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Seasonal changes of some biochemical indicators in four sheep herds from Bulgarian Dairy Synthetic Population

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Abstract: The purpose of the present study was to evaluate seasonal differences in the values of some enzymes and metabolites - ALT, AST, ALP, serum iron, glucose, as well as WBC count and N/L ratio in four herds of sheep from the *Bulgarian Dairy Synthetic Population (BDSP)*, raised at different altitudes. The research was conducted in sheep farms of the Sofia region - Ravnishte (391 m a.s.l.), IAS - Kostinbrod (548 m a.s.l.), Mirkovo (715 m a.s.l.) and Bosnek (940 m a.s.l.). Blood samples were taken from selected 24 animals from each sheep herd. Analysis of biochemical parameters was performed in extracted plasma by Semi-automatic Biochemical Analyzer BTS-350 and WBC (leucocyte subpopulations, resp.) count was performed by 5-Part-Diff Automated Haematology Analyzer.
We observed significant differences between seasons in all studied indicators except in WBC count (most in B. and R.). In the summer season there were more significant differences between sheep farms in all studied indicators compared to winter, except about the serum iron levels. All studied indices were within the reference values for sheep, except about ALP activity and serum iron levels, which were lower than limits. The lack of significant difference in total leukocyte count, but the presence of changes in subpopulations, indicated a dynamic adaptation of the immune system to seasonal factors, such as infections, nutrition, stress and parasite loads, i.e., N/L ratio, could be an indicator to seasonal (winter and summer) stress and criterion for selection for stress resistance in sheep, depending on altitude.

Keywords: Biochemical parameters; N/L ratio; altitude; sheep; Bulgarian Dairy Synthetic Population

INTRODUCTION

In our previous study (Yanchev et al., 2024), we already investigated the influence of altitude on some biochemical parameters in the same non-pregnant animals, subject of the present study during the grazing season, but in formed subgroups with high and low hematocrit levels. Continuation of this study with the analysis of the same biochemical indicators in winter season totally for the herds, combined with WBC count, can greatly contribute to supplementing the conclusions of the previous study.

Season can have a significant impact on biochemical parameters in sheep due to changes in temperature, feed availability, physiological status of the animals and other factors. In winter and early spring, when the availability of fresh pasture is limited, seasonal fluctuations in liver enzymes, serum iron and glucose are mainly related to the quality of the diet, stress, parasite load and physiological condition of the animals. Optimal nutritional management and parasite prophylaxis can minimize negative seasonal effects. Zhou et al. (2015), in their research investigated physiological and biochemical indexes of White-Tibetan sheep in Hongyuan Areas of Sichuan Province in four seasons, and found that the indexes of AST, TP, ALB, GLO and LDH in summer and autumn, were relatively higher than

those in winter and spring, although the indexes of ALT and ALP were relatively higher in spring and autumn. In continuation of this investigation, Mingliang et al. (2016) studied the seasonal changes in physiological and biochemical indexes of Tibetan sheep in Hongyuan area, and established that the indexes of ALP and PCHE in summer and autumn were extremely lower than those in winter and spring ($P<0.01$), while the indexes of AST, ALT, LDH, GLU, CHOL and CA maintained stable in all seasons.

Karthik et al. (2021), in their study investigated the effect of farming systems with reference to season on the body condition score (BCS) and adaptive profile (physiological, hemato-biochemical, hormonal, enzymatic and reproductive parameters) of *Nellore* sheep. They found that the sheep reared in intensive system showed higher glucose, total protein, albumin, cholesterol, T3, T4, calcium, and phosphorus; however, the globulin, creatinine, uric acid, aspartate amino transferase (AST), alanine amino transferase (ALT), superoxide dismutase (SOD), malondialdehyde (MDA), glutathione peroxidase, and catalase levels were elevated in extensive and semi-intensive systems. Xulu et al. (2022) as well conducted an investigation on serum biochemistry of indigenous *Zulu* sheep in different seasons (from November to June), and rangeland type and established that glucose and blood urea nitrogen were higher in rainy season ($p<0.05$).

According to many authors (Ouanes et al., 2011, Bezerra et al., 2010, Greguła-Kania et al., 2021), major priority in such studies is to define the standard parameters for non-stressed animals reared in each individual country and/or geographical and climatic zone, by building a reference table of local data for hematological and biochemical parameters, which should be updated periodically in order to made a proper comparison. Macrae (2017) recommended this for avoiding errors when conducting on-site hematological and biochemical tests in sheep and cattle.

The influence of extreme values of environmental factors on hematological and biochemical indicators can provide valuable information about the adaptation possibilities of the selected breed, so a lot of researchers investigated it (Forcada & Abecia, 2006, Rahman et al., 2018, Khalil et al., 2022). Jawasreh et al. (2009) fixed the variation in blood parameters in studied animals from *Avasi* breed in Jordan due to several factors, such as altitude, management, feeding level, age, sex, breed, health status, sampling method, hematological techniques used, daily and seasonal variations in environmental temperature and physiological status of healthy adult, non-pregnant sheep.

Rathwa et al. (2017) also found clear, reliable differences between the levels of the biochemical parameters studied, including AST, ALT, glucose, etc. during summer and winter season in native sheep in Anand, India, and Soliman (2014) found that serum ALT activity showed higher ($P<0.05$) levels in late pregnant and non-pregnant compared to early lactating *Ossimi* sheep in Egypt, with no significant difference in serum AST levels. Alomar & Zarkawi (2024) investigated the effects of temperature changes during the summer and winter seasons on hematological parameters, total cholesterol and cortisol levels in male and female Syrian *Awassi* sheep. The results showed that there were significant differences in white blood cells (WBC), monocytes (MON) and granulocytes (GRA), as well as hemoglobin (HGB) between August and January for both females and males.

Certain parameters of the metabolic profile of the blood serum of *Karakachan* sheep and the variability of their concentrations depending on age and in comparison with some other native breeds of sheep from the Balkans were investigated by Stevanović et al. (2015). They found a statistically significant difference between the average concentrations of the investigated parameters in Karakachan sheep and other breeds in total protein (*Cigai*, *Dubrovnik* and *Curly-headed* sheep), albumin (*Dalmatian*), calcium and inorganic phosphorus (*Cigai, Dubrovnik*) and AST (*Dalmatian* and *Karakachan* sheep from Bulgaria).

Another comparative study was conducted by Bozhilova-Sakova and Dimitrova (2020), about some of the most frequently studied biochemical parameters of blood serum in six Bulgarian

sheep breeds - *Karakachan, Copper Red Shumen, Bulgarian Dairy Synthetic Population, Caucasian Merino, Carnobat Merino* and *Ile de France*. They found that ALT, AST, LDH, ALP, urea and creatinine had large variations in values between different breeds and between different parameters, as they were largely influenced by the stress and general condition of the animals.

It is known that in winter, lower temperatures and increased risk of infections lead to an increase in neutrophils (NEU), which are key cells in fighting bacterial infections, along with a decrease in lymphocytes (LYM), because the immune system focuses on the innate immune response. Stress in winter can also increase corticosteroid levels, leading to a decrease in lymphocytes and an increase in neutrophils. Alsamarrae (2015), in his study examined the Neutrophil/ Lymphocyte ratio (NLR) as an indicator to seasonal (winter and summer) stress and criterion for selection for stress resistance in Iraqi sheep, and found that winter overall mean NLR were significantly higher than summer values for all groups, which indicated that winter stress, in investigated region, is more effective than summer stress. Dini et al. (2011) also established in their research that an increase in the number of leukocytes was observed during the period of autumn-winter, as well as in the percentage of neutrophils and eosinophils within the erythrocyte formula.

In the context of the studies cited above, the aim of the present study was to evaluate the values of some enzymes and metabolites - ALT, AST, ALP, serum iron serum iron and glucose, as well as WBC count and N/L ratio in four herds of sheep from the *Bulgarian Dairy Synthetic Population (BDSP)* reared on different altitude.

MATERIAL AND METHODS

The study was conducted in four sheep farms of the Sofia region at different altitudes Ravnishte village /R./ (391 m a.s.l. / a.s.l.), the experimental base of IAS - Kostinbrod /I./ (548 m a.s.l.), the village of Mirkovo /M./ (715 m a.s.l.) and Bosnek village /B./ (940 m / a.s.l.) in June, 2022, and March, 2023, and is a continuation of our previous research (Yanchev et al., 2023) on the dynamics of the red blood cell count depending on their body condition score (BCS). All four herds of BDSP sheep (age 1.5-7.5 years) were raised on manure and pasture during the summer season, with almost identical quality and quantity of concentrated feed (slightly better in B.), the sheep were milked twice. The shearing campaign of the herds was carried out more than a week before the sampling. During the winter season, the animals on all farms were fed with meadow hay ad libitum, a limited amount of alfalfa hay and a concentrate mixture on average about 800 g per day. In all four farms, the milking campaign began with two milkings and then the lambs were released for further weaning in the process of their gradual weaning.

Representative samples of 24 animals were selected from all sheep in the respective herds, matched by age and basal haematocrit level, from which blood samples were taken by jugular venipuncture in tubes with anticoagulant EDTA, which were transported in a thermo-insulated container at a temperature of +4 °C. Analysis of biochemical parameters was performed in extracted plasma by semi-automatic biochemical analyzer BTS-350 manufactured by BioSystems Ltd., Spain and WBC (leucocyte subpopulations, resp.) count was performed by 5-Part-Diff Automated Haematology Analyzer.

Results are expressed as mean values ± S.E.M. and were analyzed statistically by the analysis of variance (ANOVA) method.

RESULTS AND DISCUSSION

One of aminotransferase enzymes related to glycogen synthesis is alanine aminotransferase (ALT) and is responsible for the transfer of amino groups in the liver cells, too (Kaneko et al., 1997). ALT is a more specific indicator of liver damage than AST and can be increased even before the manifestation of typical symptoms.

In our study, ALT activity (Fig. 1) in winter season was lower than those in summer in all four

farms, but the differences between seasons in R. and M. were significant ($p<0.05$ and $p<0.001$, resp.). ALT activity in summer samples from B. was within the reference values for sheep (22-38 U/L), established by Kaneko et al. (1997) and by Radostits et al. (2000), while in the sheep from the other three farms it slightly exceeded the upper limit of the norm, the most in the animals from M. In winter ALT activity was within the reference values in all four farms. But our results are much higher than those reported by Bozhilova-Sakova and Dimitrova (2020) and Angelov et al. (2013), in similar studies with local sheep. A possible explanation could be a presence of heat stress in summer in sheep from farms located at lower altitude.

There were significant differences with different degree of freedom (dF) in summer in B. compared to other three farms, in I. compared to other two farms, but between R. and M. there was only a trend. But in winter season there was only one significant difference ($p<0.05$) – this time between R. and M.

Another aminotransferase enzyme that unlike ALT can be found not only in the liver, but also in other locations such as heart, muscle, pancreas, kidney, spleen and brain is aspartate aminotransferase (AST). High levels of AST are an indication of already acute conditions of hepatic necrosis caused by hepatitis or acute poisoning and low activity of the enzyme has no clinical significance, but can be related to malnutrition and vitamin B_6 avitaminosis (Kaneko et al., 1997).

In winter season, AST activity was lower than those in summer in R. and M., but the values in both seasons in B. and I. were almost the same (Fig. 2). The differences between seasons in B. and R. were significant ($p<0.01$ and $p<0.001$, resp.). The activity of AST in all investigated samples is within the reference values for sheep - 60-280 U/L (Kaneko et al., 1997), Radostits et al. , 2000), although closer to the upper normal limit in summer, especially in R. like the results for ALT, for AST in simmer there were a lot of significant differences between the herds with different degree of freedom – between sheep in B.&R., B.&M., I.&R., I.&M. and R.&M. The only significant difference in winter was between animals in R.&M., again ($p<0.05$). The reported values for AST activity when comparing *BDSP*

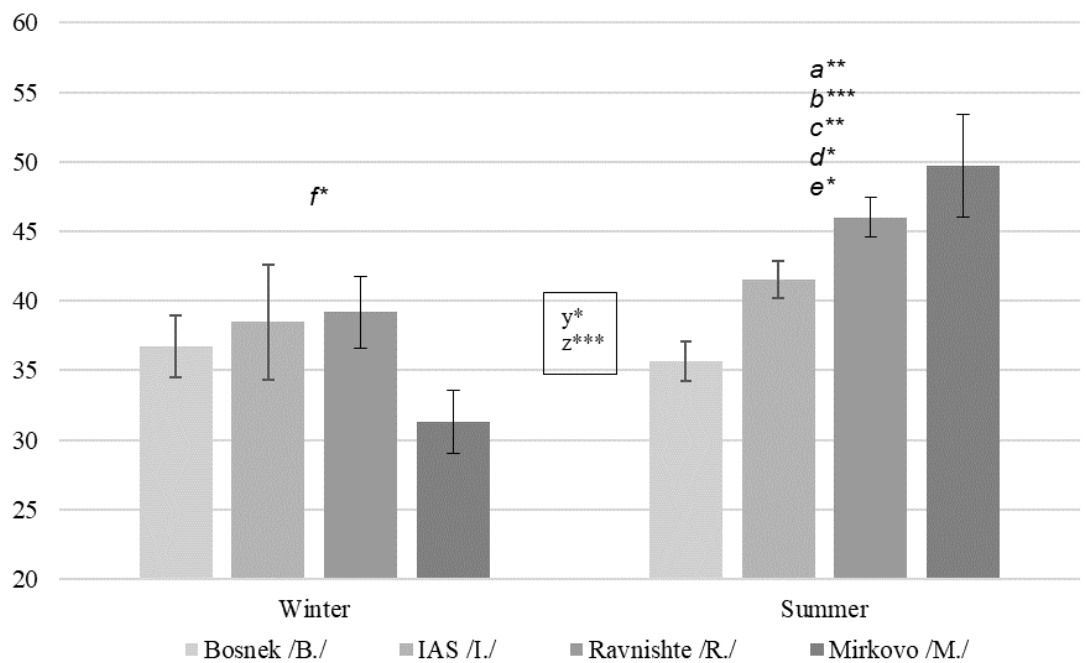

Fig. 1: ALT levels in four BDSP sheep herds (U/L)

and local breeds of sheep in Bulgaria from different studies (Angelov et al., 2013, Bozhilova-Sakova and Dimitrova, 2020), are slightly lower than ours, but they also found some gender differences. Stevanović et al. (2015) also found that the mean concentration of AST in the blood serum of *Karakachan* sheep in Serbia was significantly lower ($p < 0.001$), compared to the activity of this enzyme in the *Dalmatian* breed.

Alkaline phosphatase (ALP) is a zinc-containing metalloenzyme activated by Mg^{2+} and other divalent ions and is a hydrolase enzyme catalyzing the hydrolysis of phosphate esters with a pH-optimum in the alkaline range. ALP is widely distributed in various tissues of the body, being particularly associated with bone, small intestine, liver and especially bile, placenta and kidney. In bones, the function of ALP is associated with mineralization, probably by catalyzing the formation of phosphates from pyrophosphates, in the intestine there is evidence that it is involved in lipid transport, and variations of the enzyme are mainly observed in zinc deficiency (Kaneko et al., 1997).

According the ALP activity (Fig. 3), there were three significant differences between seasons – in B., R and M. ($p<0.05$, $p<0.01$ and $p<0.01$, resp.) The activity of ALP in the analyzed samples of the animals in summer from B., I. and M. were almost two times lower than the reference values for sheep (70-390 U/L), determined by Kaneko et al. (1997) and by Radostits et al. (2000), while that of sheep from R. was within the norm, although close to its lower limit. Four significant differences were found in summer - between the sheep from the farms in B.&R., between those in I.&R. (both at $p<0.001$), between I.&M. ($p<0.05$), and between R.&M. ($p<0.001$). In winter season there were significant difference only between R.&M. ($p<0.05$). Our results for ALP levels were also

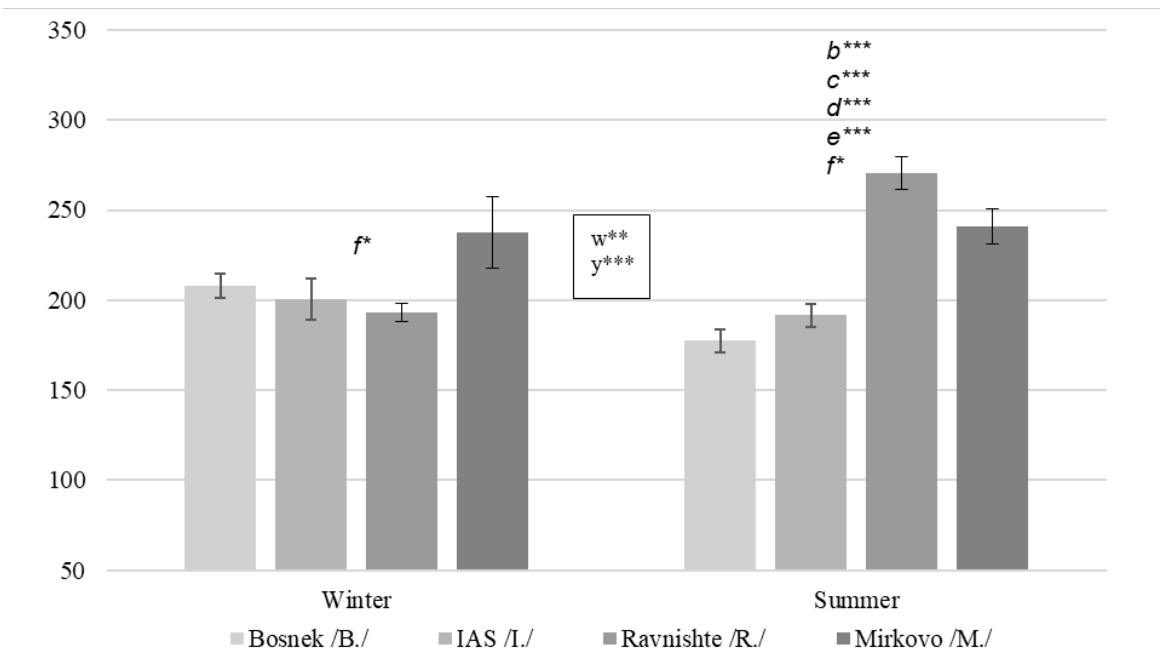

Fig. 2: AST levels in four BDSP sheep herds (U/L)

Legend:

a - significant between B. & I.
b - significant between B. & R.
c - significant between B. & M.
d - significant between I. & R.
e - significant between I. & M.
f - significant between R. & M.

w - significant between seasons in B.
x - significant between seasons in I.
y - significant between seasons in R.
z - significant between seasons in M.

** - p<0.05*
*** - p<0.01*
**** - p<0.001*

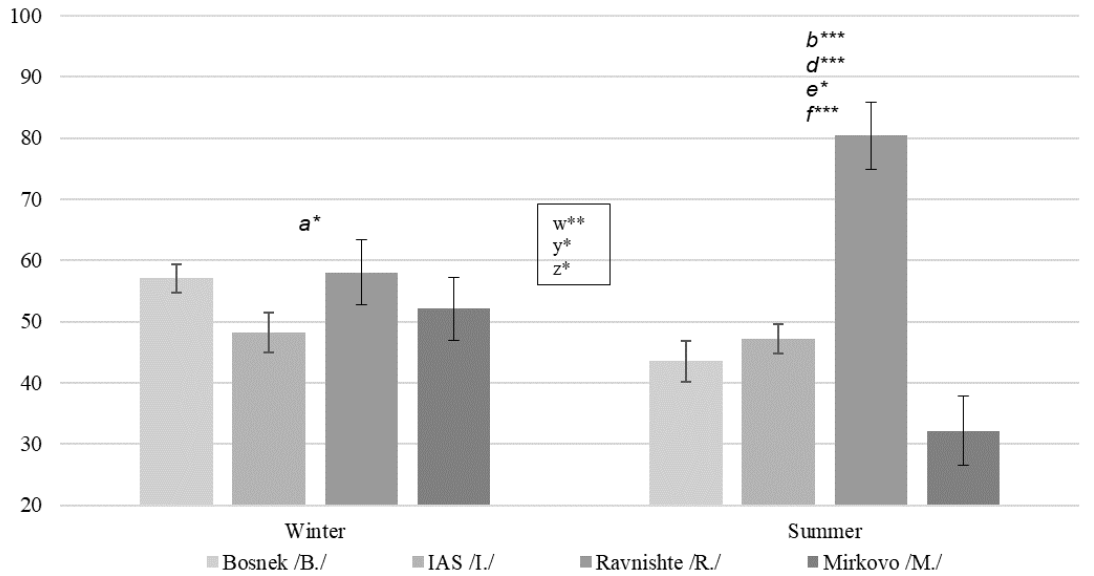

Fig. 3: ALP levels in four BDSP sheep herds (U/L)

lower than those reported by Bozhilova-Sakova and Dimitrova (2020) and Angelov et al. (2013) in their similar studies with *BDSP* sheep, with the exception of the results for the sheep from the village of Ravnishte in summer.

The concentration of iron in the body of animals and humans is strictly regulated, due to the impossibility of its excretion, therefore the levels of serum iron vary greatly during the day (Braun et al., 2010). Unbound iron is stored in the body in the form of ferritin and hemosiderin, in the blood it binds to the transport proteins transferrin, lactoferrin and mobilferin-1. Hemoglobin and myoglobin contain the highest percentage of bound iron in the form of ferrous ion, while the amounts contained in some enzymes are very small (Kaneko et al., 1997).

Seasonal differences in serum iron levels (Fig. 4) were clearly expressed in all investigated farms, where the winter values were lower than the summer ones, but the significant differences we found at farms in I. and R. ($p<0.001$ and $p<0.01$, resp.). In all investigated samples from all four farms in summer and winter, serum iron levels were under the reference values (30–40 μmol/L) determined by Radostits et al. (2000). However, sheep in B. kept higher iron level in winter, which was important to support erythropoiesis during the winter season. There weren't significant differences in summer, but in winter sheep in B. demonstrated significant differences between them and those in other three farms with a different degree of freedom.

Glucagon and insulin are the hormones responsible for regulation of blood sugar levels in the body. The first one increases glucose levels through the transformation of glycogen into glucose, and the second one lowering them through glycogen synthesis (Kaneko et al., 1997). The adrenocorticotropic hormone, adrenaline and thyroxine (synergists of glucagon) are also involved in glucose metabolism.

We found three significant differences between the seasons about the glucose levels (Fig. 5) – in B. ($p<0.01$), in R. ($p<0.01$) and in M. ($p<0.001$). Glucose levels in the samples of sheep from all farms in both seasons were normal according to the reference values (1.7-3.6 mmol/L), determined by Radostits et al. (2000), although they were close to the upper limit in all three groups and in some cases (especially in M. in summer) slightly exceeded it. In winter, there were significant differences with varying degree of freedom between animals in M. and the other those in another three

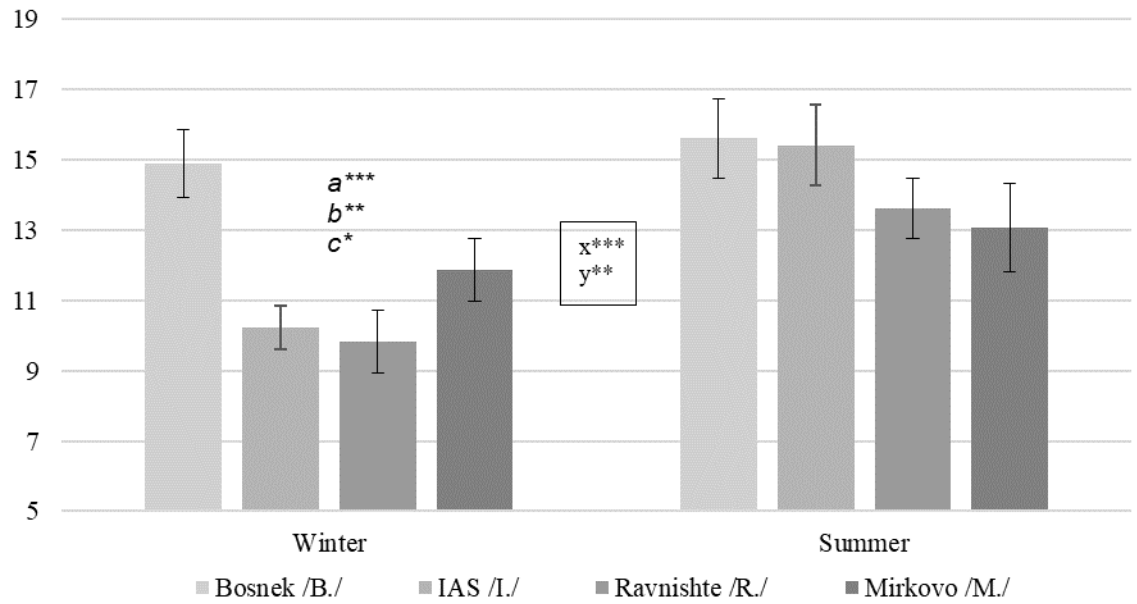

Fig. 4: Fe levels in four BDSP sheep herds (µmol/L)

Legend:

a - significant between B. & I.
b - significant between B. & R.
c - significant between B. & M.
d - significant between I. & R.
e - significant between I. & M.
f - significant between R. & M.
w - significant between seasons in B.
x - significant between seasons in I.
y - significant between seasons in R.
z - significant between seasons in M.
** - p<0.05*
*** - p<0.01*
**** - p<0.001*

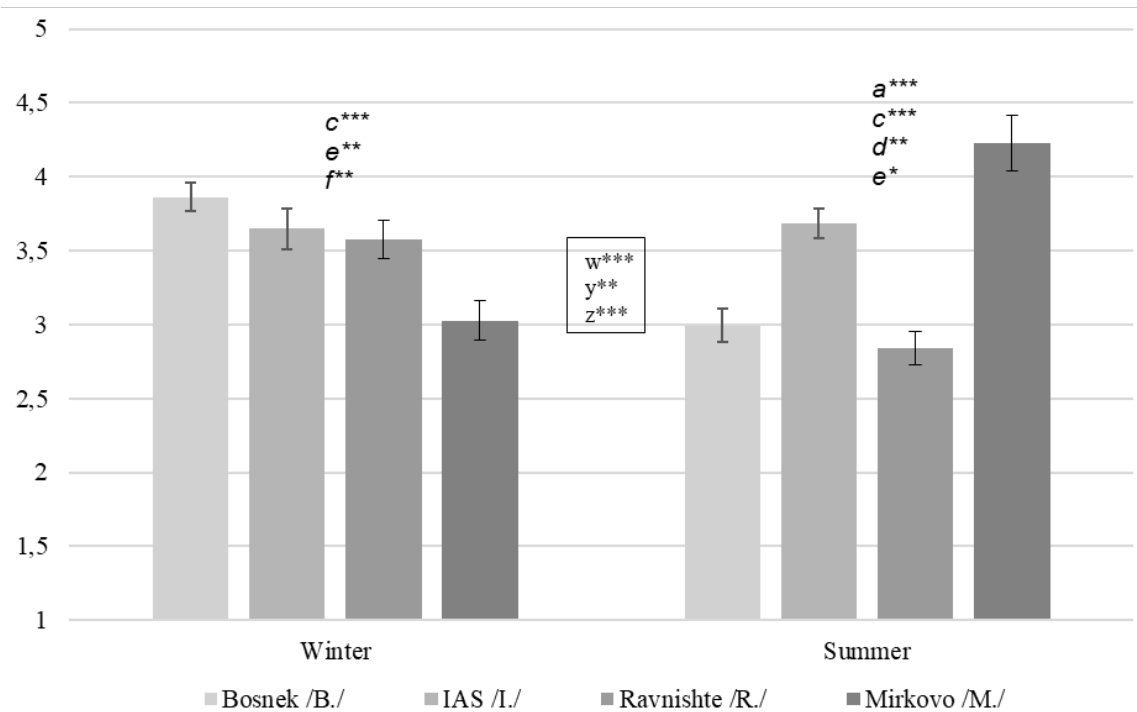

Fig. 5: Glucose levels in four BDSP sheep herds (mmol/L)

farms. In summer, there were four significant differences – between sheep in B. and those in I. and M. (both at $p<0.001$) and between I. and those in R. and M (at $p<0.01$ and 0.05, resp.).

In winter, the number of white blood cells (WBC) can increase, especially if there are stressors, such as cold or food shortages that weaken the immune system, while in spring and summer, when conditions are more favorable, the number of white blood cells is more balanced (Khalil et al., 2022). In our study we also found decreased WBC count (Fig. 6) in winter in two of the farms, while in the other two, there were no changes, but there weren't significant differences between seasons in all four farms. According to the differences between farms, only in summer we observed that those between B.&M., I.&R. and I.&M. were significant with varying degree of freedom.

Some authors (Dini et al., 2011, Alsamarrae, 2015) point out that if there is no significant difference in total leukocyte count (WBC) between seasons, but significant differences in leukocyte subpopulations are observed, this can be explained by a redistribution of different types of white blood cells in response to seasonal factors. The lack of significant difference in total leukocyte count, but the presence of changes in subpopulations, indicates a dynamic adaptation of the immune system to seasonal factors such as infections, nutrition, stress and parasite loads. This highlights the importance of qualitative analysis of the leukocyte formula, and not only the total WBC count, for a better understanding of the health status of animals.

As it was expected we found differences between seasons and between farms in both seasons (Fig. 7) in Neutrophil-to-Lymphocyte ratio (NLR). There was a high significant difference between the seasons about the NLR in B. ($p<0.001$), but this ratio was also higher in winter in the three other farms (highest in M. in both seasons). In summer, there were four significant differences between farms – in B. compared to I. and R. (both at $p<0.05$), and in M. compared to I. and R. (both at $p<0.001$). In winter, these differences with different degree of freedom were in B. compared to I. and R. and between R. and M.

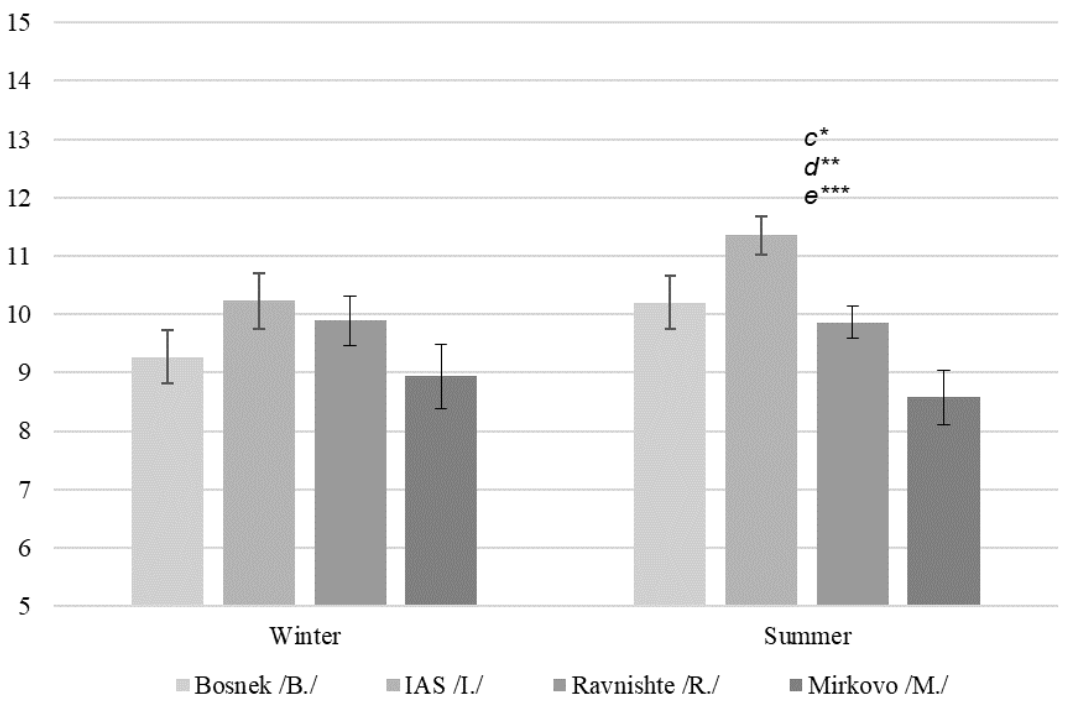

Fig. 6: WBC count in four BDSP sheep herds (10^9/L)

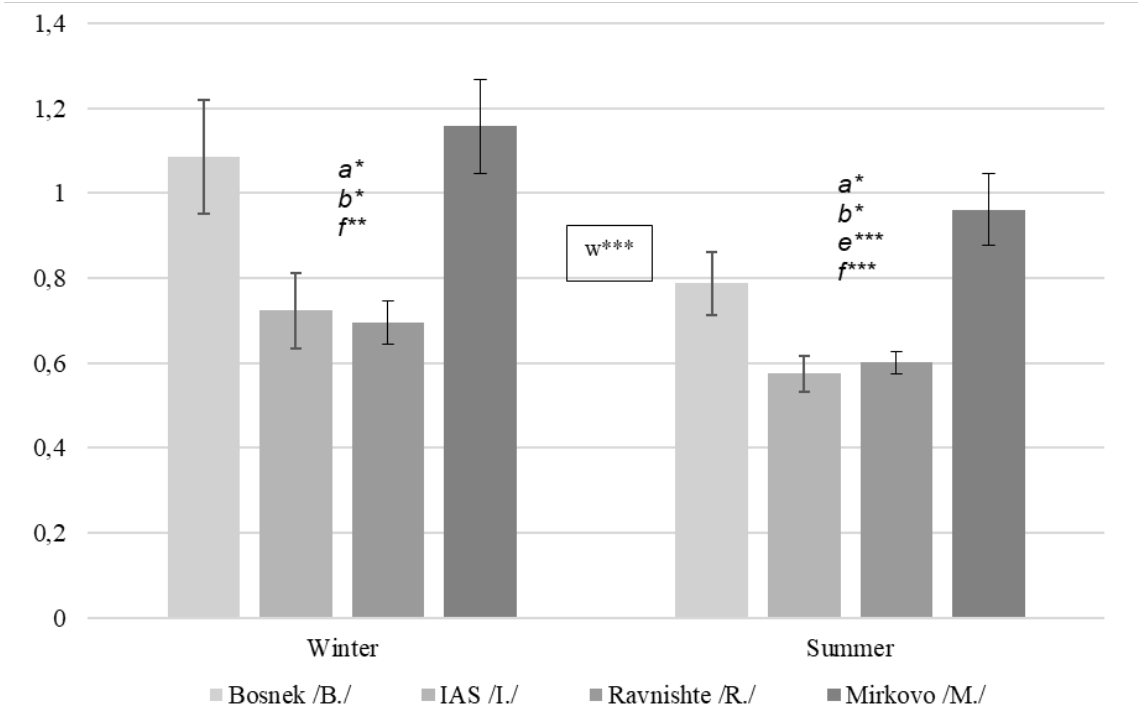

Fig. 7: N/L ratio in four BDSP sheep herds

Legend:

a - significant between B. & I.
b - significant between B. & R.
c - significant between B. & M.
d - significant between I. & R.
e - significant between I. & M.
f - significant between R. & M.

w - significant between seasons in B.
x - significant between seasons in I.
y - significant between seasons in R.
z - significant between seasons in M.

** - p<0.05*
*** - p<0.01*
**** - p<0.001*

In conclusion, we can note that the biochemical parameters and WBC plus NLR count studied by us, can provide valuable scientific information about the reaction of animals of the same breed to the factor „altitude", as the high altitude enhances seasonal changes by increasing erythropoiesis, stress response, and metabolic demands, while sheep at lower altitudes have more stable hematological and biochemical parameters, because they are not exposed to extreme oxygen and climatic stress.

CONCLUSIONS

We observed significant differences between seasons in all studied indicators except in WBC count (most in B. and R.). In the summer season there were more significant differences between sheep farms in all studied indicators compared to winter, except about the serum iron levels;

All studied indices were within the reference values for sheep, except about ALP activity and serum iron levels which were lower than limits;

The lack of significant difference in total leukocyte count, but the presence of changes in subpopulations, indicates a dynamic adaptation of the immune system to seasonal factors, such as infections, nutrition, stress and parasite loads, i.e., N/L ratio, could be an indicator to seasonal (winter and summer) stress and criterion for selection for stress resistance in sheep, depending on altitude.

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