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Original article

INFLUENCE OF AGE AND SEASON ON BLOOD BIOCHEMICAL PARAMETERS OF FREE-ROAMING KARAKACHAN HORSES

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Summary

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The present study investigated the influence of age and season on blood biochemical parameters in free-living Karakachan horses in Bulgaria. The study included 53 healthy mares divided into two age groups (young: 6 months to 3 years of age; adult: 3–10 years of age) which were followed during three seasons (spring, summer, autumn). Key blood biochemical parameters e.g. electrolytes, enzymes, proteins, and other biochemical markers were assayed using a BS-120 automatic biochemical analyzer (Mindray, China). The results showed significant differences in blood phosphorus ($P<0.05$) between age groups reflecting characteristic physiological and biochemical changes of different age. Seasonal variations influenced significantly blood parameters, with the most pronounced changes observed in blood minerals and enzyme activities. The concentrations of calcium, sodium, iron, magnesium, and potassium showed highly significant differences ($P<0.001$ to $P<0.05$) across seasons. Liver enzymes (alanine aminotransferase, alkaline phosphatase, gamma-glutamyl transferase) and other biochemical markers (triglycerides, lactate dehydrogenase, creatinine) also varied significantly indicating potential adaptive physiological responses. The obtained reference values of blood indicators can serve as a basis for monitoring the health of Karakachan horses and assist veterinary specialists in timely diagnosis and treatment, as well as in the preservation of this unique breed.

Key words: autochthonous breed, blood parameters, equine health, Karakachan horse

INTRODUCTION

The Karakachan horse breed is one of the oldest local breeds on the Balkan Peninsula, created by the semi-nomadic Karakachans, who traditionally used it to carry loads and move their herds in the mountainous regions of Bulgaria, Greece and North Macedonia. The Karakachan horse is small in stature – the average height of animals of this breed is about 130 cm, but can vary between 122–137 cm (Popova *et al.*, 2018). Their body is elongated and slightly downhill-built. The chest is large, as its circumference is greater than the height at the withers and the length of the body, which makes the animals look sufficiently massive and compact. The animals

have relatively short legs, with well-developed bones (Popova *et al.*, 2019). The breed has preserved its original endurance and adaptability to harsh climatic conditions thanks to natural selection and traditional breeding in wild conditions.

In order to ensure an effective health programme for Karakachan horses, a detailed study of their blood biological parameters and their dynamics over time is necessary. Haematological and blood biochemical indicators in horses not only support clinical diagnosis and therapeutic monitoring, but also provide valuable information on the nutritional status, biochemical processes and overall physiological state of animals (Al-Bulushi *et al.*, 2017; Shawaf, 2017; Shawaf *et al.*, 2018; Popova *et al.*, 2020). Blood composition is a dynamic indicator that reflects the body's adaptation mechanisms to environmental changes (Meyer & Harvey, 2004; Chaprazov *et al.*, 2023; Petrov *et al.*, 2023a; 2023b; 2025).

It is especially important to analyse the relationship between the blood indicators and the productivity and adaptability of horses. Blood parameters can be influenced by various factors such as the season, diet, reproductive status and geographic location (Burlikowska *et al.*, 2015; Aiello & Moses, 2016; Aros *et al.*, 2017; Popova *et al.*, 2020). Multiple studies have shown that age also plays a significant role in determining biochemical and haematological parameters (Nidl *et al.*, 2017; Shawaf *et al.*, 2018; Ono *et al.*, 2019; Popova *et al.*, 2020). The breed specificity of these parameters (Burlikowska *et al.*, 2015; Cruz *et al.*, 2017; Shawaf *et al.*, 2018; Popova *et al.*, 2020) emphasises the need to use accurate reference values, as the application of inappropriate ones may lead to wrong conclusions and inadequate veterinary interventions.

The aim of the present study was to describe and compare the key blood biochemical parameters of Karakachan horses across different ages and seasons, and to contextualise these findings within data from modern breeds. This approach will outline the characteristic variations of these parameters to facilitate future research, without committing to the establishment of definitive reference ranges for the breed.

MATERIALS AND METHODS

The study was conducted on 53 healthy Karakachan mares, raised under a free grazing system in the Sakar mountain region, Bulgaria (41°53′52.3″N; 26°33′22.1″E). The horses were not used for work or riding, and their main purpose was to protect the grassland habitats and maintain them in a good agroecological condition. In the case of death of these horses, the carcasses are transported to specialised feeding grounds managed by Green Balkans NGO to be used as food for vultures (Krastev *et al.*, 2019). Horses were divided into two age groups: young (6 months – 3 years, n=19); and adult horses (3–10 years, n=34).

Blood samples were collected from the same animals during three seasons: spring, summer and autumn to evaluate the seasonal influence on the physiological and biochemical indicators. Blood samples (10 mL) were sampled from the jugular vein into sterile vacutainers without anticoagulant. Samples were transported under refrigerated conditions and processed within 2 hours of collection. After collection, blood samples were centrifuged in a laboratory centrifuge at 3000×g for 10 min at room temperature. The serum was separated and the following biochemical parameters were analysed on a BS-120

automatic biochemical analyser (Mindray, China): calcium (Ca, mmol/L), sodium (Na, mmol/L), potassium (K, mmol/L), magnesium (Mg, mmol/L), phosphorus (P, mmol/L), iron (Fe, mmol/L), aspartate aminotransferase (AST, U/L), alanine aminotransferase (ALT, U/L), gamma-glutamyl transferase (GGT, U/L), alkaline phosphatase (ALP, U/L), albumin (ALB, g/L), globulins (GLOB, g/L), total protein (TP, g/L), glucose (GLU, mmol/L), triglycerides (TG, mmol/L), total bilirubin (TBIL, mmol/L), creatinine (CREA, mmol/L), urea (UREA, mmol/L), lactate dehydrogenase (LDH, U/L), creatine kinase (CK, U/L), amylase (AMY, U/L).

The BS-120's built-in normal ranges in the manufacturer's software were used to flag results outside the standard limits. These generic reference ranges are based on pooled data from multiple breeds and may not fully reflect Karakachan horse breed-specific norms.

Data are presented as mean ± standard deviation (SD). Non-parametric analysis of data was used due to the differences in the number of individuals in the different groups and the deviations from the normal distribution of some of the studied indicators. Mann-Whitney U test which assesses differences between two independent groups without requiring normal distribution of data was used for comparison between the age groups. Kruskal-Wallis H test allowing comparison between more than two independent groups was used for assessment of the influence of season on biochemical parameters. In the presence of statistically significant differences ($P<0.05$), Dunn's post-hoc test was performed to identify the specific groups between which significant differences existed. All statistical analyses were performed using SPSS version 21.0.

RESULTS

Table 1 presents the average values of blood indicators in young horses (6 months – 3 years) and adult horses (3–10 years) compared to reference values. Calcium (Ca) concentration was higher in adults (3.12 mmol/L) compared to young horses (2.94 mmol/L), but both remained within normal limits. Sodium (Na) values were nearly identical in both age groups (135.17 mmol/L in younger vs. 135.39 mmol/L in older horses), slightly below the reference range (136–142 mmol/L). Potassium (K) was slightly higher in young horses (4.45 mmol/L), but within normal ranges in both groups. Iron (Fe) was slightly higher in young (20.17 μmol/L) than in adult (19.32 mmol/L) horses. Phosphorus (P) was statistically significantly higher ($P<0.05$) in young horses (1.46 mmol/L) compared to adults (1.15 mmol/L), remaining within normal limits. Aspartate aminotransferase (AST) was higher in adult (345.29 U/L) than in young horses (314.27 U/L), and exceeded the reference range (152 - 294 U/L). Alanine aminotransferase (ALT) was elevated in mature individuals (23.93 U/L) compared to young ones (19.91 U/L), exceeding the normal range (4–12 U/L). Alkaline phosphatase (ALP) values were significantly high in young horses (827 U/L) and even higher in adults (1303 U/L), far exceeding the reference range (102–257 U/L). Albumin levels were significantly higher in adult than in young horses (38.52±1.99 g/L vs 32.9±7.57 g/L). Only young horses' albumin in blood was within the reference range (26–37 g/L),. Globulin levels were higher in adult horses (38.77±8.43 g/L) than in young ones (34.97 g/L). Glucose concentration was higher in young horses (4.08±3.1 mmol/L) compared to adults (2.76±0.7

Table 1. Blood biochemical parameters in the Karakachan horse breed (mean ± SD) by age groups

Blood parameters	Age groups		Reference values
	Young (6 months – 3 years)	Adult (3–10 years)	
	n=19	n=34	
Ca, mmol/L	2.94±0.38	3.12±0.23	2.65–3.25
Na, mmol/L	135.17±4.41	135.39±2.94	136–142
Mg, mmol/L	0.77±0.07	0.76±0.05	0.7–1.1
Fe, μmol/L	20.17±3.03	19.32±1.98	13–37
K, mmol/L	4.45±0.51	4.42±0.42	2.4–5.2
Total bilirubin, mmol/L	17.28±6.22	16.61±6.94	10.0–50.0
Globulins, g/L	34.97±6.6	38.77±8.43	21–38
ALT, U/L	19.91±2.51	23.93±9.82	4.0–12.0
ALP, U/L	827±258	1303±1684	102–257
Amylase, U/L	67.91±83.6	18.57±8.09	9.0–34
AST, U/L	314±89	345±79	152–294
Albumin, g/L	32.9±7.57	39.5±1.99	26–37
Phosphorus, mmol/L	1.46±0.28	1.15±0.23	0.7–1.5
Creatinine, μmol/L	91.36±27.26	88.14±10.83	40–150
Triglycerides, mmol/L	0.25±0.22	0.19±0.15	0.1–0.5
LDH, U/L	815±128	958±210	140–460
Glucose, mmol/L	4.08±3.1	2.76±0.7	4.2–7.1
Total protein, g/L	68.63±11.1	77.29±7.97	52–79
GGT, U/L	16.36±4.8	19.14±9.3	9.0–25
CK, U/L	421±104	414±238	113–333
Urea, mmol/L	7.79±2.02	6.88±1.20	2.5–8.3

AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma-glutamyl transferase; ALP: alkaline phosphatase; LDH: lactate dehydrogenase; CK: creatine kinase.

mmol/L); the average levels of both groups were below the reference range (4.2–7.1 mmol/L). Amylase in young horses (67.91 U/L) exceeded the normal range (9.0–34 U/L). Lactate dehydrogenase (LDH) was elevated in both age groups; similarly creatine kinase (CK) levels were above the reference range in both age groups.

Table 2 presents the average values of studied blood indicators in Karakachan horses in spring, summer and autumn, compared with the reference values. In autumn, Ca levels (3.41 mmol/L) exceeded the upper reference limit (3.25 mmol/L) whereas in spring (2.92 mmol/L) and summer (3.04 mmol/L), the values remained within normal ranges. Sodium levels in spring and summer were below the lower reference limit, while in autumn (142.43 mmol/L), reached the upper reference limit (136–142 mmol/L). Magnesium values remained within normal limits (0.7–1.1 mmol/L) across all seasons, with a slight decrease in summer. Potassium decreased in autumn (4.28 mmol/L) compared to spring (4.81 mmol/L) and summer (4.43 mmol/L), yet within normal limits. Iron (Fe) levels were the highest in

Table 2. Blood indicators in Karakachan horses (mean ± SD; n=53) in spring, summer and autumn, compared to the reference values

Blood parameter	Spring	Summer	Autumn	Reference values
Ca, mmol/L	2.92±0.07	3.04±0.31	3.41±0.2	2.65–3.25
Na, mmol/L	133.26±1.73	135.3±3.58	142.4±6.24	136–142
Mg, mmol/L	0.87±0.07	0.77±0.06	0.80±0.07	0.7–1.1
Fe, µmol/L	27.14±5.1	19.7±2.48	18.76±4.47	13–37
K, mmol/L	4.81±0.43	4.43±0.45	4.28±0.4	2.4–5.2
Total bilirubin, mmol/L	13.62±7.79	16.9±6.51	22.73±5.61	10.0–50.0
Globulins, g/L	42.19±3.97	37.1±7.77	43.59±7.67	21–38
ALT, U/L	13.77±4.88	22.16±7.68	10.93±2.06	4.0–12.0
ALP, U/L	199±57	1094±1273	612±152	102–257
Amylase, U/L	–	40.28±59.77	12.29±3.73	9.0–34
AST, U/L	342±97	332±84	324±46	152–294
Albumin, g/L	32.14±2.02	36.05±5.84	37.88±3.38	26–37
Phosphorus, mmol/L	1.04±0.3	1.29±0.29	0.96±0.24	0.7–1.5
Creatinine, µmol/L	68.69±10.78	89.56±19.38	92.79±9.72	40–150
Triglycerides, mmol/L	0.95±1.64	0.22±0.18	0.27±0.09	0.1–0.5
LDH, U/L	403±69	895±190	907±223	140–460
Glucose, mmol/L	4±1	3.34±2.17	3.49±0.58	4.2–7.1
Total protein, g/L	74.31±3.84	73.48±10.25	–	52–79
GGT, U/L	17.86±12.56	17.92±7.64	10.57±2.31	9.0–25
CK, U/L	210±49	417±188	420±104	113–333
Urea, mmol/L	–	7.28±1.64	5.11±0.72	2.5–8.3

AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma-glutamyl transferase; ALP: alkaline phosphatase; LDH: lactate dehydrogenase; CK: creatine kinase.

spring (27.14 µmol/L) than in summer and autumn (19.7 µmol/L; 18.76 µmol/L).

The influence of age of the horses and season of blood samples collection is shown in Table 3. The analysis of biochemical parameters between the two age groups showed statistically significant differences only in phosphorus levels ($P<0.05$). The remaining parameters did not show statistically significant differences between the different age groups. The analysis of biochemical indicators revealed also significant seasonal differences. In the comparison between autumn and spring, statistically significant differences are observed for Mg ($P<0.05$), K and TBIL ($P<0.01$) and highly significant differences ($P<0.001$) were demonstrated for Ca, Na, Fe, K, ALP, albumin, creatinine, triglycerides, LDH, GGT and CK. In the comparison between autumn and summer, significant differences were registered for total bilirubin, globulins, triglycerides and glucose ($P<0.05$); ALP and amylase ($P<0.01$) and highly significant differences at $P<0.001$ for Ca, Na, ALT, P, GGT and Urea. While comparing spring and summer seasons, statistically significant differences are observed for K, P, bilirubin and globulins ($P<0.05$) and for Mg, Fe, ALT, ALP, albumin, creatinine, triglycerides, LDH, CK ($P<0.001$).

Table 3. Influence of age of the horses and season of blood samples' collection

Blood parameter	Age	Seasons		
	P-value	Autumn:Spring; P-value	Autumn:Summer P-value	Spring:Summer P-value
Ca, mmol/L	0.572	*0.000****	*0.000****	0.053
Na, mmol/L	0.697	*0.000****	*0.000****	0.063
Mg, mmol/L	0.896	*0.020**	0.138	*0.000****
Fe, µmol/L	0.993	*0.000****	0.160	*0.000****
K, mmol/L	0.571	*0.002***	0.278	*0.028**
Total bilirubin, mmol/L	0.889	*0.002***	*0.010**	*0.042**
Globulins, g/L	0.540	0.872	*0.029**	*0.024**
ALT, U/L	0.278	0.079	*0.000****	*0.000****
ALP, U/L	0.299	*0.000****	*0.004***	*0.000****
Amylase, U/L	0.451	–	*0.003***	–
AST, U/L	0.584	1.000	0.714	0.872
Albumin, g/L	0.430	*0.000****	0.736	*0.000****
Phosphorus, mmol/L	*0.015**	0.629	*0.001****	*0.020**
Creatinine, µmol/L	0.941	*0.000****	0.235	*0.000****
Triglycerides, mmol/L	0.897	*0.000****	*0.013**	*0.000****
LDH, U/L	0.897	*0.000****	0.872	*0.000****
Glucose, mmol/L	0.831	0.223	*0.018**	*0.002***
Total protein, g/L	0.076	–	–	0.965
GGT, U/L	0.243	*0.000****	*0.000****	0.368
CK, U/L	0.062	*0.000****	0.529	*0.000****
Urea, mmol/L	0.161	–	*0.000****	–

*P<0.05; **P<0.01; ***P<0.001; AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma-glutamyl transferase; ALP: alkaline phosphatase; LDH: lactate dehydrogenase; CK: creatine kinase.

DISCUSSION

In equine practice, blood biochemical parameters are an indispensable tool both for the clinical diagnosis of organic, infectious, and parasitic diseases and for monitoring recovery during treatment (Ricketts, 1987; Lassen and Swardson, 1995; Messer, 1995; Čebulj-Kadunc *et al.*, 2002). Interpretation of reference ranges must account for multiple influencing factors including breed, age, reproductive status, feeding time, diurnal rhythms, recent exercise, and even handling during blood collection, as each can provoke splenic contraction and acute shifts in circulating cell and protein concentrations (Schalm *et al.*, 1975; Hodgson & Rose, 1994; Lassen & Swardson, 1995; Čebulj-Kadunc *et al.*, 2002). In our study, statistically significant age-related differences in biochemical parameters were limited to serum phosphorus (Table 2), underscoring its fundamental role in bone metabolism and energy homeostasis. This significant variation may reflect age-associated physiological shifts, dietary influences, or alterations in renal phosphorus handling, whereas the stability of other parameters

suggests effective homeostatic control or insufficient stratification among age cohorts; larger studies with more age categories are needed to clarify these observations.

Age-related differences in serum biochemistry (Table 1) were the most pronounced for phosphorus, reflecting its pivotal role in osteogenesis and energy metabolism in young versus adult Karakachan horses. Regarding the electrolyte and mineral panels, adult horses exhibited marginally high calcium consistent with stable bone mineralisation, whereas young horses had elevated phosphorus, in line with active skeletal growth. Sodium values fell slightly below normal in both groups, likely due to limited salt intake or cutaneous losses in hot conditions, a phenomenon also described in endurance, polo, and stalled sedentary horses (Adamu *et al.*, 2010; Akinniyi *et al.*, 2024). Similarly, higher potassium and iron concentrations in young horses may reflect increased muscular activity and haematopoietic demand during development.

Among hepatic enzymes, adult animals showed modest AST and ALT elevations, suggestive of cumulative physical exertion and hepatic metabolic stress rather than overt pathology (Remmers & Kaijot, 1963; Nishioka *et al.*, 2019; Seifi *et al.*, 2002; Chikhaoui *et al.*, 2023). Alkaline phosphatase levels were markedly high in both groups, expected in growing foals and attributable to bone remodelling or hepatic load in adults, mirroring findings in Japanese Noma and Kiso breeds (Ono *et al.*, 2019) but contrasting with stable ALP activities in Polish Koniks (Witkowska-Piłaszewicz *et al.*, 2021).

The protein profile of mature horses was characterised by increased albumin and globulins, reflecting lower anabolic demands and a more robust immune status; similar trends have been observed in donkeys under nutritional stress (Gupta *et al.*, 1999) and in various equine disciplines (Adamu *et al.*, 2010; Akinniyi *et al.*, 2024). Total protein concentration in blood remained consistent across ages, supporting tight homeostatic regulation (Bauer *et al.*, 1984; Stockham, 1995).

Energy-related parameters exhibited age-related shifts: both groups showed lower glucose, potentially indicating high energy turnover which parallels reports in Polish Koniks and high-performance endurance horses (Adamu *et al.*, 2010; Witkowska-Piłaszewicz *et al.*, 2021), while amylase elevations in foals lacked clear diagnostic relevance (Stockham, 1995).

Finally, muscle-associated enzymes demonstrated that CK is a sensitive indicator of skeletal muscle stress rising post-exercise or with sample handling artifacts (Fayolle *et al.*, 1992; Castejon *et al.*, 2006) whereas increased LDH likely reflects nonspecific cellular turnover or subclinical myocardial strain, as documented in Purebred Arabian (Stockham, 1995, Japanese Noma (Ono *et al.*, 2019) and East Bulgarian horses (Popova *et al.*, 2020).

Collectively, these age-dependent biochemical variations underscore the need for age-specific normal ranges in Karakachan horses and establish a baseline for future studies integrating haematological and longitudinal data.

The seasonal blood chemistry dynamics in free-range Karakachan horses (Table 2) can be traced to the interplay of pasture composition, water availability and environmental stress. In spring, the onset of fresh, protein- and mineral-rich forage elevates serum triglycerides: a form of benign hypertriglyceridemia documented in equids without progression to ponies' hyperlipidemia syndrome

(Dunkel *et al.*, 2014; Ragno *et al.*, 2021; Kellon & Gustafson, 2023) and drives peak iron concentrations, reflecting iron-dense vegetation (Latham *et al.*, 2024). As summer advances, forage desiccation and reduced moisture intake contribute to modest declines in magnesium (Pickering *et al.*, 2020) and phosphorus absorption, while high temperatures and prolonged foraging lead to potassium losses via sweating (Knochel, 1977).

In autumn, cooler temperatures and renewed forage growth coincide with significant rises in calcium and sodium (Table 2), probably reflecting both dietary shifts and mild dehydration (Gupta *et al.*, 1999' Adamu *et al.*, 2010; Schawaf, 2017; Popova *et al.*, 2020; Akinniyi *et al.*, 2024). Phosphorus levels remain within normal limits across seasons but exhibit a reproducible summer peak and autumn decline (Etemadi *et al.*, 2023), consistent with stable bone remodelling demands. Meanwhile, liver-associated enzymes (ALT, ALP and GGT) and total bilirubin showed consistent seasonal patterns: bilirubin elevation suggests increased haemoglobin turnover after the summer stress, whereas reduced summer ALP implies diminished hepatic or osteoblastic activity under heat impact.

These patterns of electrolyte, mineral, lipid, and enzyme variation highlight the adaptive physiology of Karakachan horses to a variable grazing environment. By demonstrating distinct biochemical signatures of spring growth, summer drought and autumn renewal, our results highlight the critical need for season-specific normal ranges when interpreting diagnostic panels in this breed. Incorporating these refined ranges into herd-health protocols will improve nutritional management and early detection of maladaptive responses in extensive grazing systems.

Seasonal effects, as detailed in Table 3, emerged across multiple indicator groups. Electrolytes and minerals including calcium, sodium, magnesium, potassium, iron, and phosphorus exhibited pronounced seasonal dynamics. The autumnal elevations in calcium and sodium likely resulting from changes in diet and hydration status, while the midsummer decline in iron is possibly related to heat-induced losses or reduced intake. Liver enzymes (ALT, ALP, GGT) and total bilirubin also varied significantly, with higher autumn bilirubin suggesting increased haemoglobin turnover and lower summer ALP – adaptive modulation of hepatic or bone metabolic activity. The protein profile (globulins, albumin, total protein) demonstrated seasonal immune and osmotic adjustments, as evidenced by globulin shifts between summer and the other seasons and elevated albumin in autumn. Renal markers (creatinine and urea) rose in autumn, consistent with enhanced protein catabolism or mild dehydration during periods of declining forage quality. Finally, triglycerides and LDH declined in spring and summer, a pattern that likely reflects increased energy expenditure and biochemical activity in warmer months.

Together, these results highlight the critical importance of considering both age and season effects when interpreting biochemical data in Karakachan horses and the need from age- and season-specific normal ranges.

CONCLUSION

In the present study, age-related differences in Karakachan horses were primarily manifested by significant fluctuations in serum phosphorus levels, reflecting differences in bone metabolism between young and adult individuals. Conversely,

seasonal changes had a significant impact on a wide range of biochemical parameters including electrolytes (Ca, Na, Mg, K), iron, and liver enzymes (ALT, ALP, GGT) underscoring the dynamic physiological adaptation to variations in diet and environmental conditions. The findings highlight the need to apply breed-specific reference ranges when interpreting biochemical analyses and provide a foundation for future research incorporating haematological parameters and long-term population monitoring.

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