Analysis of metabolomic patterns in thoroughbreds before and after exercise

Hyun-Jun Jang¹,²,a, Duk-Moon Kim³,a, Kyu-Bong Kim¹, Jeong-Woong Park⁴, Jae-Young Choi⁴, Jin Hyeog Oh⁵, Ki-Duk Song², Suhkmann Kim⁵,*, and Byung-Wook Cho⁴,*

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 ¹H 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 ¹H 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 glyconeogenesis, 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.
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\textsubscript{2max}) and maximal exercise (>85% VO\textsubscript{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 ¹H or ¹³C 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 ¹H 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\textsubscript{2}O 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 ¹H NMR spectroscopy analysis. Briefly, 45 μL of the samples was used with 5 μL of deuterium oxide (D\textsubscript{2}O) containing 20 mM of the reference material trimethylsilylpropionate (TSP); 20 mg of the skeletal muscle samples was analyzed with 25 μL of D\textsubscript{2}O containing 2 mM of TSP, and 630 μL of the urine samples was mixed with 70 μL of D\textsubscript{2}O 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 acetocetate, 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)
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-glyceryl-3-phosphocholine |
| Plasma only          | 3     | Formate, histidine, propionate                                              |
| Urine only           | 13    | Acetoacetate, allantoin, benzoate, citrate, citrulline, glutarate, hippurate, homocitrulline, inosine, methylsuccinate, phenylacetylglucose, 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, glyceralin                                              |
| 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) ($R^2_X$: 0.977; $R^2_Y$: 0.852; $Q^2$: -0.142) (A) and variable importance plots (VIPs) for the muscle (D). OPLS-DA ($R^2_X$: 0.889; $R^2_Y$: 0.883; $Q^2$: -1.33) (B) and VIPs for the plasma (E). OPLS-DA ($R^2_X$: 0.987; $R^2_Y$: 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, phenylacetylglucose, 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 (R²X: 0.977; R²Y: 0.852; Q²: −0.142), plasma (R²X: 0.889; R²Y: 0.883; Q²: −1.33), and urine (R²X: 0.987; R²Y: 1; Q²: 0.971) before and after exercise.

| Var ID (Primary) | Muscle | Plasma | Urine |
|------------------|--------|--------|-------|
| Lactate          | 4.33964| 4.09219| 4.90527|
| Creatine         | 2.86407| 3.37581| 2.43861|
| Taurine          | 1.15696| 0.67732| 0.830247|
| Cysteine         | 1.07798| 1.11629| 0.73167|
| Proline          | 0.95002| 1.15483| 0.348348|
| O-Phosphoethanolamine | 0.76878| 1.08083| 0.335367|
| Glutamine        | 0.75791| 0.404849| 0.482387|
| Glucose          | 0.71287| 0.576654| 0.330525|
| Choline          | 0.65500| 0.984574| 0.305149|
| Carnitine        | 0.59744| 1.45509| 0.20250|
| Betaine          | 0.56076| 0.471686| 0.189586|
| Methionine       | 0.50935| 0.76717| 0.135353|
| Threonine        | 0.490596| 0.62621| 0.129172|
| Ethanol          | 0.489648| 0.81646| 0.117822|
| Pyruvate         | 0.421163| 1.43033| 0.121098|
| Succinate        | 0.419546| 1.43253| 0.103247|
| Arginine         | 0.40325| 0.71139| 0.101376|
| Leucine          | 0.390458| 0.208214| 0.0948408|
| Serine           | 0.339834| 0.873955| 0.0927904|
| Valine           | 0.274741| 1.8082| 0.0869787|
| Fumaric acid     | 0.221147| 0.15998| 0.085139|
| Isoleucine       | 0.19769| 1.37773| 0.127546|
| Glutamate        | 0.151078| 1.67088| 0.0585096|
| Asparagine       | 0.147928| 1.74206| 0.0564183|
| Phenylalanine    | 0.147539| 0.487178| 0.0424139|
| O-Phosphocho line | 0.129257| 1.55877| 0.127546|
| Glycine          | 0.117228| 0.76393| 0.0243009|
| myo-Inositol     | 0.113193| 0.541967| 0.0617341|
| Alanine          | 0.0848435| 1.37882| 0.0955172|
| sn-Glycerol-3-phosphocholine | 0.0792956| 0.543817| 0.0491691|
| Glycerol         | 0.0652003| 1.38621| 0.0113337|
| Aspartate        | 0.0604699| 1.36912| 0.0459365|
| Acetate          | 0.0461708| 0.938492| 0.073076|
| Tyrosine         | 0.032664| 0.755125| 0.0305876|
| Lysine           | 0.0203432| 0.702034| 0.133487|

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.
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, transcriptomic, 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-
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 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 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    | 3    | 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                            | 1     | 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.

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