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Analysis of the season-dependent component in the evaluation of morphological and biochemical blood parameters in Shetland ponies of both sexes during exercise

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Abstract

Introduction: Determination of morphological and biochemical blood indices facilitates assessment of the health and welfare of horses, their nutrient demand, the effects of training already undertaken, and the horses' suitability for exercise. Identification of the season-dependent components and the effects of sex and exercise on changes in frequently referenced haematological and biochemical parameters was the main goal of the current study. **Material and Methods:** The blood morphology of 21 healthy adult Shetland ponies (11 mares and 10 stallions) aged 6.5 ± 1.4 years from the central Pomeranian region in Poland was analysed. Blood samples were taken once per season for one year. **Results:** No statistically significant season-dependent differences were found in the blood morphology parameters in either mares or stallions before or after exercise. Beta-coefficient results revealed the strength and type of the relationship of red blood cell distribution width (RDW) and granulocyte count (GRA) with the season, of red blood cell count (RBC), haematocrit, mean corpuscular volume and mean platelet volume with the sex, and of RDW, white blood cell count, GRA and RBC with the exercise factor. Biomarkers demonstrating the relationship between aerobic and anaerobic levels of energy metabolism in the blood did not show any sex dependency in regression analysis. **Conclusion:** The sex-independence of energy metabolism biomarkers may indicate the universality of these parameters. Both seasonality itself and its combination with the exercise factor took part in the formation of effective adaptive reactions for maintenance of morphological blood indices in the ponies during exercise.

Keywords: seasonal changes, Shetland ponies, haematological parameters, aminotransferases, lactate dehydrogenase, lactate-to-pyruvate ratio.

Introduction

Determination of haematological and biochemical indices of horses' blood facilitates assessment of their state of health and welfare, nutrient demand, the effects of training already undertaken, and the horses' suitability for a specific type of exercise (5, 17). Blood parameters in the horse are influenced by a number of factors, including the animal's age, sex, breed, physiological state, reproductive status, regularity, intensity and recentness of physical exercise, and a set of factors that make up the concept of the season. The literature increasingly often reports variations in haematological and biochemical parameters as well as blood chemical elements in horses which depend on such environmental factors as atmospheric pressure, temperature, light, and other characteristics of seasons (13).

The locomotor activity of horses in classical equestrian sports and recreation imposes physical and mental stress. The species-specific and physiological characteristics of horses suit them to use in riding sports, but participation requires sensitivity to the horse's welfare from riders and owners (31). Training, testing, monitoring and using horses in the conditions of equestrian competition needs to follow a modern scientifically based system. Assessing the improvement

of the horse's body gained through training needs to be guided by an objective method (36). It is possible to meet these needs only through an in-depth study of the metabolic processes underlying the adaptive reactions in the horse. Therefore, studies of the processes of adaptation of horses of different breeds to systematic physical activity and research to ensure timely correction of functional disorders caused by overtraining of horses remain very relevant.

The blood system is one of the most important integral systems of the body, and its elements are sensitive to various external influences. Therefore, analysis of the composition of peripheral blood is an important component of the veterinary examination of horses (33). Previous studies have shown that haematological parameters and markers of oxidative stress in the blood of horses of different breeds differed significantly (1, 5). This may indicate the adaptive reactions in order to maintain homeostasis coming under greater or lesser strain depending on the breed; however, such factors as season and type of exercise may also affect the efficacy of adaptation.

In our earlier studies, we investigated photoperiod-induced changes and effects of physical exercise on oxidative stress biomarkers, total antioxidant activity, and antioxidant defence activities together with biomarkers of metabolic changes, such as glucose, urea, uric acid, and lactate dehydrogenase activity in the blood of Shetland pony mares and stallions ridden recreationally (15). This research is now pursued further by identifying the impact of the photoperiod component and the effects of sex and exercise on red and white blood cell parameters and basic biochemical indices that are often used in the evaluation of horse welfare during exercise.

It should be noted that there are scant literature data from this line of research and they are mostly single clinical examples from veterinary studies. Besides addressing this knowledge gap, an equally important reason for the research was that this horse breed is increasingly being used in hippotherapy for children in many countries, especially in Poland (3). Therefore, the aim of our study was to determine the effect of environmental conditions related to changes in the season on haematological and biochemical blood parameters in both sexes of Shetland ponies ridden recreationally before and after exercise.

Material and Methods

Animals. The experiments were conducted in compliance with the Guidelines of the European Council and the current laws in Ukraine and Poland and approved by the ethical committees of Gdańsk University, Poland and the T. H. Shevchenko National University "Chernihiv Colehium", Chernihiv, Ukraine. Twenty-one healthy adult Shetland ponies (11 mares and 10 stallions) aged 6.5 ± 1.4 years from the central Pomeranian region in Poland (Strzelinko, N54º30′48.0″ E16º57′44.9″) were used in the current study. All were used in recreational riding. The animals were housed in individual boxes, with hay and oats provided twice a day at 8.00 am and 6.00 pm and water available *ad libitum*. All ponies were thoroughly examined clinically and screened for haematological, biochemical and vital parameters, which were within reference ranges. The females were non-pregnant.

Exercise session. The exercise started at 10:00 am, lasted one hour, and consisted of a cross country ride with walking (5 min), trotting (15 min), walking (10 min), trotting (10 min), walking (5 min), galloping (5 min), and walking (10 min). These were a selection of routine physical demands in typical durations for Shetland ponies and in combination they were exertion which did not exhaust the animals.

Blood samples. Blood was drawn from the jugular veins of the animals in the morning, 90 min after feeding while the horses were in the stables (between 8:30 and 10 am), and immediately after the exercise test (between 11 am and 12 pm). Blood samples were taken once per season for one year: in spring (3 April), summer (5 July), autumn (1 October) and winter (25 January). Blood was stored in tubes with K_3-ethylenediaminetetraacetic acid and 3.8% sodium citrate and kept on ice until centrifugation at 3,000 rpm for 10 min. The plasma was removed. The erythrocyte suspensions (one volume) were washed with five volumes of phosphate-buffered saline (pH 7.35) three times and centrifuged at 3,000 rpm for 5 min.

Morphological blood parameters. Routine haematological parameters (haematocrit (HCT), haemoglobin concentration (HGB), red blood cell count (RBC), white blood cell count (WBC), platelet count (PLT), leucogram, mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), red cell distribution width (RDW), platelet distribution width (PDW), and mean platelet volume (MPV)) were measured and counted with the use of an Abacus Junior Vet automated Hematology Analyzer (Diatron Medical Instruments, Budapest, Hungary). The leucogram was assessed as the count of lymphocytes (LYM); granulocytes comprising neutrophils, monocytes, eosinophils and basophils (GRA); miscellaneous identified debris (MID) cells (including less frequently occurring and rare cells correlating to monocytes, eosinophils, basophils, blasts and other precursor white cells that fell in a particular size range), and their percentages (LYM%, MID% and GRA%).

Assay of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity. On the one hand, these aminotransferases play a central role in protein metabolism, carrying out oxidative deamination of amino acids indirectly through glutamic acid; on the other hand, they are useful markers for evaluation of the degree of cell damage. This is determined by their localisation: ALT is predominantly in the cytoplasm and AST is in the cytoplasm and mitochondria. Analysis of ALT and AST activity was carried out in plasma with

the standard colorimetric procedure using a Randox Alanine Aminotransferase (ALT) Kit (Cat. No. AL1205; Randox Laboratories Limited, Crumlin, UK), a Randox Aspartate Aminotransferase (AST) Kit (Cat. No. AS3804) and a Randox RX Monza Clinical Chemistry Analyser. The Randox assay gave within-run precision of <4.96%.

Assay of lactate dehydrogenase (LDH) activity. Aerobic and anaerobic energy metabolism have indicators in specific enzymes and substrates comprising a group widely represented in the literature, and one mainly associated in horses with strenuous physical exercise. One relevant enzyme among these indicators is lactate dehydrogenase. The activity of this enzyme was measured in plasma with the standard procedure using a Randox Lactate Dehydrogenase P-L (UV) Kit (Cat. No. LLD3818) and the RX Monza analyser. The measuring range was 42.3–1191 U/L. The absorbance was read at 37°C° in the analyser in a 1-cm light path cuvette.

Assay of lactate and pyruvate levels. The energy metabolism substrates associated with exertion in equines are lactate and pyruvate. Their concentrations were measured according to the procedure described by Herasimov and Plaksina (12). The absorbance was measured at 420 nm. A mixture with 0.5% p-dimethylaminobenzaldehyde and 25% NaOH was used as a blank. For the pyruvate concentration assay, the resulting supernatant was resuspended in 0.1 mL of 10% copper (II) sulphate, 4 mL of concentrated H_2SO_4, and 0.1 mL of 20% hydroquinone (dissolved in alcohol), which was then heated in a water bath at 95°C for 15 min. The absorbance was measured at 430 nm. Calibration curves of lactate (0.1–5 mM) and pyruvate (0.1–5 mM) were used, and the results were expressed in mmol lactate per L or mmol pyruvate per L. The ratio of lactate to pyruvate as a marker of energy exchange in the blood of the horses was also calculated.

Statistical analysis. The Statistica 13.3 package (TIBCO Software, Palo Alto, CA, USA) was used for statistical analysis. Significant differences between the means were measured using a multiple-range test with P-values < 0.05 assumed to denote significance. Data that did not have a normal distribution were log-transformed. The data were tested for homogeneity of variance using Levene's test, and normality was checked with the Kolmogorov–Smirnov test.

Differences between the values obtained before and after exercise, in mares and stallions, and in the four photoperiods were analysed by two-way ANOVA and Bonferroni's post-hoc test. The use of multivariate significance tests of the main effects (photoperiod, gender, exercise and their combined effects) on the morphological and main biochemical blood parameters often used in studies evaluating different exercises helped to determine statistically significant relationships for all values. In the model approach, to combine the impact of three factors (season, gender and exercise), we adopted a three-way classification model using four tests, *i.e.* Wilks', Pillai's, Hotelling's and Roy's, for estimation of the value of the dependent variable, the mean, the main effect with the effect of their interaction, and the experimental random error with the F test and its significance.

The multivariate analysis of variance (MANOVA) analysis data were also confirmed by the results of the sum-of-squares test (total SS model) *vs* residual SS regarding the values of the multiple correlation analysis (R), the coefficient of determination (R^2), and its corrected form reduced by random errors (R^2 adjusted) in the data analysis. The coefficient of determination is considered to be the main indicator of the quality of the regression model and to describe the relationship between dependent and independent variables in the statistical model.

For assessment of the multivariate dependencies of the influence of the analysed predictors in the model of adult Shetland ponies before exercise and after it relative to the two sexes, standardised β-coefficients of regression to compare the overall effect of each predictor on the dependent variable with the effect of each other predictor were used. This helped to compare the effects of each of the main factors as well as their combined effects on each of the morphological and biochemical parameters studied in the blood of the Shetland ponies (32). The analysis results were expressed as means ± standard deviation.

Results

Blood morphology. Tables 1–4 present the measurements of the standard parameters, excluding the aerobic and anaerobic pathway biomarkers ALT and AST. The leucogram was assessed as the count of LYM, GRA, MID, and LYM%, MID% and GRA%. The statistical analysis of these data revealed no statistically significant differences related to the season or the type of physical exercise in the morphological blood parameters in either sex of the Shetland pony.

Biomarkers of aerobic and anaerobic pathways. The measurements of ALT and AST activity are shown in Fig. 1. Maximum values of ALT activity were found in the spring period in both mares and stallions after exercise. These values were significantly lower in the autumn and especially in the winter photoperiod, compared not only to the spring but also to the summer values. It should be noted that there were statistically significant differences in the ALT activity before and after exercise between the sexes in the spring, autumn and winter periods. In spring, there was higher ALT activity in the mares and stallions after exercise, while in the autumn and winter this enzyme's activity was statistically significantly lower after exercise in both sexes. Thus, a pronounced season, sex and exercise dependence was noted for ALT activity (Fig. 1).

Table 1. Blood morphological parameters in Shetland pony mares (M) and stallions (S) before and after exercise in spring

Parameters	Sex	Before exercise	After exercise	Sex	Before exercise	After exercise
RBC,·10^{12}/L	M	8.88 ± 1.58	9.12 ± 1.23	S	8.10 ± 0.73	7.93 ± 1.02
HGB, g/dL	M	15.37 ± 2.84	16.19 ± 1.98	S	13.74 ± 1.54	12.55 ± 1.78
HCT, %	M	42.56 ± 6.26	45.96 ± 2.37	S	38.11 ± 4.24	35.27 ± 4.84
MCV, fL	M	46.03 ± 6.83	47.83 ± 2.42	S	45.89 ± 3.43	44.45 ± 2.09
MCH, pg	M	17.48 ± 2.79	17.87 ± 2.01	S	17.01 ± 2.04	15.82 ± 0.77
MCHC, g/dL	M	36.14 ± 4.40	35.10 ± 2.60	S	36.17 ± 3.19	35.56 ± 0.59
RDW, %	M	17.73 ± 1.61	17.13 ± 2.70	S	18.02 ± 1.41	17.21 ± 3.25
PLT, ·10^9/L	M	295.36 ± 65.90	285.45 ± 42.69	S	293.58 ± 43.66	306.70 ± 68.19
MPV, fL	M	11.27 ± 0.21	11.45 ± 0.44	S	10.63 ± 0.75	11.31 ± 1.35
PDW, %	M	37.26 ± 0.64	37.60 ± 0.78	S	34.51 ± 4.37	34.29 ± 3.07
WBC,·10^9/L	M	7.65 ± 2.00	9.89 ± 3.24	S	8.02 ± 1.42	8.57 ± 1.82
LYM,·10^9/L	M	2.30 ± 0.74	2.23 ± 0.65	S	2.31 ± 0.87	2.03 ± 0.37
MID,·10^9/L	M	0.23 ± 0.13	0.21 ± 0.20	S	0.31 ± 0.22	0.25 ± 0.15
GRA,·10^9/L	M	4.95 ± 1.94	7.03 ± 1.87	S	5.63 ± 1.10	6.17 ± 1.33
LY%	M	32.00 ± 9.41	22.81 ± 5.74	S	26.52 ± 9.87	24.21 ± 5.29
MI%	M	2.78 ± 1.52	2.14 ± 1.99	S	3.98 ± 3.18	2.90 ± 1.65
GR%	M	63.21 ± 13.27	70.13 ± 13.02	S	70.69 ± 9.42	72.68 ± 5.92

RBC – red blood cell count; HGB – haemoglobin; HCT – haematocrit; MCV – mean corpuscular volume; MCH – mean corpuscular haemoglobin; MCHC – mean corpuscular haemoglobin concentration; RDW – red cell distribution width; PLT – platelets; MPV – mean platelet volume; PDW – platelet distribution width; WBC – white blood cell count; LYM – lymphocytes; MID – miscellaneous identified debris; GRA – granulocytes; LY% – lymphocyte percentage; MI% – miscellaneous identified debris percentage; GR% – granulocyte percentage

Table 2. Blood morphological parameters in Shetland pony mares (M) and stallions (S) before and after exercise in summer

Parameters	Sex	Before exercise	After exercise	Sex	Before exercise	After exercise
RBC,·10^{12}/L	M	8.01 ± 1.45	8.59 ± 0.53	S	7.71 ± 1.03	8.99 ± 1.64
HGB, g/dL	M	14.44 ± 2.5	16.78 ± 1.78	S	13.62 ± 1.52	14.04 ± 1.99
HCT, %	M	43.13 ± 6.12	45.24 ± 4.59	S	39.57 ± 3.35	39.77 ± 5.67
MCV, fL	M	46.53 ± 2.95	45.95 ± 2.47	S	43.63 ± 3.58	44.74 ± 2.93
MCH, pg	M	18.05 ± 1.22	19.48 ± 1.04	S	15.98 ± 1.77	15.74 ± 1.23
MCHC, g/dL	M	33.44 ± 2.52	37.18 ± 2.83	S	30.87 ± 0.95	35.34 ± 1.55
RDW, %	M	17.77 ± 2.36	20.50 ± 0.49	S	17.52 ± 2.31	20.45 ± 1.22
PLT, 10^9/L	M	250.18 ± 78.26	266.54 ± 43.30	S	262.30 ± 49.45	305.20 ± 56.66
MPV, fL	M	11.08 ± 0.38	11.76 ± 0.61	S	11.32 ± 0.86	10.74 ± 0.93
PDW, %	M	36.95 ± 0.86	37.51 ± 0.96	S	33.34 ± 3.02	34.84 ± 3.34
WBC, 10^9/L	M	7.23 ± 1.58	7.80 ± 1.39	S	7.04 ± 1.32	8.25 ± 1.91
LYM, 10^9/L	M	2.34 ± 0.86	2.39 ± 0.46	S	2.22 ± 0.72	2.22 ± 0.92
MID, 10^9/L	M	0.17 ± 0.13	0.20 ± 0.15	S	0.16 ± 0.13	0.14 ± 0.21
GRA, 10^9/L	M	4.75 ± 0.99	5.24 ± 1.82	S	4.69 ± 0.95	5.28 ± 1.53
LY%	M	30.29 ± 8.14	32.12 ± 8.45	S	29.62 ± 8.09	25.50 ± 8.06
MI%	M	2.33 ± 1.75	2.68 ± 2.11	S	2.32 ± 1.77	2.35 ± 3.27
GR%	M	67.38 ± 7.84	65.00 ± 8.66	S	68.07 ± 7.60	71.06 ± 7.23

RBC – red blood cell count; HGB – haemoglobin; HCT – haematocrit; MCV – mean corpuscular volume; MCH – mean corpuscular haemoglobin; MCHC – mean corpuscular haemoglobin concentration; RDW – red cell distribution width; PLT – platelets; MPV – mean platelet volume; PDW – platelet distribution width; WBC – white blood cell count; LYM – lymphocytes; MID – miscellaneous identified debris; GRA – granulocytes; LY% – lymphocyte percentage; MI% – miscellaneous identified debris percentage; GR% – granulocyte percentage

Table 3. Blood morphological parameters in Shetland pony mares (M) and stallions (S) before and after exercise in autumn

Parameters	Sex	Before exercise	After exercise	Sex	Before exercise	After exercise
RBC,·10^{12}/L	M	8.27 ± 0.83	8.93 ± 0.60	S	7.46 ± 1.36	7.75 ± 1.55
HGB, g/dL	M	15.18 ± 2.02	16.64 ± 2.59	S	12.04 ± 2.97	12.69 ± 3.47
HCT, %	M	43.22 ± 5.93	45.21 ± 5.09	S	33.59 ± 6.89	37.75 ± 7.90
MCV, fL	M	47.09 ± 3.13	44.91 ± 2.30	S	43.73 ± 3.12	47.25 ± 3.11
MCH, pg	M	18.45 ± 2.52	18.70 ± 3.08	S	16.03 ± 1.38	16.26 ± 1.97
MCHC, g/dL	M	35.21 ± 1.93	36.82 ± 4.32	S	35.68 ± 2.50	33.28 ± 2.19
RDW, %	M	19.88 ± 0.68	20.83 ± 0.69	S	19.96 ± 0.69	20.08 ± 1.24
PLT, 10^9/L	M	277.63 ± 83.59	321.72 ± 61.29	S	276.20 ± 61.49	289.80 ± 82.93
MPV, fL	M	11.19 ± 0.31	11.44 ± 0.46	S	10.34 ± 0.58	10.72 ± 0.82
PDW, %	M	37.03 ± 0.68	36.96 ± 0.92	S	33.49 ± 2.34	34.28 ± 3.05
WBC, 10^9/L	M	7.56 ± 1.34	8.66 ± 2.40	S	8.57 ± 3.10	7.06 ± 1.31
LYM, 10^9/L	M	2.34 ± 0.90	2.82 ± 1.25	S	2.22 ± 0.41	2.27 ± 0.71
MID, 10^9/L	M	0.30 ± 0.13	0.37 ± 0.37	S	0.31 ± 0.17	0.18 ± 0.13
GRA, 10^9/L	M	4.84 ± 0.96	5.75 ± 1.24	S	5.49 ± 1.95	4.64 ± 0.98
LY%	M	30.92 ± 7.76	26.73 ± 10.84	S	27.41 ± 6.74	30.26 ± 8.36
MI%	M	4.01 ± 1.63	3.79 ± 2.43	S	4.14 ± 2.48	2.58 ± 1. 79
GR%	M	62.30 ± 8.99	70.37 ± 10.60	S	69.60 ± 8.55	67.16 ± 8.03

RBC – red blood cell count; HGB – haemoglobin; HCT – haematocrit; MCV – mean corpuscular volume; MCH – mean corpuscular haemoglobin; MCHC – mean corpuscular haemoglobin concentration; RDW – red cell distribution width; PLT – platelets; MPV – mean platelet volume; PDW – platelet distribution width; WBC – white blood cell count; LYM – lymphocytes; MID – miscellaneous identified debris; GRA – granulocytes; LY% – lymphocyte percentage; MI% – miscellaneous identified debris percentage; GR% – granulocyte percentage

Table 4. Blood morphological parameters in Shetland pony mares (M) and stallions (S) before and after exercise in winter

Parameters	Sex	Before exercise	After exercise	Sex	Before exercise	After exercise
RBC, 10^{12}/L	M	8.55 ± 1.40	9.10 ± 1.12	S	7.59 ± 1.53	7.37 ± 1.38
HGB, g/dL	M	15.08 ± 2.71	16.52 ± 1.66	S	12.25 ± 2.89	16.08 ± 3.29
HCT, %	M	42.50 ± 5.14	45.74 ± 1.11	S	34.15 ± 7.85	32.20 ± 5.42
MCV, fL	M	45.59 ± 2.48	46.31 ± 2.66	S	43.58 ± 3.73	44.01 ± 3.45
MCH, pg	M	17.86 ± 3.08	18.29 ± 1.89	S	16.13 ± 2.09	18.08 ± 5.22
MCHC, g/dL	M	35.37 ± 3.53	36.10 ± 3.32	S	35.86 ± 1.24	38.40 ± 1.10
RDW, %	M	17.18 ± 2.86	21.30 ± 0.68	S	18.07 ± 3.03	19.93 ± 1.10
PLT, ·10^9/L	M	303.81 ± 63.11	278.54 ± 26.08	S	241.80 ± 55.29	262.30 ± 61.32
MPV, fL	M	11.03 ± 0.40	11.33 ± 0.45	S	10.95 ± 0.89	10.48 ± 1.03
PDW, %	M	36.23 ± 0.88	37.59 ± 0.82	S	33.42 ± 2.59	32.35 ± 4.73
WBC, 10^9/L	M	7.00 ± 1.26	8.46 ± 2.94	S	6.90 ± 1.68	8.01 ± 1.65
LYM, 10^9/L	M	1.76 ± 0.32	2.34 ± 0.91	S	2.05 ± 0.57	2.44 ± 0.93
MID, 10^9/L	M	0.13 ± 0.08	0.28 ± 0.14	S	0.16 ± 0.09	0.24 ± 0.16
GRA, 10^9/L	M	4.96 ± 1.28	4.83 ± 0.97	S	4.84 ± 1.39	5.23 ± 1.74
LY%	M	24.50 ± 5.12	29.87 ± 7.74	S	27.67 ± 6.90	31.38 ± 10.66
MI%	M	2.08 ± 1.37	3.71 ± 1.93	S	2.47 ± 1.66	3.01 ± 1.83
GR%	M	73.40 ± 5.75	67.27 ± 9.39	S	69.84 ± 7.42	66.49 ± 10.79

RBC – red blood cell count; HGB – haemoglobin; HCT – haematocrit; MCV – mean corpuscular volume; MCH – mean corpuscular haemoglobin; MCHC – mean corpuscular haemoglobin concentration; RDW – red cell distribution width; PLT – platelets; MPV – mean platelet volume; PDW – platelet distribution width; WBC – white blood cell count; LYM – lymphocytes; MID – miscellaneous identified debris; GRA – granulocytes; LY% – lymphocyte percentage; MI% – miscellaneous identified debris percentage; GR% – granulocyte percentage

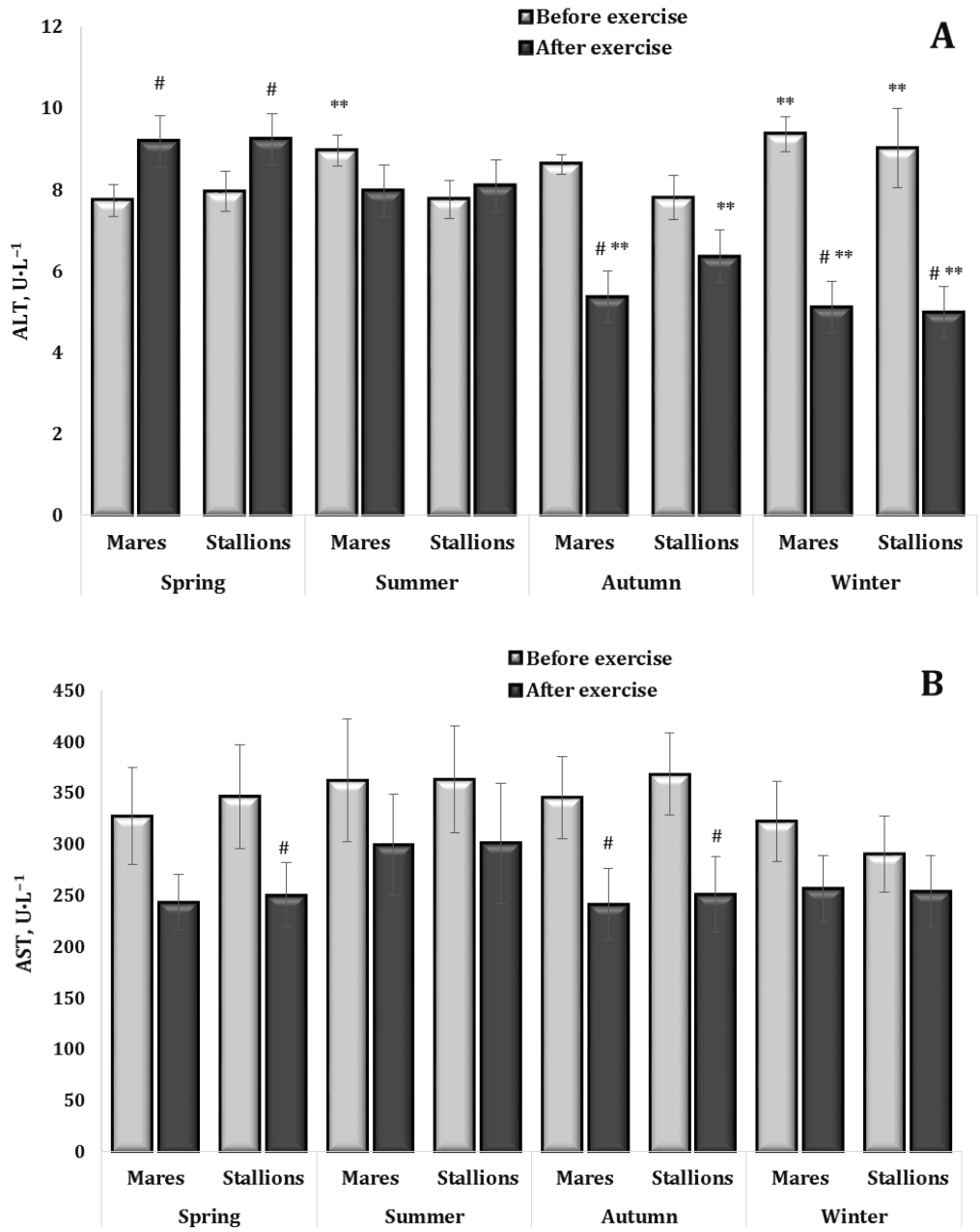

Fig. 1. Activities of aminotransferases in the blood of Shetland pony mares and stallions before and after exercise in different photoperiods. A – activity of alanine aminotransferase; B – activity of aspartate aminotransferase
– statistically significant difference between the parameter before and the parameter after exercise (P-value < 0.05); ** – statistically significant difference between different seasons (P-value < 0.05). Results are expressed as mean ± standard deviation

The activity of AST in these conditions showed less variation than that of ALT. It was found that there were no season-dependent differences in its activity. Differences were observed between the value before and the value after exercise in mares in winter and in both sexes in autumn. Thus, the identified patterns of AST activity were observed to be a component of adaptive changes in energy metabolism during exercise.

The trends in LDH activity were evident as seasonal changes in stallions before exercise in summer and in stallions after exercise in autumn (data not shown). The levels measured of the particular enzymes and substrates involved in energy metabolism, namely LDH and lactate, are presented in Fig. 2A. The importance of anaerobic pathways was also confirmed by the lack of significant statistical differences in the pyruvate values presented in Fig. 2B. It was noted that the lactate-to-pyruvate ratio exhibited statistically significant dependencies in the summer period in both stallions and mares after exercise. The lactate-to-pyruvate ratio was statistically significantly higher in the winter period in both stallions and mares after exercise. Thus, the energy-related patterns of metabolism had seasonal dependencies, even if the exercise was not exhausting but routine for this group of animals.

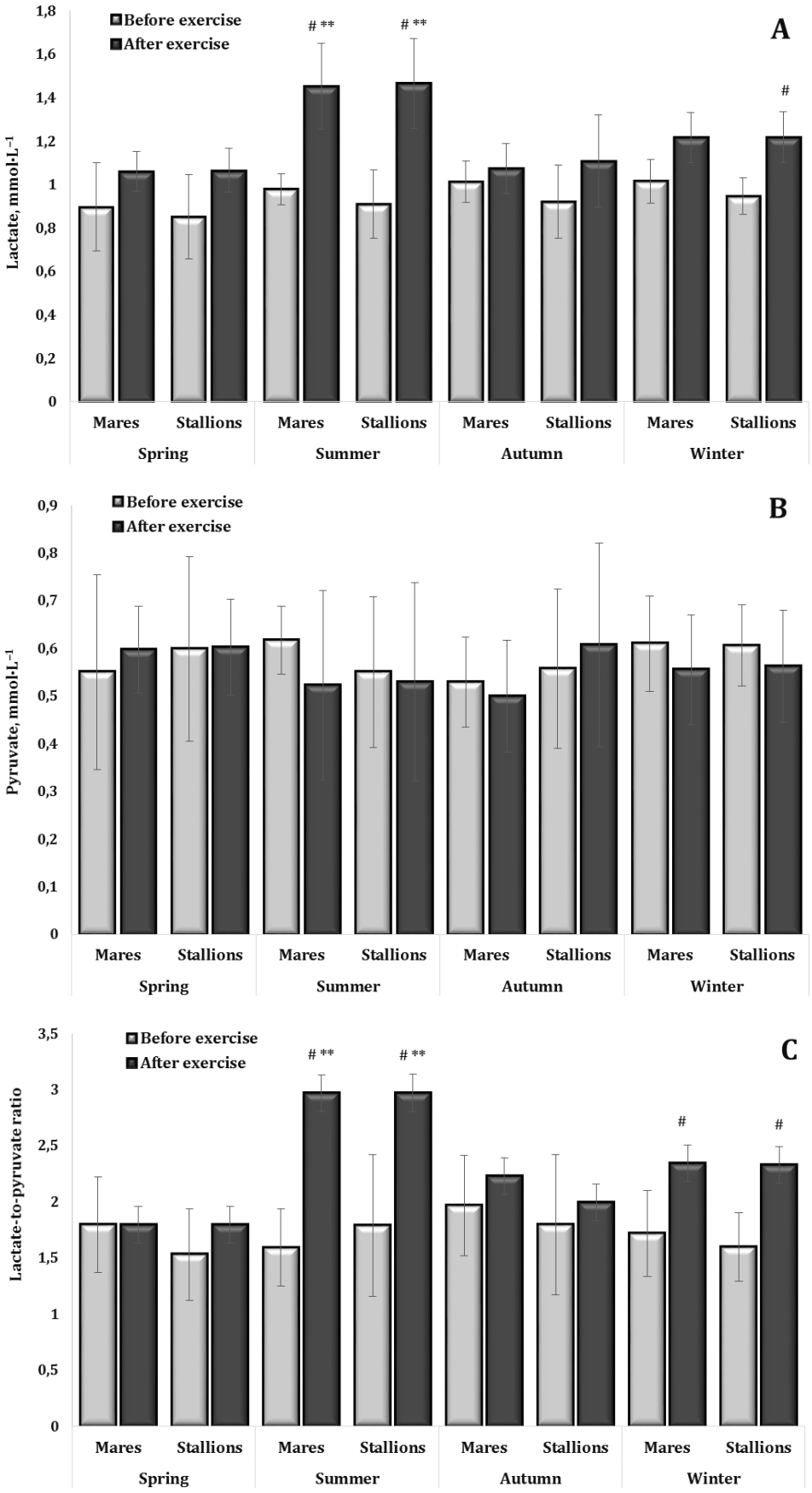

Fig. 2. Lactate and pyruvate dynamics in the blood of Shetland pony mares and stallions before and after exercise depending on the photoperiod. A – lactate; B – pyruvate; C – lactate-to-pyruvate ratio
– statistically significant difference between the parameter before and the parameter after exercise (P-value < 0.05);
** – statistically significant difference between different seasons (P-value < 0.05). Results are expressed as mean ± standard deviation

Table 5. Standardised β-coefficients of the levels of haematological and biochemical parameters in Shetland ponies in relation to photoperiod, sex and exercise as factors bearing influence, as well as *t*-values with their significance (P-value). Parameterisation with σ-constants

Parameter	Factor	β-coefficient ± standard error of the mean	*t*	P-value
RBC, 10^{12}/L	Sex	−0.408 ± 0.096	−4.254	0.000
RBC, 10^{12}/L	Exercise	0.406 ± 0.191	2.116	0.035
HCT, %	Sex	−0.947 ± 0.029	−9.196	0.000
MCV, fL	Sex	−0.811 ± 0.260	−3.116	0.002
RDW, %	Seasons	0.596 ± 0.146	4.064	0.000
RDW, %	Exercise	0.433 ± 0.327	4.370	0.000
MPV, fL	Sex	0.276 ± 0.054	5.094	0.000
WBC, 10^9/L	Exercise	0.865 ± 0.313	2.763	0.006
GRA, 10^9/L	Seasons	−0.276 ± 0.100	−2.746	0.006
GRA, 10^9/L	Exercise	0.519 ± 0.225	2.306	0.022
ALT, U·L^{-1}	Seasons	−0.538 ± 0.089	−6.007	0.000
ALT, U·L^{-1}	Exercise	−0.378 ± 0.200	−6.883	0.000
AST, U·L^{-1}	Exercise	−0.568 ± 0.09	−8.725	0.000
Lactic acid, mmol·L^{-1}	Exercise	0.264 ± 0.070	3.734	0.000
Lactate-to-pyruvate ratio	Exercise	0.580 ± 0.153	3.774	0.000

RBC – red blood cell count; HCT – haematocrit; MCV – mean corpuscular volume; RDW – red cell distribution width; MPV – mean platelet volume; WBC – white blood cell count; GRA – granulocytes; ALT – alanine aminotransferase; AST – aspartate aminotransferase

Table 6. Test of total sum-of-squares model *vs* sum-of-squares residual model of the levels of haematological and biochemical parameters in Shetland ponies as well as *t*-values with their significance (P-value). The models account for the influence of photoperiod, sex and exercise factors

Parameter	R	R^2	Adj R^2	F	P-value
RBC, 10^{12}/L	0.449	0.201	0.123	2.559	0.002
HCT, %	0.653	0.427	0.370	7.561	0.000
RDW, %	0.632	0.400	0.340	6.756	0.000
MPV, fL	0.487	0.237	0.162	6.856	0.000
PDW, %	0.635	0.403	0.344	6.856	0.000
GRA, 10^9/L	0.415	0.172	0.090	2.113	0.012
ALT, U·L^{-1}	0.915	0.838	0.822	52.685	0.000
AST, U·L^{-1}	0.651	0.424	0.368	7.488	0.000
LDH, U·L^{-1}	0.592	0.350	0.286	5.477	0.000
Lactate-to-pyruvate ratio	0.420	0.176	0.095	2.173	0.009

R – multiple correlation coefficient; R^2 – coefficient of determination; Adj R^2 – its corrected form to account for random error; F – F coefficient (F-test) in MANOVA to identify significant differences between the three groups within complex data sets (photoperiod, sex and exercise); RBC – red blood cell count; HCT – haematocrit; RDW – red cell distribution width; MPV – mean platelet volume; PDW – platelet distribution width; GRA – granulocytes; ALT – alanine aminotransferase; AST – aspartate aminotransferase; LDH – lactate dehydrogenase

Model outputs from blood morphological parameters. Multivariate tests of significance were used to analyse the data using the T-coefficient (T-statistic) to assess differences between group mean values in multivariate analysis of variation (MANOVA) and the F coefficient (F-test) in MANOVA to identify significant differences between the three groups within complex datasets (photoperiod, pony sex and physical activity level). The results of the analysis of the main factors showed that the effect of the photoperiod in our statistical model was statistically significant, giving test values of 2.380–0.188 (F = 4.04–8.00, P-value = 0.000). The effects of sex were also statistically significant, with values of T = 1.507–0.399 (F = 8.52, P-value = 0.000). The effects of exercise in the statistical model were as follows: T = 2.052–0.328 (F = 11.60, P-value = 0.000). The tests also revealed statistically significant correlations in the effect of the combination of the season × exercise main factors (T = 3.921–0.132, F = 18.60–4.15, P-value = 0.000). Also, the combination of the sex × exercise effects was found to be statistically significant, but less significant than in the previous test (T = 0.248–0.752, F = 1.86, P-value = 0.016). The same level of statistical significance was demonstrated for the effects of the combination of the three factors season × sex × exercise (T = 0.784–0.406, F = 1.44–2.33, P-value = 0.016). The analysis of the effects revealed the leading role of the photoperiod and exercise factors as well as their combined effect on morphological blood parameters, where the dynamics in these indicated the formation of effective adaptive reactions in the ponies during exercise.

Regression analysis. Values for each independent variable were calculated to represent the strength and type of the relationship of the independent variable with the dependent variable. Table 5 presents the standardised β-coefficients of the levels of haematological and biochemical parameters in the ponies with reference to the influence of the analysed factors and the *t*-value with its significance as indicated by P-value. The analysis of only statistically significant dependencies in our statistical model revealed the highest β-coefficients for HCT (negative for sex), MCV (negative for sex) and WBC (positive for exercise). Smaller but statistically significant β-coefficients were established for RDW (positive), GRA (negative) and, especially for the season factor.

The results of the analysis to determine coefficients of determination are presented in Table 6. This approach was used to analyse the photoperiod, sex and exercise factors in the Shetland ponies. The coefficient of determination indicates how much of the variation in a particular variable is accounted for and is due to the influence of the factors included in the statistical model. The specification of the coefficient of determination and its adjusted form allowed the following relationships for statistically significant changes to be constructed: HCT > PDW > RDW > MPV > RBC > GRA, and ALT > AST > LDH > lactate-to-pyruvate ratio.

Discussion

This study assesses the functional status of morphological blood parameters in Shetland ponies during different periods of the year, taking into account such factors as the sex of the animals and whether they had been recently exercised physically. Although studies of this type exist which are focused on physiological (9, 10) and pathological conditions associated with various diseases (25), our research contains new elements of analysis, which have not been previously presented in the literature. Four main directions of analysis are salient.

Firstly, the four MANOVA tests used helped to estimate differences between the three main factors of photoperiod, sex and exercise as well as the role of each parameter in our statistical model. It was revealed that photoperiod and exercise outweighed sex in triggering the changes in the parameters studied. It is important that factors in combination (season × exercise, sex × exercise and season × sex × exercise) were effective in mediating adaptive reactions to maintain or restore blood chemistry homeostasis in the ponies during exercise. Thus, it is clear from our results that both seasonality itself and its combination with the exercise factor have an important influence on the mechanisms of stable homeostasis in the blood of horses.

Previously, this dependence was presented in the literature for different species of animals and especially horses of different breeds (8). It is considered that conditioning to the season is a critical ability of most organisms. At temperate latitudes, the photoperiod is the main synchroniser of seasonal functions. Seasonal changes in physiology and behaviour typically are innately timed long-term processes, requiring weeks or months to wax and wane. Therefore, in addition to photoperiodic readout mechanisms, living creatures have evolved endogenous long-term timing devices, which allow them to anticipate forthcoming season changes. In the most extreme cases, cycles of about 365 days recur for years in animals kept under constant photoperiods; such so-called circannual rhythms exist in a variety of birds and longer-lived mammals (16).

Characteristics of the stable ultradian structure of behaviour were compared by other authors between ruminants and a group of Przewalski's horses (30). A less stable adaptive temporal structure was established in Przewalski's horses. The authors singled out the activity parameter as a multipurpose behaviour. In this case, the activity was variable, and feeding had a clear rhythmic and harmonic structure. The percentage of the circadian component and harmonic ultradian components relative to all rhythmic components of the spectrum was high in well-adapted, healthy, and undisturbed individuals. However, these values decreased during periods of adaptation, illness, or social interactions (30).

Secondly, the statistical analysis revealed no statistically significant season-related differences in blood morphology parameters during exercise in either sex of Shetland pony. The absence of any seasonal effect may be explained by the level of exercise in horses having been adequate, not excessively exhausting and causing no severe stress that could be associated with significant changes in the blood morphology. The evolutionarily established pattern in the control of blood morphology and homeostasis is one of the powerful mechanisms of functional stability under physiological stress.

Exercise remains a key aspect of improving a horse's endurance and performance (14). These traits in horses result from a number of physiological adaptations. In some cases, these adaptative mechanisms are independent of training, such as the lung size, while others change in response to training, such as the blood volume. The athletic performance of horses is attributed to their high aerobic capacity, large intramuscular stores of energy substrates and especially glycogen, a large volume of mitochondria in the muscles, their ability to increase the oxygen capacity of the blood at the beginning of training by contracting the spleen, gait efficiency, and effective thermoregulation (14).

Several studies have shown that acute and chronic exercise is associated with changes in haematological, biochemical, immunological and hormonal parameters (26). Exercise has different effects on haematological parameters depending on its duration and intensity (short-term high-intensity or maximal exercise or alternatively long-term low or submaximal exercise), the animal's fitness and extent of training already undertaken, and environmental conditions (26). However, many physiological and biochemical

changes in the blood caused by exercise are those that can ultimately affect its reduction-oxidation status. Erythrocytes (mainly because of their number) and leukocytes (mainly because of their acute activation during exercise) can be the main generators of reactive particles in the blood during exercise (20).

Systematic and adequate physical activity of horses increases the capacity of the organism to adapt to oxidative stress with changes in metabolism, in particular. Horses adapt to oxidative stress with a gradual increase in mitochondrial biogenesis, which increases the functional capacity of the cardiovascular and muscular systems (18). This has been reported in a previous study (21). All metabolic reactions are carried out by enzymes; therefore, the regulation of metabolism during exercise leads to the regulation of their activity and *de novo* synthesis. Measuring biochemical and haematological blood parameters in animals during exercise facilitates determination of the functional state of the organism and changes in the process of adaptation to systematic physical activity (7, 22). Also beyond gaining understanding of the equine response to exertion, haematological studies of adaptive capabilities and monitoring of welfare and performance remain important in modern equine physiology management (29).

It has been suggested that the dynamics of humoral and cellular immunity and the intensity of free radical oxidation processes during physical activity indicate a dose-dependent effect of such loads on the immune system, *i.e.* physical activity with maximum intensity suppresses the immune system, while exercise with intermediate intensity normalises and stimulates this system (4). At the same time, immune system dysfunction may be one of the leading factors limiting physical performance. The main mechanisms for improving the immunological status comprise the complement system and the formation of immunoglobulins in the blood, mobilisation of the reticular-endothelial system, and increased free radical–induced oxidation. It has been established that muscular activity accelerates the migration of some lymphocytes to the bone marrow, thereby stimulating its haematopoietic function and releasing excess red blood cells and lymphocytes into the bloodstream (37).

During moderate physical activity, when stress is not a significant damaging factor, there is an initiation of adrenergic regulation adequate to the intensity of and the animal's capacity for physical activity, with corresponding energy expenditure and the development of physiological hypoxia. To optimise tissue oxygenation, the structural and functional state of red blood cells is of great importance, as it determines their shape, size, ability to deform and degree of aggregation. Tissue hypoxia that accompanies intense physical activity causes the release of erythropoietin and stimulation of the erythroid part of haematopoiesis (19).

Thirdly, the results of the MANOVA statistical analysis using the β-coefficient presented a series of relationships: of RDW and GRA with the season; of RBC, HCT, MCV and MPV with sex; and of RBC, RDW, WBC and GRA with exercise. It should be noted that the movement of horses around pasture which is only possible in the warm seasons also affects the animals' haematological parameters. This movement ensures the intake of fodder with a sufficient nutrient composition and content of mineral compounds. The intake and digestion of different types of feed have a significant effect on changes in some biochemical and haematological blood parameters. For example, increased concentrations of glucose, Ca, Mg, Fe and inorganic phosphorus have been observed in the serum (11). Conversely, prolonged starvation may result in a decrease in such haematological blood parameters as RBC and WBC and a decrease in the HGB concentration (35). Similar changes in the peripheral blood of horses related to the nutritional process may also result from a shortage of water or overwatering due to hot weather or/and intense exercise in these conditions, which is associated with weather peculiarities of the season (34). Monitoring of morphological and biochemical blood parameters can be used as a tool to assess the overall health and welfare of horses. By having analysed blood parameters during exercise, the study can provide breeders with insights into how different seasons affect the physiological responses of horses to exercise. This information can guide breeders in optimising exercise programmes for their ponies throughout the year. This understanding may help breeders to anticipate and manage seasonal changes in equine health and performance.

Seasonal changes in the environment influence the physiological responses of animals. Changes in such haematological parameters as RBC are of value in determining the adaptation of animals to the environment (28). In spring, water intake with grazing may limit the effect of haemoconcentration induced by physical activity and/or thermolysis (27). The greater ALT activity observed in mares and stallions after exercise in spring and lesser activity in this phase in autumn and winter can also be explained by the period of transition to additional grazing following winter. Satué and *et al.* (28) observed an increase in erythrocytes, HCT and PLT counts in Carthusian broodmares in summer. The higher temperatures prevailing in summer months may contribute to the development of an adaptive response to heat stress (27). These high environmental temperatures activate thermoregulatory mechanisms, with loss of water through sweating and evaporation through respiratory mucosa. This in turn may lead to a decrease in plasma volume and an increase in the HCT level (27, 28).

It has been noted that intense muscle activity is accompanied by significant changes in metabolic and haematological parameters (23). As a physical training regimen intensifies, the attendant biochemical changes indicate how the main energy substrates in the horse are mobilised and utilised and what destructive changes are taking place (22). Markers of such metabolic changes include the activity of energy metabolism enzymes such as LDH and ALT and AST, which are involved in the transfer of amino groups from amino acids to ketoacids,

and also include blood lactate and pyruvate levels. Changes in the activity and level of these biomarkers in the dynamics of physical activity reflect specific transformations in the metabolism of horses. In trained horses, the content of lactic acid after physical exertion in response to exhausting but systematic exercise is significantly lower than in untrained animals. In response to intensive systematic training loads, the dynamics of changes in the levels of lactate and pyruvate in the blood of sports horses remain almost the same, and their ratio determines the intensity of anaerobic processes (6).

The physiological increase in the activities of ALT and AST has been shown to occur without any tissue destruction. The degree of this increase depends on the type of exercise. As a muscle-derived enzyme, AST is commonly used as an indicator of muscle damage in athletes or sports horses. Levels of AST in horses have been reported to increase immediately after a 160-km endurance race (24). After four and six months of training, there were no differences in pre-training performance. Increased muscle mass and increased activity of certain metabolic enzymes, combined with changes in the expression of structural proteins, are known to promote significant adaptations in muscles and metabolism, leading to improvements in muscle strength, endurance, and overall metabolic efficiency (7). These adaptations are often observed in response to regular physical activity, exercise training, and other physiological stimuli. They play a fundamental role in enhancing athletic performance, maintaining muscle health, and supporting metabolic homeostasis.

Fourthly, the role of biomarkers demonstrating the relationship between aerobic and anaerobic levels of energy metabolism in the blood should be mentioned, as they clarify the relationships in the regression analysis in our statistical model for LDH activity, levels of lactate and pyruvate, lactate-to-pyruvate ratios, and ALT and AST activity. Importantly, none of these parameters in our regression analysis showed any dependence on sex, which may indicate the universality of these parameters in such types of dependencies. In this case, statistically significant β-coefficients were obtained for photoperiod and exercise, the determining factors for ALT, which confirms their important role.

Defence against the lack of oxygen in hypoxia-tolerant animals implicates a reduction in energy turnover and/or an improvement of energetic efficiency in other metabolic processes. An increase in LDH, creatine kinase or AST activity has been observed in exercised race horses, after endurance rides and after show-jumping competitions (2). Lactate is widely used to examine the effects of training, diagnose positive performance and assess the level of fitness in sports horses (22).

Conclusion

Identification of the season-dependent components and the effects of sex and exercise on the changes in haematological and biochemical parameters that are often used in equine physiological assessment was the main goal of the current study. The analysis of these effects revealed the leading role of the photoperiod and exercise factors and of their combination on the formation of effective adaptive reactions for maintenance of morphological blood indices in the ponies during exercise. There were no statistically significant differences in the blood morphology parameters in either Shetland pony mares or stallions depending on the season or the undertaking of recent exercise. Maximum ALT activity was found in the spring period in both mares and stallions after exercise. This activity was significantly weaker in the autumn and especially in the winter photoperiod, compared not only to the result in spring but also to that in summer. Lactate dehydrogenase activity exhibited seasonal changes in the stallions before exercise in summer and after exercise in autumn. The lactate-to-pyruvate ratio was statistically significantly higher in the winter period in both mares and stallions after exercise.

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