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## **ACTIVITY OF ANTIOXIDANT ENZYMES IN THE BLOOD OF SHETLAND PONY MARES AND STALLIONS INVOLVED IN RECREATIONAL HORSEBACK RIDING: ROLE OF PHOTOPERIOD AND EXERCISE**

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*This study investigated seasonal, sex and exercise-induced variations in the activity of key antioxidant enzymes [superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)] in Shetland ponies. region (Pomeranian Voivodeship, northern part of Poland). Twenty-one healthy adult Shetland ponies (11 mares and 10 stallions) aged  $6.5 \pm 1.4$  years were used in this study. All horses participated in recreational riding. The training session started at 10:00 am, lasted 1 hour and consisted of a cross-country ride including walking (5 min), trotting (15 min), walking (10 min), trotting (10 min), walking (5 min), galloping (5 min) and walking (10 min). Blood was collected from the animals' jugular veins in the morning, 90 min after feeding, while the horses were in the stable (between 8.30 and 10 am) and immediately after the exercise test (between 11 am and 12 am). Blood samples were taken once per season for one year. The research highlights the complex interplay between environmental factors and physiological stressors in modulating antioxidant defence mechanisms. Significant seasonal variations in the activities of SOD, CAT and GPx were observed, with the enzymes showing increased sensitivity to exercise, particularly during the colder months. Mares showed a more pronounced exercise-induced decrease in SOD activity compared to stallions, especially during autumn and winter. Conversely, stable enzyme activity was observed in spring and summer, indicating reduced oxidative stress during milder seasons. Statistical analysis revealed significant seasonal differences in SOD and GPx activities, with higher coefficients of determination for SOD ( $R^2 = 0.45$ ) compared to CAT and GPx. The study suggests that mares have a greater oxidative response to exercise in colder seasons, highlighting sex-specific differences in antioxidant defences. These findings contribute to the understanding of how seasonal and exercise-related stressors influence antioxidant enzyme activity and highlight the role of environmental adaptations in equine health and performance. The results have practical implications for optimising exercise regimes and antioxidant supplementation in equine management. Further research is needed to explore the underlying mechanisms of these differences and their wider implications for animal health.*

**Keywords:** *superoxide dismutase, catalase, glutathione peroxidase, exercise, seasonal alterations, Shetland ponies, mares and stallions*



## АКТИВНІСТЬ АНТИОКСИДАНТНИХ ФЕРМЕНТІВ У КРОВІ КОБИЛ ТА ЖЕРЕБЦІВ ШЕТЛАНДСЬКОГО ПОНІ, ЯКІ БЕРУТЬ УЧАСТЬ У РЕКРЕАЦІЙНІЙ ВЕРХОВІЙ ЇЗДІ: РОЛЬ ФОТОПЕРІОДУ ТА ФІЗИЧНОГО ТРЕНІНГУ

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У цьому дослідженні оцінювали сезонні та статеві зміни активності ключових антиоксидантних ферментів [супероксиддисмутази (SOD), каталази (CAT) і глутатіонпероксидази (GPx)] в крові кобил і жеребців шетландських поні до і після тренування. Ми проаналізували вплив фотоперіоду і фізичних вправ на рівні активності антиоксидантних ферментів у крові кобил і жеребців шетландських поні, які беруть участь у рекреаційній верховій їзді в центральній частині Поморського регіону (Поморське воєводство, північна частина Польщі). Двадцять один здоровий дорослий шетландський поні (11 кобил і 10 жеребців) віком  $6,5 \pm 1,4$  років були використані в цьому дослідженні. Усі коні брали участь у рекреаційній верховій їзді. Тренування розпочиналося о 10:00, тривало 1 годину і складалося з кросу ходьбою (5 хв), риссю (15 хв), ходьбою (10 хв), риссю (10 хв), ходьбою (5 хв), галопом (5 хв) і ходьбою (10 хв). Кров брали з яремної вени тварин вранці, через 90 хвилин після годування, під час перебування коней у стайні (між 8:30 та 10 ранку) та відразу після тесту з фізичним навантаженням (між 11 ранку та 12 ранку). Проби крові відбирали один раз за сезон протягом року. Дослідження підкреслює складну взаємодію між факторами навколишнього середовища та фізіологічними стресовими факторами в модулюванні механізмів антиоксидантного захисту. Спостерігалися значні сезонні коливання активності SOD, CAT і GPx, при цьому ферменти демонстрували підвищену чутливість до фізичних вправ, особливо в холодні місяці року. Кобилиці показали більш виражене зниження активності SOD, спричинене фізичним навантаженням, порівняно з жеребцями, особливо восени та взимку. Навпаки, стабільна активність ферменту спостерігалася навесні та влітку, що вказує на зниження окиснювального стресу в більш м'які сезони. Статистичний аналіз виявив значні сезонні відмінності в активності SOD і GPx, з вищими коефіцієнтами детермінації для SOD ( $R^2 = 0,45$ ) порівняно з CAT і GPx. Дослідження показує, що кобилиці мають інтенсивнішу окиснювальну реакцію на фізичні вправи в холодну пору року, підкреслюючи відмінності в антиоксидантному захисті залежно від статі. Ці висновки сприяють розумінню того, як сезонні та пов'язані з фізичними навантаженнями стресори впливають на активність антиоксидантних ферментів, і підкреслюють роль адаптації навколишнього середовища для здоров'я та продуктивності коней. Результати мають практичне значення для оптимізації режимів фізичних вправ і антиоксидантних добавок у догляді за конями. Потрібні подальші дослідження, щоб дослідити основні механізми цих відмінностей та їхній ширший вплив на здоров'я тварин.



**Ключові слова:** супероксиддисмутаза, каталаза, глутатіонпероксидаза, фізичні навантаження, сезонні зміни, шетландські поні, кобили та жеребці.

**Introduction.** Oxidative stress, characterised by an imbalance between the production of reactive oxygen species (ROS) and the antioxidant defence system, is a critical factor affecting animal health and performance (Durand D. et al., 2022). Antioxidant enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) play a key role in mitigating oxidative stress by neutralising ROS and maintaining cellular homeostasis (Bhattacharyya A. et al., 2014; Kurutas E. B., 2016). In equine physiology, the activity of these enzymes can be influenced by various intrinsic and extrinsic factors, including exercise, environmental conditions and sex (Kirschvink N. et al., 2006; Andriichuk A. and Tkachenko H., 2017; Bażanów B. A. et al., 2020; Kurhaluk N. et al., 2022).

Recreational riding, a popular activity with Shetland ponies, involves varying degrees of physical exertion which may affect oxidative metabolism (Kurhaluk N. et al., 2022). Exercise-induced oxidative stress is well documented in horses, leading to increased ROS production through increased metabolic activity (Marañón G. et al., 2008; Yavari A. et al., 2015). Seasonal variations, such as changes in photoperiod and temperature, further influence metabolic and physiological processes and may modulate the antioxidant defence system (Chainy G. B. et al., 2016). The interplay between these factors remains an area of active research, particularly in smaller breeds such as Shetland ponies, which are often used for recreational purposes.

The Shetland pony, known for its resilience and adaptability, provides an excellent model for studying the effects of environmental and physiological stressors on antioxidant mechanisms (Brinkmann L. et al., 2012, 2014). Despite their robust nature, little is known about how photoperiod and exercise affect the activity of key antioxidant enzymes in mares and stallions. Understanding these dynamics is essential to optimise management practices and ensure the health and welfare of these animals.

This study aims to investigate the activity of antioxidant enzymes [superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)] in the blood of Shetland pony mares and stallions involved in recreational riding in the central Pomeranian region (Pomeranian Voivodeship, northern part of Poland). Specifically, we are investigating the effects of photoperiod (seasonal variation) and exercise on enzyme activity, providing insights into the adaptive responses of these animals to environmental and physical challenges. By identifying sex- and season-specific patterns, this research contributes to the growing body of knowledge on oxidative stress and its management in equine species.

#### **Materials and methods.**

**Horses.** The study followed the guidelines of the Council of the European Union and current legislation. Twenty-one healthy adult Shetland ponies (11 mares and 10 stallions) from the Central Pomeranian region of Poland (Strzelinko, N54°30'48.0" E16°57'44.9"), aged  $6.5 \pm 1.4$  years, were included. All ponies were involved in recreational riding. They were individually housed in box stalls, fed twice daily (hay and oats) at 8:00 am and 6:00 pm, and had unlimited access to water. Comprehensive clinical examinations and assessments of haematological, biochemical and vital parameters confirmed that all horses were within normal reference ranges. The mares were not in heat and were non-pregnant.

**Training protocol.** Exercise sessions started at 10:00 am, lasted 1 hour, and included the following sequence: walking (5 minutes), trotting (15 minutes), walking



(10 minutes), trotting (10 minutes), walking (5 minutes), galloping (5 minutes), and walking (10 minutes).

**Blood sample collection.** Blood samples were collected from the jugular vein in the morning, 90 minutes after feeding, while the horses were in their stalls (between 8:30 and 10:00) and immediately after the exercise session (between 11:00 and 12:00). Samples were taken once per season over one year: spring, summer, autumn, and winter. Blood was stored in tubes containing K<sub>3</sub>-EDTA or 3.8% sodium citrate and kept on ice until centrifugation at 3,000 rpm for 10 minutes. The plasma was separated and the erythrocyte suspensions were washed three times with five volumes of PBS (pH 7.35) and centrifuged at 3,000 rpm for 5 minutes.

**Assay of superoxide dismutase activity.** The Randox kit method (RANSOD, Cat. N SD 125, Randox Laboratories Limited, UK) was used to measure plasma superoxide dismutase (SOD) activity using xanthine and xanthine oxidase (XOD). The method proposed by Woolliams J. A. et al. (1983) and Suttle N. F. and McMurray C. H. (1983) was used. This method is based on the use of xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. The results were calculated and expressed as U per mL.

**Assay of catalase activity.** Catalase (CAT) activity was determined by measuring the reduction of H<sub>2</sub>O<sub>2</sub> in the reaction mixture according to the method developed by Koroliuk M. A. et al. (1988). One unit of CAT activity was defined as the amount of enzyme required to degrade 1 μmol H<sub>2</sub>O<sub>2</sub> per minute per mL.

**Assay of glutathione peroxidase activity.** Glutathione peroxidase (GPx) activity was measured in blood by the standard method using a Ransel Glutathione Peroxidase Assay Kit (RX Monza, RS 504, Randox Laboratories Limited, UK). This method is based on GPx catalysis of the oxidation of glutathione by cumene hydroperoxide. All procedures followed the methodology proposed by Paglia D. E. and Valentine W. N. (1967) and Kraus R. J. and Ganther H. E. (1980) with our modification. The decrease in absorbance at 340 nm was measured on an Rx Monza analyser in a 1 cm cuvette light path at +37°C and expressed as U per mL.

**Statistical analysis.** Results are presented as mean ± S.D. Significant differences between means were determined using a multiple range test with a threshold of at least  $p < 0.05$ . Data that did not follow a normal distribution were log-transformed. Statistical tests with 95% confidence intervals ( $\alpha = 0.05$ ) were used to assess the significance of differences between the studied parameters (Stanisz A., 2006, 2007). Homogeneity of variance was assessed using Levene's test and normality was assessed using the Kolmogorov-Smirnov test.

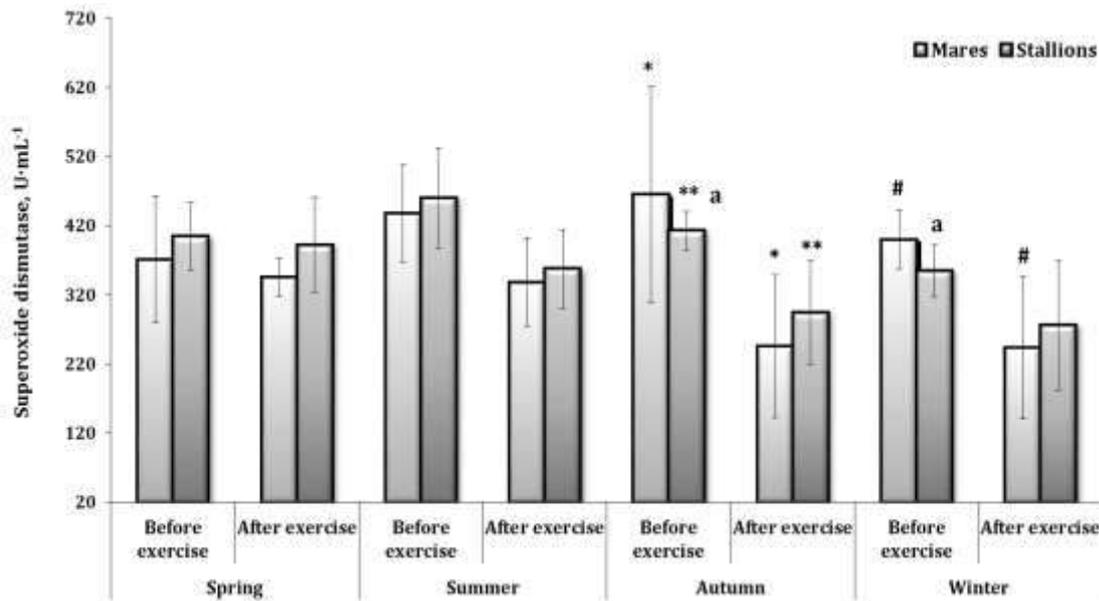
Parametric correlations were analysed using Pearson's regression analysis within the multiple regression module. The results of the MANOVA analysis were further supported by the sum of squares test (total SS model) versus residual SS, taking into account the multiple correlation analysis (R), the coefficient of determination (R<sup>2</sup>) and the adjusted coefficient of determination (R<sup>2</sup> adjusted), which takes into account random errors.

Basic statistical analyses, including significance of regression slopes and analysis of variance, were performed using the STATISTICA 13.3 software package (TIBCO Software Inc., USA). The SS test was used to quantify the contributions of all analysed biomarkers of oxidative stress and biochemical parameters in the assessment of antioxidant defences, using the F test and its significance (Stanisz A., 2006, 2007).

**Results.** We examined the activity of antioxidant enzymes in the blood of Shetland ponies under the influence of three factors: photoperiod, sex, and exercise. The



enzyme activity in the blood of Shetland pony mares and stallions, both before and after exercise, during spring, summer, autumn, and winter, is illustrated in Figures 1–3.



**Fig. 1. Superoxide dismutase activity in the blood of Shetland pony mares (n = 11) and stallions (n = 10) before and after exercise in spring, summer, autumn, and winter.**

Statistically significant differences ( $p < 0.05$ ) in the following dependency groups according to the ANOVA post-hoc Tukey (HSD) test.

\* – between values obtained before and after exercise in mares in autumn;

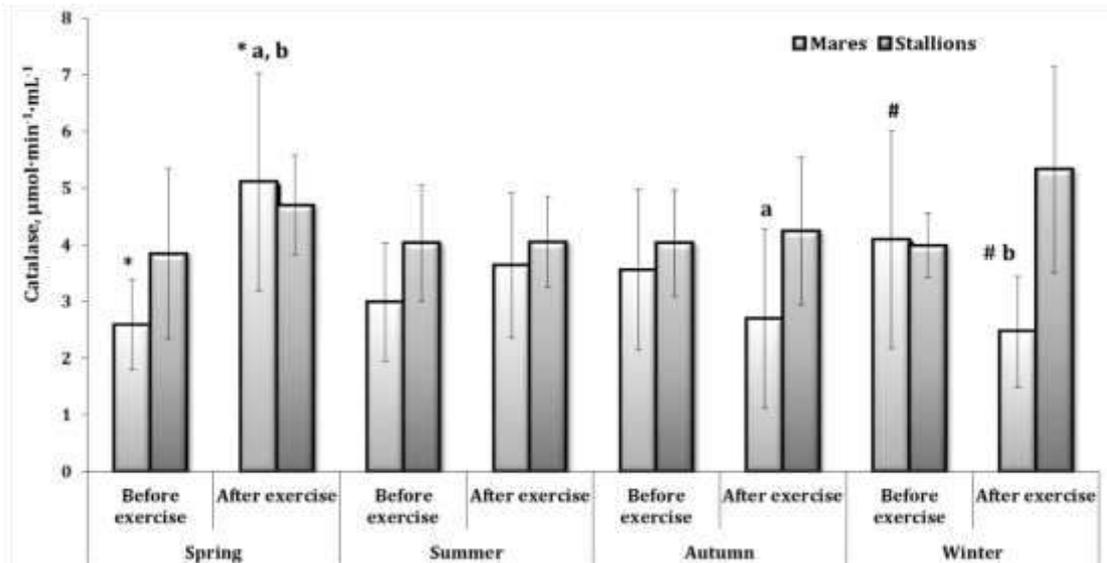
\*\* – between values obtained before and after exercise in stallions in autumn;

# – between values obtained before and after exercise in mares in winter;

a – between values obtained in stallions before exercise in winter and autumn.

The results of our study showed that the blood SOD activity of Shetland pony mares in autumn was statistically significantly decreased after exercise to ( $245.7 \pm 102.89 \text{ U} \cdot \text{mL}^{-1}$ ) compared to the state before exercise ( $465.01 \pm 156.10 \text{ U} \cdot \text{mL}^{-1}$ ). On the other hand, a statistically significant decrease in blood SOD activity was observed in stallions after exercise ( $294.38 \pm 75.52 \text{ U} \cdot \text{mL}^{-1}$ ) compared to the state before exercise ( $413.06 \pm 27.95 \text{ U} \cdot \text{mL}^{-1}$ ). The percentage decrease was 47.2% ( $p < 0.05$ ) for mares and 28.7% ( $p < 0.05$ ) for stallions. Similarly, in winter, SOD activity in the blood of mares of Shetland ponies was statistically significantly decreased after exercise to ( $243.60 \pm 103.10 \text{ U} \cdot \text{mL}^{-1}$ ) compared to the state before exercise ( $399.89 \pm 42.80 \text{ U} \cdot \text{mL}^{-1}$ ). The percentage decrease was 39.1% ( $p < 0.05$ ). Blood SOD activity in Shetland pony mares was statistically significantly lower before exercise in autumn ( $399.89 \pm 42.80 \text{ U} \cdot \text{mL}^{-1}$ ) compared to before exercise in winter ( $465.01 \pm 156.10 \text{ U} \cdot \text{mL}^{-1}$ ). The percentage decrease was 14% ( $p < 0.05$ ). After exercise, SOD activity in the blood of both mares and stallions of summer and spring was statistically non-significantly lower than before exercise. Higher levels of SOD activity were observed in the blood of mares compared to stallions in autumn and winter (Fig. 1).

Catalase activity in the blood of Shetland pony mares and stallions before and after exercise in spring, summer, autumn, and winter were illustrated in Figure 2.



**Fig. 2. Catalase activity in the blood of Shetland pony mares (n = 11) and stallions (n = 10) before and after exercise in spring, summer, autumn, and winter.**

Statistically significant differences ( $p < 0.05$ ) in the following dependency groups according to the ANOVA post-hoc Tukey (HSD) test.

\* – between values obtained before and after exercise in mares in spring;

# – between values obtained before and after exercise in mares in winter;

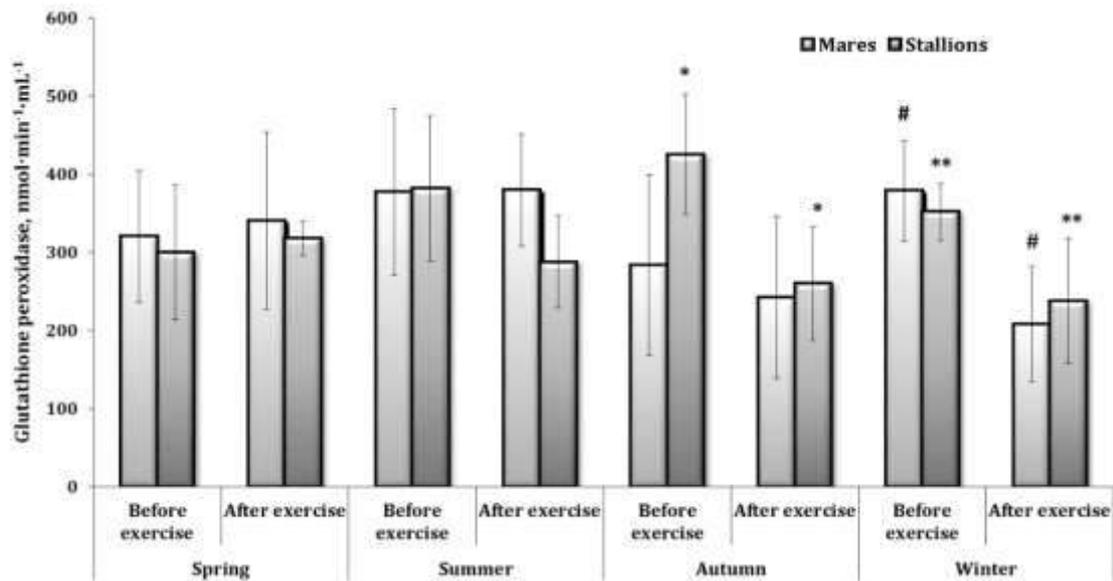
a – between values obtained in mares after exercise in spring and autumn;

b – between values obtained in mares after exercise in spring and winter.

The results of our study showed that catalase activity in the blood of Shetland pony mares in autumn was statistically significantly increased after exercise to ( $5.11 \pm 1.92 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mL}^{-1}$ ) compared with the pre-exercise state ( $2.59 \pm 0.79 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mL}^{-1}$ ). The percentage of increase was 97.3% ( $p < 0.05$ ). Conversely, in winter, catalase activity in the blood of mares of Shetland ponies was statistically significantly decreased after exercise to ( $2.47 \pm 0.98 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mL}^{-1}$ ) compared with the pre-exercise state ( $4.09 \pm 1.92 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mL}^{-1}$ ). The percentage reduction was 39.6% ( $p < 0.05$ ). Blood catalase activity in Shetland pony mares was statistically significantly lower after exercise in autumn ( $2.70 \pm 1.58 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mL}^{-1}$ ) and winter ( $2.47 \pm 0.98 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mL}^{-1}$ ) than before exercise in spring ( $5.11 \pm 1.92 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mL}^{-1}$ ). The percentage decreases were 47.2% ( $p < 0.05$ ) and 51.7% ( $p < 0.05$ ) respectively. In summer and spring, catalase activity in the blood of both mares and stallions after exercise was not statistically significantly higher than before exercise. Higher levels of catalase activity were observed in the blood of stallions compared to mares in all seasons (Fig. 2).

GPx activity in the blood of Shetland pony mares and stallions before and after exercise in spring, summer, autumn, and winter were illustrated in Figure 3.

The results of our study showed that GPx activity in the blood of Shetland pony stallions in autumn was statistically significantly decreased after exercise to ( $260.13 \pm 72.71 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mL}^{-1}$ ) compared with the pre-exercise state ( $425.54 \pm 77.07 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mL}^{-1}$ ). The percentage reduction was 38.9% ( $p < 0.05$ ). Similarly, in winter, GPx activity in the blood of mares of Shetland ponies was statistically significantly decreased after exercise to ( $208.31 \pm 73.82 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mL}^{-1}$ ) compared with the pre-exercise state ( $379.12 \pm 64.39 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mL}^{-1}$ ). The percentage reduction was 45.1% ( $p < 0.05$ ). Blood GPx activity in Shetland pony mares and stallions was at the same level during exercise in both spring and summer (Fig. 3).



**Fig. 3. Glutathione peroxidase activity in the blood of Shetland pony mares (n = 11) and stallions (n = 10) before and after exercise in spring, summer, autumn, and winter.**

Statistically significant differences ( $p < 0.05$ ) in the following dependency groups according to the ANOVA post-hoc Tukey (HSD) test.

- \* – between values obtained before and after exercise in stallions in autumn;
- \*\* – between values obtained before and after exercise in stallions in winter;
- # – between values obtained before and after exercise in mares in winter.

**Discussion.** The results of this study demonstrate distinct seasonal, sex and exercise-induced variations in the activity of key antioxidant enzymes (SOD, catalase and GPx) in the blood of Shetland ponies. These variations highlight the complex interplay of environmental and physiological factors in modulating antioxidant defence mechanisms in horses.

This study identifies GPx, CAT and SOD as key markers of enzymatic antioxidant defence. These enzymes are considered part of the first-line antioxidant defense system as they rapidly neutralize free radicals or molecules with the potential to form free radicals (Jomova K. et al., 2024). SOD activity showed a marked decrease in both mares and stallions following exercise in autumn and winter, with mares showing a more pronounced reduction than stallions (Fig. 1). This suggests that mares may have an increased oxidative response to exercise during these colder seasons. The seasonal comparison showed that SOD activity was significantly lower in autumn than in winter before exercise, suggesting possible seasonal adaptations in oxidative stress management. Conversely, no significant changes in SOD activity were observed in spring and summer, probably reflecting a more stable oxidative state during these milder seasons (Fig. 1). In our study, the activity of this enzyme showed higher statistically significant variability ( $F_{15,152} = 8.34$ ;  $p = 0.000$ ) compared to the activities of CAT ( $F_{15,152} = 4.18$ ;  $p = 0.000$ ) or GPx ( $F_{15,152} = 5.95$ ;  $p = 0.000$ ). This finding was further supported by the correlation (R) and determination coefficients, as well as the corrected form of the latter used in the statistical analysis. In our study, SOD activity was more strongly influenced by the sex and exercise variables, but not by the photoperiodic factor. However, the differences in SOD activity were statistically significant in the total SS model versus the residual SS, with correlation coefficient values ( $R = 0.67$ ), coefficient of determination ( $R^2 = 0.45$ ), and the adjusted form ( $R^2_{adj} = 0.40$ ) at  $F = 8.34$  ( $p = 0.000$ ).



In the study of Bażanów B. A. et al. (2020), these researchers observed significantly higher activities of total SOD, MnSOD, and CuZnSOD in blood samples from Hucul horses compared to Arabian horses. The superoxide dismutase (SOD) family serves as the first line of defense against ROS (Wang Y. et al., 2018). Changes in SOD activity are known to protect hosts against microorganisms, bacteria, and parasites, with such variations influenced by physical activity, diet, and chemical factors (Rahal A. et al., 2014).

SOD activity was positively associated with long-term improvements in maximal oxygen uptake in horses following a 12-week conditioning programme in the study by de Moffarts B. et al. (2004). However, during a single exercise session, SOD activity does not appear to change significantly during short duration, high intensity work. In Standardbreds undergoing a 14-minute high-intensity treadmill test, no changes in resting, peak or recovery SOD activity were observed, although a reduction in reduced glutathione levels was noted (de Moffarts B. et al., 2004). Similarly, Ji L. L. et al. (2001) found no significant changes in SOD, catalase or glutathione peroxidase activity in response to acute exercise when measured at rest, 2 minutes and 30 minutes after a 12-minute high-intensity treadmill session.

The response of SOD activity to prolonged, lower intensity exercise is less well understood. Balogh N. et al (2001) reported no significant differences in SOD activity when measured 24 hours before, immediately after and 24 hours after exercise in horses competing in a pentathlon. This event involved two consecutive rounds of 20-minute warm-up followed by 1-minute jumping sessions for each horse, although no measurements were taken during the recovery period after the second round. The response of SOD activity to low-intensity standardised exercise tests remains unclear. In addition, the nutritional status of the horses used in these studies was not reported, which could influence the functionality of their antioxidant systems.

A longitudinal cohort study was conducted by Bollinger L. et al. (2023) with 49 healthy horses participating in the 160 km endurance ride at the 2016 World Endurance Riding Championships in Samorin, Slovakia. The study of Bollinger L. et al. (2023) found that lower pre-ride SOD levels were associated with better race results. It is possible that SOD levels are altered primarily after prolonged exercise, such as endurance rides over 120 km (Ono K. et al., 1990). This is consistent with the findings of Kinnunen S. et al. (2005) who reported that general resting markers of oxidative stress – such as oxygen radical scavenging capacity, vitamin E levels, lipid hydroperoxide (LPO) concentrations and glutathione-related enzyme activities - were higher in endurance horses than in trotters. However, even prolonged exercise, such as an 80 km ride, did not induce oxidative stress in endurance horses (Kinnunen S. et al., 2005).

Although it was initially expected that higher pre-ride SOD levels would reduce the likelihood of elimination, the opposite was observed. SOD levels are known to increase after prolonged exercise, suggesting that horses with higher pre-ride SOD levels may have experienced sustained oxidative stress prior to sampling. This stress could be due to overtraining or inadequate recovery periods (Balogh N. et al., 2001; Ferrareso R. L. et al., 2012). Blood SOD levels reflect current SOD activity and oxidative stress defence rather than stored SOD reserves (Bollinger L. et al., 2023).

After a race, lower SOD availability in failed horses compared to successful horses may make them more susceptible to oxidative damage (Bollinger L. et al., 2023). Further studies, possibly including muscle biopsies to assess muscle SOD levels prior to competition, may provide more detailed insights. Currently, there is limited data on pre-ride or resting SOD levels in endurance horses, apart from a small sample studied by Fraipoint A. et al. (2011). As a result, it remains unclear which levels are indicative of a



healthy resting state and which are indicative of increased SOD activity due to ongoing oxidative stress.

CAT is a cytoplasmic protein that plays a crucial role in reducing hydrogen peroxide by catalyzing its conversion into water and oxygen. The efficiency of CAT in metabolizing hydrogen peroxide is dependent on the level of hydrogen peroxide accumulation (Nandi A. et al., 2019). Catalase activity showed contrasting seasonal patterns (Fig. 2). In autumn, exercise induced a significant increase in catalase activity in mares, suggesting an upregulated response to counter oxidative stress. In winter, however, catalase activity decreased significantly after exercise, possibly indicating a reduced capacity for antioxidant defence during the colder months. Stallions had consistently higher catalase activity in all seasons, which may reflect sex differences in enzymatic antioxidant defences (Fig. 2). Plasma CAT activity showed statistically significant differences only in the group of mares after exercise during the spring-summer and spring-winter photoperiods. The following coefficients were calculated for CAT activity: correlation coefficient ( $R = 0.54$ ), coefficient of determination ( $R^2 = 0.29$ ), and its adjusted form ( $R^2_{adj} = 0.22$ ) at  $F = 4.18$  ( $p = 0.000$ ). For GPx activity, the statistical analysis revealed the following values:  $R = 0.61$ ,  $R^2 = 0.37$ , and  $R^2_{adj} = 0.31$  at  $F = 5.94$  ( $p = 0.000$ ). Therefore, the activities of the selected antioxidant enzymes showed the following relationships in the statistical model:  $SOD > GPx > CAT$ . Siqueira R. F. et al. (2014) reported that prolonged endurance exercise had no effect on CAT activity in racehorses.

Another enzyme involved in maintaining redox balance is GPx, which removes  $H_2O_2$  by converting it to water and reducing lipid peroxides to their corresponding alcohols. This function is essential for the prevention of lipid peroxidation (Pei J. et al., 2023). GPx activity followed similar trends, with significant decreases observed in stallions during autumn and in mares during winter after exercise (Fig. 3). These findings highlight the sensitivity of GPx to both seasonal and exercise-induced oxidative challenges, particularly during the colder months when environmental stressors may exacerbate oxidative demands. The consistent GPx levels observed in spring and summer further support the notion of seasonal stability of antioxidant enzyme activity during these periods (Fig. 3). The observed seasonal and gender differences in enzyme activity may be due to variations in metabolic demands, hormonal influences and environmental conditions, including photoperiod and temperature. Exercise-induced oxidative stress appears to be modulated differently by season and sex, reflecting the dynamic adaptability of Shetland ponies to their environment.

This finding is consistent with previous research suggesting that antioxidant enzyme activity may decrease during intense exercise due to increased ROS production, but may return to higher levels during recovery (Fisher-Wellman K. and Bloomer R. J., 2009). Conversely, Balogh N. et al. (2001) reported no significant changes in SOD or GPx activities in pentathlon horses between blood samples taken before, immediately after and 24 hours after exercise. Ott E. C. et al. (2022) observed unchanged GPx activity after prolonged exercise in horses. However, their study reported an increase in SOD activity, another key marker of enzymatic antioxidant defence.

In our previous study (Kurhaluk N. et al., 2022), we aimed to determine the photoperiod-induced variations and the effects of exercise on oxidative stress biomarkers (2-thiobarbituric acid reactive substances [TBARS], aldehyde [AD] and ketone [KD] derivatives of oxidatively modified proteins [OMP], total antioxidant capacity [TAC], and biomarkers of metabolic changes (glucose, urea and uric acid, and lactate dehydrogenase [LDH] activity) in the blood of Shetland pony mares and stallions involved in recreational riding. A MANOVA analysis showed that photoperiod played a



predominant role in the changes in these biomarkers, while exercise and sex had a lesser effect. Lipid peroxidation biomarkers, such as plasma TBARS levels, showed the highest adjusted coefficient of determination ( $R^2_{adj} = 0.77$ ). Pre-exercise (resting) plasma TBARS levels in stallions and mares were lowest during the spring and summer photoperiods and highest during the autumn and winter photoperiods. A statistically significant reduction in the levels of both aldehyde and ketone derivatives of OMP was observed in the blood of ponies during the autumn and winter periods; in addition, the level of ketone derivatives of OMP decreased after exercise in the spring. TAC was statistically significant in the spring and winter photoperiods, both before and after exercise. Photoperiod and exercise-induced changes in markers of oxidative stress and antioxidant defences may play a role in the adaptation of animals to exercise, with sex differences observed. Seasonal variations in antioxidant defences and energy metabolism substrates in the blood of mares and stallions as a function of exercise capacity may be crucial in understanding how animals' endogenous adaptive mechanisms prepare for environmental changes associated with different seasons (Kurhaluk N. et al., 2022).

**Conclusions.** This study highlights the significant influence of photoperiod, sex and exercise on antioxidant enzyme activity in Shetland ponies. Antioxidant enzyme activity was generally more affected during autumn and winter, indicating increased oxidative stress during these seasons. Stable enzyme activity in spring and summer suggests reduced oxidative stress during these periods. Mares were more sensitive to exercise-induced oxidative stress in colder seasons, while stallions had higher overall catalase activity. Exercise consistently modulated antioxidant enzyme activity, with the magnitude and direction of change influenced by both season and sex.

These results provide valuable insights into the physiological adaptability of Shetland ponies to environmental and physical stressors. The findings may inform strategies for optimising equine management practices, including seasonally adapted exercise regimes and dietary supplementation to support antioxidant defences. Future research should explore the underlying mechanisms driving these variations and assess their implications for equine health and performance.

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### References

- Andriichuk, A., & Tkachenko, H. (2017). Effect of gender and exercise on haematological and biochemical parameters in Holsteiner horses. *Journal of animal physiology and animal nutrition*, 101(5), e404–e413. <https://doi.org/10.1111/jpn.12620>.
- Balogh, N., Gaál, T., Ribiczeyné, P. S., & Petri, A. (2001). Biochemical and antioxidant changes in plasma and erythrocytes of pentathlon horses before and after exercise. *Veterinary clinical pathology*, 30(4), 214–218. <https://doi.org/10.1111/j.1939-165x.2001.tb00434.x>.
- Balogh, N., Gaál, T., Ribiczeyné, P. S., & Petri, A. (2001). Biochemical and antioxidant changes in plasma and erythrocytes of pentathlon horses before and after exercise. *Veterinary clinical pathology*, 30(4), 214–218. <https://doi.org/10.1111/j.1939-165x.2001.tb00434.x>.
- Bażanów, B. A., Chelmecka, E., Romuk, E., & Stygar, D. M. (2020). Basic Studies on the Oxidative Stress Markers in Two Types of Horse Breed: Semi-isolated Population of Huculs Is Different from Commercially Used Arabian Horses. *BioMed research international*, 2020, 7542384. <https://doi.org/10.1155/2020/7542384>.



- Bhattacharyya, A., Chattopadhyay, R., Mitra, S., & Crowe, S. E. (2014). Oxidative stress: an essential factor in the pathogenesis of gastrointestinal mucosal diseases. *Physiological reviews*, 94(2), 329–354. <https://doi.org/10.1152/physrev.00040.2012>.
- Bollinger, L., Bartel, A., Weber, C., & Gehlen, H. (2023). Pre-Ride Biomarkers and Endurance Horse Welfare: Analyzing the Impact of the Elimination of Superoxide Dismutase,  $\delta$ -Aminolevulinic-Dehydratase, Thiobarbituric Acid Reactive Substances, Iron, and Serum Amyloid A Levels in Elite 160 km Endurance Rides. *Animals: an open access journal from MDPI*, 13(10), 1670. <https://doi.org/10.3390/ani13101670>.
- Brinkmann, L., Gerken, M., & Riek, A. (2012). Adaptation strategies to seasonal changes in environmental conditions of a domesticated horse breed, the Shetland pony (*Equus ferus caballus*). *The Journal of experimental biology*, 215(Pt 7), 1061–1068. <https://doi.org/10.1242/jeb.064832>.
- Brinkmann, L., Gerken, M., Hambly, C., Speakman, J. R., & Riek, A. (2014). Saving energy during hard times: energetic adaptations of Shetland pony mares. *The Journal of experimental biology*, 217(Pt 24), 4320–4327. <https://doi.org/10.1242/jeb.111815>.
- Chainy, G. B., Paital, B., & Dandapat, J. (2016). An Overview of Seasonal Changes in Oxidative Stress and Antioxidant Defence Parameters in Some Invertebrate and Vertebrate Species. *Scientifica*, 2016, 6126570. <https://doi.org/10.1155/2016/6126570>.
- de Moffarts, B., Kirschvink, N., Art, T., Pincemail, J., Michaux, C., & Lekeux, P. (2004). Effect of exercise intensity and training on oxidative stress in Standardbreds. *Equine and comparative exercise physiology*, 1(3), 211–220.
- Durand, D., Collin, A., Merlot, E., Baéza, E., Guilloteau, L. A., Le Floc'h, N., Thomas, A., Fontagné-Dicharry, S., & Gondret, F. (2022). Review: Implication of redox imbalance in animal health and performance at critical periods, insights from different farm species. *Animal: an international journal of animal bioscience*, 16(6), 100543. <https://doi.org/10.1016/j.animal.2022.100543>.
- Ferraresso, R. L., de Oliveira, R., Macedo, D. V., Nunes, L. A., Brenzikofer, R., Damas, D., & Hohl, R. (2012). Interaction between overtraining and the interindividual variability may (not) trigger muscle oxidative stress and cardiomyocyte apoptosis in rats. *Oxidative medicine and cellular longevity*, 2012, 935483. <https://doi.org/10.1155/2012/935483>.
- Fisher-Wellman, K., & Bloomer, R. J. (2009). Acute exercise and oxidative stress: a 30 year history. *Dynamic medicine: DM*, 8, 1. <https://doi.org/10.1186/1476-5918-8-1>.
- Fraipont, A., Van Erck, E., Ramery, E., Richard, E., Denoix, J. M., Lekeux, P., & Art, T. (2011). Subclinical diseases underlying poor performance in endurance horses: diagnostic methods and predictive tests. *The Veterinary record*, 169(6), 154. <https://doi.org/10.1136/vr.d4142>.
- Ji, L. L., Dillon, D. A., Bump, K. D., & Lawrence L. M. (2001). Antioxidant response to exercise in equine erythrocytes. *Journal of equine veterinary science*, 10(5), 380–383.
- Jomova, K., Alomar, S. Y., Alwasel, S. H., Nepovimova, E., Kuca, K., & Valko, M. (2024). Several lines of antioxidant defense against oxidative stress: antioxidant enzymes, nanomaterials with multiple enzyme-mimicking activities, and low-molecular-weight antioxidants. *Archives of toxicology*, 98(5), 1323–1367. <https://doi.org/10.1007/s00204-024-03696-4>.



- Kinnunen, S., Atalay, M., Hyuypä, S., Lehmuskero, A., Hänninen, O., Oksala, N. (2005). Effects of prolonged exercise on oxidative stress and antioxidant defense in endurance horse. *Journal of sports science and medicine*, 4, 415–421.
- Kirschvink, N., de Moffarts, B., Farnir, F., Pincemail, J., & Lekeux, P. (2006). Investigation of blood oxidant/antioxidant markers in healthy competition horses of different breeds. *Equine veterinary journal. Supplement*, (36), 239–244. <https://doi.org/10.1111/j.2042-3306.2006.tb05546.x>.
- Koroliuk, M. A., Ivanova, L. I., Maïorova, I. G., & Tokarev, V. E. (1988). Metod opredeleniia aktivnosti katalazy [A method of determining catalase activity]. *Laboratornoe delo*, (1), 16–19.
- Kraus, R. J., & Ganther, H. E. (1980). Reaction of cyanide with glutathione peroxidase. *Biochemical and biophysical research communications*, 96(3), 1116–1122. [https://doi.org/10.1016/0006-291x\(80\)90067-4](https://doi.org/10.1016/0006-291x(80)90067-4)
- Kurhaluk, N., Lukash, O., & Tkachenko, H. (2022). Photoperiod-dependent changes in oxidative stress markers in the blood of Shetland pony mares and stallions involved in recreational horseback riding. *Chronobiology international*, 39(11), 1419–1434. <https://doi.org/10.1080/07420528.2022.2115922>.
- Kurutas E. B. (2016). The importance of antioxidants which play the role in cellular response against oxidative/nitrosative stress: current state. *Nutrition journal*, 15(1), 71. <https://doi.org/10.1186/s12937-016-0186-5>.
- Marañón, G., Muñoz-Escassi, B., Manley, W., García, C., Cayado, P., de la Muela, M. S., Olábarri, B., León, R., & Vara, E. (2008). The effect of methyl sulphonyl methane supplementation on biomarkers of oxidative stress in sport horses following jumping exercise. *Acta veterinaria Scandinavica*, 50(1), 45. <https://doi.org/10.1186/1751-0147-50-45>.
- Nandi, A., Yan, L. J., Jana, C. K., & Das, N. (2019). Role of Catalase in Oxidative Stress- and Age-Associated Degenerative Diseases. *Oxidative medicine and cellular longevity*, 2019, 9613090. <https://doi.org/10.1155/2019/9613090>.
- Ono, K., Inui, K., Hasegawa, T., Matsuki, N., Watanabe, H., Takagi, S., Hasegawa, A., Tomoda, I. (1990). The changes of antioxidative enzyme activities in equine erythrocytes following exercise. *Nihon Juigaku Zasshi*, 52, 759–765.
- Ott, E. C., Cavinder, C. A., Wang, S., Smith, T., Lemley, C. O., & Dinh, T. T. N. (2022). Oxidative stress biomarkers and free amino acid concentrations in the blood plasma of moderately exercised horses indicate adaptive response to prolonged exercise training. *Journal of animal science*, 100(4), skac086. <https://doi.org/10.1093/jas/skac086>.
- Paglia, D. E., & Valentine, W. N. (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *The Journal of laboratory and clinical medicine*, 70(1), 158–169.
- Pei, J., Pan, X., Wei, G., & Hua, Y. (2023). Research progress of glutathione peroxidase family (GPX) in redoxidation. *Frontiers in pharmacology*, 14, 1147414. <https://doi.org/10.3389/fphar.2023.1147414>.
- Rahal, A., Kumar, A., Singh, V., Yadav, B., Tiwari, R., Chakraborty, S., & Dhama, K. (2014). Oxidative stress, prooxidants, and antioxidants: the interplay. *BioMed research international*, 2014, 761264. <https://doi.org/10.1155/2014/761264>.
- Siqueira, R. F., Weigel, R. A., Nunes, G. R., Mori, C. S., & Fernandes, W. R. (2014). Oxidative profiles of endurance horses racing different distances. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, 66, 455–461. <https://doi.org/10.1590/1678-41625760>.



- Stanisz A. 2006, 2007. An affordable course of statistics using STATISTICA PL on examples from medicine. Vol. 1-3. Basic Statistics. StatSoft Polska, Krakow, 2006, 2007. – 532 p., ISBN 83-88724-18-5.
- Suttle, N. F., & McMurray, C. H. (1983). Use of erythrocyte copper:zinc superoxide dismutase activity and hair or fleece copper concentrations in the diagnosis of hypocuprosis in ruminants. *Research in veterinary science*, 35(1), 47–52.
- Wang, Y., Branicky, R., Noë, A., & Hekimi, S. (2018). Superoxide dismutases: Dual roles in controlling ROS damage and regulating ROS signaling. *The Journal of cell biology*, 217(6), 1915–1928. <https://doi.org/10.1083/jcb.201708007>.
- Woolliams, J. A., Wiener, G., Anderson, P. H., & McMurray, C. H. (1983). Variation in the activities of glutathione peroxidase and superoxide dismutase and in the concentration of copper in the blood in various breed crosses of sheep. *Research in veterinary science*, 34(3), 253–256.
- Yavari, A., Javadi, M., Mirmiran, P., & Bahadoran, Z. (2015). Exercise-induced oxidative stress and dietary antioxidants. *Asian journal of sports medicine*, 6(1), e24898. <https://doi.org/10.5812/asjasm.24898>.