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Article

Untargeted Metabolomics Profiling Reveals Exercise Intensity-Dependent Alterations in Thoroughbred Racehorses' Plasma after Routine Conditioning Sessions

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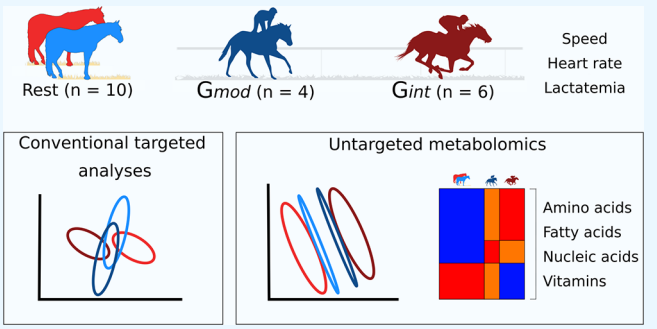
ABSTRACT: Thoroughbred (TB) racehorses undergo rigorous conditioning programs to optimize their physical and mental capabilities through varied exercise sessions. While conventional investigations focus on limited hematological and biochemical parameters, this field study employed untargeted metabolomics to comprehensively assess metabolic responses triggered by exercise sessions routinely used in TB conditioning. Blood samples were collected pre- and post-exercise from ten racehorses, divided into two groups based on exercise intensity: high intensity (n = 6, gallop at ± 13.38 m/s, 1400 m) and moderate intensity (n = 4, soft canter at ± 7.63 m/s, 2500 m). Intensity was evaluated through monitoring of the speed, heart rate, and lactatemia. Resting and 30 min post-exercise plasma samples were analyzed using ultraperformance liquid chromatography coupled with high-resolution mass spectrometry. Unsupervised principal component analysis revealed exercise-induced metabolome changes, with high-intensity exercise inducing greater alterations. Following high-intensity exercise, 54 metabolites related to amino acid, fatty acid, nucleic acid, and vitamin metabolism were altered versus 23 metabolites, primarily linked to fatty acid and amino acid metabolism, following moderate-intensity exercise. Metabolomics confirmed energy metabolism changes reported by traditional biochemistry studies and highlighted the involvement of lipid and amino acid metabolism during routine exercise and recovery, aspects that had previously been overlooked in TB racehorses.



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INTRODUCTION

Thoroughbred (TB) flat racehorses have undergone rigorous selective breeding for their speed capabilities.[1] In France, flat racing is a highly popular and competitive equestrian sport that requires TB to attain speeds exceeding 16.66 m/s over distances ranging from 900 to 4000 m on various track surfaces.[2] To optimize the performance and mitigate the risk of injuries, conditioning programs are individualized to each horse based on career objectives, abilities, and response to conditioning. These programs encompass diverse types of exercise repeated at varying frequencies, intensities, and duration to elicit specific adaptations, associated with adequate recovery time.[3,4]

Exercises performed by TB flat racehorses are highly demanding in energy. Races covering a distance of at least 900 m cannot solely rely on the anaerobic pathway and require contributions of the aerobic pathway.[5−7] Different energetic substrates are used to provide the continuous energy replenishment necessary to sustain muscle contraction. The balance between aerobic and anaerobic pathways for energy production is subject to multiple influencing factors. These factors encompass the type, intensity, and duration of exercise as well as the oxygen and energetic substrate availability, muscle fiber type composition, enzyme activity, mitochondrial function, nutritional status, and conditioning regimen.[7,8]

Previous studies on metabolic responses to exercise have primarily focused on a limited number of targeted parameters, such as specific energetic substrates, metabolic intermediates, or enzymes involved in energy production and substrate utilization, using conventional individual analysis methods.[9−16] With the emergence of metabolomics, there is an opportunity to expand the understanding of the metabolic response to exercise by investigating a broader range of metabolites,

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thereby enabling the exploration of underexplored metabolic pathways.

Metabolomics is a field that focuses on a wide range of low-molecular-weight molecules (i.e., <1500 Da) present in a biological sample using advanced analytical chemistry techniques.[17] Untargeted metabolomics is a high-throughput analysis aiming at capturing as many features as possible in a single analysis,[18] providing a global "snapshot" of the metabolic state at a specific moment. This technique offers insights into the underlying mechanisms of a condition, leading to the generation of hypotheses, and the potential discovery of metabolic signatures.[19−21]

While recent metabolomics studies have provided valuable insights into the energetic pathways and substrate utilization during exercise, these investigations have predominantly focused on endurance racehorses utilizing a nuclear magnetic resonance spectrometry-based approach[22−25] and more recently liquid chromatography coupled to mass spectrometry (LC−MS)-based approach.[26] These investigations have provided valuable insights into previously overlooked metabolites, such as proteins and lipids, during endurance races. In the alignment with the importance of lipid metabolism during exercise, Nolazco Sassot and collaborators (2019) used untargeted lipidomics to comprehensively profile the lipidome of TB racehorses before and after supramaximal treadmill exercise.[27] It is worth noting that only a limited number of metabolomics studies have investigated the response of TB racehorses to exercise, with a predominant focus on scrutinizing the alterations induced by actual race events.[28−30] Recently, metabolomics investigations have been expanded to TB racehorses undergoing conditioning exercises, exploring changes in the plasma metabolome associated with exercise on varying surface hardness.[31]

Previous metabolomics studies have explored the impact of exercise on the plasma metabolome; however, the specific effects of different exercises commonly integrated into routine conditioning programs remain unclear. Understanding how these exercises influence metabolites is crucial to characterizing the metabolic pathways activated to sustain and facilitate recovery from the various exercises used by trainers. This understanding holds the potential for customizing conditioning programs to optimize performance and mitigate injury and fatigue risks, thereby ensuring the welfare of racehorses. Identifying metabolites influenced by exercise also offers valuable insights for monitoring fitness levels and training adaptations throughout the conditioning program as well as for adjusting feeding composition and making informed breeding decisions.

This field study aimed to employ an untargeted metabolomics approach to characterize the blood metabolome changes resulting from various exercise sessions frequently incorporated into the conditioning regimen of TB racehorses. We hypothesized that this approach would offer additional insights into the exercise-induced response, surpassing the limitations of conventional targeted analysis, which typically focuses on a limited number of biochemical parameters.

EXPERIMENTAL SECTION

Population—Inclusion and Exclusion Criteria. The study was approved by the Ethics Committee of the University of Liège (Approval no. 21-2403) and the owners' informed consent was obtained via the trainer. Ten TB flat racehorses trained in Chantilly (France) were included in the study. The recruited horses consisted of six mares and four geldings with an average age of 2.9 ± 0.9 years. Each horse received a body condition score determined through visual and palpation assessments, utilizing the 1−9 scale established by Henneke et al., 1983, where 1 represents extreme emaciation and 9 signifies extreme fatness.[32] The horses presented an average body condition score of 4.5 ± 0.5 on the 9-point scale.[32] The horses had an average number of starts of 10.8 ± 9.7. Population characteristics are displayed in Table S1.

Horses followed an individualized conditioning protocol (6 days per week) designed by the trainer. Horses were housed under identical environmental conditions and received a similar diet. Both the trainer and the stable veterinarian evaluated and confirmed the horses' fitness and readiness for racing. On the day of the experiment, a comprehensive clinical examination was conducted, revealing no abnormalities. Additionally, the horses had not participated in any races within the preceding 7 days. Shortly after the study, Horse 1 was diagnosed with bilateral dorsal metacarpal disease.

Exercise Session Monitoring and Blood Sample Collection. The experiment took place during a conditioning session in December. The weather was overcast, with the temperature ranging between 5 and 8 °C and humidity levels at 85%. Exercise sessions, consistent with each horse's regular conditioning program (i.e., usual riders, surfaces, distances, speeds, etc.), were monitored using a heart rate (Polar, Finland) and global positioning system monitor (Waook Equi-Test, France). Exercise and track surfaces were deliberately not standardized to mirror the customized conditioning programs implemented by the trainer, enabling an examination of natural responses that arise during racehorse training. Thus, two uneven groups were constituted retrospectively according to the exercise intensity performed. Exercise intensity was classify based on the monitored heart rate, speed, distance run and post-exercise lactatemia as high-intensity exercise corresponding to a "gallop" (Group intense, Gint; n = 6) and moderate-intensity exercise corresponding to a "soft canter" according to the terminology of the discipline (Group moderate, Gmod; n = 4).

Venous blood samples were collected in the morning at rest prior to feeding (T0; approximately 6:00 AM), 5 min (T1), 30 min (T2), and 120 min (T3) after the exercise session via jugular venipuncture using a 20G needle and tube holder (BD Vacutainer, UK). No alcohol was used prior to the blood collection. Vacuum blood collection tubes (BD Vacutainer, UK) included plain tubes for biochemistry analysis, lithium heparin tubes for untargeted metabolomics, ethylene diamine tetraacetic acid tubes for hematological analysis, and sodium fluoride/potassium oxalate tubes for lactate measurement. At T1, only sodium fluoride/potassium oxalate tubes were collected, and lactate levels were immediately measured in whole blood using a portable analyzer (Lactate Scout 4, EKF Diagnostics, UK). The plasma was centrifuged immediately after blood collection at 1000g, while the serum was centrifuged 1 h after collection at 3000g at room temperature. The resulting plasma and serum were immediately frozen and kept at −80 °C until further analysis.

Standard hematological and biochemical parameters were determined using flow cytometry and chemiluminescence immunoassay (SYNLAB diagnostic laboratory, Liège, Belgium). Untargeted metabolomics was performed using ultra-performance liquid chromatography (UPLC, 1290 Infinity, Agilent Technologies, USA) coupled with high-resolution mass

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spectrometry (6540 UHD Accurate-Mass Q-TOF, Agilent Technologies, USA). The untargeted metabolomics workflow applied in this study adhered to the established protocol outlined in prior research conducted by Wouters and collaborators,[33] which had previously been employed for investigating equine plasma.

Untargeted Metabolomics. *Sample Preparation.* Heparin lithium plasma samples of the 10 horses, collected at rest (T0) and T2 (i.e., 30 min after exercise), were thawed at 4 °C. The sample preparation protocol was conducted independently for positive and negative ionization mode analyses, and technical triplicates were performed. 100 mL of each sample was mixed with 250 μL of internal standards solution and vortexed for 20 s. Internal standards solution composed by acetonitrile with the carbon isotope with a mass number of 13 (^{13}C)-labeled carnitine, ^{13}C-labeled caffeine, ^{13}C-labeled diclofenac, nicotine, phthalate, and eight labeled acylcarnitines was used as internal standards for positive ionization mode analysis. A solution of acetonitrile with ^{13}C-labeled diclofenac was used for negative ionization mode analysis. The sample was then placed on ice for 10 min before being centrifuged at 20,000*g* at 4 °C for 25 min. The supernatant was evaporated using the low boiling point mode of an EZ2 GENEVAC (Biopharma Technologies, France) for 2 h. The resulting supernatant was resuspended in 250 μL of an aqueous solution containing 0.1% formic acid for positive ionization mode analysis or 1 mM acetic acid for negative, ionization mode analysis. The samples were then vortexed for 15 min, filtered using a PVDF filter (PVDF 0.45 μM, 13 mm, Merck Millipore, USA) and transferred to injection vials with inserts. Quality controls (QCs) composed of 10 μL of each extracted sample in a vial, extraction solvent blanks, and solutions of pure acetonitrile and diclofenac were prepared.

Batch Composition. 5 μL of each sample was analyzed independently for both positive and negative ionization modes. A batch of analysis was initiated with injection of five acetonitrile samples, followed by five acetonitrile samples containing ^{13}C diclofenac to flush out residual metabolites from the column and 15 QC samples to condition the system. The triplicate samples and extraction solvent blanks were then injected in a randomized order and separated every five samples by a QC sample. To finalize the batch, five acetonitrile samples and five acetonitrile samples containing ^{13}C diclofenac were injected to rinse the column.

Liquid Chromatography Separation. The metabolites were separated using UPLC (model 1290 Infinity, Agilent Technologies, USA). Metabolites passed through a reverse-phase precolumn C18 (2.1 mm × 50 mm BEH 1.7 μm particle, Waters Corp., USA) and a high-performance C18 column (2.1 mm × 150 mm BEH 1.7 μm particle, Waters Corp., USA). 5 mL of the sample was loaded at 0.4 mL/min in ultrapure water (eluent A) and eluted with acetonitrile (eluent B). Formic acid (0.1%) or acetic acid (1 mM) was added to the eluents for both positive and negative ionization modes, respectively. The elution process was performed over a period of 11 min, followed by a 5 min re-equilibration period prior to analyzing the next sample. The elution gradient was as follows: 0 min, 99% A; 0.6 min, 98% A; 2 min, 80% A; 4 min, 40% A; 4.3 min, 30% A; 6.5 min, 10% A; 7.5 min, 5% A; 9 min, 2% A; and 11 min, 98% A. A re-equilibration step was performed for the five remaining minutes.

Mass Spectrometry Analysis. Mass spectrometry analysis was performed using a high-resolution mass spectrometer and a quadrupole time-of-flight with electrospray ionization (6540 UHD, Agilent Technologies, USA). The capillary temperature was kept at 350 °C with a drying gas flow rate of 11 L/min. The nebulizer pressure was set at 40 psig, and the nitrogen gas flow rate was 12 L/min. A spray voltage of 4 kW was used for the positive mode and 3.5 kW was used for the negative mode. The instrument scanned masses in the range of 100−1000 mass to charge ratio (m/z).

Acquisition Validation. Agilent MassHunter Qualitative Analysis software (version B.07.00) was used to evaluate the pressure of the system along the analysis, the detection of the pure solution of diclofenac, the quality of the extraction solvent blanks, and the detection of the internal standards. The internal standards were used to monitor and evaluate the performance of the instruments.

Data Processing. The data processing was performed independently for each ionization mode. Raw data were converted to mzXML format using MsConvert (version: 3.0.18205, ProteoWizzard). The processing was done on the Galaxy web interface using Workflow4metabolomics (version: 22.05).[34,35] The peak intensity matrix was generated after filtration, peak detection and grouping, and retention time (RT) correction using the XCMS R package (R version 4.1.2).[36] A comprehensive overview of the detailed parameters employed at each step of the Workflow4metabolomics processing is provided in Table S2. Chromatographic peaks were detected using the CentWave algorithm with a maximal m/z deviation of 5 ppm in consecutive scans, peak width between 5 and 30 s, signal-to-noise threshold of 6, and a minimal m/z difference of 0.001 ppm for peaks with overlapping RTs. The m/z centers of the chromatographic peaks were calculated using the m/z value at the apex. Peaks were grouped within and between samples using the PeakDensity method with a bandwidth of 8 before RT correction and 5 after. The minimal fraction of the sample the peaks had to be present to be considered as the peak was set at 0.5 and the minimal number of samples to 1. The width of the overlapping m/z slices was set to 0.01 ppm. The maximum number of groups to identify in a single milli-second slice was set to 50. RT correction was achieved through alignment of features (peak groups) present in most samples using a loess smooth method with 0.2° of smoothing and Gaussian fitting. Further processing involved the integration of missing peaks using the fillPeaks and annotation using the Camera annotate function. Features eluted within the first 40 s (i.e., leftovers) and ions with a ratio lower than 1.5 between sample and blank were filtered out.

Extraction blanks were removed. Batch correction was performed using a locally quadratic (loess) regression model on the sample value to correct intensities for signal drift and batch-effect.[37] After that, the QC samples were removed for analysis.

Missing values were imputed using the *k*-nearest neighbors 10 algorithm (KNN-10)[38,39] to ensure robust statistical analysis via the VIM R package.[39] Normality of the data was improved after a log-10 transformation. The median intensity of biological triplicates was kept for each feature to reduce the analytical bias.

Unsupervised Analysis. Multilevel principal component analysis (PCA)[40] was performed using R Studio (version: 4.1.2) on the median intensity of biological triplicates for each detected feature, utilizing samples from all horses (Gint and Gmod) taken before (pre) and after (post) exercise. Multilevel

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argument was used to consider the repeated measurement on each horse, before and after exercise with the mixOmics R package.[41]

Feature Selection. Subsequently, feature selection was performed separately for Gint and Gmod horses using the respective samples collected both at rest and after exercise. This analysis aimed to evaluate the modifications in the metabolome induced by high-intensity and moderate-intensity exercise.

The feature selection process was performed independently for both groups using the MetaboAnalyst (version 5.0)[42,43] web interface. The process included univariate (i.e., Student paired *t*-test and fold change) and multivariate statistical analysis [i.e., orthogonal partial least-squares discriminant analysis, variable importance in projection score (VIP)]. The *p*-value is corrected for multiple testing using the false discovery rate method (*p*FDR).[44] In order to select the features most modified by exercise, features were selected based on one of the following criteria: a Student paired *t*-test *p*FDR ≤ 0.05, a fold change (FC) ≥ 3 (upregulated) or $\leq 1/3$ (downregulated), or a VIP score > 1.5.

Metabolite Identification. Features were identified using the in-house library of LABÉO (Caen, France) containing information about more than 700 standard metabolites. Information such as mass to charge ratio (m/z) and RT was collected by injection of all standard metabolites using the same LC−MS method that is described in the present study. The in-house library contained the MSMLS library (IROA Technologies, USA), acylcarnitines, and drugs commonly used in equine medicine. Metabolites were identified when m/z and RT matched standard metabolite characteristics, with a tolerance of 0.005 Da and 6 s, for m/z and RT, respectively. The resulting identified metabolites were manually evaluated, and features leading to an ambiguous identification were removed from the analysis. The identifications were classified as level 1 of confidence according to the Metabolomics Standard Initiative (MSI) classification.[45]

Metabolite Classification. Metabolite classes were investigated using chemical classification (superclass, class, and subclass) from online databases ClassyFire[46] and the online human metabolome database.[47] Then, according to their chemical classes, metabolites were categorized into six distinct groups based on their involvement in different metabolisms, i.e., amino acid, carbohydrate, cofactor and vitamin, energy, lipid, and nucleotide metabolism. Metabolites related to chemical compounds and drugs commonly used in equine medicine as well as two metabolites unrelated to animal metabolism (i.e., *trans*-cinnamaldehyde and *trans*-cinnamate) were removed from the analysis. Metabolic pathway analyses and metabolite set enrichment analysis were performed with MetaboAnalyst (version 5.0) using the KEGG pathway and the metabolites main chemical class set library, respectively. The hypergeometric test was employed as an enrichment method and the relative-betweenness centrality for topology analysis.

Heatmap and Hierarchical Clustering Representation. Metabolites selected independently for Gint and Gmod were used together as a metabolic signature of the response to exercises. In order to visualize changes of the metabolic signature after the exercise sessions, a heatmap was generated using MetaboAnalyst (version 5.0) with autoscale features, the Euclidean distance measure, and the average clustering method.[42,43] Hierarchical clustering was used to order the rows and columns of the heatmap regarding the similarities of the observations.

STATISTICAL ANALYSES

Statistical analyses were performed using R Studio (version: 4.1.2). For continuous data, comparisons between groups were made using the Wilcoxon−Mann−Whitney test, while Wilcoxon signed-rank tests were utilized to compare paired pre- and post-exercise conditions within a group (R function "wilcox.test"). For categorical data, a Fisher exact test was used (R function "fisher.test"). The *p*-value was corrected for multiple comparisons using the FDR correction.[44] A *p*-value ≤ 0.05 was considered significant.

RESULTS

Characterization of the Exercises Performed. Six horses performed a gallop (i.e., Gint; horse 1, 2, 4, 7, 8, 9) and four a soft canter (i.e., Gmod; horse 3, 5, 6, 10) according to parameters listed in Table 1. Analysis of the exercise session was structured into three distinct phases: warm up, exercise, and recovery. Due to technical issues, the physiological parameters of horse 7 were not recorded. Detailed exercise monitoring data are displayed in Table S3.

Table 1. Groups' and Exercises' Characterization[a]

Population	Gint $n = 6$	Gmod $n = 4$
female	3	3
gelding	3	1
mean age (years)	2.5 ± 0.6	3.5 ± 1
mean body condition score (/9)	4.5 ± 0.55	4.5 ± 0.58
race experience (years)	0.80 ± 1.00	2.50 ± 1.00*
number of starts	5.33 ± 6.62	19 ± 7.87*
total duration of the session (s)	3528.00 ± 920.17	3000.00 ± 244.95
Warm-Up Phase		
warm-up duration (s)	173.80 ± 34.91	76.25 ± 17.86*
warm-up mean speed (m/s)	4.40 ± 0.31	3.23 ± 0.40*
warm-up mean heart rate (bpm)	129.85 ± 27.05	86.99 ± 22.4
Exercise Phase		
work duration (s)	107.40 ± 38.37	323.00 ± 26.09*
work distance (m)	1440 ± 510	2460 ± 230*
work mean speed (m/s)	13.38 ± 0.31	7.63 ± 0.64*
work maximal speed (m/s)	16.94 ± 0.90	9.93 ± 0.35*
work mean heart rate (bpm)	203.09 ± 7.55	158.75 ± 12.32*
work maximal heart rate (bpm)	209.25 ± 8.77	181.5 ± 4.65*
immediate post-exercise lactatemia (mmol/L)	8.10 ± 1.48	0.25 ± 0.00*
30 min post-exercise lactatemia (mmol/L)	3.12 ± 2.20	0.25 ± 0.00*
Recovery Phase		
recovery duration (s)	1560.00 ± 328.63	1425.00 ± 150.00
recovery mean heart rate (bpm)	132.97 ± 29.15	100.67 ± 9.66
recovery mean speed (m/s)	1.08 ± 0.19	1.68 ± 0.48
heart rate after 5 min recovery (bpm)	120.00 ± 26.94	82.00 ± 6.38*

[a]Gint: high-intensity exercise, Gmod: moderate-intensity exercise, bpm = beats per minute. The results are displayed as mean ± standard deviation. * indicates significant differences between the groups (*p*FDR ≤ 0.05, Wilcoxon−Mann−Whitney test).

The mean age was not significantly different between Gint and Gmod horses, but Gint horses had significantly less

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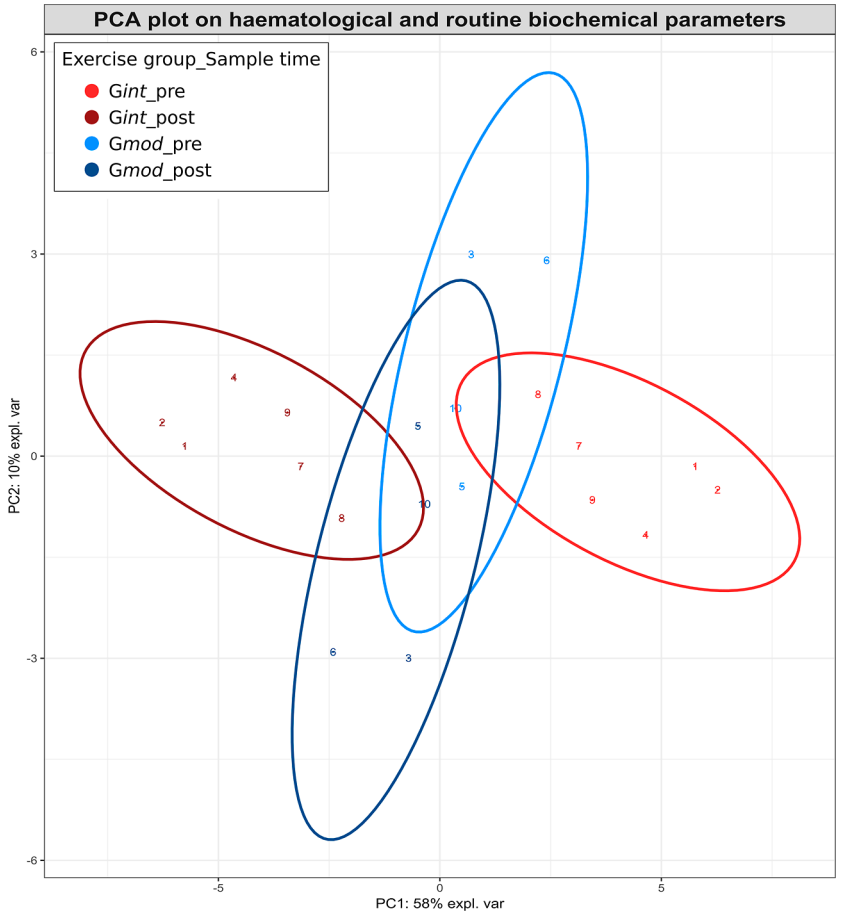

Figure 1. Multilevel PCA visualization of hematological and biochemical parameters measured on pre- and post-exercise samples of horses from group intense and group moderate. Ellipses represent the group dispersion at a confidence level of 95%. Gint: group intense (high-intensity exercise); horses 1, 2, 4, 7, 8, 9. Gmod: group moderate (moderate-intensity exercise); horse 3, 5, 6 10. T0: rest/pre-exercise sample. T2: 30 min post-exercise sample. PC1: principal component 1. PC2: principal component 2.

experience in racing than Gmod horses, with an average number of starts of 5.33 ± 6.62 and 19 ± 7.87, respectively (p-value = 0.04032, Wilcoxon−Mann−Whitney test).

Horses from Gint performed a faster (work mean speed: 13.38 ± 0.31 m/s) and shorter (work distance: 1440 ± 510 m) exercise leading to a higher heart rate (work mean heart rate: 203.09 ± 7.55 bpm) and post-exercise lactatemia (immediate post-exercise lactatemia: 8.1 ± 1.48 mmol/L) compared to Gmod (work mean speed: 7.63 ± 0.64 m/s—work distance: 2460 ± 230 m—work mean heart rate: 158.75 ± 12.32 bpm—immediate post-exercise lactatemia: 0.25 ± 0.00) (p-value = 0.0375, Wilcoxon−Mann−Whitney test).

Hematological and Targeted Biochemical Analyses. One horse from Gmod (horse 3) presented abnormal values of serum amyloid A at rest (211 mg/L). Despite this, the horse was evaluated as "fit to race" by both the stable veterinarian and trainer, and therefore, his results were not excluded from the study. Hematological and biochemical parameters results were not significantly different at rest between the two groups (p-value > 0.05).

Hematological and routine biochemical results were explored using a PCA model to represent the variation of the data set (Figure 1). Clear discrimination between pre- and post-exercise samples was observed for Gint; however, for Gmod, the hematological and biochemical parameters did not result in distinct sample clustering, suggesting that these parameters did not show consistent patterns or significant changes in this group, as confirmed by the Wilcoxon signed-rank test.

Hemoglobinemia, red blood cell count, creatinemia, triglyceridemia, insulinemia, cortisolemia, aspartate aminotransferase activity, gamma-glutamyl transferase, and creatine kinase (CK) activity increased, whereas β-hydroxybutyric acid decreased 30 min after a high-intensity exercise (Table 2). These parameters were significantly modified when examined individually (p-value ≤ 0.05, Wilcoxon signed-rank test). However, after the FDR correction was applied for multiple comparisons, the observed differences were no longer statistically significant (pFDR > 0.05). Hematology and routine biochemistry results of each sample are displayed in Table S4.

Reference Ranges,[48] Available online: https://www.rossdales.com/laboratories/reference-ranges.

Whole Metabolome Assessment. A total of 4260 and 3417 features were detected for positive and negative ionization modes, respectively. Based on the unsupervised PCA representation of all detected features, samples taken before and after exercise were separated. This separation was greater in Gint. The pattern of separation was more distinct using metabolites detected in positive rather than negative ionization modes (Figure 2). Comparison of resting samples

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Table 2. Mean Concentration of Hematological and Routine Biochemical Parameters before and after Exercise for Group Intense and Group Moderate[a]

parameters (units)	RIs from SYNLAB diagnostic laboratory	RIs of 3-year-old TB horses in conditioning (Rossdales laboratories)	Gint		Gmod	
			pre-exercise	post-exercise	pre-exercise	post-exercise
hemoglobin (g/100 mL)	11−19	13.0−16.1	15.07 ± 1.63	**16.80± 1.31***	15.00 ± 0.48	14.68 ± 0.67
erythrocytes (10^6/mm^3)	6.5−12.5	8.6−10.5	9.66 ± 1.19	**11.03 ± 0.87***	9.35 ± 0.65	9.38 ± 0.69
hematocrit (%)	32−52	35−43	43.67 ± 4.18	49.33 ± 3.98	43.50 ± 1.29	43.25 ± 1.71
serum amyloid A (mg/L)	<5	0−20	1.42 ± 1.09	1.37 ± 0.54 (T3)	53.05 ± 105.24	44.03 ± 85.52 (T3)
urea (mg/dL)	10−38	10.9−17.9	22.50 ± 1.76	**23.83 ± 1.72***	21.75 ± 3.59	22.50 ± 3.32
creatinine (mg/dL)	1−1.8	1.3−1.9	1.15 ± 0.11	**1.46 ± 0.16***	1.19 ± 0.04	1.29 ± 0.04
uric acid (mg/dL)	<1		0.05 ± 0.00	2.10 ± 1.80	0.50 ± 0.00	0.50 ± 0.00
glutamate-oxaloacetate-transaminase (UI/L)	160−400	289−630	339.33 ± 52.81	**382.17 ± 65.06* (T3)**	348.50 ± 30.77	365.25 ± 29.92 (T3)
alkaline phosphatase (UI/L)	80−300	277−541	140.83 ± 32.28	143.67 ± 32.92	122.50 ± 22.58	122.25 ± 20.61
γ-glutamyl transferase (UI/L)	10−40	13−47	26.50 ± 11.38	**29.67± 12.61***	18.75 ± 3.50	18.50 ± 3.42
creatine kinase (UI/L)	60−330	156−875	201.50 ± 47.28	**375.33± 170.77* (T3)**	158.75 ± 16.82	221.75 ± 42.41 (T3)
troponin I (ng/L)	10−30	100−360	3.33 ± 5.72	7.95 ± 7.15 (T3)	1.00 ± 0.00	1.00 ± 0.00 (T3)
glycemia (mg/dL)	75−115	61.3−106.3	94.67 ± 4.93	127.17 ± 35.02	89.00 ± 3.92	89.25 ± 6.13
total protein (g/L)	52−79	58−67	55.33 ± 3.50	56.17 ± 3.31	57.25 ± 3.20	58.25 ± 2.22
albumin (g/L)	29−41	35−40	33.67 ± 1.51	34.83 ± 1.17	35.00 ± 1.63	35.75 ± 0.50
β-hydroxybutyrate (μmol/L)			152.57 ± 26.41	**107.65± 30.63***	124.90 ± 13.58	109.1 ± 19.99
insulin (pmol/L)	<145	55.6−329.9	15.17 ± 4.88	**26.50 ± 6.02***	10.00 ± 4.90	14.75 ± 12.26
triglycerides (mg/dL)	<50	23.0−230.1	20.83 ± 6.85	**98.17 ± 42.75***	16.75 ± 4.35	15.25 ± 7.14
non-esterified fatty acids (mmol/L)			0.16 ± 0.16	0.22 ± 0.20	0.26 ± 0.09	0.39 ± 0.15
cortisol (μg/dL)	2.3−11.4	2.6−8.7	4.12 ± 0.82	**6.33 ± 0.58***	3.45 ± 0.93	4.32 ± 0.26

[a]Gint: high-intensity exercise, Gmod: moderate-intensity exercise, RIs: Reference Intervals. Data are displayed as mean ± standard deviation. Samples were obtained at rest and after exercise (30 min post-exercise as default, or T3: 120 min post-exercise when specified) from six horses performing a high-intensity exercise (Gint) and four horses performing a moderate-intensity exercise (Gmod). Pre- and post-exercise values were compared within each group. * indicates significant differences between pre- and post-exercise values within each group (p-value ≤ 0.05, Wilcoxon signed-rank test).

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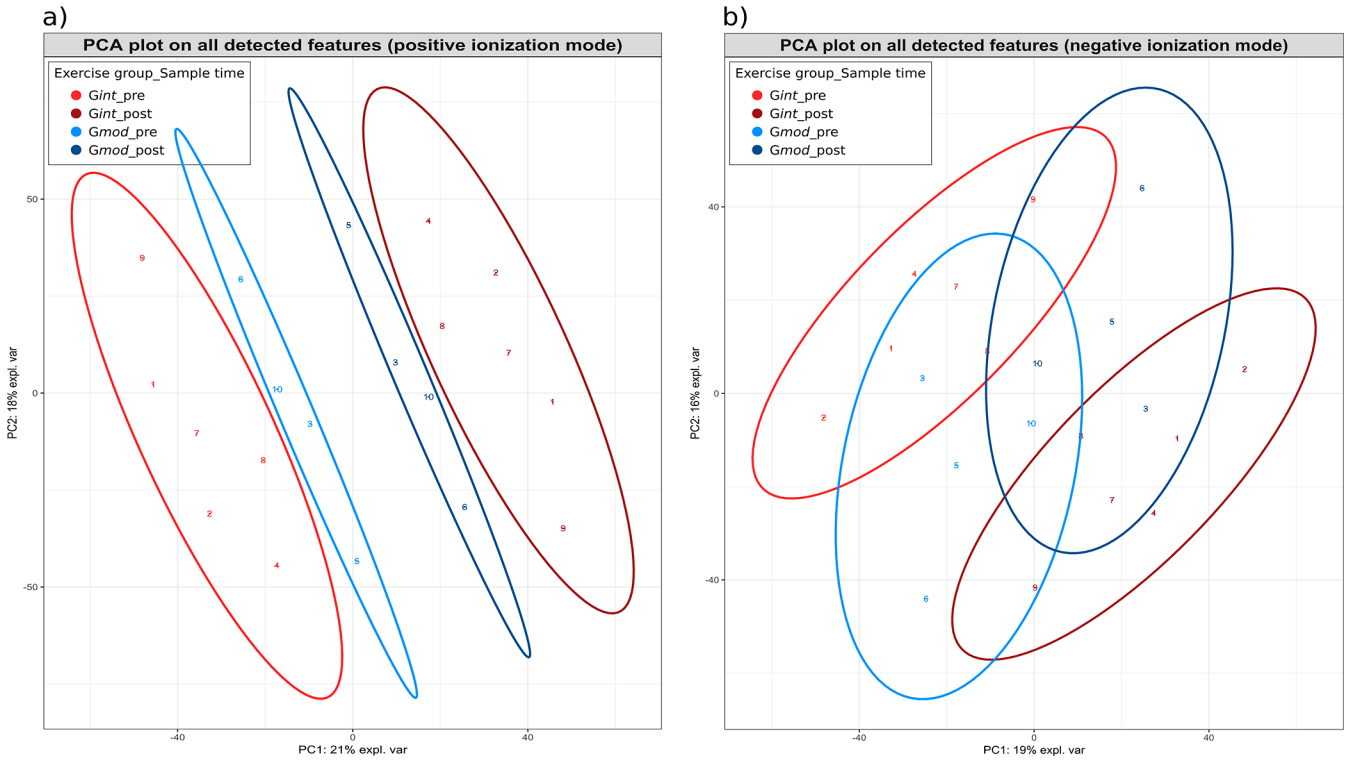

Figure 2. Multilevel PCA visualization of all metabolite intensities detected on pre- and post-exercise samples of horses from group intense and group moderate in (a) positive and (b) negative ionization modes. Ellipses represent group dispersion at a confidence level of 95%. Gint: group intense (high-intensity exercise); horses 1, 2, 4, 7, 8, 9. Gmod: group moderate (moderate-intensity exercise); horses 3, 5, 6 10. T0: rest/pre-exercise sample. T2: 30 min post-exercise sample. PC1: principal component 1. PC2: principal component 2.

did not reveal any significant differences between groups of different exercise intensity.

Selection of the Features Most Modified by Exercise. To evaluate how different exercise intensities affect the metabolic responses, we independently selected discriminant features for both groups (Gint and Gmod) using a combination of univariate and multivariate analyses. We compared the samples collected before and after the exercise sessions, for the high-intensity exercise group and then for the moderate-intensity exercise group.

For Gint, a total number of 1164 (640 and 524 for positive and negative ionization modes, respectively) features were selected using the criteria mentioned above in both ionization modes. For Gmod, a total number of 1100 (617 and 483 for positive and negative ionization modes, respectively) features were selected using the criteria of inclusion in both ionization modes.

Metabolite Identification and Classification. The selected features were identified with an annotation confidence level of 1 according to the MSI classification[45] through standard comparison using two parameters (m/z and RT). Details regarding the statistical selection criteria, the identification, and the intensity of the selected metabolites are provided in Tables S5−S7, respectively.

For Gint, among the 1164 selected features (including both ionization modes) 54 features were identified, leading to the identification of 4.7% of the features. Fifty-nine percent of metabolites (32 metabolites) were linked to amino acid metabolism, whereas 15% (8 metabolites) were linked to lipid metabolism, 13% (7 metabolites) to nucleotide metabolism, and 7% (4 metabolites) to cofactors and vitamin metabolism. Other 3 metabolites were associated with the tricarboxylic acid cycle and carbohydrate metabolism. Based on the enrichment analysis (Table S8), amino acids and peptides, fatty acids and conjugates, pyrimidines, purines, alkanolamines, pyridoxines, carboximidic acids, furanones, sulfonic acids, butyrophenones, and tricarboxylic acid cycle were enriched (i.e., pFDR < 0.05). Amimoacyl-tRNA biosynthesis, D-glutamine and D-glutamate metabolism, and arginine biosynthesis were the main relevant pathways affected by high-intensity exercise (Table S9).

For Gmod, among the 1100 selected features (including both ionization modes), 23 features were identified, leading to the identification of 2.1% of the features. Two metabolites were identified in both ionization modes (4-pyridoxate, taurine). Forty-three percent of metabolites (10 metabolites) were linked to amino acid metabolism, 39% (9 metabolites) to lipid metabolism, and 9% (2 metabolites) to nucleotide metabolism. Other 2 metabolites were associated with tricarboxylic acid cycle (TCA) and cofactor and vitamin metabolism. Enrichment analysis (pFDR <0.05) in Table S10 indicated enrichment in amino acids and peptides, fatty acids and conjugates, alkanolamines, sulfonic acids, and fatty esters. The primary affected pathways due to moderate-intensity exercise were identified as biosynthesis of unsaturated fatty acids and linoleic acid metabolism, as shown in Table S11.

Eight metabolites were influenced by both types of exercise, whereas 46 metabolites were modified only after high-intensity exercise and 15 metabolites were modified only after moderate-intensity exercise. Among the eight common metabolites, three were related to amino acid metabolism (e.g., lysine, ophthalmate, taurine), three to lipid metabolism (e.g., acetylcarnitine, diethanolamine, palmitoylcarnitine), one to cofactor and vitamin (e.g., 4-pyridoxate) and one to nucleotide metabolism (e.g., allantoin).

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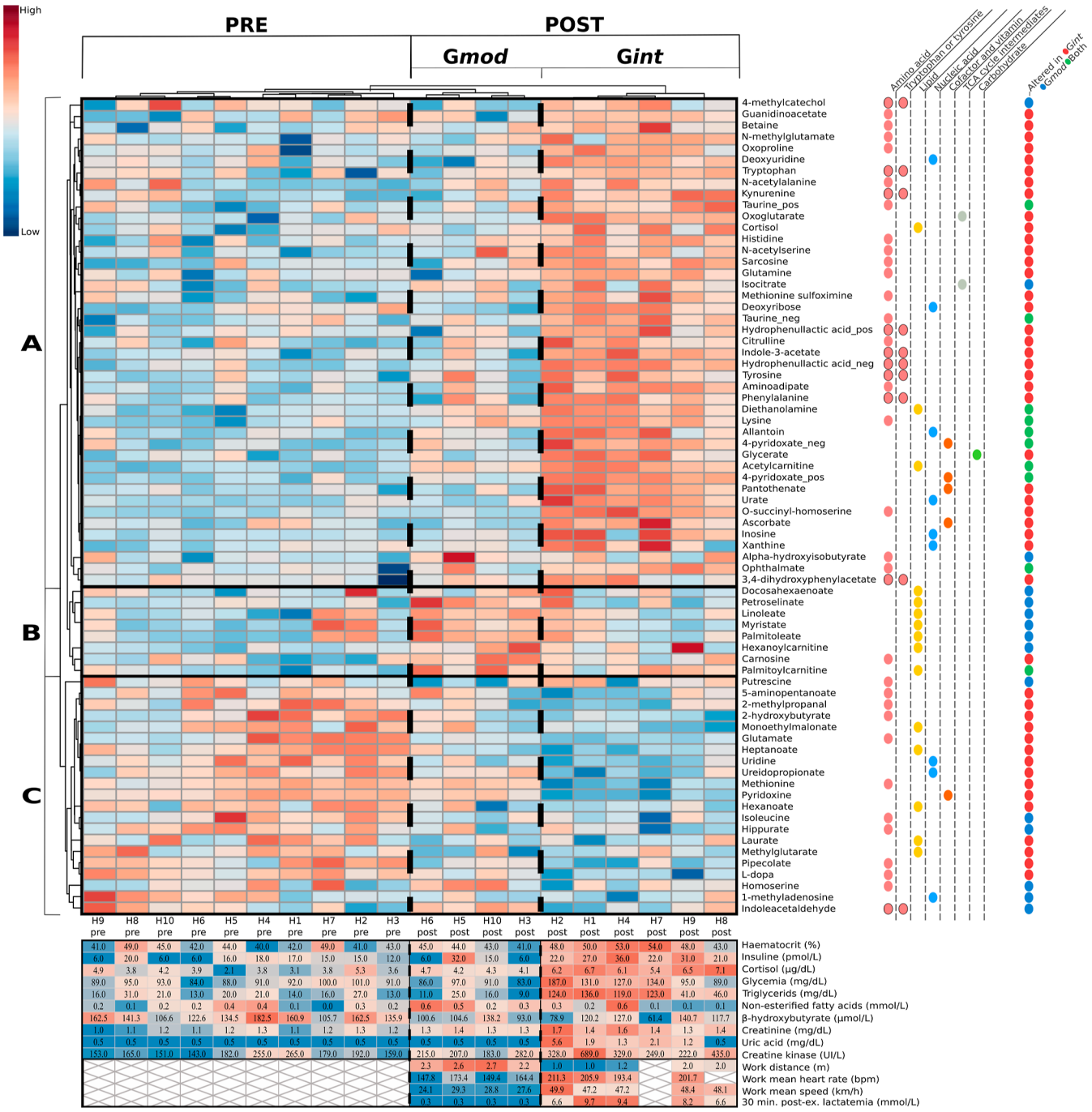

Figure 3. Heatmap and hierarchical clustering of the selected metabolites (in row) from the 10 horses sampled at rest and 30 min after exercise (in column). Groups of individuals sharing an analogous pattern of metabolites are separated with dotted lines and correspond to rest and 30 min post-exercise samples for Gint (high-intensity exercise) and Gmod (moderate-intensity exercise). Metabolites with similar abundance across samples are displayed close to each other in the heatmap, group of under- (C) and overabundant (A,B) metabolites are highlighted. Colors have been assigned to each metabolite depending on the primary metabolism they are associated with. Metabolites altered by the high-intensity exercise, the moderate-intensity exercise, or both exercises, are highlighted with in red, blue, and green, respectively. Additionally, individual values of physiological parameters and routine blood parameters are included at the bottom of the heatmap.

Heatmap and Hierarchical Clustering Analyses. Metabolites that were modified by exercise (intense or moderate) were pooled together, giving a metabolic signature, making a total number of 72 metabolites. Modifications of this metabolic signature with exercises are visualized using heatmap with hierarchical clustering, two unsupervised tools (Figure 3).

Based on the relative intensities of each metabolite, hierarchical clustering revealed the presence of distinct clusters of individuals sharing a similar metabolite pattern (separated with dotted lines). These clusters corresponded to samples collected at rest and 30 min post-exercise, with a further subdivision of horses that performed with high intensity or moderate intensity. The hierarchical clustering displayed groups of metabolites underabundant and overabundant in samples (separated with plain lines). In the group formed by post-exercise Gint samples, it appears that the two last

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individuals present a distinct metabolic profile. These two horses performed a high-intensity exercise on a fiber-sand track surface, while the other four horses performed a high-intensity exercise on a sand track with a final slope.

Three distinct clusters of metabolites are depicted in Figure 3, each exhibiting specific intensity patterns; increased intensity levels following high-intensity exercise (group "A"), increased intensity levels after moderate-intensity exercise (group "B"), and decreased intensity levels, particularly following high-intensity exercise (group "C").

DISCUSSION

In the context of routine conditioning practices, various practical constraints emerge, primarily stemming from the inherent variability among horses in terms of age, racing experience, and health status within the training center. Moreover, the necessity for customized conditioning programs tailored to each horse's state of fitness and career objectives introduces added complexity. Consequently, the implementation of a crossover design was not feasible in the context of this field study. Despite these inherent variations, it is worth noting that the study benefited from a consistent environment under the guidance of a single trainer. By not standardizing the exercise across all horses in our study, diverse responses that naturally arise during the conditioning of racehorses were underlined.

Significant changes in hematological and routine biochemical parameters were only observed following a high-intensity exercise. Significant elevation in post-exercise lactatemia indicates that high-intensity exercise relies on anaerobic lactic metabolism to support energy production but not solely as a significant increase in triglycerides was also observed. In contrast to previous studies, no significant changes were observed in nonesterified fatty acids (NEFA).[10,49,50] These plasma levels are the result of lipolysis, oxidation, and triglycerides synthesis.[15,50] A previous study by Pösö and collaborators (1989) reported a positive correlation between plasma glycerol concentration and exercise intensity but found no correlation between NEFA and exercise intensity.[50] Interestingly, the same study indicated that NEFA concentrations following a high-intensity exercise were lower than those after low- and medium-intensity exercise. In our study, the increase in triglyceride level and remaining low plasma NEFA level following high-intensity exercise suggests that NEFA are used to a greater extent in Gint compared to Gmod. While standard laboratory parameters measured in this study indicated the mobilization of lipids, these analyses were insufficient to assess the relative proportion of NEFA used as oxidative substrates or utilized for re-esterification into triglycerides.

The unsupervised analyses uncovered exercise-induced metabolome changes that were intensity-dependent. The findings from this study indicate changes in the metabolome following exercise, with certain metabolites demonstrating a decrease in abundance, while others exhibited an increase. The relative proportions of these metabolites varied according to the exercise intensity. These exercise conditions likely resulted in the recruitment of different muscle fibers, leading to a different metabolic profile. Exercise intensity is one factor that can influence these modifications. It is important to acknowledge that other factors, including diet, sex, age, training stage, metabolic state, and overall health, may also exert a substantial influence. The metabolic profile evaluated at a specific point in time, within specific environmental conditions, is determined by various factors that impact homeostasis. Notably, extensive documentation in human studies demonstrates the influence of both internal and external factors of the metabolome.[51−53] Moreover, the plasma metabolome provides a "snapshot" of the fluxes (i.e., uptake and release processes) of different organs. The observed relative changes in plasma metabolites induced by exercise reflect dynamic alterations and additional methods such as arteriovenous metabolite gradient measurements or isotope tracing could be employed to delve deeper into the understanding of the uptake and release processes between the circulation and other organs.[54] Therefore, the observed alterations in metabolites should be considered as a complex interplay of multiple factors, necessitating careful interpretation and further exploration to determine their physiological implication. Samples collected 30 min post-exercise were analyzed; therefore, the observed metabolites' modifications could be attributed to a combination of exercise-induced changes as well as ongoing recovery processes.

Supervised analyses were performed with criteria adapted to the nature of this field study. Due to the limited number of recruited horses and the large number of parameters investigated, the power of statistical analyses was reduced. Furthermore, the expected changes in metabolites induced by exercise are relatively low compared to what could be observed under pathological conditions, as exercise response is a physiological process occurring mainly for a short period of time. Due to the aforementioned factors, significance thresholds were not reached for any feature following moderate-intensity exercise. To extract the biological relevance of the data, exercise-induced changes in the metabolome were assessed using a VIP score threshold of 1.5, leading to the selection of metabolites that were the most modified by exercise. The selected metabolites were identified using an in-house library. Therefore, a high confidence level[45] can be attributed to the identification, but this led to the identification of a small number of features (4%) and was limited to the metabolites present in the library. Supervised analysis highlighted the importance of metabolites related to amino acid, lipid, nucleic acid, cofactors, and vitamin metabolism and key intermediates in the TCA cycle.

Nucleic Acid Metabolism. Nucleic acid metabolism was found to be altered following high-intensity exercise, indicating substantial degradation of purine derivative molecules as well as pyrimidines derivatives. Changes in metabolites downstream of purine metabolism (inosine, xanthine, urate, allantoin) align with previous studies that reported similar changes in purine metabolites after exercise and attributed them to adenosine triphosphate (ATP) consumption during exercise.[14,55] Given the high energy demand associated with high-intensity exercise, these findings are expected after racing.[29,30] None of the above-mentioned studies have reported modifications in pyrimidine metabolites. Beyond their role in energy metabolism, both purine and pyrimidine metabolites may serve as regulatory molecules in response to exercise.[56]

Tricarboxylic Acid Cycle Intermediates. The elevated levels of oxoglutarate and isocitrate, key intermediates in the TCA cycle, following high-intensity exercise, suggest the activation of this metabolic pathway. The TCA cycle can be fueled by various pathways, including glycolysis, β-oxidation of free fatty acids, or amino acid catabolism. In our study, lipid and amino acid metabolisms were modified in response to exercise, suggesting their involvement as contributors to the

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TCA cycle and the subsequent production of energy. However, there were no changes in carbohydrates metabolites, as would have been expected with high-intensity exercise where ATP production primarily relies on carbohydrate metabolism. Carbohydrate metabolites accounted for only 6% of the total metabolites identified in the present analysis. The limited number of carbohydrate-related metabolites identified could be attributed to the LC−MS methodology used or to the identification strategy employed (i.e., relatively low number of carbohydrates metabolites in the in-house library), which may explain why no exercise-induced modifications in carbohydrates metabolites were observed. However, Ohnuma and collaborators (2022) did not find changes in carbohydrate-related metabolism after racing,[29] whereas Wang and collaborators (2023) observed significant increases in carbohydrates metabolites such as glucose, glucoheptuloside, tuliposide, and oxymatrine immediately after exercise at approximately 10 m/s.[31] The difference in sampling time after exercise varies between these studies, which may also contribute to the observed variation in the carbohydrate metabolites.

Lipid Metabolism. The untargeted analysis revealed a decreased level in saturated medium chain fatty acid (heptanoate, hexanoate, and laurate) and an increased level of unsaturated long chain fatty acids (docosahexaenoate, petroselinate, linoleate, and palmitoleate) following exercise. Myriaste, a saturated long chain fatty acid, also showed an increase after exercise. Previous studies reported similar findings in endurance horses[23,27] and suggested that the changes may be attributed to either lipolysis of adipose tissue (i.e., unsaturated/saturated ratio is higher in adipose compared to resting plasma NEFA)[57] or of the preferential utilization of specific fatty acids during exercise.[23] Further examination of our data revealed a more pronounced decrease in medium chain saturated fatty acids after high-intensity exercise, whereas the moderate-intensity exercise group exhibited a more prominent increase in the long chain unsaturated fatty acid. These findings may arise from variation in the mobilization of fatty acids through lipolysis or an increased utilization of particular fatty acid chains for energy production or re-esterification into triglycerides. Untargeted metabolomics indicates the importance of lipid metabolism associated with exercise in our study. Assessing the activity of enzymes involved in fatty acid oxidation, uptake, and release was beyond the scope of this study but could provide additional insights. To gain a more comprehensive understanding of the contribution of lipids to energy production in muscle, further studies are warranted and the development of techniques such as lipidomics is of primary importance. Recently, the lipidome of racehorses was characterized following treadmill exercise in a limited number of horses, showing promise in unravelling the involvement of lipids in horse racing.[27]

Amino Acid Metabolism. High-intensity exercise had a significant impact on various amino acid levels, while fewer changes were observed after moderate-intensity exercise, indicating that exercise intensity, duration, and type may affect the plasma amino acid levels as previously suggested by Pösö and collaborators (1991).[13] Amino acids derived from the degradation of structural and functional proteins have the potential to contribute to energy production. Certain amino acids serve as intermediates in the TCA cycle, while others are utilized for gluconeogenesis. Moreover, amino acids play major roles in muscle development and recovery and synthesis of neurotransmitters and hormones, among others. It has been documented that the rate of protein synthesis declines during exercise, concomitant with an increase in protein breakdown.[13] Previous studies have reported modifications in plasma amino acid levels in response to exercise.[58,59] Specifically, aromatic amino acids, such as phenylalanine and tyrosine, have been recognized as markers of protein degradation, as they undergo neither oxidization nor synthesis within the muscle.[13,60] In our study, an increase in plasma levels of tyrosine and phenylalanine was observed following high-intensity exercise, indicating the occurrence of protein degradation. Many amino acids exhibited changes after a high-intensity exercise, highlighting the importance of protein degradation and amino acid metabolism. Amino acids can serve as energy sources or contribute to protein synthesis for muscle repair and growth. These findings provide evidence of the dynamic changes in amino acid metabolism during exercise-induced physiological stress and highlight the importance of understanding protein turnover processes in response to exercise.

Specifically, metabolites linked to the urea cycle, such as citrulline, putrescine, glutamate, and glutamine, exhibited changes in plasma levels following a high-intensity exercise. This indicates activation of the urea cycle. Furthermore, metabolites associated with the creatine-phosphocreatine metabolic pathway, including guanidinoacetate, sarcosine, and betaine, were modified by exercise. The urea cycle plays a crucial role in maintaining the nitrogen balance and preventing the accumulation of toxic ammonia. During such intense exercise, ammonia production can be attributed to the breakdown of proteins and amino acids as well as the breakdown of adenosine monophosphate, as indicated by the increased levels of adenine nucleotide degradation metabolites. The urea cycle also indirectly contributes to the synthesis of creatine, which is converted to phosphocreatine to replenish the ATP stores necessary for muscle contraction.

The increase in taurine and carnosine levels indicates that routine exercise sessions induce muscle damage as indicated by the increased activity of creatine kinase after exercise. Taurine and carnosine levels are presented as valuable diagnostic tools to assess damage in type I and type II muscle fibers.[61] Changes in the plasma carnosine level have been attributed to muscle cell leakage,[62] with slight post-exercise variations reported. The findings presented here suggest that the intensity, duration, and type of exercise can result in varying degrees of damage to different muscle fiber types. High-intensity and short duration exercise recruits a greater proportion of type II muscle fibers in TB,[63] which may explain the significant increase in carnosine plasma level observed after completion of the high-intensity exercise.

Moreover, exercise intensity influenced the tyrosine as well as the tryptophan pathway, suggesting the potential influence of exercise on neurological and inflammatory processes. Tyrosine metabolism serves multiple functions, acting as an indicator of muscular protein catabolism, contributing to protein synthesis, and playing a crucial role in energy production and regulation. Specifically, tyrosine is involved in the synthesis of catecholamines (dopamine, epinephrine, norepinephrine), thyroid hormone synthesis, and intermediate products of tyrosine metabolism can provide energy to the TCA cycle through glucogenic or ketogenic.[64] Tryptophan serves as a precursor for various metabolites that exert effects on multiple systems, including the gastrointestinal, immune, and nervous systems.[65] In mammals, tryptophan is metabolized

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through the kynurenine and the serotonin pathways, leading to the production of NAD^+ or serotonin and melanin.[65,66] The metabolites derived from kynurenine and serotonin pathways have various biological activities, including neurological effects, anti-inflammatory properties, and energy homeostasis.[66−68] Previous studies have reported that exercise intensity influences tryptophan metabolism.[66,67,69,70] Tryptophan, being an essential amino acid, is derived from the diet or the breakdown of endogenous proteins. In the context of high-intensity exercise, there is a suggested increased breakdown of endogenous proteins, surpassing its utilization. A significant increase in plasma kynurenine levels was observed in our study, suggesting that the kynurenine pathway is enhanced by high-intensity exercise. Kynurenine is known to have neurotoxic and depressive effects, among others.[71] Previous studies have concluded a neutral effect on the health status of racehorses, with tryptophan metabolism leading to the production of both neurotoxic and neuroprotective metabolites.[66,67] However, an investigation of exercise-induced responses in other downstream kynurenine metabolites is warranted to complement these findings, confirm the activation of this pathway, and evaluate the health effects of these resulting compounds. The "tryptophan-serotonin-central fatigue" theory suggests that increased transport of tryptophan to the brain could lead to serotonin production, resulting in an increased central fatigue,[69,70,72,73] and potentially impacting aerobic performances.[74]

Cofactor and Vitamin Metabolism. Significant alterations in cofactors and vitamin-related metabolites were evident following high-intensity exercise, in contrast to the absence of such changes after moderate-intensity exercise. These findings suggest that the response to enhance energy production and antioxidant defenses varies depending on the exercise's intensity, duration, and type. A significant decrease in pyridoxine level and a concurrent increase in 4-pyridoxate, a downstream metabolite, was observed following high-intensity exercise, suggesting that pyridoxine is utilized during high-intensity exercise with subsequent metabolite accumulation in plasma. Vitamin B6 (pyridoxine) acts as a cofactor for enzymes involved in proteins and amino acids catabolism, lactate to glucose conversion, muscle glycogen breakdown, carnitine synthesis and the synthesis of oxygen transporters like hemoglobin.[75] A significant increase in vitamin B5 (pantothenate) levels after high-intensity exercise was observed, indicating mobilization of vitamin B5 stores to support energy metabolism through optimal coenzyme A availability. Following high-intensity exercise, vitamin C (ascorbate) levels were found to increase, consistent with previous studies.[29,31] Notably, Wang, and collaborators (2023) suggested that vitamin C levels could serve as an indicator of track surface hardness, as higher levels were observed after exercising on hard surfaces.[31] Vitamin C (ascorbate) is a nonenzymatic antioxidant, protecting against oxidative stress, which also plays a role in carnitine and collagen synthesis.[76]

CONCLUSIONS

Exercises commonly used in the conditioning of TB racehorses are of varying intensities, durations, and types and have a significant impact on their metabolome. Untargeted metabolomics approach was successfully used to characterize the blood metabolome changes of racehorses for the first time in field conditions during conditioning sessions. This approach complemented routine hematological and biochemical analyses. This allowed a more comprehensive exploration of the exercise-induced response to exercises used in conditioning and the determination of a metabolic signature discriminating exercises of different intensities. Findings previously reported in energy metabolism following exercise in TB racehorses were confirmed, and involvement of lipid and amino acid metabolism during such exercises and recovery were emphasized, aspects that have been largely overlooked until now. In summary, our study highlights the pivotal role of exercise intensity in modulating metabolic pathways. This underscores the need for a deeper understanding of equine exercise physiology, which in turn can inform the creation of individualized conditioning programs that optimize performance while concurrently minimizing the potential for injury development and fatigue in equine athletes.

ASSOCIATED CONTENT

Data Availability Statement

The mass spectrometry metabolomic data have been deposited to the MetaboLights database, with the access number NTBLS8285. MetaboLights study: MTBLS8285: https://www.ebi.ac.uk/metabolights/reviewer98fa4326-9920-42a8-9309-aaf3a96bde1b.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c08583.

Characteristics of the population; data processing parameters used in Workflow4metabolomics; exercise monitoring data; hematological and routine biochemical data; selected metabolites—details on statistical selection criteria; selected metabolites—details on metabolite identification; selected metabolites—details on metabolite intensity for each sample; metabolite set enrichment analysis of the metabolites influenced by high-intensity exercise; pathway analysis of the metabolites influenced by high-intensity exercise; metabolite set enrichment analysis of the metabolites influenced by moderate-intensity exercise; and pathway analysis of the metabolites influenced by moderate-intensity exercise (XLSX)

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Maëlle M. Bonhomme, Florence Patarin, Clovis P. Wouters and Dominique-Marie Votion designed this study with the scientific and technical support of Anne Couroucé and Claire Leleu. Maëlle M. Bonhomme, Florence Patarin, Caroline-J. Kruse, Anne-Christine François, Benoît Renaud, Jérôme Seignot, and Dominique-Marie Votion collected samples and performed clinical examinations. François Boemer performed some targeted analysis. Maëlle M. Bonhomme, Eric A. Richard, and Marie-Pierre Toquet performed the untargeted analysis. Maëlle M. Bonhomme and Clovis P. Wouters analyzed the data. Maëlle M. Bonhomme wrote the manuscript draft. Maëlle M. Bonhomme, Clovis P. Wouters, and Dominique-Marie Votion made revisions to the manuscript.

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Notes

The authors declare no competing financial interest.

The study adhered to ethical guidelines, and approval was obtained from the Animal Ethics Committee of the University of Liege. All procedures were conducted in accordance with the national and international guidelines for animal welfare. As the entire procedure was part of the routine medical follow-up of racehorses in conditioning, overseen by the stable veterinarian, it was deemed not to fall under the legal definition of an experiment. The study was assigned the identifier number 21-2403. Informed consent was obtained from the owners for the inclusion of their horses in the research.

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■ ABBREVIATIONS

^{13}C	carbon isotope with a mass number of 13
ATP	adenosine triphosphate
CK activity	creatine kinase activity
Da	Daltons
FC	fold change
Gint	group intense
Gmod	group moderate
LC−MS	liquid chromatography coupled to mass spectrometry
m/*z*	mass to charge ratio
MSI	metabolomics standard initiative
NEFA	nonesterified fatty acids
OPLS-DA	orthogonal partial least-squares discriminant analysis
PC1	principal component 1
PC2	principal component 2
PCA	principal component analysis
*p*FDR	*p*-value corrected for multiple testing using the false discovery rate method
Post	blood sample collected after exercise
Pre	blood sample collected before exercise
QC	quality control
Ris	reference intervals
RT	retention time
T0	blood sample collected in the morning at rest prior feeding
T1	blood sample collected 5 min after exercise
T2	blood sample collected 30 min after exercise
T3	blood sample collected 120 min after exercise
TB	thoroughbred
TCA cycle	tricarboxylic acid cycle
UPLC	ultraperformance liquid chromatography
VIP	variable importance in projection score

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