaximal intensity exercise, lipids also contribute to produce muscle energy with glycogen [10]. After exercise, supplementation of muscle glycogen can slowly take up to 72 h in horses [11]. Previous studies have shown that muscle glycogen supplement after exercise was enhanced by certain processes such as intravenous glucose infusion, oral acetate administration, and rehydration with hypotonic electrolyte solutions in horses [12]. In addition, the buffering capacity that prevents muscle acidosis by lactate is higher in horses than in other species, probably because of high carnosine content [9]. Some studies have also suggested that equine adaptation to exercise could improve both aerobic and anaerobic capacities [9]. However, the mechanism underlying equine metabolism in response to exercise is still unclear.

Recently, multivariate approaches of metabolomic analysis have been used to understand biological mechanisms [13]. With respect to biological endpoints, quantifications of metabolomes could elucidate biological phenomena with other omics studies such as genomics, transcriptomics, and proteomics. For the acquisition of metabolic data, high-resolution ^{1}H or ^{13}C nuclear magnetic resonance (NMR) spectroscopy and mass spectroscopy have been used along with other spectroscopic methodologies [14,15]. The acquired data can be interpreted using multivariate statistical analysis, such as hierarchical cluster analysis, principal component analysis, different types of partial least square analysis, and subsequent modeling with new regression algorithms [16,17].

In this study, we analyzed the metabolic profiles of equine muscle, plasma, and urine before and after exercise by using ^{1}H NMR spectroscopy. On the basis of the analysis results, commonly or specifically expressed metabolites were selected from the muscle, plasma, and urine, and they reflected the effects of exercise. Subsequently, we suggested metabolic pathways related to those metabolites. Our study could contribute to understanding fluctuations in equine metabolism because of exercise.

MATERIALS AND METHODS

Horses and ethical statement

Three Thoroughbred were used in this study. The Pusan National University-Institutional Animal Care and Use Committee approved the study design (Approval Number: PNU-2015-0864).

Sample collection

Blood, muscle, and urine samples were collected from each horse before and after exercise (30 min). Briefly, venous blood samples were collected using a-50 mL syringe and transferred to heparin-containing tubes and centrifuged at 5,000 rpm for 15 min to obtain plasma. The plasma samples were stored at −20°C until NMR sample preparation. For skeletal muscle biopsy, local anesthesia was administered to the gluteus medius, and a biopsy collection syringe was used to obtain the muscle samples before and after exercise. The samples were stored in liquid nitrogen until analysis. Urine was collected from the subjects before and after exercise and centrifuged to remove solids. An 600 μL aliquot of the supernatant was added to a micro centrifuge tube containing 70 μL of D_2O solution with 5 mM dextran sulphate sodium (DSS) and 10 mM imidazole. The DSS was used as the qualitative standard for the chemical shift scale. In addition, 30 μL of 0.42% sodium azide was added. The urine samples were stored at −70°C until analysis.

Nuclear magnetic resonance spectroscopy

The skeletal muscle and plasma samples were subjected to ^{1}H NMR spectroscopy analysis. Briefly, 45 μL of the samples was used with 5 μL of deuterium oxide (D_2O) containing 20 mM of the reference material trimethylsilylpropionate (TSP); 20 mg of the skeletal muscle samples was analyzed with 25 μL of D_2O containing 2 mM of TSP, and 630 μL of the urine samples was mixed with 70 μL of D_2O containing 20 mM of TSP before NMR measurement.

We conducted high-resolution magic angle spinning NMR for the skeletal muscle and plasma samples. The spinning rate was 2,050 Hz. To analyze the skeletal muscle, plasma, and urine samples, the Carr-Purcell-Meiboom-Gill pulse sequence was used to remove the water peak and macromolecular peak signal. The acquisition time was 1.704 s, and the relaxation delay was 1.0 s. Each sample was scanned 128 times, and the total analysis time was 8 min and 13 s.

Chenomx NMR Suite 7.1 (Chenomx Inc., Edmonton, AB, Canada) and SIMCAp+12.0 (Umetrics, Umea, Sweden) software were used to minimize the errors of the measured spectrum and statistical analysis, respectively. In this study, we quantified 22 metabolites in the plasma, while 33 metabolites were investigated in the skeletal muscle in both groups. We used TSP as the standard and measured the absolute concentrations of the metabolites to normalize the samples; the relative concentration of each metabolite was measured. The multivariate statistical analysis method was used to calculate the amount of metabolites present in the samples.

Orthogonal partial least square discriminant analysis

All data were converted from the NMR software format to the Microsoft Excel format. One-dimensional NMR spectra data were imported into SIMCA-P (version 12.0, Umetrics Inc., Kin-

nelon, NJ, USA) for multivariate statistical analysis, to examine intrinsic variations in the data set. These data were scaled using centered scaling prior to the orthogonal partial least square discriminant analysis (OPLS-DA). For the scaling process, the average value of each variable was calculated and then subtracted from the data. OPLS-DA score plots were used to interpret intrinsic variations in the data.

Statistical analysis

Means and standard deviations of the metabolites were calculated using Microsoft Excel. The statistical significance ($p<0.05$, $p<0.01$, or $p<0.001$) of apparent differences in metabolite concentrations before and after exercise was assessed using analysis of variance, followed by the *t*-test (Prism 5.01, San Diego, CA, USA).

RESULTS

Differentially expressed metabolites and metabolic patterns before and after exercise in horses

The metabolite analyses before and after exercise showed that 35, 25, and 34 metabolites were detected in the muscle, plasma, and urine, respectively. Sixteen metabolites were commonly changed among the muscle, plasma, and urine after exercise, and 11, 3, and 14 metabolites were specifically changed in the muscle, plasma, and urine, respectively, after exercise (Figure 1, Table 1). The relative levels of the metabolites after exercise in the muscle, plasma, and urine were measured and compared with the corresponding levels of before exercise. The results showed that aspartate, betaine, choline, cysteine, ethanol, and threonine were increased over 2-fold in the muscle; propionate and trimethylamine were increased over 2-fold in the plasma; and alanine, glycerol, inosine, lactate, and pyruvate were increased over 2-fold and acetoacetate, arginine, citrulline, creatine, glutamine, glutarate, hippurate, lysine, methionine, phenylacetylglycine, taurine, trigonelline, trimethylamine, and trimethylamine *N*-oxide were decreased below 0.5-fold in the urine (Figure 1).

OPLS-DA and variable important plots of the metabolites before and after exercise

OPLS-DA showed clear separation of the metabolic patterns before and after exercise in the muscle, plasma, and urine (Figure 2A, 2B, 2C). Subsequently, when variable important plots (VIPs)

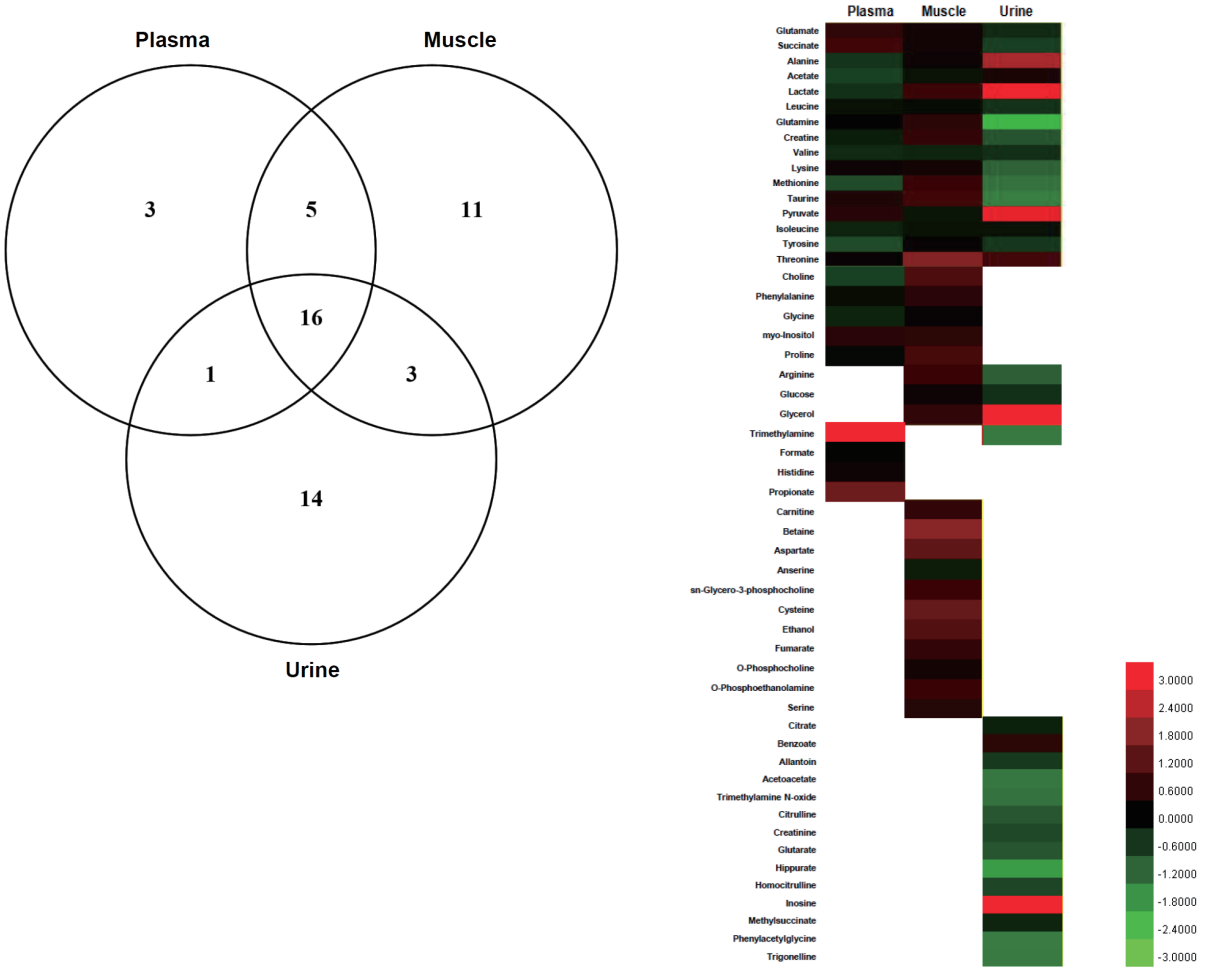

Figure 1. Metabolic clustering (left) and heatmap analysis of the differentially expressed metabolites (right) among the muscle, plasma, and urine.

Table 1. Metabolic clustering among the muscle, plasma, and urine

Clustering	Total	Metabolites
Muscle only	11	Anserine, aspartate, betaine, carnitine, cysteine, ethanol, fumarate, o-phosphocholine, o-phosphoethanolamine, serine, sn-glycero-3-phosphocholine
Plasma only	3	Formate, histidine, propionate
Urine only	13	Acetoacetate, allantoin, benzoate, citrate, citrulline, glutarate, hippurate, homocitrulline, inosine, methylsuccinate, phenylacetylglycine, trigonelline, trimethylamine n-oxide
Muscle and plasma	5	Choline, glycine, myo-inositol, phenylalanine, proline
Plasma and urine	1	Trimethylamine
Urine and muscle	3	Arginine, glucose, glycerol
Muscle, plasma, and urine	16	Lactate, creatine, taurine, glutamine, methionine, threonine, pyruvate, succinate, leucine, valine, isoleucine, glutamate, alanine, acetate, tyrosine, lysine

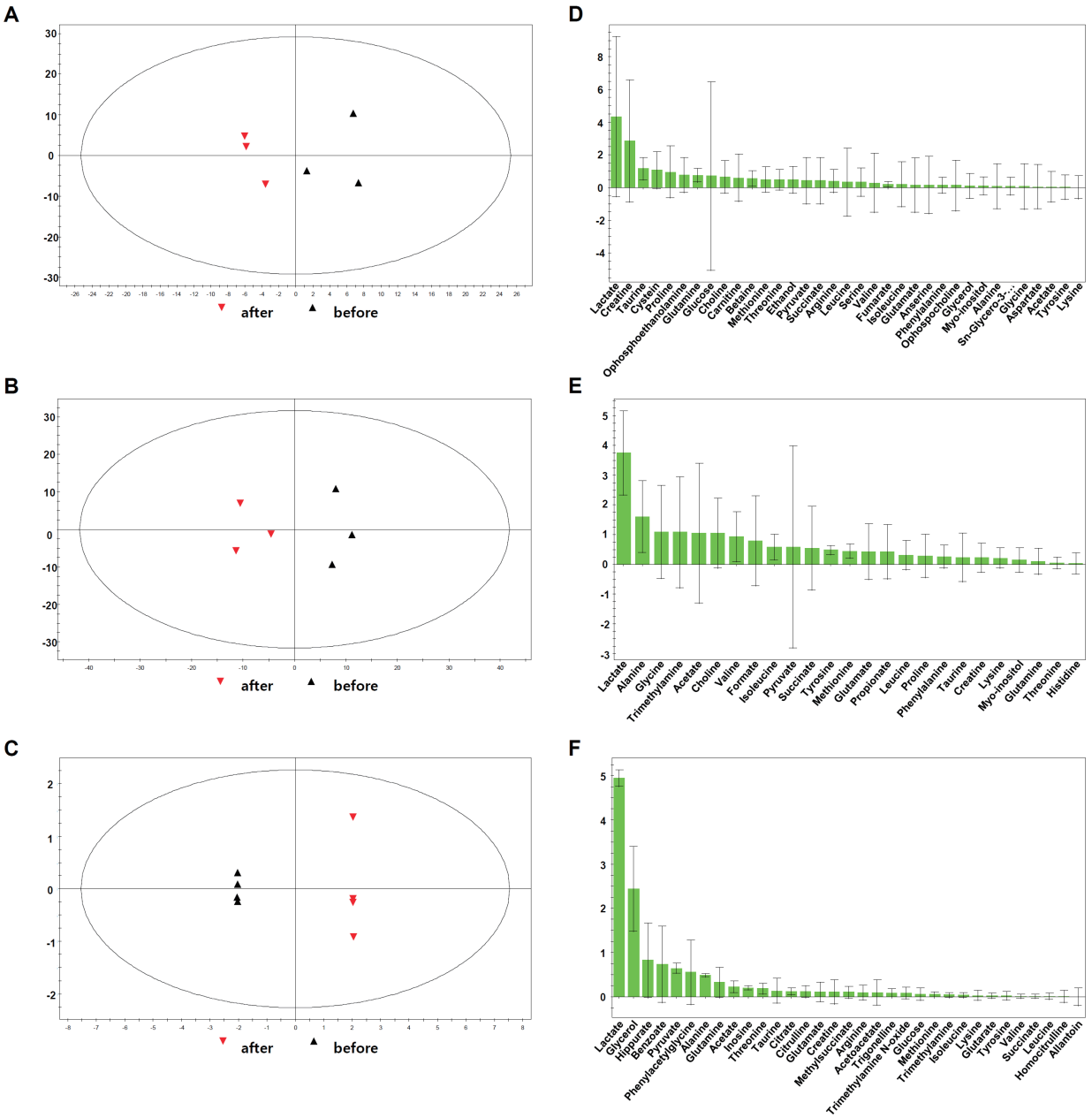

Figure 2. Analysis of the metabolic patterns in equine muscle, plasma, and urine before and after exercise. Orthogonal partial least square discriminant analysis (OPLS-DA) (R^2X: 0.977; R^2Y: 0.852; Q^2: –0.142) (A) and variable importance plots (VIPs) for the muscle (D). OPLS-DA (R^2X: 0.889; R^2Y: 0.883; Q^2: –1.33) (B) and VIPs for the plasma (E). OPLS-DA (R^2X: 0.987; R^2Y: 1; Q^2: 0.971) (C) and VIPs for the urine (F).

were derived from OPLS-DA for the metabolic patterns before and after exercise, the detected metabolites that contributed to separating the clusters in the respective samples were scored to reflect their priorities (Figure 2D, 2E, 2F). Lactate, creatine, taurine, and cysteine had VIP scores >1 in the muscle; lactate, alanine, glycine, trimethylamine, acetate, and choline had VIP scores >1 in the plasma; and lactate and glycerol had VIP scores >1 in the urine (Table 2).

Metabolites that responded to exercise

When the levels of the differentially expressed (fold change >2 or <0.5) and high-VIP-score (VIP score >1) metabolites were collectively analyzed in the muscle, plasma, and urine, the expressed levels were observed to be significantly changed in the urine after exercise, while no significant differences were detected in the muscle and plasma before and after exercise. After exercise, acetoacetate, arginine, glutamine, hippurate, phenylacetylglycine trimethylamine, trimethylamine *N*-oxide, and trigonelline were significantly decreased by 38.8%, 44.6%, 19.6%, 22.7%, 33.8%, 30.6%, 37.8%, and 30.8%, respectively, while alanine, glycerol, inosine, lactate, and pyruvate were significantly increased by 436.7%, 2,184.4%, 1,008.8%, 8,347.9%, and 726.5%, respectively, in the urine ($p<0.05$; Figure 3). With respect to the commonly detected metabolites, alanine, glutamine, lactate, and pyruvate showed significantly different expressions in the urine after exercise ($p<0.05$; Figure 4); the concentrations of alanine, lactate, and pyruvate in the plasma were significantly higher than in the muscle and urine, whereas the concentration of glutamine was not signifi-

Table 2. VIP scores show the list of metabolites that contributed to the separation of the clustering in the muscle (R^2X: 0.977; R^2Y: 0.852; Q^2: –0.142), plasma (R^2X: 0.889; R^2Y: 0.883; Q^2: –1.33), and urine (R^2X: 0.987; R^2Y: 1; Q^2: 0.971) before and after exercise

Muscle			Plasma			Urine		
Var ID (Primary)	VIP	VIPcvSE	Var ID (Primary)	VIP	VIPcvSE	Var ID (Primary)	VIP	VIPcvSE
Lactate	4.33964	4.90219	Lactate	3.75706	1.41472	Lactate	4.95072	0.185054
Creatine	2.86407	3.73581	Alanine	1.60664	1.205	Glycerol	2.43861	0.961256
Taurine	1.15696	0.677321	Glycine	1.08877	1.57026	Hippurate	0.830247	0.844768
Cysteine	1.07798	1.11629	Trimethylamine	1.08766	1.87254	Benzoate	0.733784	0.860513
Proline	0.950802	1.58483	Acetate	1.05611	2.35361	Pyruvate	0.64467	0.110574
O-Phosphoethanolamine	0.768788	1.08083	Choline	1.05136	1.17688	Phenylacetylglycine	0.553367	0.73167
Glutamine	0.757915	0.408429	Valine	0.927083	0.844553	Alanine	0.483287	0.0354834
Glucose	0.712887	5.76654	Formate	0.788366	1.50607	Glutamine	0.330252	0.337596
Choline	0.655007	0.984574	Isoleucine	0.590047	0.434523	Acetate	0.225269	0.12487
Carnitine	0.597443	1.45509	Pyruvate	0.588535	3.40277	Inosine	0.2025	0.0420699
Betaine	0.560761	0.476186	Succinate	0.54452	1.40882	Threonine	0.185985	0.128163
Methionine	0.509355	0.76717	Tyrosine	0.485224	0.149815	Taurine	0.133553	0.276668
Threonine	0.490596	0.624621	Methionine	0.442662	0.236588	Citrate	0.121972	0.0814875
Ethanol	0.489648	0.81646	Glutamate	0.423751	0.939892	Citrulline	0.117822	0.128122
Pyruvate	0.421163	1.43033	Propionate	0.422877	0.92142	Glutamate	0.112898	0.216093
Succinate	0.419546	1.43253	Leucine	0.320238	0.485677	Creatine	0.103247	0.271346
Arginine	0.40325	0.711309	Proline	0.289082	0.731382	Methylsuccinate	0.101376	0.134033
Leucine	0.340958	2.08214	Phenylalanine	0.272284	0.387797	Arginine	0.0948408	0.16968
Serine	0.339834	0.873955	Taurine	0.235986	0.816538	Acetoacetate	0.0927904	0.284629
Valine	0.274741	1.8082	Creatine	0.225572	0.492157	Trigonelline	0.0869787	0.0891645
Fumarate	0.221147	0.15998	Lysine	0.217242	0.344466	Trimethylamine N-oxide	0.0845139	0.127546
Isoleucine	0.19769	1.37773	myo-Inositol	0.151193	0.41876	Glucose	0.0585096	0.144095
Glutamate	0.151078	1.67088	Glutamine	0.109232	0.445521	Methionine	0.0564183	0.0567537
Anserine	0.147928	1.74206	Threonine	0.0471337	0.192853	Trimethylamine	0.0424139	0.0581431
Phenylalanine	0.147539	0.487178	Histidine	0.0349163	0.347014	Isoleucine	0.0411947	0.0560735
O-Phosphocholine	0.129257	1.55877				Lysine	0.0279116	0.11095
Glycerol	0.117228	0.76393				Glutarate	0.0243009	0.0617341
myo-Inositol	0.113193	0.541967				Tyrosine	0.0228266	0.0955172
Alanine	0.0848435	1.37882				Valine	0.0115851	0.0491691
sn-Glycero-3-phosphocholine	0.0792956	0.543817				Succinate	0.0113337	0.0459536
Glycine	0.0652003	1.38621				Leucine	0.0106587	0.073076
Aspartate	0.0604699	1.36912				Homocitrulline	0.00670114	0.133487
Acetate	0.0461708	0.938492				Allantoin	0.000348166	0.206484
Tyrosine	0.032664	0.755125						
Lysine	0.0203432	0.702034						

VIP, variable important plots; VIPcvSE, variables associated with lower standard errors in relation to VIP.
Bold box indicates major metabolites that have a VIP score of more than 1 according to orthogonal partial least square discriminant analysis.

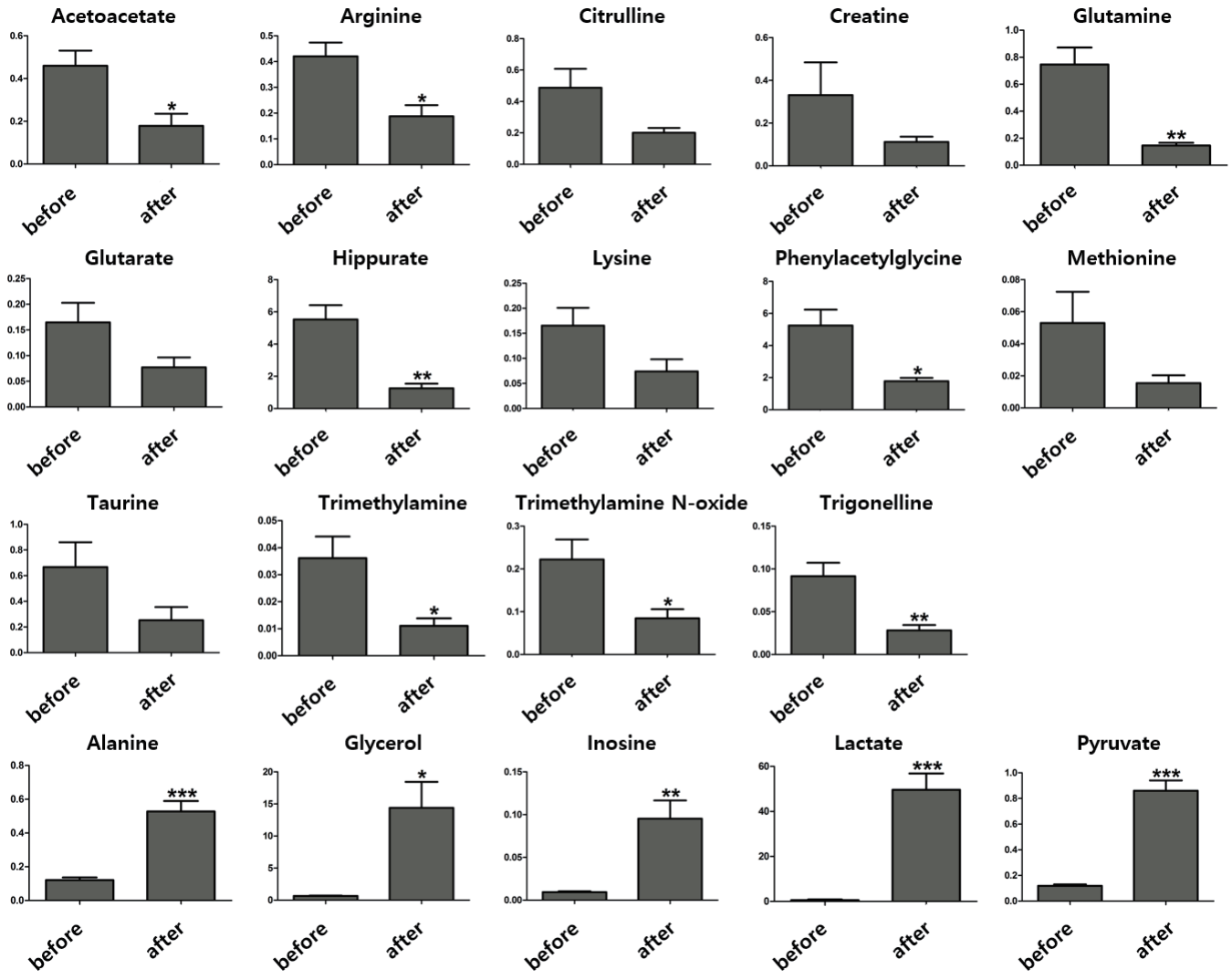

Figure 3. On the basis of the differentially expressed (fold change >2 or <0.5) or high-variable importance plots (VIPs)-score (VIP >1) metabolites, concentration of the metabolites in the urine before and after exercise. Error bars are expressed as standard deviation; * p<0.05; ** p<0.01; *** p<0.001.

cantly different between the muscle and plasma (p<0.05; Figure 4).

Enrichment analyses of metabolic pathways that responded to exercise

Enrichment analyses for the differentially expressed (fold change >2 or <0.5) and high-VIP-score (VIP score >1) metabolites were performed using MetaboAnalyst 3.0 [18], and 36 pathways were predicted (Table 3).

DISCUSSION

Metabolic alteration reflects biological responses to various genetic, transcriptiomic, proteomic, and environmental influences [19-21]. Many metabolic studies have applied to characterize metabolic patterns derived from altered gene function in plants [22,23], explore microbial metabolism [24], assess drug toxicity [25] and diagnostic applications [26], and discover biomarkers for animal health and disease [21,27,28]. Therefore, metabolic biomarkers are regarded as a promising tool for improving animal health and welfare.

Since domestication, horses have been selected for superior athletic traits related to strength, endurance, and speed. In particular, racehorses have undergone artificial structural and functional adaptations for athletic performances. As a result, racehorses developed maximal aerobic capacity, intramuscular energy stores, mitochondrial volume in the muscle, and oxygen-carrying capacity in the blood [29]. From the unique physiological properties, most of the metabolic studies on exercising horses focused on glycogen stores, whereas only a few studies have addressed muscle triglyceride or protein stores. During intensive short-term exercise, muscle glycogen stores may be depleted by 20% to 35%, and prolonged exercise results in a decline in muscle glycogen by 50% to 100% [30]. However after cessation of exercise, the rate of glycogen repletion is much lower in horses than in other ani-

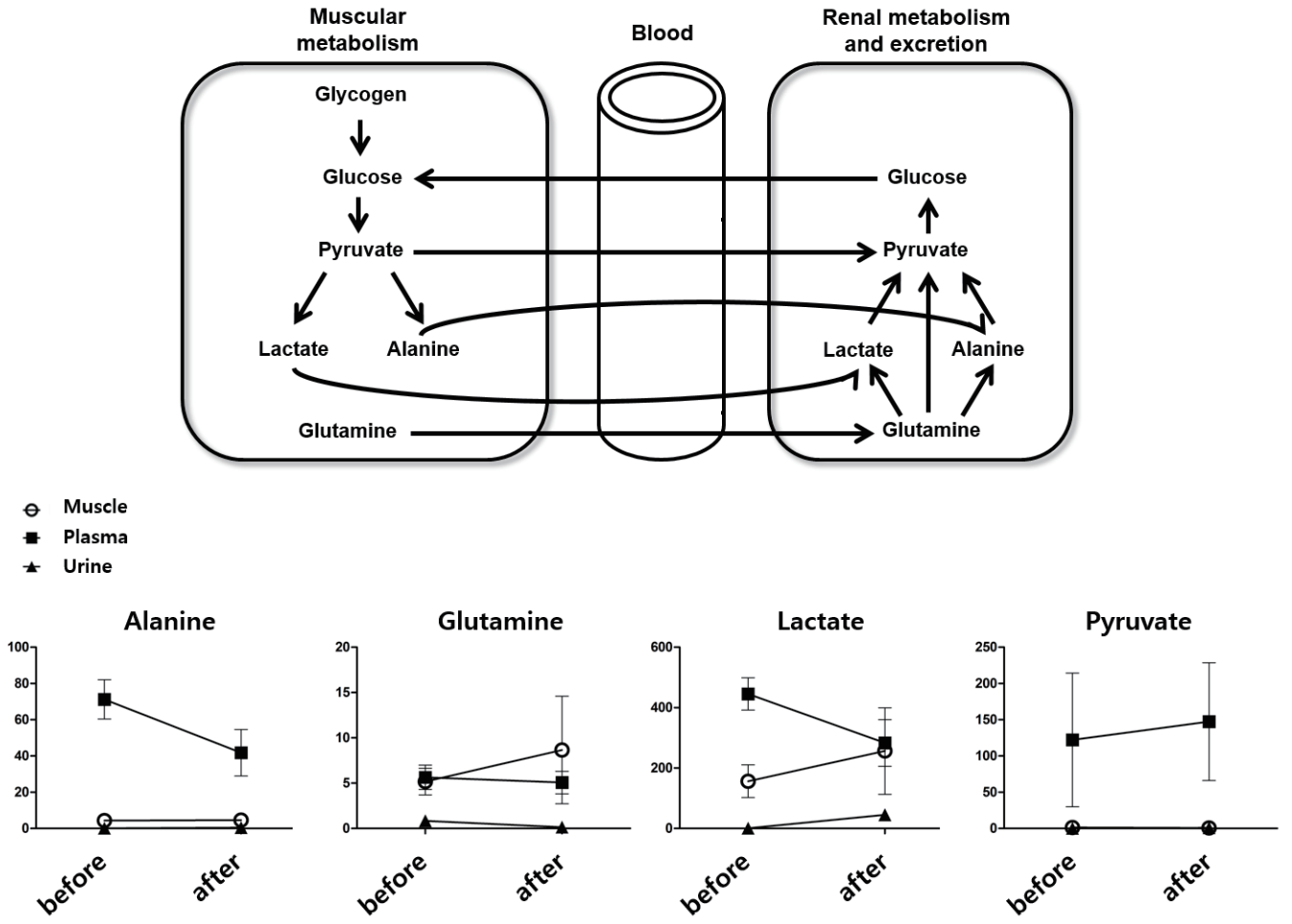

Figure 4. The metabolic cycles for alanine, glutamine, lactate, and pyruvate from the muscle to the kidney, and the concentrations of alanine, glutamine, lactate, and pyruvate in the muscle, plasma, and urine before and after exercise.

mal species and human athletes [31]. In addition, exercise induces changes in the amino acid profile in the blood and muscle. An increase in branched-chain amino acids, such as leucine, isoleucine, and valine, has been observed during prolonged sub-maximal exercise in horses [32], and it may have been due to increased output by the liver in which proteolysis has been shown to accelerate during exercise [33]. Furthermore, certain amino acids are believed to be oxidized for energy production in the muscle [34], although the contribution of proteins to energy expenditure in horses during exercise is still unknown. Recently, exercise in young horses was associated with lipid metabolism, including choline and glycerol; carbohydrate metabolism, including lactate, fumarate, and glucose; and amino acid metabolism, including creatine, creatinine, phenylalanine, tyrosine, and glutamate [35]. Collectively, our results showed the consistency of the differentially expressed metabolites in relation with the enrichment analysis of the metabolic pathways. We also suggested additional metabolic changes during equine exercise.

Alanine, glutamine, lactate, and pyruvate, which were commonly detected among the muscle, plasma, and urine, showed significantly different expressions in the urine after exercise ($p< 0.05$). During exercise, muscle glycogen, which is a primary energy source, is sequentially processed to pyruvate and pyruvate and can be used to produce ATP aerobically or anaerobically through glycolysis [36]. When the muscle cannot use enough oxygen for aerobic glycolysis at high-exercise intensities, anaerobic glycolysis produces ATP in the cytosol of the muscle by the incomplete breakdown of glucose into lactate [36]. Subsequently, muscle lactate is excreted into the blood for the balance of production rate and removal [37]. Once in the bloodstream, lactate can be taken up by exercising or non-exercising skeletal muscles, kidney, or liver, where it is converted to pyruvate for gluconeogenesis [38]. Concurrently, when muscles degrade amino acids for energy needs, the resulting nitrogen is transaminated to pyruvate to produce alanine. This alanine is transported to the liver, where nitrogen enters the urea cycle and pyruvate is used to produce glucose [39]. In addition, glutamine is primarily synthesized from glutamate and glutamic acid in the skeletal muscle. Glutamine is considered important for the maintenance of the renal tubules, contributing to the healthy functioning of the kidneys. Glutamine

Table 3. List of metabolic pathways obtained using enrichment analysis for the differentially expressed (fold change >2 or <0.5) and high-VIP-score (VIP score >1) metabolites

Related metabolic pathway	Total	Expected	Hits	Raw p	Holm p	FDR
Protein biosynthesis	19	0.622	5	0.000217	0.0174	0.0113
Urea cycle	20	0.655	5	0.000283	0.0224	0.0113
Glycine, serine and threonine metabolism	26	0.851	5	0.00105	0.0817	0.0279
Ammonia recycling	18	0.589	4	0.00204	0.157	0.0408
Arginine and proline metabolism	26	0.851	4	0.00832	0.632	0.133
Pyruvate metabolism	20	0.655	3	0.0246	1	0.328
Betaine metabolism	10	0.327	2	0.0395	1	0.4
Methionine metabolism	24	0.785	3	0.04	1	0.4
Aspartate metabolism	12	0.393	2	0.0556	1	0.495
Biotin metabolism	4	0.131	1	0.125	1	0.999
Alanine metabolism	6	0.196	1	0.181	1	1
Taurine and hypotaurine metabolism	7	0.229	1	0.208	1	1
Gluconeogenesis	27	0.884	2	0.22	1	1
Cysteine metabolism	8	0.262	1	0.235	1	1
Malate-aspartate shuttle	8	0.262	1	0.235	1	1
Butyrate metabolism	9	0.295	1	0.26	1	1
Glutathione metabolism	10	0.327	1	0.284	1	1
Ketone body metabolism	10	0.327	1	0.284	1	1
Glucose-alanine cycle	12	0.393	1	0.331	1	1
Beta-alanine metabolism	13	0.425	1	0.353	1	1
Phenylalanine and tyrosine metabolism	13	0.425	1	0.353	1	1
Lysine degradation	13	0.425	1	0.353	1	1
Glycerolipid metabolism	13	0.425	1	0.353	1	1
Purine metabolism	45	1.47	2	0.44	1	1
Propanoate metabolism	18	0.589	1	0.454	1	1
Glutamate metabolism	18	0.589	1	0.454	1	1
Phospholipid biosynthesis	19	0.622	1	0.472	1	1
Insulin signalling	19	0.622	1	0.472	1	1
Bile acid biosynthesis	49	1.6	2	0.485	1	1
Glycolysis	21	0.687	1	0.507	1	1
Porphyrin metabolism	22	0.72	1	0.524	1	1
Citric acid cycle	23	0.753	1	0.54	1	1
Galactose metabolism	25	0.818	1	0.57	1	1
Valine, leucine and isoleucine degradation	36	1.18	1	0.706	1	1
Pyrimidine metabolism	36	1.18	1	0.706	1	1
Tyrosine metabolism	38	1.24	1	0.726	1	1

Raw p, raw p value; FDR, false discovery rate.

in the kidneys contributes to the elimination of acids from the blood, and it is lysed to glutamate, aspartate, pyruvate, lactate, alanine, and citrate through a series of metabolic reactions [40]. Collectively, we suggest that the fluctuations in alanine, glutamine, lactate, and pyruvate are potentially associated with exercise in the muscle, blood, and urine of Thoroughbred horses (Figure 4). The balances of these metabolites in equine biofluid could be utilized as an effective indicator of feeding and management to maintain optimal racing performance.

In conclusion, we first tried to analyze the integrated metabolic patterns and enrichment of metabolic pathways in the muscle, plasma, and urine of racehorses before and after exercise. Our results could contribute to understanding metabolic regulation and development of metabolic markers for equine exercise.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

ACKNOWLEDGMENTS

This study was supported by grants from the Next Generation Bio Green 21 Program (No. PJ01117301, PJ01104401), Rural Development Administration, Republic of Korea.

REFERENCES

1. Ball D. Metabolic and endocrine response to exercise: sympathoadrenal integration with skeletal muscle. J Endocrinol 2015;224:R79-95.

2. Hargreaves M. Skeletal muscle metabolism during exercise in humans. Clin Exp Pharmacol Physiol 2000;27:225-8.
3. Van Hall G, Jensen-Urstad M, Rosdahl H, et al. Leg and arm lactate and substrate kinetics during exercise. Am J Physiol Endocrinol Metab 2003;284:E193-205.
4. Lewis GD, Farrell L, Wood MJ, et al. Metabolic signatures of exercise in human plasma. Sci Transl Med 2010;2:33ra7.
5. Pierce GL, Donato AJ, LaRocca TJ, et al. Habitually exercising older men do not demonstrate age-associated vascular endothelial oxidative stress. Aging Cell 2011;10:1032-7.
6. Henderson GC, Alderman BL. Determinants of resting lipid oxidation in response to a prior bout of endurance exercise. J Appl Physiol 2014;116:95-103.
7. Kjaer M, Galbo H. Effect of physical training on the capacity to secrete epinephrine. J Appl Physiol 1988;64:11-6.
8. Olesen J, Gliemann L, Bienso R, et al. Exercise training, but not resveratrol, improves metabolic and inflammatory status in skeletal muscle of aged men. J Physiol 2014;592:1873-86.
9. Rivero JL, Hill EW. Skeletal muscle adaptations and muscle genomics of performance horses. Vet J 2016;209:5-13.
10. Geor RJ, Hinchcliff KW, McCutcheon LJ, Sams RA. Epinephrine inhibits exogenous glucose utilization in exercising horses. J Appl Physiol 2000;88:1777-90.
11. Brojer J, Holm S, Jonasson R, Hedenstrom U, Essen-Gustavsson B. Synthesis of proglycogen and macroglycogen in skeletal muscle of standardbred trotters after intermittent exercise. Equine Vet J Suppl 2006;38:335-9.
12. Waller AP, Heigenhauser GJ, Geor RJ, Spriet LL, Lindinger MI. Fluid and electrolyte supplementation after prolonged moderate-intensity exercise enhances muscle glycogen resynthesis in Standardbred horses. J Appl Physiol 2009;106:91-100.
13. Jang HJ, Kim JW, Ryu SH, et al. Metabolic profiling of antioxidant supplement with phytochemicals using plasma 1H NMR-based metabolomics in humans. J Funct Foods 2016;24:112-21.
14. Barding GA, Jr., Salditos R, Larive CK. Quantitative NMR for bioanalysis and metabolomics. Anal Bioanal Chem 2012;404:1165-79.
15. van den Berg RA, Hoefsloot HC, Westerhuis JA, Smilde AK, van der Werf MJ. Centering, scaling, and transformations: improving the biological information content of metabolomics data. BMC Genomics 2006;7:142.
16. Zang Q, Keire DA, Wood RD, et al. Class modeling analysis of heparin 1H NMR spectral data using the soft independent modeling of class analogy and unequal class modeling techniques. Anal Chem 2011;83:1030-9.
17. Kim KB, Um SY, Chung MW, et al. Toxicometabolomics approach to urinary biomarkers for mercuric chloride ($HgCl_2$)-induced nephrotoxicity using proton nuclear magnetic resonance (^{1}H NMR) in rats. Toxicol Appl Pharmacol 2010;249:114-26.
18. Xia J, Sinelnikov IV, Han B, Wishart DS. MetaboAnalyst 3.0-making metabolomics more meaningful. Nucleic Acids Res 2015;43:W251-7.
19. German JB, Hammock BD, Watkins SM. Metabolomics: building on a century of biochemistry to guide human health. Metabolomics 2005;1:3-9.
20. Oresic M, Vidal-Puig A, Hanninen V. Metabolomic approaches to phenotype characterization and applications to complex diseases. Expert Rev Mol Diagn 2006;6:575-85.
21. Moore RE, Kirwan J, Doherty MK, Whitfield PD. Biomarker discovery in animal health and disease: the application of post-genomic technologies. Biomark Insights 2007;2:185-96.
22. Weckwerth W. Metabolomics in systems biology. Annu Rev Plant Biol 2003;54:669-89.
23. Schauer N, Fernie AR. Plant metabolomics: towards biological function and mechanism. Trends Plant Sci 2006;11:508-16.
24. Kell DB. Metabolomics and systems biology: making sense of the soup. Curr Opin Microbiol 2004;7:296-307.
25. Nicholson JK, Connelly J, Lindon JC, Holmes E. Metabonomics: a platform for studying drug toxicity and gene function. Nat Rev Drug Discov 2002;1:153-61.
26. Brindle JT, Antti H, Holmes E, et al. Rapid and noninvasive diagnosis of the presence and severity of coronary heart disease using ^{1}H-NMR-based metabonomics. Nat Med 2002;8:1439-44.
27. Whitfield PD, Noble PJM, Major H, et al. Metabolomics as a diagnostic tool for hepatology: validation in a naturally occurring canine model. Metabolomics 2005;1:215-25.
28. Dumas ME, Canlet C, Vercauteren J, Andre F, Paris A. Homeostatic signature of anabolic steroids in cattle using ^{1}H-^{13}C HMBC NMR metabonomics. J Proteome Res 2005;4:1493-502.
29. Evans DL. Physiology of equine performance and associated tests of function. Equine Vet J 2007;39:373-83.
30. Hodgson DR, Rose RJ. The athletic horse. Principles and practice of equine sports medicine. Philadelphia, PA, USA: Saunders; 1994.
31. Vervuert I. Energy metabolism of the performance horse. In: Proceedings of the 5th European Equine Nutrition & Health Congress 2011; 2011 Apr 15-16: Waregem, Belgium: The Scientific Committee of the European Equine Health and Nutrition Congress; 2011.
32. Vervuert I, Coenen M, Watermülder E. Metabolic responses to oral tryptophan supplementation before exercise in horses. J Anim Physiol Anim Nutr (Berl) 2005;89:140-5.
33. Dohm GL, Tabscott EB, Kasperek GJ. Protein degradation during exercise and recovery. Med Sci Sports Exerc Suppl 1987;189:166-71.
34. Strüder KH, Hollmann W, Platen P, et al. lterations in plasma free tryptophan and large neutral amino acids do not affect perceived exertion and prolactin during 90 min of treadmill exercise. Int J Sports Med 1996;17:73-9.
35. Luck MM, Le Moyec L, Barrey E, et al. Energetics of endurance exercise in young horses determined by nuclear magnetic resonance metabolomics. Front Physiol 2015;6:198.
36. Adeva-Andany M, Lopez-Ojen M, Funcasta-Calderon R, et al. Comprehensive review on lactate metabolism in human health. Mitochondrion 2014;17:76-100.
37. Hubbard JL. The effect of exercise on lactate metabolism. J Physiol 1973;231:1-18.
38. Katz J, Tayek JA. Recycling of glucose and determination of the Cori Cycle and gluconeogenesis. Am J Physiol 1999;277:E401-7.

39. Shulman GI. Cellular mechanisms of insulin resistance in humans. Am J Cardiol 1999;84:3J-10J.

40. Leguina-Ruzzi A. Therapeutic targets of glutamine in parenteral nutrition: a medical science review. Int J Prev Treat 2015;4:34-9.