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Chemical elements accumulated in the mosses (*Sphagnum* sp., *Hylocomium splendens*, *Palustriella commutata*) collected in the Belianske Tatra Mountains

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Abstract. Mosses are often used for the analysis of chemical elements and air pollutants, due to their high ability to accumulate in tissues. In this study, we focused on moss species such as *Palustriella commutata*, *Hylocomium splendens* and *Sphagnum* sp. These species can be suitable indicators of the accumulation of selected elements such as S, Cl, K, Ca, Ti, Cr, Mn, Fe, Cu, Zn, Rb, Sr, Zr, Mo, Cd, Sb, Ba and Pb, due to their wider occurrence (water environment, decaying wood, waterlogged soil) in the Tatras. We collected a total of 278 samples (*Hylocomium splendens* - 76, *Palustriella commutata* - 115 and *Sphagnum* sp. - 87) at six locations (1083-1566 m a. s. l.), at regular monthly intervals during two years between October 2019 and December 2021. Individual elements were measured by an XRF spectrometer and levels of Cd by AAS. The results confirmed the difference in accumulation between species, wherein *Palustriella commutata* accumulated the most elements. The elements S, K and Ca, which are the main plant nutrients, showed the highest values. Differences between sites were also confirmed, which can be influenced by many factors, including rainfall, geology, and altitude, whereas Cd exhibited a clear positive cumulation trend with altitude. Significant seasonal differences were confirmed only in the species *Hylocomium splendens* and *Sphagnum* sp. which can be considered a suitable species for tracking seasonality and the cycle of elements in the ecosystem. Levels of Cl and K were the highest in summer, and Fe, Pb and Zn gradually accumulated during the spring and summer. The lowest values were measured in fall (Zn and Pb) and winter (Cl, Ti, and Cr).

Key words: chemical elements, heavy metals, mosses, Tatra mountains

Introduction

Interactions between humans and natural components represent an open system that is constantly evolving, so it is important to monitor. Generally, as urbanization and industrialization have increased, emissions from anthropogenic activities have increased as well. Mountain ecosystems are vulnerable and can be more exposed to pollution due to their barrier effect on air masses and the higher amounts of precipitation transported over great distances (Miśkowiec 2022). Though mountain ecosystems are protected (national parks), it is impossible to shield them from anthropogenic influences that occur outside of this specific region (Maňkovská *et al.* 2003; Bodiš *et al.* 2011). The biggest anthropogenic pollution sources in the atmosphere or on the ground include mining, burning fossil fuels, metallurgical industries, coal ash deposits, traffic deposits, and the application of chemicals such as fertilizers and pesticides in agricultural activities (Sandeep *et al.* 2019). However, the most dangerous pollutants are heavy metals (HM), which are mostly classified into non-essential and essential heavy metals based on their influence on organisms. When these elements increase beyond their allowed limits, they become harmful to organisms. Some heavy metals such as Cu, Co, Fe, Mn, Mo, Ni and Zn are essential for biota (Wintz *et al.* 2002) and are thus classified as essential micronutrients (Baker *et al.* 2020). On the other hand, elements like As, Cd, Cr, Hg, and Pb are considered non-essential and can be potentially toxic to plants. Additionally, these metals can be harmful to the ecosystem as a whole as they persist in the food chain for a long time (Szyczewski *et al.* 2009). Other elements in the environment like calcium, potassium and sulphur are also essential nutrients for plant life (Tripathi *et al.* 2014), but they can also be connected to pollution (Šoltés *et al.* 2014).

Due to unique morphological and physiological characteristics, species of the Bryopsida class have been found to be quite effective in both absorbing HM and monitoring the environment (Brown and Buck 1985). They are helpful in investigation of biomonitoring of various environmental characteristics due to their varying sensitivity to airborne pollutants (Ruhling and Tyler 1970). Mosses have a high ability to absorb and retain heavy metals through rainfall as well as dry deposition. As they lack a root system or skin layer, mineral absorption occurs throughout their whole surface area (Rfihling and Tyler 1968). There are many studies focused on the use of mosses in biomonitoring chemical elements worldwide (Frontasyeva *et al.* 2004; Bing *et al.* 2019; Oishi 2022) as well as in Europe specifically (Achotegui-Castells *et al.* 2013; Vuković *et al.* 2015; Kłos *et al.* 2018; Shetekauri *et al.* 2018).

Since 1990, several European countries have used a moss biomonitoring technique on a regular basis every 5 years (Frontasyeva *et al.* 2014; Harmens *et al.* 2015; Schröder *et al.* 2016). Results showed that mosses with higher heavy metal loads are in Eastern and South-eastern Europe (Harmens *et al.* 2013, 2015). These studies are not limited to individual moss families (Bing *et al.* 2019) but the most investigated chemical elements are As, Cd, Cr, Pb, Sb, Zn. Mosses are often used to study atmospheric deposition in Mountain environments (Gerdol 2002; Frontasyeva *et al.* 2004; Shetekauri *et al.* 2015; Oishi 2022; Bozau and Zupančič 2019). In the Tatra National Park, studies on concentrations of elements present in mosses have also been conducted (Šoltés 1992; Samecka-Cymerman *et al.* 2007; Barančoková *et al.* 2009; Šoltés and Gregušková 2013; Maňkovská *et al.* 2017; Korzeniowska *et al.* 2021).

In this study we analysed seasonal accumulation of elements (S, Cl, K, Ca, Ti, Cr, Mn, Fe, Cu, Zn, Rb, Sr, Zr, Mo, Cd, Sb, Ba and Pb) in three species of Bryophyta (*Palustriella commutata*, *Hylocomium splendens* and *Sphagnum* sp.) in the Belianske Tatra Mountains, Slovakia. Hypothesize that species experience differing abilities to accumulate elements, due to the influence of water environment, decaying wood, waterlogged soil, or elevation; and that accumulation of elements is seasonally different.

Material and Methods

Investigated moss species and study sites

For purpose of this study, we focused on three moss species: *Palustriella commutata*, *Hylocomium splendens* and *Sphagnum* sp., which are easily identifiable, commonly widespread in the study area, and representatives of different environmental conditions, including water environment, decaying wood, and waterlogged soil.

The research was done in six locations starting with the Javorova valley in the Tatra Mountains. Javorinka stream continues through Meďodolský potok stream in the Zadné Meďodoly valley to the shore of tarn Kolové pleso (Fig. 1 and 2). The research sites were carefully chosen according to the presence of the investigated species in areas with diverse geology and terrain. This allowed us to element accumulation in relation to different environments.

First location – L1 (N49.2503061°, E20.1560308°) is situated in Javorova valley, in montane zone at 1,083 m a.s.l., and *Palustriella commutata*, grows mainly in the stream on wet rocks. In the second location – L2 (N49.2435478°, E20.1614089°) at 1,134 m a.s.l. the stream bed is wider and *Hylocomium splendens* can be found on decaying wood, while *Palustriella commutata* is found on wet rocks. The third location – L3 (N49.2421589°, E20.1627122°) at 1,148 m a.s.l. is a spring with waterlogged soil. All three types of moss can be found here. The fourth location – L4 (N49.2360147°, E20.1802686°) at 1,325 m a.s.l. is situated in a dry, dead forest (caused by bark beetle) near Meďodolský stream. In this area all species of studied moss can be found. The fifth location – L5 (N49.2338058°, E20.1912122°) at 1,390 m a.s.l. is a spring, but the occurrence of species is different, with only *Palustriella commutata* present. The sixth location – L6 (N49.2205692°, E20.1927411°) in the alpine zone at 1,566 m a.s.l. is on the shore of Kolové pleso lake, where *Sphagnum* sp. can be found.

Sampling and processing

Moss species were collected monthly from October 2019 until December 2021. All samples were collected in plastic bags. The total number of samples collected was 278, including 76 samples of *Hylocomium splendens*, 115 samples of *Palustriella commutata* and 87 samples of *Sphagnum* sp.

In the laboratory, samples were cleaned with water to remove debris (e.g., needles, soil, sand, rocks and other natural materials), then stored in Petri dishes to dry at room temperature. The green, upper parts of the plants were used and the rest was removed. Every sample was milled into powder using a Cryomill (Retsch GmbH, Germany) for 40 seconds at 30 Hz. For non-destructive analysis of chemical elements an ED-XRF spectrometer DELTA Professional with XRF WorkStation was used (Olympus, Innov-X, Woburn, Massachusetts, USA). Every sample was placed in a plastic vial in a thin layer of 1 cm, measured three times for five minutes and then averaged. The results were in ppm (parts per million) units. The study was focused on the following elements: S, Cl, K, Ca, Ti, Cr, Mn, Fe, Cu, Zn, Rb, Sr, Zr, Mo, Cd, Sb, Ba and. Pb. As well as P, Co, Ni, As, Se, Ag, Sn and Hg but their concentrations were under the detection limits. Additionally, samples of *Sphagnum* sp. from locations L2, L4 and L6 were selected for measuring of Cd by Atomic Absorption Spectrometry. This procedure included the Microwave Digestion System MARS 6 (CEM Corporation, USA). Samples were weighed in PTFE containers and sample weights varied from 0.0995

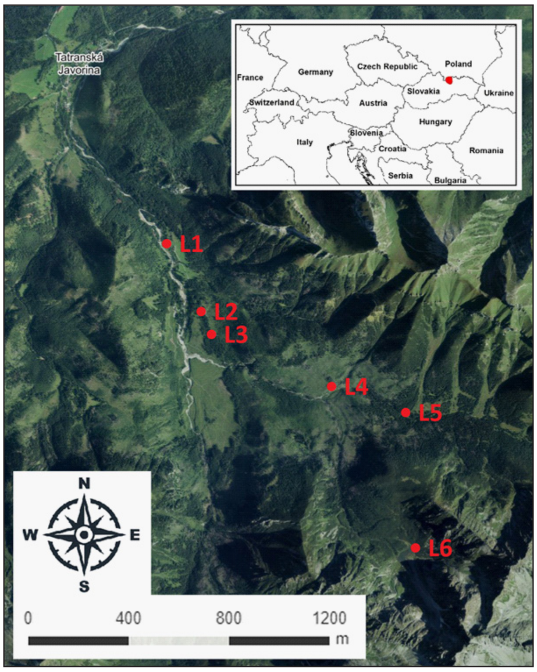

Fig. 1. Study sites in northeast part of Tatra Mountains (Source: GKÚ 2022).

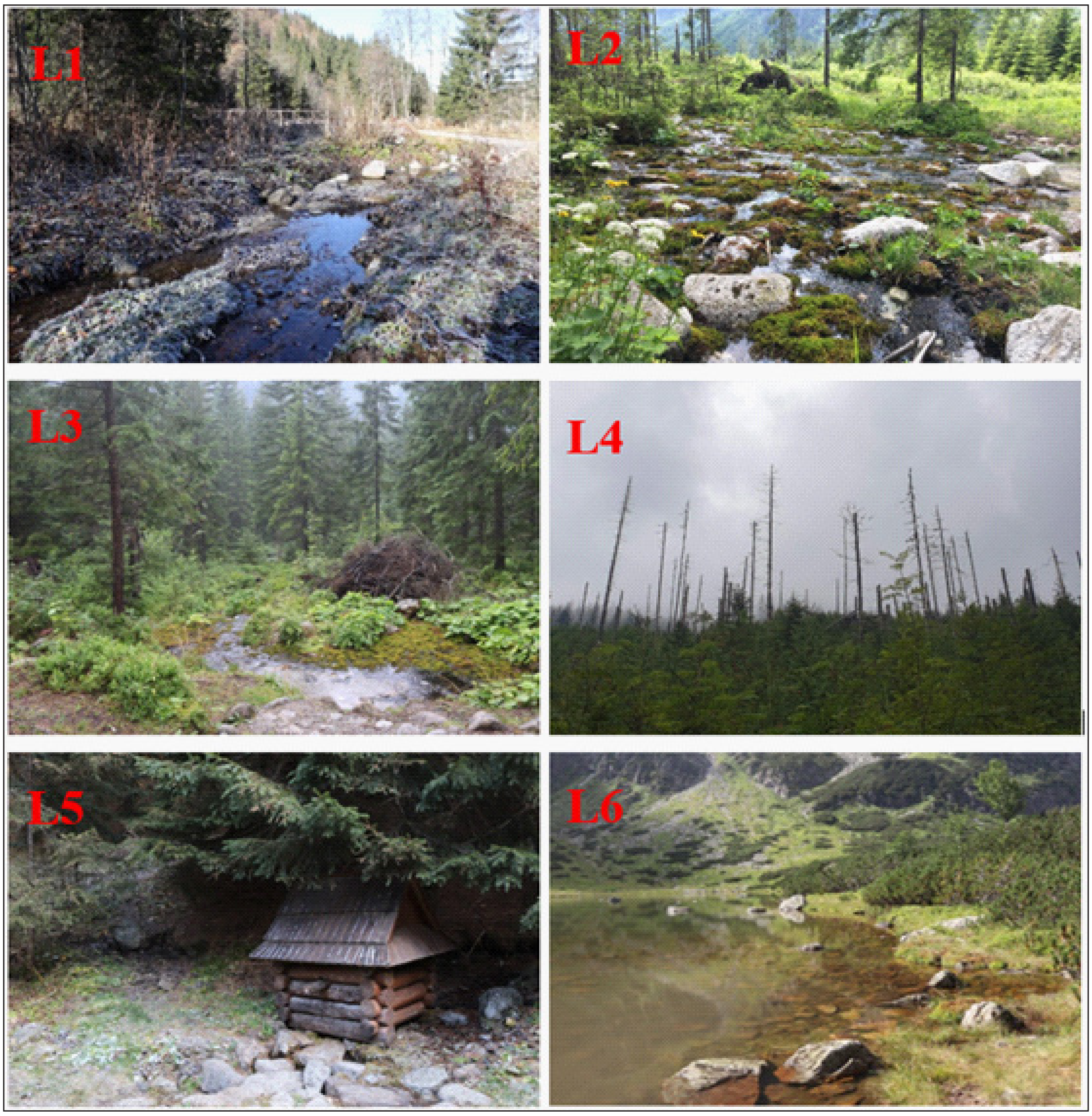

Fig. 2. Study sites. L1 and L2 are sites in valley Javorova dolina. L2- L5 are sites in valley Zadné Meďodoly. L6 is in valley Kolová dolina (Foto: L. Lackovičová, 2022).

g to 3.050 g depending on availability. Next we added 10 ml of 65% HNO_3 and left it to dissolve over a second day in closed PTFE containers. The following day the PTFE containers were stored in the Microwave at 200 °C at 800 psi for 15 minutes, then the temperature slowly decreased for another 15 minutes. Samples were diluted with ultrapure water (Elga PureLab, VWS Deutschland, Germany) to 50 ml. Samples were measured in AAS ZEEnit 650 (Analytik Jena, Germany). Certified reference material Polish Virginia Tobacco Leaves (INCT-PVTL-6) was used as an analytical control. All laboratory glassware and equipment were cleaned in HNO_3.

For statistic evaluation we used Statistica 8 (StatSoft, USA). According to the Shapiro–Wilk normality test, our data did not have normal distribution. Therefore, we used nonparametric statistical methods (such as the Kruskal–Wallis H test (K-W H)) for estimation of accumulation differences between mosses, sites, and seasons. Individual differences for pairs of mosses were calculated using the Mann-Whitney U test (M-W U).

Results

Accumulation differences in investigated mosses

Differences in the accumulation of some elements (except Mn, Cu, Zn and Cd) in *Palustriella commutata*, *Hylocomium splendens* and *Sphagnum* sp. were confirmed (Table 1). Generally, *Palustriella commutata* represented the species in which the elements accumulate the most. The following elements were found to be significantly different in mosses: S, Cl, K, Ca, Ti, Cr, Fe, Rb, Sr, Sb, Ba and Pb. The only element deemed to be non-significant was Mo. The highest mean levels of S were measured in *Sphagnum* (3 098.59 mg/kg), Cl in *Palustriella* (1 526.30 mg/kg), K in *Sphagnum* (16 567.87 mg/kg), Ca in *Palustriella* (42 021.23 mg/kg), Ti in *Palustriella* (470 mg/kg), Cr in *Hylocomium* (176.16 mg/kg), Fe in *Palustriella* (4858.62 mg/kg), Rb in *Sphagnum* (12.57 mg/kg), Se in *Palustriella* (54.86 mg/kg), Sb in *Hylocomium* (20.79 mg/kg), Ba in *Palustriella* (233.35 mg/kg)

Elements	Species	N	Mean	SE	K-W H	K-W H p - value	M-W U 1	M-W U 2
S	*Sphagnum*	87	3098.59	102.72	10.4235	0.0055		
	Palustriella	115	2757.72	105.47			***	
	Hylocomium	76	2948.13	96.45			-	**
Cl	*Sphagnum*	87	1233.47	78.85	70.1127	0.0001		
	Palustriella	115	1526.30	96.41			**	
	Hylocomium	72	691.62	52.87			***	***
K	*Sphagnum*	87	16,567.87	559.94	46.0257	0.0001		
	Palustriella	115	12,117.30	475.06			-	
	Hylocomium	76	12,600.92	516.68			***	-
Ca	*Sphagnum*	87	14,725.13	862.88	179.3915	0.0001		
	Palustriella	115	42,021.23	1813.71			-	
	Hylocomium	76	14,593.28	599.13			-	***
Ti	*Sphagnum*	13	122.77	20.82	49.4542	0.0001		
	Palustriella	90	470.00	36.34			***	
	Hylocomium	34	125.06	12.02			-	***
Cr	*Sphagnum*	87	111.44	4.57	23.9042	0.0001		
	Palustriella	115	155.82	9.74			**	
	Hylocomium	76	176.16	11.06			***	***
Fe	*Sphagnum*	87	1024.57	58.76	102.4834	0.0001		
	Palustriella	115	4858.62	404.05			-	
	Hylocomium	76	1640.87	77.88			***	***
Rb	*Sphagnum*	87	12.57	0.34	88.3172	0.0001		
	Palustriella	115	11.30	0.33			***	
	Hylocomium	76	8.37	0.19			***	***
Sr	*Sphagnum*	84	22.51	3.52	141.8056	0.0001		
	Palustriella	115	54.86	2.72			-	
	Hylocomium	74	8.82	0.54			-	***
Mo	*Sphagnum*	14	1.08	0.02	4.0284	0.1334		
	Palustriella	35	1.17	0.02			*	
	Hylocomium	21	1.14	0.03			-	-
Sb	*Sphagnum*	23	18.43	0.59	12.1425	0.0023		
	Palustriella	38	16.87	0.51			***	
	Hylocomium	14	20.79	1.31			-	***
Ba	*Sphagnum*	87	204.77	12.27	10.6571	0.0049		
	Palustriella	115	233.35	9.84			***	
	Hylocomium	76	184.54	4.38			-	***
Pb	*Sphagnum*	86	18.16	0.47	12.0370	0.0024		
	Palustriella	113	20.85	0.59			***	
	Hylocomium	75	18.29	0.44			-	***

Table 1. Differences of elements accumulation in mosses collected from Tatra Mountains (the highest mean values in bold). K-W H – Kruskal-Wallis H test; significant values $p < 0.05$ in bold; M-W U – Mann-Whitney U Test *** $p < 0.01$; ** $p < 0.03$; * $p < 0.05$; SE – standard error of mean, 1 – *Sphagnum*; 2 – *Palustriella*.

and Pb in *Palustriella* (20.85 mg/kg). Significant differences of accumulation between individual pairs of species were confirmed in Cl, Cr and Rb. Significant differences between *Palustriella* and *Sphagnum* were found with S, Ti, Sb, Ba and Pb. Significant differences between *Palustriella* and *Hylocomium* were found with S, Ca, Ti, Fe, Sr, Sb, Ba and Pb. Significant differences between *Sphagnum* and *Hylocomium* were only found with K and Fe. We can conclude that the most differences were found in accumulation levels in *Palustriella* compared to either *Hylocomium* or *Sphagnum*.

Accumulation site differences

The differences between sites were assessed individually for each species (Table 2). The significant site differences for all investigated moss species were in levels of Mn, Zn and Rb. Elements such as S and Mo did not vary in accumulation based on site. *Palustriella* was the species with the most elements that were different by site. *Hylocomium* differed in its accumulation of K, Cr, Mn, Zn, Rb and Sb based on the site. Whereas *Sphagnum* showed differentiation between sites in accumulation of Cl, Ca, Mn, Zn, Rb, Sr, Ba and Pb. Additional measurements of Cd by AAS in selected *Sphagnum* samples from locations L2, L4 and L6 confirmed significant differences at those sites (Fig. 3 A). The lowest mean value was in L2, and the highest mean value in L6. Thus, it is evident that Cd values in *Sphagnum* increase with increasing altitude. A comparable situation was not observed in the case of Pb (Fig. 3 B). *Hylocomium* cumulated a higher level of Pb in lower-altitude sites (L1-2), and *Palustriella* at site L4. *Sphagnum* had the highest mean value of Pb at L6 and the lowest at L4.

Seasonal trends

Seasonal differences were also assessed individually for each species (Table 3). Significant differences in element accumulation was not found in *Palustriella*. On the other hand, *Hylocomium* and *Sphagnum* showed significant seasonal differences of element accumulation with respect to K, Fe and Zn (Fig. 4 A and 5A, B). *Sphagnum* had significant variation in Cl, and *Hylocomium* showed significant variation in Ti, Cr and Pb.

Seasonal mean concentrations of K were higher in *Sphagnum* during all seasons (Fig. 4 A). Values decreased between fall and spring both for *Sphagnum* and for *Hylocomium*. Between spring and summer, all mean concentrations increased again, which is why the values were highest in summer for both species. The mean Cl concentrations in *Sphagnum* appeared highest during summer, gradually decreased in autumn, and the lowest mean levels were measured in winter. In the spring, their mean level rose again (Fig. 4 B). The mean concentrations of Fe were higher in *Hylocomium* in all seasons (Fig. 5 A). *Sphagnum* has the lowest values in autumn, the concentration gradually increases from autumn to spring, and decreases again in summer. *Hylocomium* has the lowest value in winter, the mean concentration increases during spring and is the highest in summer. The trend of the mean concentration of Zn during the seasons is relatively the same for both monitored species (Fig. 5 B). For both, the lowest value occurred in the fall, the concentration increased during the winter, and the values were the highest in the spring. During the summer, Zn values decreased again in both cases. The mean concentrations of Ti in *Hylocomium* show that the highest values occurred during spring and gradually decreased, with the lowest value present in winter (Fig. 6 A). The mean values of Cr in *Hylocomium* show that Cr accumulates the most during summer, decreases in autumn and has the

Elements	*Sphagnum* sp.	*Palustriella commutata*	*Hylocomium splendens*
S	0.1688	0.2020	0.8031
Cl	**0.0007**	**0.0001**	0.9951
K	0.0602	**0.0001**	**0.0062**
Ca	**0.0001**	**0.0001**	0.4010
Ti	0.3084	**0.0001**	**0.0152**
Cr	0.2821	**0.0001**	**0.0001**
Mn	**0.0001**	**0.0001**	**0.0014**
Fe	0.1673	**0.0001**	0.5909
Cu	1.0000	**0.0326**	0.0768
Zn	**0.0001**	**0.0001**	**0.0484**
Rb	**0.0001**	**0.0001**	**0.0196**
Sr	**0.0001**	**0.0001**	1.0000
Zr	1.0000	**0.0033**	0.3424
Mo	0.5300	0.1332	1.0000
Sb	0.3999	0.1252	**0.0244**
Ba	**0.0001**	**0.0001**	0.0709
Pb	**0.0189**	**0.0001**	1.0000
Cd AAS	**0.0001**	-	-

Table 2. Site differences of elements accumulation in mosses collected from Tatra Mountains. Values represent level p of Kruskal-Wallis H test; significant values $p < 0.05$ in bold.

Elements	*Sphagnum* sp.	*Palustriella commutata*	*Hylocomium splendens*
S	0.1248	0.9286	0.2083
Cl	**0.0474**	0.3645	0.1765
K	**0.0019**	0.2277	**0.0015**
Ca	0.2526	0.4332	0.7102
Ti	0.3305	0.1448	**0.0093**
Cr	0.0709	0.2446	**0.0013**
Mn	0.1709	0.2869	0.6872
Fe	**0.0051**	0.8800	**0.0005**
Cu	-	0.7025	0.2567
Zn	**0.0199**	0.9814	**0.0022**
Rb	0.3885	0.7787	0.6281
Sr	0.6810	0.3352	0.4059
Zr	-	0.5469	-
Mo	0.2063	0.1522	0.3952
Sb	0.3163	0.2433	0.3096
Ba	0.3471	0.4751	0.2145
Pb	0.2341	0.9984	**0.0336**
Cd AAS	0.4084	-	-

Table 3. Seasonal differences of elements accumulation in mosses collected from Tatra Mountains. Values represent level p of Kruskal-Wallis H test; significant values $p < 0.05$ in bold.

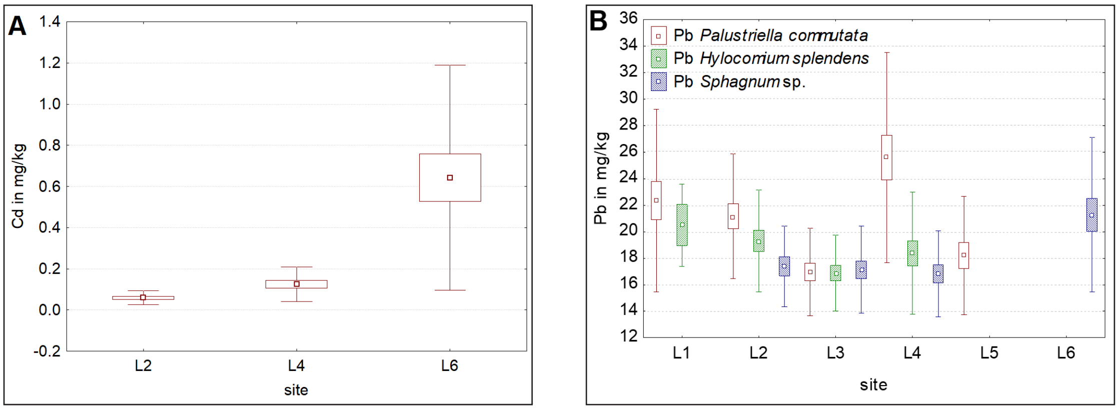

Fig. 3. **A** - Concentrations of Cd (in mg/kg) in *Sphagnum* sp. in relation to study sites (L2, 4 and 6). K-W H (2, 62) = 23.2501, p = 0.0001; **B** - Concentrations of Pb (in mg/kg) in *Palustriella commutata* (K-W H (4, 113) = 30.3965, p = 0.0001), *Hylocomium splendens* (K-W H (2, 71) = 5.2944, p = 0.0709) and *Sphagnum* sp. (K-W H (4, 113) = 30.3966, p = 0.0001) in relation to study sites (L1-L6). Point: Mean; Box: Standard error of mean; Whisker: Standard deviation.

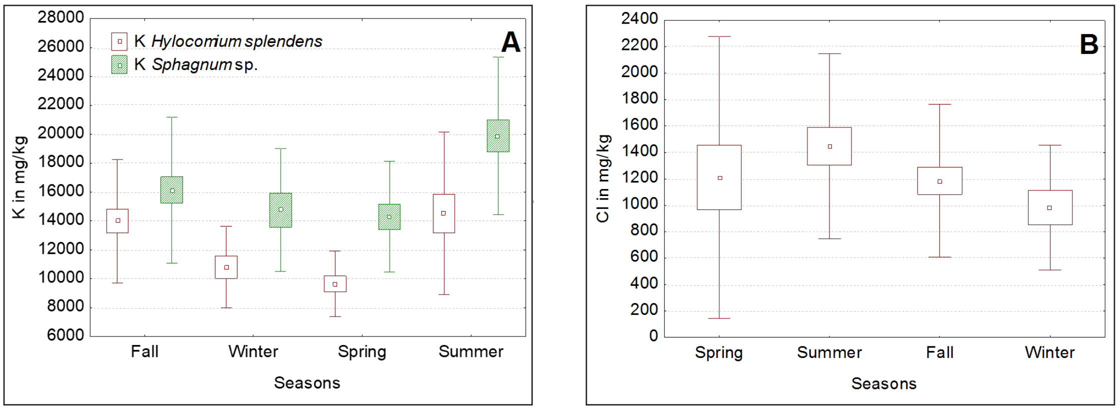

Fig. 4. A - Concentrations of K (in mg/kg) in *Hylocomium splendens* (K-W H (3, 76) = 15.4215, p = 0.0015) and *Sphagnum* sp. (K-W H (3, 87) = 14.9255, p = 0.0019) depending on the seasons. **B** - Concentrations of Cl (in mg/kg) in *Sphagnum* sp. (K-W H (3, 87) = 7.9329, p = 0.0474) depending on the seasons. Point: Mean; Box: Standard error of mean; Whisker: Standard deviation.

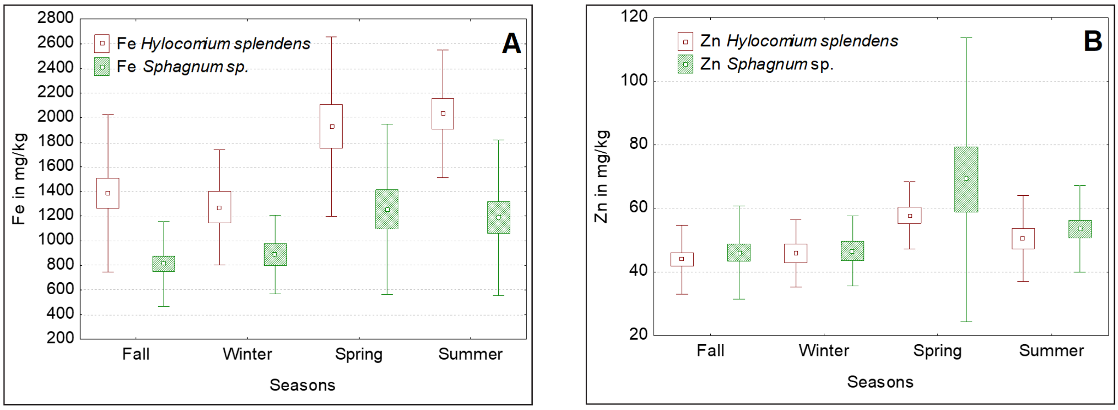

Fig. 5. **A** - Concentrations of Fe (in mg/kg) in *Hylocomium splendens* (KW-H (3, 76) = 17.8494, p = 0.0005) and *Sphagnum* sp. (K-W H (3, 87) = 12.8026, p = 0.0051) depending on the seasons. **B** - Concentrations of Zn (in mg/kg) in *Hylocomium splendens* (K-W H (3, 76) = 14.5731, p = 0.0022) and *Sphagnum* sp. (K-W H (3, 87) = 9.8529, p = 0.0199) depending on the seasons. Point: Mean; Box: Standard error of mean; Whisker: Standard deviation.

lowest values in winter (Fig. 6 B). In the spring, the concentration starts to increase again. The mean concentrations of Pb in *Hylocomium* show that lead accumulates most in spring, decreases during summer, then continues to decrease to its lowest levels in the autumns, then begins to accumulate again in the winter (Fig. 7).

Discussion

The chemical elements S, Cl, K, Ca, Ti, Cr, Mn, Fe, Cu, Zn, Rb, Sr, Zr, Mo, Cd, Sb, Ba and Pb were measured in this study. The highest mean values were measured for calcium (Ca), potassium (K) and sulphur (S). These elements are among the essen-

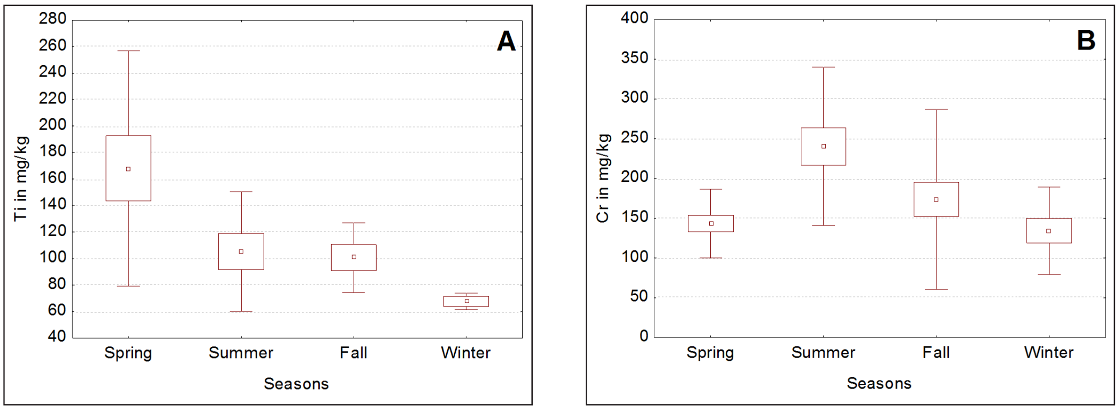

Fig. 6. A - Concentrations of Ti (in mg/kg) in *Hylocomium splendens* (K-W H (3,34) = 11.5085, p = 0.0093) depending on the seasons. **B** - Concentrations of Cr (in mg/kg) in *Hylocomium splendens* (K-W H (3,76) = 15.6931, p = 0.0013) depending on the seasons. Point: Mean; Box: Standard error of mean; Whisker: Standard deviation.

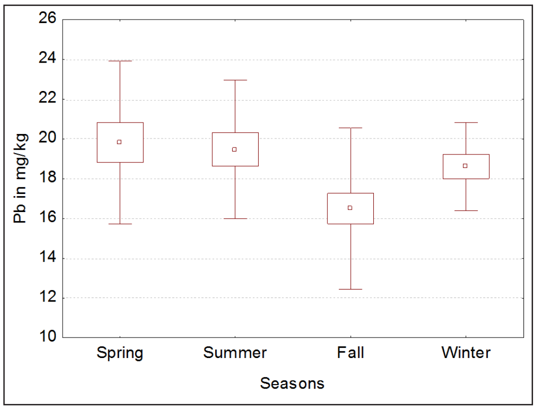

Fig. 7. Concentrations of Pb (in mg/kg) in *Hylocomium splendens* (K-W H (3, 75) = 8.6948, p = 0.0336) depending on the seasons. Point: Mean; Box: Standard error of mean; Whisker: Standard deviation.

tial nutrients of plants (Tripathi *et al.* 2014) and that is why they can occur in such enormous quantities. Similarly, high values of these elements were also recorded in studies by Šoltés and Gregušková (2013) and Tuchyňa (2022). The study by Šoltés and Gregušková (2013) that the highest concentrations occurred in 1-year leaves, which in Bryophyta grow over the years. On the other hand, the concentration of these elements in stems were highest in the first year and then lowered. Tuchyňa (2022) found that algae in aquatic environments had a growing concentration of Ca over the course of the years. In our samples of *Palustriella*, an aquatic moss, we recorded a similar phenomenon of high calcium concentrations. Concentration of Ca and K are can be predicted by water chemistry (Hájek *et al.* 2014) due to bedrock. The Belianske Tatra Mountains are mainly composed of limestone and dolomite (Sedláková 2008) and these rocks are rich in Ca and K (Bodiš *et al.* 2011). *Hylocomium* did not exhibit site differences as it was collected mainly on dead and decaying wood (Andersson and Hytteborn 1991) where there is no connection with bedrock.

Potassium (P) levels showed significant differences between sites in both *Palustriella* and *Hylocomium*. Among the main nutrients, P is mainly present in soil. According to Kobza *et al.* (2008) there is about 2% of potassium in rendzinas. The amount of P in the soil also depends on its thickness and overall soil moisture. In general, the wetter the soil is, the higher a plant's ability to absorb P (Mouhamad *et al.* 2016). Likewise, significant differences were also shown in seasonal trends in *Sphagnum* and *Hylocomium*. This may be influenced by the mobility of the element from the soil to the moss and its water solubility in the spring. In the spring, there is a larger amount of water in the mountains from melting snow and spring precipitation, and it is because of this that P could have been washed out and stored during the summer in the intercellular space. Experimental studies (Vázquez *et al.* 1999, 2000; Figueira and Ribeiro 2005) show that intracellular accumulation of P is higher than extracellular in mosses and decreases in more acidic environments with higher metal content. Significantly different P accumulations within seasonality with an increase during summer coincide with the study of Bargagli *et al.* (1995), where element values were compared in *Hylocomium splendens* in Italy and Antarctica. In *Sphagnum* sp., seasonal differences could also be influenced by the physiological status of plant parts (ageing segments), as older segments lose most of their P accumulation (Hájek and Adamec 2009).

Sulphur (S) accumulated the most in *Sphagnum* sp. Seasonal differences and site differences were not noted, but it accumulated differently between individual moss species. There is a strong assumption that *Sphagnum* sp. can accumulate more S because it can absorb nutrients from water, sludge, and soil, since it grows in waterlogged locations. In the past, high S concentrations were recorded from emissions in the Tatras, as reported by Šoltés *et al.* (2014). Along with heavy metals, S remains in the ecosystem for an exceptionally long time and their permissible values are still exceeded. S emissions are often a result of industrial activity such as metallurgy and engineering production. In *Palustriella*, S concentrations were the lowest as it occurs in an aquatic environment that is constantly dynamic and can wash out S from its tissue structures.

Zinc (Zn) came out to be significantly different between locations in all types of moss. The seasonal assumption was confirmed for *Sphagnum* and *Hylocomium*, where in both cases the lowest value was in autumn and the highest was in spring. It is possible that the increase in the Zn level was influenced by temperature. Comparable results were reported in a study by Martins *et al.* (2004), which compared Zn levels in samples of *Fontinalis antipyretica*. The moss showed higher levels of Zn with increasing temperature and the maximum value reached was at 30°C. It is possible that *Palustriella* did not show this trend because the aquatic environment provides a stable and dynamic place where deposits do not accumulate. It can also be influenced by the difference in locations and how much Zn is in the environment. Low precipitation rates can decrease levels of Zn in environments and mosses. Similar results were recorded in Chopok during the years 1987-2009, where the analysis of Zn concentration in precipitation revealed a significant decreasing trend (Sitár *et al.* 2016). Based on a study by Čeburnis *et al.* (1999), it was found that Zn levels are not as affected by air pollution and dust particles as leaching from the canopy of trees, whereas mosses take up trace metals from soil through several pathways, including upward capillary movement of soil water, raindrops, and windblown soil particles (Bargagli *et al.* 1995). In a study by Say *et al.* (1981), the highest Zn amounts were found in mosses collected downstream. The relationship between Zn and altitude was also confirmed by Šoltés *et al.* (2014).

Iron (Fe) is a micronutrient necessary for metabolic activities (Nagajyoti *et al.* 2010). Significant differences in Fe accumulation between sites were found in *Palustriella*, while significant differences in terms of seasonality were confirmed in *Sphagnum* and *Hylocomium*. Compared to the study of Šoltés and Gregušková (2013), which compared one-year leaves and older leaves of the moss *Polytrichum commune*, the values measured in *Palustriella* were notably higher. Fe can also be related to the geological bedrock, with aquatic environment likely to be richer in Fe. Samples from sites L1, L4 and L5 were collected directly at the mountain stream. The differences between these sites are mostly attributed to altitude. Based on the results, Fe values in *Palustriella* gradually increased from L5, L4 to L1, from which it can be assumed that the lower-altitude the location, the more iron-rich the water is. Although there are mainly limestones here, which are characterized by a lower Fe content, in the Belianske Tatras there is a Carpathian Keuper layer, which is rich in Fe, as observed by Barančoková *et al.* (2009). In a study that points to the content of Fe in sediments in Javorova dolina valley (Zacher 2022), Fe concentrations appeared higher in the parts where the Meďodolský stream flowed into Javorinka. It can therefore be assumed that iron in the stream is gradually collected and washed out from higher-altitude positions to lower ones. On the other hand, the increased iron content observed by Berg and Steinnes (1997) is most likely due to dry deposition of soil particles on the mosses, instead of better uptake capability of these elements in the mosses.

The highest mean lead (Pb) value was noted in *Palustriella*. Significant site differences were noted in *Sphagnum* and *Palustriella*. In terms of seasonality, significant differences were found in *Hylocomium*. It is likely that *Hylocomium* had the lowest Pb values in autumn samples due to reduced precipitation ratios. In September and November 2020 and in September, October and November 2021, precipitation values were below the long-term normal (SHMÚ 2023), indicating that atmospheric deposition could influenced these seasonal differences. In spring, Pb concentrations were the highest, which may be due to an increase in precipitation. Seasonal differences were not reflected in the aquatic environment in *Palustriella*. The lowest Pb concentrations in *Palustriella* were recorded at sites L3 and L5, which represent a spring and a well, and thus the water is probably cleaned of deposits and atmospheric lead. The highest mean value for the highest deviations were recorded for *Palustriella*, sampled in the Meďodolský stream at a higher altitude. The effect of elevated Pb with altitude was noted by Šoltés (1992) in various moss species in Tatra Mountains. On the other hand, geological composition of parent rock could influence Pb levels in aquatic mosses as mentioned by Samecka-Cymerman *et al.* (2007). When compared to Šoltés and Gregušková (2013), the measured Pb values were lower.

According to the XRF spectrometer, cadmium (Cd) was not measured in any monitored moss due to its detection limit, but after using the AAS ZEEnit 650 absorption spectrophotometer in the additional measurement of *Sphagnum* sp. in L2, L4, L6 sites its presence was confirmed. A dependence with increasing altitude was confirmed, with L2 and L4 representing waterlogged sites with flowing water and L6 representing a site with stagnant water. The effect of elevated Cd with altitude was also observed by Šoltés (1992) in the Tatra Mountains. Thera are some indications from comparative heavy metal values from across Europe (Harmens *et al.* 2004), that elevated Cd values were influenced by industrial areas such as the Silesian Black Coal Mine and Ostrava. In Slovakia, Cd values were higher in the High Tatras and Beskydy Mountains because of the influence of industrial zones and the long-distance transmission of pollution. Concentrations of heavy metals were also increased in areas without emission sources in the Scandinavian region, which confirms long-distance transport of substances.

From the observed concentrations of metals in selected species of mosses in the Tatras, it can be concluded that they are useful and effective for monitoring for heavy metal pollution and that their impact is affected by air pollution over long distances (Harmens *et al.* 2004; Šoltés *et al.* 2014) even in areas far from the source. However, on the other hand, it is also likely that soil particles trapped in the mosses resulted in an increased concentration of elements (Bargagli *et al.* 1995). As the species used in this work are common throughout Europe (Berg *et al.* 1995; Berg and Steinnes 1997; Harmens *et al.* 2004; Cowden and Aherne 2019) the values measured can be used for further comparison.

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Site- and age-related changes in blood count parameters in *Apodemus flavicollis* during short-term monitoring

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Abstract. Blood count values vary between individuals of the same species due to individual predispositions, as well as in response to exogenous factors. In addition to individual components of blood count, red blood cells respond to these environmental changes by their size and shape. However, these changes are less visible and occur more slowly. In this study, we focused on comparing the blood counts of yellow-necked mice (*Apodemus flavicollis*) during a month-long study (August 2018) in the vicinity of the city of Žilina. The first group of individuals came from the polluted site of a heating plant tailings impoundment, this is a waste fly ash storage site. The control site was selected based on habitat similarity approximately 1 km from the tailings pond. Morphological parameters of individuals, blood count parameters and erythrocyte size (cell length, cell width) were analyzed using principal component analysis (PCA). The resulting factor score was compared with location, age and sex. While no significant differences were found by gender, locality and age were significant variables that correlated with the factors. Animals at the tailings site showed higher HGB, MCHC and PLT values (Factor 1) and similarly, animals at the tailings site were smaller, had fewer granulocytes and had higher lymphocyte values (Factor 2). Age was significantly related to Factors 2 and 4. Two-year-old animals were larger, had fewer granulocytes, and more lymphocytes (Factor 2). Two-year-olds with longer paws and longer ears have higher MCH and MCV, but fewer WBCs (Factor 4). Although RBC size and width were related to two factors found (Factors 3 and 6), these factors did not related with location, age or sex..

Key words: yellow-necked mouse, blood count, pollution, age

Introduction

Animals are often used in the investigation and assessment of adaptative mechanisms in the environment, and thus, their blood count values are important in the overall assessment of these changes. Analysis of blood parameters is one of the simplest ways to assess the health and physiological status of a wild vertebrate population (Ardia and Schat 2008; Maceda-Veiga *et al.* 2015; Kophamel *et al.* 2022). Hematological analyses extend ecological (Jensen *et al.* 2003; Davis *et al.* 2008) or ecotoxicological studies (Gorriz *et al.* 1996; Bersenyi *et al.* 2003; Maceda-Veiga *et al.* 2015; Tête *et al.* 2015) and complete a picture of the influence of external factors on the population under study (Ovuru and Ekweozor 2004). External factors include environmental conditions (Večerek *et al.* 2002), elevation (Basak *et al.* 2021), season (Sealander 1964; Ono *et al.* 2021), and pollution (Gorriz *et al.* 1996). Endogenous factors include age, sex and reproductive status, and weight, among others (Doubek *et al.* 2003). Hematological data are also an important indicator of the status of individuals and wildlife populations that are affected by toxins or disease (Rostal *et al.* 2012).

Hematological parameters can be useful indicators of both the physiological state, as well as the condition and state of immunological resistance in animals (Etim *et al.* 2013; Pessini *et al.* 2020). Red blood cell (RBC) parameters reflect the oxygen-carrying capacity of the blood, (i.e., the general metabolic ability of the organism (Wolk and Kozlowski 1989)).

The red blood cell count, concentration of hemoglobin and hematocrit (HCT), and red blood cell distribution width (RDW) reflects the oxygen-carrying capacity of blood (Wolk and Kozlowski 1989; Pérez–Suárez *et al.* 1990; Tersago *et al.* 2004). The RDW is a measure of the extent of variability in RBC volume (Nah *et al.* 2018). However, some disorders cause significantly increased variability in RBC size. Higher RDW values indicate greater size differences (Nah *et al.* 2018). RDW test results are often used in conjunction with mean corpuscular volume (MCV) results to determine the causes of anemia. Variations in red blood cell size (anisocytosis) can be quantified and expressed as red blood cell distribution width (RDW) or as an index of red blood cell morphology. Hemoglobin (HGB) is an iron-containing protein that binds oxygen and is found in red blood cells (erythrocytes). When hemoglobin is deficient, there is inadequate tissue oxygenation (Sakalová *et al.* 1995). The most common cause of anemia is iron deficiency, which leads to reduced heme synthesis. RBCs in iron deficiency anemia are hypochromic (lacking red hemoglobin pigment) and microcytic (smaller than normal). Hematocrit values represent the percentage of RBCs in the blood volume. Higher HCT levels represent a higher capacity for oxygen transport (Birchard 1997), but can also occur in the case of dehydration, shock, or congenital heart and lung diseases. It may also reflect an increase in red blood cell fraction (e.g., an increase in erythropoietin) or may reflect a decrease in the plasma component of the blood (Dean 2005). Conversely, a low hematocrit represents a decrease in RBC production in the bone marrow, consistent with bone marrow disease (damage from toxins or cancer) or a decrease in erythropoietin, a hormone secreted by the kidneys that stimulates RBC production (Sakalová *et al.* 1995). The mean corpuscular volume (MCV) is the average volume of RBCs. It is part of the blood count and one of the values that indicates anemia. It is occasionally found in acute conditions such as blood loss or hemolysis (Doubek *et al.* 2003). Mean corpuscular hemoglobin (MCH) or "mean cellular hemoglobin" is the average mass of hemoglobin per RBC in a blood sample. It is provided as part of a standard complete blood count. The MCH value is reduced in hypochromic anemias. MCH is reduced when HGB synthesis is reduced or when RBCs are smaller than normal, as in the case of iron deficiency anemia. The mean corpuscular hemoglobin concentration (MCHC) describes the average hemoglobin concentration in each volume of RBCs. MCV, MCH and MCHC are referred to as red blood cell indices, and these values are useful in elucidating the etiology of anemias. Red cell indices can be calculated if the values of hemoglobin, hematocrit (packed cell volume), and red blood cell count are known.

White blood cell (WBC) parameters reflect the level of immunological resistance, with extreme values indicating pathological conditions (Wolk and Kozlowski 1989). The number of leukocytes, lymphocytes, monocytes, and granulocytes reflect the immunological resistance of the organism. If their values are extremely high, they indicate a pathological condition (Wolk and Kozlowski 1989). If their values are low, the organism is exposed to infections. WBCs (leucocytes) are divided into several types, with each type having specific functions and morphologically differing from other types of leukocytes (Greer *et al.* 2013). Leukocytes are cells of the immune system that are involved in protecting the body from infectious diseases and foreign agents. They include three main types: granulocytes, lymphocytes, and monocytes (Doubek *et al.* 2003; Jenkins 2008). Granulocytes are further divided into three groups: neutrophils, eosinophils, and basophils.

Platelets or thrombocytes are a component of blood whose function is to respond to bleeding when blood vessels are injured by clumping and forming blood clots (Laki 1972). Changes in platelet count (PLT), mean platelet volume (MPV), and platelet crit (PTC) may be due to inflammatory disease, iron deficiency, or underdevelopment (Weiss *et al.* 2010).

Changes in erythrocyte size and shape in mammals are most often described because of ontogeny and shunting (Kostelecka-Myrcha 1973; Pis 2008; Grenat *et al.* 2009; Starostová *et al.* 2013) or because of seasonal influences (Kostelecka-Myrcha 1967; Van Voorhies 1996; Ruiz *et al.* 2004). Variability in RBC size and shape is also a response to external conditions and metabolism-related changes during an individual´s annual cycle, as shown by previous studies of avian RBCs (Janiga *et al.* 2017; Janiga and Haas 2019; Haas and Janiga 2020).

Due to their wide distribution, small mammals are often used as bioindicators of environmental pollution (Talmage and Walton 1991; Shore and Douben 1994; González *et al.* 2008; Blagojević *et al.* 2012). They are widespread in both contaminated and uncontaminated areas and accumulate larger amounts of pollutants (Blagojević *et al.* 2012). They are closely adapted to their environment and are therefore good monitors of pollution and heavy metal concentrations (Sawicka-Kapusta *et al.* 2007). They are also suitable because of their small body size, ease of capture, limited range of territory, short lifespan, and close relationship to their environment (Martiniaková *et al.* 2010, 2012). Populations of small mammals may also be exposed to various chemicals. Chemicals accumulate in organs and can have a negative impact on the organism (Tête *et al.* 2015). The reaction of mice from the polluted area may indicate physiological stress due to diminished environmental quality.

Suitable bioindicators of pollution are mice of the genus Apodemus, which belongs to the family Muridae. The yellow-necked mouse (*Apodemus flavicollis* Melchior, 1834) is often used in bioindication studies (Martiniaková *et al.* 2010). Changes in physiology usually involve the blood and blood-forming organs, genital organs, digestive tract, and respiratory system (Jančová *et al.* 2006). The yellow-necked mouse is a common species with a wide range, mainly in the more mountainous parts of western Europe, except for Scandinavia, southern Spain, western France, and Ireland. It is primarily a woodland species, living in the marginal parts of the forest, but it also occurs in scrubland, fences, orchards and plantations. It lives throughout Slovakia, from the lowlands to the alpine zone (Dungel and Gaisler 2002).

The main objective of this study was to determine the size and shape of erythrocytes in *A. flavicollis* individuals and to determine whether these changes in erythrocytes are directly related to other blood parameters, environmental conditions, and individual characteristics of the individuals. The study is an extension of the research on the impact of the tailing's impoundment (a thermal power plant fly ash dump) on the local biota. The results of the research have already been published separately (see Pogányová *et al.* 2022), this study extends the research to erythrocyte morphometry of the *A. flavicollis* population. We assume that the impact of direct pollution of the tailings pond will be reflected in the blood count values. Changes in HGB content and RDW may also be related to changes in the size and shape of blood cells.

Material and Methods

Field sampling

Mice (n = 28) were captured during August 2018 at the Rosina tailings pond (N 49.180450°; E 18.748783°; Žilina district, Slovakia), where waste fly ash from the burning of lignite in the adjacent heating plant is stored. The control site was selected at approximately 1 km from the tailings pond (N 49.170783°; E 18.747583°) in a forest-meadow habitat. At the control site, 33 individuals were caught. Capture of animals occurred continuously throughout the month for 4 to 5 consecutive days each week, depending on the weather. Mice were captured using Sherman traps filled with pieces of fruit, oatmeal, and dry grass to provide thermoregulation during the night. The distance between traps was 10 m. Traps were checked in the early morning and evening on each trapping day.

Mice were anesthetized by brief inhalation of chloroform vapor. A cotton swab was dipped in chloroform and placed in a plastic bag, in which the mouse was then placed for a short time. When the activity slowed down and the mouse was put to sleep, it was immediately weighed, and body length, tail length, earlobe length, and hind leg paw length were measured.

Subsequently, blood was collected from the orbital sinus using a hematocrit capillary. Bleeding was stopped with a cotton swab. The age of the mice was determined based on the progression of tooth growth and tooth wear (Wolk and Kozlowski 1989), and the individuals were divided into two age classes, 1-year-old and 2-year-old animals. Morphological measurements also provided information about the age class of the animal. The sex of the animals was determined based on the distance between the anus and the papilla. In males, the papilla is further from the anus, while in females this distance is shorter. In addition, sexual activity was recorded based on the condition of the testes in males and nipples in females.

Laboratory analysis

Collected blood samples (in heparinized tubes) were transported to the laboratory (Institute of High Mountain Biology ŽU, T. Javorina) for further analyses. Samples were analysed 4-5 hours after collection, at room temperature. In the laboratory, a blood smear was made from the blood by smearing on a microscope slide. The remaining blood was used for blood count analysis. All blood samples were analysed using a BC-2800Vet Auto Hematology Analyzer (Mindray Bio-medical Electronics Co., Ltd, China). Blood that was not mixed with anticoagulant was loaded into the analyzer. The instrument determined the folliwing parameters: white blood cells (WBC), lymphocytes (Lymph), monocytes (Mon), granulocytes (Gran), percentage of lymphocytes, monocytes, and granulocytes, red blood cells (RBC), hemoglobin (HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width (RDW), hematocrit (HCT), platelets (PLT), mean platelet volume (MPV), and platelet distribution width (PDW).

Blood smears for microscopic analysis were stained according to Pappenheim (Doubek *et al.* 2003). They were examined microscopically at 1000x magnification. For each individual examined, 50 undeformed erythrocytes were randomly selected, and the following parameters were measured: erythrocyte length (transverse line through the erythrocyte, larger value) erythrocyte width (perpendicular line to the first measurement, smaller value), and erythrocyte perimeter. All measurements were performed using LAS software (Leica Application Suite; ver. 4.5.0; Leica Microsystems CMS GmbH, Switzerland). The slides were viewed meanderingly, and the presence of blood parasites and the general condition of the cells were also monitored. Some cells were affected by hemolysis (cell disintegration).

Statistical analysis

The morphological parameters of the individuals, blood count parameters and erythrocyte size (cell length, cell width) were analyzed by principal component analysis (PCA). Six factors whose percentage of total variance was greater than 5% were selected. For each factor, the dependence on the location (tailing pond, control locality), age (1-year-old, 2-year-old), and sex of individuals was detected. The Kruskal-Wallis H test was used for the analysis, and the confidence level was set at 95% ($p < 0.05$). Statistica software (Ver. 8) was used for the analyses.

Results

Hematology of mice in relation to environment, sex and age of individuals

Principal component analysis (PCA) factor coordinates were calculated based on the individual variables of the study subjects, the mean hematological values of the blood counts and the mean erythrocyte size measured by digital microscopy (Table 1). Five principal factors were selected whose percentage contribution to the total variance was greater than 5%. These factors were further correlated with capture location, sex ,and age of the individual.

Factor 1 (F1) represents a joint increase in the three blood count parameters HGB, MCHC and PLT. Factor 2 (F2) is bipolar and represents a group of animals whose body parameters (body length, tail length and weight) and granulocytes decrease while lymphocyte count increases. Factor 3 (F3) represents the individual erythrocyte shape (length, width), which increases with increasing HCT. Factor 4 (F4) is also a bipolar factor of the morphology of individuals, it is also related to the hematological parameter MCH. When the values of these parameters increase, WBC decreases at the same time. This means that animals with longer paws and ears have higher MCH values, but at the same time have lower WBC values. Factor 5 (F5) is a bipolar factor that is also related to the size of the individuals, this time size is reflected in the size of the paws and tail, and the number of lymphocytes, decreasing if and only if PDW, MCH and MPV increase. Factor 6 (F6) is related to erythrocyte size and shape, is also a bipolar factor, and manifests as an increase in erythrocyte length and width, with a concomitant decrease in monocyte count.

Factors 1 and 2 were significantly related to the location of capture. Animals captured directly at the tailings pond had higher HGB, MCHC and PLT values (F1) than animals from the control site (Fig. 1). Individuals from the tailings site had more lymphocytes, but at the same time fewer granulo-

	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6
Weight	-0.191	**-0.756**	0.070	0.353	-0.176	-0.085
Total length	-0.207	**-0.806**	0.172	0.227	-0.142	-0.056
Tail	0.165	**-0.649**	-0.123	0.423	**-0.337**	-0.107
Paw	0.244	-0.277	-0.048	**0.659**	**-0.402**	-0.066
Ear	0.006	-0.040	0.221	**0.549**	-0.176	-0.059
WBC	0.612	-0.336	0.405	**-0.458**	-0.178	0.049
Lymph #	0.275	0.226	0.636	-0.291	**-0.396**	-0.005
Mon #	0.501	-0.325	0.423	-0.392	-0.195	-0.230
Gran #	0.583	-0.578	0.031	-0.363	0.069	0.093
Lymph %	-0.420	**0.745**	0.243	0.217	-0.293	0.017
Mon %	-0.080	-0.248	-0.100	-0.202	0.005	**-0.565**
Gran %	0.391	**-0.712**	-0.297	-0.173	0.324	0.084
RBC	-0.422	-0.016	0.059	0.055	-0.048	0.185
HGB	**-0.780**	-0.258	0.332	-0.257	0.084	-0.224
HCT	0.228	0.256	**0.529**	0.051	-0.26	0.014
MCV	0.160	-0.041	0.433	0.403	0.248	-0.260
MCH	0.179	-0.180	0.209	**0.493**	**0.510**	0.094
MCHC	**-0.798**	-0.281	0.324	-0.236	0.078	-0.193
RDW	0.518	-0.202	-0.113	-0.028	0.038	0.162
PLT	**-0.722**	-0.274	0.319	-0.140	0.067	-0.271
MPV	0.434	0.347	0.385	0.037	**0.376**	-0.294
PDW	0.461	0.262	0.359	0.323	**0.520**	-0.340
PCT	0.486	0.203	0.431	0.082	-0.232	0.019
Cell length (mean)	-0.215	-0.229	**0.643**	0.019	0.130	**0.513**
Cell width (mean)	-0.321	-0.340	**0.510**	0.078	0.248	**0.494**
Eigenvalue	4.650	4.216	2.992	2.462	1.737	1.519
Total variance	**17.9 %**	**16.2 %**	**11.5 %**	**9.5 %**	**6.7 %**	**5.8 %**

Table 1. Factor coordinates of variables by correlation and percentage of variation in PCA. The most significant factor scores are listed in bold.

cytes and were also smaller (F2, Fig. 2). The second (F2) and fourth factor (F4) were significantly related to the age of individuals. One-year-old individuals were smaller and had fewer granulocytes, but at the same time had more lymphocytes (Fig. 3).

Factor 4 represents a group of individuals that have longer paws and ears and have higher MCH values but fewer WBCs. This factor was significantly related to two-year-old individuals (Fig. 4). No factor was significantly related to the individual's gender.

Discussion

Environmental pollution from anthropogenic sources affects the physiological responses of wildlife, but knowledge of how animals respond to these factors is limited (Gottdenker *et al.* 2014). Levels of hematological parameters are based on the general physiology of the organism, but they also respond to environmental pollution (Gorriz *et al.* 1996). In this study, we focused on the correlation of blood count, morphological characteristics, and erythrocyte size in yellow-necked mouse (*Apodemus flavicollis*), with environmental factors represented by two sites - a tailings pond, which represents a source of pollution, and a control site with no direct source of pollution. We evaluated these variables along with age and sex of individuals.

The variables were evaluated against each other using principal component analysis (PCA) based on the use of a correlation matrix. Using this analysis, it is possible to better compare different relationships between variables, especially when large data sets are difficult to interpret. PCA is a technique to reduce the dimensionality of large data sets, thereby increasing interpretability but minimizing information loss. It does this by creating new uncorrelated variables that gradually maximize the variance. The search for such new variables (principal components) is reduced to solving the eigenvalue/eigenvector problem, and the new variables are defined by the data set, not a priori, making PCA an adaptive data analysis technique. It is also adaptive in another sense, as variants of this technique have been developed to

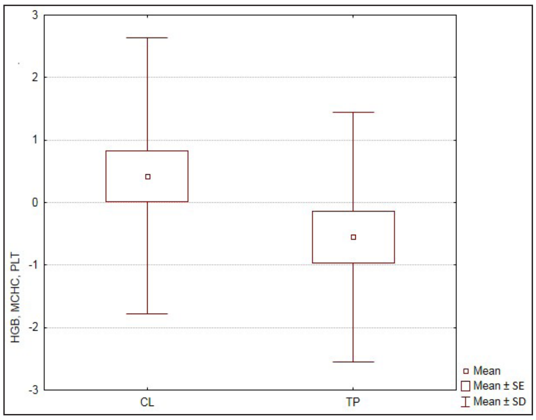

Fig. 1. F1 manifesting based on HGB, MCHC and PLT showed significant differences between sampling sites, with individuals from the tailing pond showing significantly higher values in these blood parameters. KW-H(1;53) = 4.0399; p = 0.0444. CL – control locality; TP – tailing pond; HGB – hemoglobin; MCHC – mean corpuscular hemoglobin concentration; PLT – platelet.

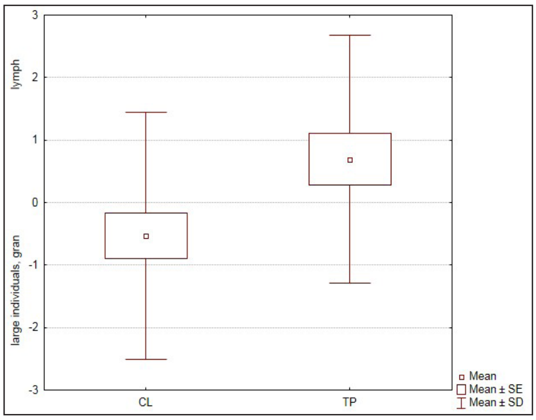

Fig. 2. Animals near the tailing ponds have more lymphocytes but fewer granulocytes and are smaller compared to animals from the control site (F2). KW-H(1;53) = 4.6377; p = 0.0313. CL – control locality; TP – tailing pond, gran – granulocytes; lymph – lymphocytes, large individuals – body length, tail length, weight.

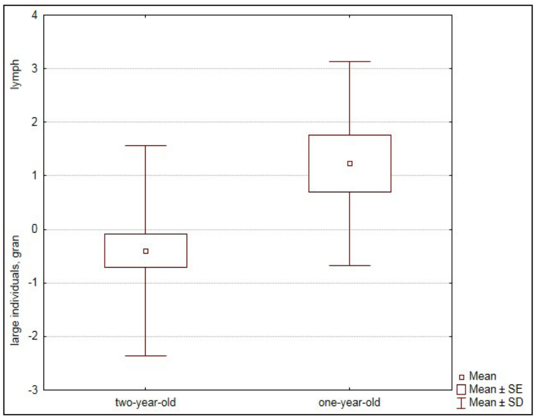

Fig. 3. Difference between ages of individuals on F2. One-year-old animals have more lymphocytes, at the same time they are smaller, and they have fewer granulocytes. KW-H(1;53) = 6.5709; p = 0.0104, gran – granulocytes; lymph – lymphocytes, large individuals – body length, tail length, weight.

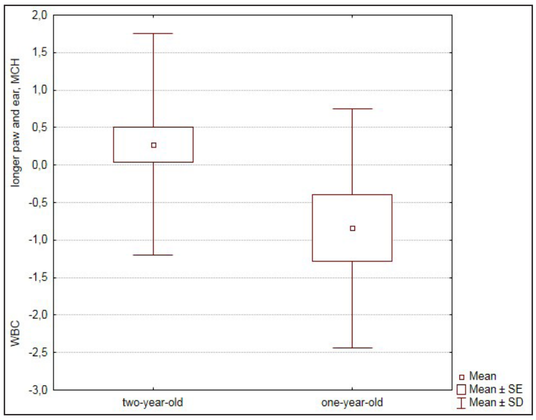

Fig. 4. Difference between ages of individuals and on F4. The older individuals (two-year-old) have greater values of MCH, have longer paws and ears, but have lower values of WBC. KW-H(1;53) = 5.2654; p = 0.0218. MCH – mean corpuscular hemoglobin, WBC – white blood cell.

suit different types of data and structures (Jolliffe and Cadima 2016). In the results of this study, we evaluated the 6 most significant factors for morphometric measurements and blood count results, then compared them to determine the dependence on other variables represented by location, age, and gender of individuals.

Of the six significant factors, only the first two factors (F1 and F2) were related to the sites. We found that animals from the tailings impoundment had higher levels of HGB, MCHC, PLT (F1) than animals captured at the control site. Also, animals at the tailings site had more lymphocytes but fewer granulocytes and were smaller (F2). Some authors have linked the increase of HGB in mice with increased metabolic activity during the winter months (Sealander 1962; Kostelecka-Myrcha 1967). This assumption is closely related to food intake, which requires increased caloric intake (Sealander 1962). Although our research was conducted in summer, when we can rule out an increased energy load due to low ambient temperatures, the increase in HGB may be influenced by an increase in metabolism. A high hemoglobin level is also caused by a low blood oxygen level (hypoxia) that persist for prolonged periods of time. The hemoglobin level is a good measure of the body's ability to carry oxygen. When oxygen is deficient, the body's energy metabolism becomes less economical (Auvinen *et al.* 2021). MCHC is also related to oxygen transfer, and increasing this value could also improve oxygen transfer to cells and tissues. According to Kubota *et al.* (1991), the results confirmed that hemoglobin concentration, red blood cell count, and hematocrit values decrease with increasing age in elderly individuals, and it can be assumed that one of the causes of this phenomenon is decreased protein intake. Since the decline in these blood factors occurs at the control site where there were more two-year-old subjects, these values may also be age related.

Studies (e.g., Rogival *et al.* 2006; Tête *et al.* 2015; Hondda *et al.* 2017) have shown that when exposed to environmental stress in the form of increasing concentrations of heavy metals in the environment, tissues and blood in mammals have been shown to cause a decrease in red blood cells, hematocrit, hemoglobin, MCV and/or MCH. Contrary to these claims, our results document an increase in HCT and MCHC in what we considered to be a polluted environment - the tailings impoundment. The tailings pond with stored fly ash from the thermal power plant represents a load in the environment that releases particulate matter (PM) in addition to potential heavy metals into the environment. The study by Siebel de Moraes *et al.* (2020), reported that no significant changes were observed in terms of hematological and biochemical parameters with particulate matter. Based on our results, we can assume that individuals from the control location are metabolically more active, which is related to increased HGB and MCHC values. What other factor this increased metabolism is further related to could be the subject of further research. However, it may also be related to the age of individuals, which conditions other factors discussed below.

The second factor explains that larger individuals have lower levels of lymphocytes and higher levels of granulocytes, thus representing opposite values of agranulocytes and granulocytes. Animals from the tailings pond were smaller, had lower granulocytes but higher levels of lymphocytes compared to animals from the control site. The increase in the number of lymphocytes (T and B lymphocytes) is mainly related to the body's immune response to infections (bacterial, viral, other) and inflammation. An increase in granulocyte count is related to infection, but also to the development of some autoimmune diseases causing persistent (chronic) inflammation. The number of circulating granulocytes also increases in stress situations in birds and mammals (Apanius 1998; Beldomenico *et al.* 2008). In mice, lymphocyte counts can decrease with handling or other stressors (Schwab *et al.* 2005), as well as with age, when neutrophil counts increase (Jain 1993; Boillinger *et al.* 2010), and the number of lymphocytes begins to decrease (Siegel and Walton 2020). Based on these findings, we can assume that the mice from the control site were exposed to more stressful conditions than the individuals from the tailings pond. Since this factor (F2) was also related to age and was significant for two-year-old individuals, we can assume that there were more one-year-old individuals in the group of individuals from the tailings pond. In a study by Tête *et al.* (2015), the authors observed an unexpected increase in white blood cells in wood mice in both polluted and unpolluted sites. The authors hypothesized that this increase may be due to site characteristics such as unexpected sources of pollution or high parasite abundance. Therefore, it is also possible that the increase in lymphocytes is related to factors other than pollution.

Similarly, factor 4 (F4) was also related to the age of the mice. Two-year-old individuals had higher MCH values, longer paws and ears, and fewer WBCs. Older individuals have greater morphometric predispositions than younger individuals, as indicated by F4 (longer paws, ears) and F2 (larger individuals). Although there is an increase in leukocytes with age in mice, this shift is mainly due to an increase in neutrophils; lymphocytes and eosinophils do not show this trend (Boggs *et al.* 1986; Jain 1993). As rodents age, the proportion of lymphocytes decreases, while the proportion of neutrophils increases (Lindstrom *et al.* 2015). Magnani *et al.* (1988) and similarly Restell *et al.* (2014) observed slightly higher lymphocyte counts in older mice than in younger individuals, which contrasts with our results. In addition to diseases related to inflammatory processes, an increase in lymphocytes in the blood occurs with physical exertion or stress (Schwab *et al.* 2005), but also with inadequate intake of substances important for hematopoiesis, such as vitamin B (Jelinek and Koudela 2003). It is therefore likely that although F4 in mice correlates with age, it is also influenced by other factors that were not considered in this research, in particular other environmental conditions or food sources.

The results of our research did not confirm the correlation of the factors with the sex of the individuals. Also, the factors that were correlated with RBCs size (F3 and F6) which we hypothesized to be related to hemoglobin level or hemolysis, were not significantly confirmed. We conclude that changes in cell size and shape cannot be evaluated in a short-term study and given that the lifespan of erythrocytes in mice is approximately 50 days (Khandelwal and Saxena 2007; Makley *et al.* 2010), the factors influencing the change in cell size are manifested over a longer time frame.

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Male European robins (*Erithacus rubecula*) and mercury transference in the Tatra Mountains of Slovakia

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Abstract. Pollutants such as heavy metals are increasingly prevalent in the environment. For example, Organic methylmercury is known for its persistence and bio-accumulative capacity, and thus for its high toxicity in various organisms. Birds are often used as bioindicators for measuring and monitoring the concentrations of contaminants in the environment. In this report, the mercury content in different tissues of 32 male robins (*Erithacus rubecula*) was investigated. The level of mercury contamination in 29 of 32 birds was within tolerable limits. Significantly more mercury was found in feathers than in muscle tissues ($\alpha = 0.05$). Migratory robins showed slightly higher Hg levels in soft tissues, while residential birds showed higher Hg levels in their plumage. The latter could be an effect of increased Hg deposition in mountain ecosystems, while the higher amounts of Hg ingested by robins in wintering areas may be excreted near the northern montane nesting areas, contributing to increased Hg exposure.

Key words: Erithacus rubecula, Hg exposure, migratory robins, residential robins, Slovakia

Introduction

Since the Industrial Revolution, pollutants such as the heavy metal mercury (Hg), are entering the environment to a higher degree (Sonke *et al.* 2023). Their emission as recurrent waste products of agricultural and industrial activities results in contamination of surrounding areas (Costa *et al.* 2011; Grúz *et al.* 2018). Although elemental Hg is considered a mutagen, teratogen, and carcinogen, potentially affecting behaviour, physiology, and reproductive success, it has no metabolic function with relatively low toxicity (Eisler 1987; Tsipoura *et al.* 2008). Its methylated organic form MeHg, on the other hand, has the potential to accumulate and magnify in organisms and along trophic levels throughout the food web.

To monitor mercury concentrations and contamination in the environment, bio indicative species are studied. Birds have been used in numerous cases as bioindicators for the assessment of heavy metal contamination (e.g., Ackerman *et al.* 2016; Condon and Cristol 2009; Frederick *et al.* 2002; Low *et al.* 2020). Exposure levels differ between species according to direct contact, food and foraging type, habitat area, and moulting (Bianchi *et al.* 2008). Higher levels of contamination are expected for species that feed on soil- or water-borne food and live in wet, open habitats near industrial sites and along contaminated water bodies. Due to the bio accumulative nature of Hg, the highest levels are expected at the end of the food chain, where it biomagnifies, for example, in fish- and bird-eating raptors (Ackerman *et al.* 2016; Grúz *et al.* 2019; Solonen and Lodenius 1990; Shrum 2009). An acute dietary intake of 4-40 mg/kg total Hg is considered lethal for birds (Eisler 1987). The threshold in feathers, which has been shown to have adverse effects such as reproductive performance, is 5.0-5.43 mg/kg (Burger and Gochfeld 1997; Eisler 1987; Grúz *et al.* 2019). A minimum of 70% of a bird's Hg burden is accumulated in the feathers. This is due to the high affinity of Hg to the thiol group in the keratin of bird feathers (Tsipoura *et al.* 2008). During feather growth, the feather is supplied with blood through which Hg is transported. The Hg in feathers corresponds to the level circulating in the blood during the period of feather growth, reflecting the acute dietary intake and the amount already stored in other tissues (Solonen and Lodenius 1990). Once feather growth is complete, the Hg in the feather remains physically and chemically stable, even after death (Condon and Cristol 2009; Low *et al.* 2020). The moult allows birds to excrete the stored mercury and to partially detoxify their bodies on a regular basis (Grúz *et al.* 2018; Poláček and Haas 2018). This could be the reason why Hg tolerance in birds is relatively high compared to mammals (Shrum 2009). Despite the presence of Hg in many tissues, it is usually highest in concentration in feathers, followed by the liver, as a storage organ and a major site for selenium-induced demethylation of Hg (Eisler 1987; Low *et al.* 2020). Muscle tissue usually contains the smallest amount of Hg in examined tissues (Solonen and Lodenius 1990; Zaman *et al.* 2022). Feathers can be obtained non-invasively from the ground, from nests or even from deceased specimens (Bianchi *et al.* 2008; Parmar *et al.* 2016; Solonen and Lodenius 1990). The Hg levels in rectrices (tail feathers) tends to be less than in primaries (long flight feathers) but more than in secondary flight feathers (Furness *et al.* 1986).

The mercury content in various tissues of 32 male European robins (*Erithacus rubecula*), found deceased in more than 10 montane areas in Slovakia was examined in this research. The robin is a typical forest passerine species belonging to the family Muscicapidae, with a wide distribution in the western Palearctic (Bianchi *et al.* 2008; Cramp 1988). It is mainly territorial and only forages over a small area, leading to a firm association with an area and the level of contamination present within it (Costa *et al.* 2011). Robins mainly feed on worms and insects on the ground, but may also consume berries when these other food sources are unavailable (Miller and Harley 1996). In addition to a relatively high metabolic rate, robins have a higher potential to accumulate Hg than other passerines (Costa *et al.* 2011). Examination of their feathers is thus considered to be a reliable method to evaluate Hg contamination during periods of feather growth and between moults. Moult is indicated by the length of day and/or exposure to daylight (Payne 1972). It may thus vary between migratory species or individuals. Robins are considered partly migratory, with males spending winters close to their nesting site, while females and young migrate further away, (e.g., to southern Slovakia and Hungary) (Janiga 2021). In general, the moulting period is between May or June, and September (Bianchi *et al.* 2008). Due to the physiology of moulting and Hg storage, the first moulted primaries should have the highest Hg content in the plumage of passerine birds (Furness *et al.* 1986).

The robins studied in this report were collected in alpine and mountainous areas of the Tatra Mountains in Slovakia. Mountain ecosystems are exposed to higher atmospheric Hg concentrations, which varies among biotopes and may depend on the type of forest cover (Poláček and Haas 2018). MeHg availability in birds is linked to atmospheric Hg deposition in montane areas (Rimmer *et al.* 2005). There is a higher concentration of pollutants in the High Tatra Mountains (Martinková *et al.* 2019). The nearest coal power plants, (i.e., Hg emission sources) are in south Poland. Poland is one of Europe's largest Hg emitters, and southern Poland is considered an air pollution "hot-spot" (Jędruch *et al.* 2021). The elevated Hg levels in the mountains of south-eastern Poland, bordering the High Tatras in Slovakia, are also due to the naturally elevated Hg content in their bedrock. Much of the Hg deposited on the land accumulates in moist, organically enriched soils, is washed out and eventually enters water bodies as runoff. The Hg transported via air and deposited among mountain ranges is exacerbated by increased precipitation and humidity (Martinková *et al.* 2019).

The main objective of this study was to compare Hg concentrations in the feathers of individuals to determine mercury concentrations. The impact of migrating behavior on the accumulation of Hg in different tissues was also investigated. The results were compared with Hg concentrations from other studies and with toxic reference values (TRV) to determine trends in the current contamination risk in the samples area within the Tatra Mountains of Slovakia.

Material and Methods

Sampling sites and sample collection

Between the 2000 and 2021, 32 deceased specimens of *E. rubecula* were collected. 14 samples were found in 7 different areas of the High Tatras and 18 were collected in 3 different areas of the Low Tatras. 16 of the latter were found deceased on the same day in southern Chopok. Two additional specimens were collected from an unknown locality. The birds were packed in individual sterile plastic bags and stored in a freezer until analysis was performed.

Morphological measurements were taken, including weight in a half-frozen state and length of tarsus, bill, wings, and tail. Heart and liver, as well as breast and thigh muscle tissues were dissected. Internal organs were examined for the presence of intestinal parasites. All tissues were dried in a laboratory incubator (IF 160 Plus) (Memmert, Germany) at 40 °C, with 30% air circulation, for 48 hours. After drying, samples were homogenized in a cryomill (Retsch, Germany).

Laboratory analyses

Mercury: the 1st and 3rd primary feather and the 4th and 5th tail feather (rectrices) from the left side were used. A piece weighing approximately 0.0010-0.0016 g was cut with the use of sterile steel scissors and scalpels. Exact weight determination of each sample (tissues and feathers) needed for mercury analysis was performed using an accuracy of 0.001 g on a Kern 770 balance scale (Kern and Sohn, Germany). Mercury concentrations in different sample types were measured with the direct mercury analyzer DMA-80 (DMA-80 Dual Cell, Milestone srl., Italy), according to the manufacturer's instructions.

Sex determination: For the determination of gender, 1 mm of the very tip and top of the calamus (quill) were cut off each feather of each individual with use of a sterile scalpel, and stored in an Eppendorf tube. DNA was then extracted from the cut-offs using commercially available DNeasy Blood & Tissue Kits (QIAGEN, Germany), according to the manufacturer's instructions. Amplification of a fragment of the CHD gene by PCR with use of P2 and P8 primers and GoTaq® Hot Start Polymerase (Promega, USA) was done (Griffiths *et al.* 1998). PCR was performed using a C1000 Touch ThermalCycler (Bio-Rad, USA). The PCR cycling conditions were as follows: 95 °C for 3 min, followed by 34 cycles of 95 °C for 30 s, an annealing temperature of 48 °C for 45 s and 72 °C for 45 s, then one final cycle of 72 °C for 5 min. Amplified products were visualized using electrophoresis for 40 mins at 7-10 V/cm in 2% agarose gel stained with EliDNA PS Green (Elisabeth Pharmacon).

Statistical analysis

All measured values were recorded in a data sheet in Microsoft© Excel 2016. Mean values (MV), as well as median and standard deviation (SD) were calculated using the commands "Stabw" (SD), "Median", and "Mittelwert" (MV) in the German version of Excel. Diagrams and statistical tests (analysis of variance, one- sided ANOVA, Tukey's test, Mann-Whitney U test) were generated using IBM© SPSS (version 29.0.0.0) with a significance level of $\alpha = 0.05$. Multivariate principal component analysis (PCA) for more easily accessible and comprehensive visualisation of complex data was performed using Past software (version 4.13).

Results

Sex determination revealed that all sampled *E. rubecula* specimens (n = 32) were indeed male. The concentration of mercury (Hg) was measured in the sampled rectrices (T) and primaries (W) of all 32 robins (Fig. 1). The median concentration in rectrices was 1.26 mg/kg, with 29 values under 4.0 mg/kg. The median concentration in primaries was 1.16 mg/kg, with all but three values under 3.0 mg/kg. The samples ER7 and ER23 were statistical extremes with Hg levels above the toxic reference values (TRV) of 5.0 mg/kg in both feather tissues. There was no statistical difference in the concentration levels between feather tissues.

The Hg concentrations in heart and lung tissues, as well as femoral and pectoral muscle tissue were obtained for 28 of the birds (Fig. 2). ER2, ER6, ER19, and ER24 were excluded from further analysis because Hg concentrations could not be measured in all tissues. No parasites were found in the organs or muscle tissues. Only one specimen had a measured concentration of higher than 5.0 mg/kg. Median concentration in liver tissue was 0.425 mg/kg, 0.326 mg/kg in heart tissue, and 0.198 mg/kg and 0.185 mg/kg in femoral and pectoral muscle tissues, respectively. Samples ER12, ER16, and ER32 were statistical extremes and had the highest levels of Hg in all four soft tissues. No differences in Hg concentration between tissue types were significantly different from on another.

A one-sided analysis of variance in all tissues examined suggested a significant difference in the Hg concentration values. Using the Tukey post-hoc test at a significance level of $\alpha = 0.05$, it was found that both feather tissues differed significantly from both muscle tissues in Hg concentrations. Furthermore, Hg levels in rectrices also differed significantly from those in heart tissues. High conformities ($\alpha > 0.9$) of Hg contents were found between muscle and heart tissues, as well as between heart and liver tissues.

To investigate whether the concentration of Hg in the sampled *E. rubecula* differed according to their spatial wintering behaviour, Mann-Whitney U tests were conducted for each tissue type (Fig. 4). Migratory individuals had higher mercury levels in all examined soft tissues than residential individuals. The biggest difference between the two groups was observed in heart tissues, with a significance of 0.06, followed by liver tissues and pectoral muscles, each with a significance of 0.09. Conversely, residential birds showed higher levels of Hg in both feather tissues. Both clear trends were not significant, but can be visualized using multivariate principal component analysis (PCA). It was, for example, evident that migrating birds had accumulated significantly more Hg in their livers (as in all soft tissues), while resident birds had accumulated a comparatively higher proportion of Hg in or on their primary feathers (as well as in/on their rectrices) (Fig. 3).

Discussion

Given that all sampled individuals were male, differences in Hg accumulation and excretion based on gender could be eliminated for this study. However, the literature is not clear on whether sex has an influence on Hg levels of bird tissues (Low *et al.* 2020), whether the observation of differences in Hg levels of different sexes is tissue-dependent, (Vizuete *et al.* 2022), or whether sex is related to Hg accumulation at all (Grúz *et al.* 2019). Our sampling of only male specimens may be attributed to

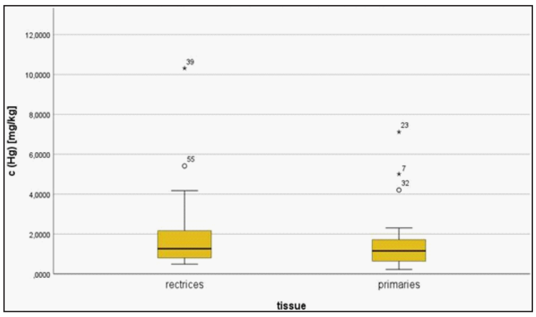

Fig. 1. The concentration of mercury (Hg) was measured with the DMA-80 in the barbs of the 1st and 3rd primary feather and 4th and 5th rectrices of n = 32 European robins (*E. rubecula*). The dotted line marks the threshold at 5.0 mg/kg, above which adverse effects of Hg have been reported in birds (Eisler 1987). Most concentrations were within this limit. Samples ER7 (7, 39), ER23 (23, 55), and ER32 (32) are statistical extremes in this diagram. The mercury concentration in rectrices and primaries was not statistically different but was slightly higher in rectrices than primaries.

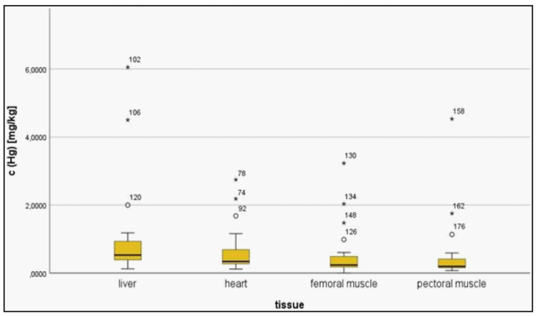

Fig. 2. The concentration of mercury (Hg) was measured with the DMA-80 in the tissues of liver, heart, femoral, and pectoral muscle of n = 28 European robins (*E. rubecula*). The dotted line marks the threshold at 5.0 mg/kg, above which adverse effects of Hg have been reported in birds (Eisler, 1987). All but one of the measured concentrations lay within this limit. The samples ER12 (74, 102, 130, 158), ER16 (78, 106, 134, 162), and ER32 (92, 120, 148, 176) were statistical extremes displaying the highest levels in all 4 tissues. None of the differences in mercury concentration per tissue were significantly different.

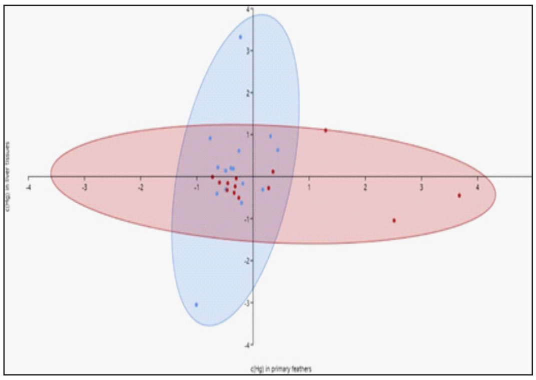

Fig. 3. Multivariate principal component analysis (PCA) of the mercury concentration in primaries and liver tissue of migratory (blue) and residential (red) male *E. rubecula*. The distribution clearly shows opposing trends of the two groups. Migrating birds show higher accumulation of Hg in the liver as resident specimens. The latter show higher accumulation of Hg in the primaries. Created with the software Past (version 4.03).

the territorial nature of the species, with females remaining near males during breeding season (Adriaensen and Dhondt 1990).

The relative amount of Hg was highest in feathers, followed by liver tissue. Concentrations in heart tissue were higher than in muscles, where levels were lowest. This order of accumulated Hg concentrations per tissue is consistent with the literature (e.g., Low *et al.* 2020; Tsipoura *et al.* 2008; Solonen and Lodenius 1990), though the highest concentration were found in rectrices instead of primaries (Furness *et al.* 1986). The levels of Hg contamination in 29 of 32 birds were < 2.40 mg/kg, and thus within tolerable limits (Grúz *et al.* 2019). Three birds had elevated levels, two of them above the threshold of 5.43 mg/kg in wing and tail feathers, which has been shown to impair reproductive performance (Eisler 1987; Grúz *et al.* 2019). These samples were all found in relatively urbanized areas in montane northern Slovakia than specimens with mercury levels well below the threshold. The amount of Hg measured in plumage may be locally impacted by external factors. As mentioned above, aquatic and mountain habitats are particularly vulnerable to pollution from Hg deposition (Rimmer *et al.* 2005). Ground-feeding insectivorous passerines such as the robin are particularly vulnerable to elevated Hg levels. The increased amount of Hg deposition in the mountains could also be reflected in increased external Hg levels on the feathers of individuals that exhibit year- round site fidelity. External and internal Hg content was not distinguished in the analytical method used. This may be accounted for based on differences in acute dietary intake, delayed accumulation in the feathers during and shorty after moult, and on the feathers due to atmospheric deposition.

Hg concentrations were usually highest in the rectrices, which usually moult first, and thus start to accumulate Hg before other feathers and may contain more Hg overall (Jenni and Winkler 2020). According to Bianchi *et al.* (2008), the moulting period in robins takes place in September. It is not clear whether feathers accumulate Hg from other body tissues during growth (Furness *et al.* 1986, Solonen and Lodenius 1990), although the opposite has been shown to occur (Whitney and Cristol 2017). This could reduce the Hg concentration in the body during and shortly after feather growth in autumn. The same phenomena apply to lower concentrations observed in feathers shortly after the typical moulting period ends in September, when feathers have not completely regrown, and have thus not accumulated their full load of Hg yet.

In organ tissue, the highest Hg concentrations were measured in ER12 and ER16, exceeding the values of comparable samples. Both individuals were found in April in southern Chopok. This area is in the Low Tatras. A total of 16 robins were found frozen to death on the same day in mid-April 2013. It is likely that this group was on its migration route. They likely wintered in more southern locales and were returning to their breeding grounds, in northern Slovakia. Male robins tend to overwinter near nesting sites and these birds are rare finds of scientific interest (Janiga 2021). Significantly higher levels of Hg were detected in soft tissues of the migratory group. Orally accumulated Hg takes about 2-3 months to degrade and be excreted (Rimmer *et al.* 2005). It can, therefore, be assumed that they ingested an increased amount of Hg in their diet in their wintering habitat.

Mercury may be excreted into the environment by birds through excrement, glandular secretions, eggshells, and moulting (Bianchi *et al.* 2008; Costa *et al.* 2011; Grúz 2019). While Hg remains stable in feathers even after moult, Hg can enter the environment via these other pathways (Low *et al.* 2020). Although these amounts are small in contrast to the amounts released by human activity, the excretions of migratory birds represent an additional Hg load in nesting areas.

Statistically significant differences in Hg concentration in the sampled male robins were most clearly observed between those in feathers and those in muscle tissues. This distribution is consistent with migratory behavior and what is known about deposition patterns in mountainous regions to date. Individuals that migrate to areas with elevated Hg content (e.g., in food) during winter, transport additional Hg to the nesting site when they return during breeding season. This increases exposure and risk of poisoning for adults, hatchlings, and higher trophic levels of the food web. Furthermore, mercury concentrations increased rather than decreased during the sampling period from 2000 until 2021. Certainly, climate change will have further implications on the distribution of heavy metals such as mercury, and additional, more detailed studies on avifauna as bioindicators could begin to show us the extent of its influence. With this additional information, appropriate responses could be identified and implemented at an early stage.

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The use of principal component analysis to identify factors affecting mercury concentrations in *Apodemus flavicollis* and *Apodemus sylvaticus*

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Abstract. This study deals with the extent to which a spring and autumn moult and the type of seasonally preferred food can affect mercury concentrations in the body of mice. Samples of 5 types of tissues (blood, hair, liver, brain, kidney) were obtained from 102 dead mice of the genus *Apodemus*. Data on the concentration of total Hg in the sample were obtained by a DMA-80 analyzer. PCA revealed 5 factors involved in influencing concentration of Hg in tissues. The origin of these factors and potential explanations for phenomena that are paradoxical are discussed. Comparing the seasonality and effect of the factor that impacts the concentration of Hg in the blood revealed an increase in mercury contamination level in a season where animals experience lower food intake. Exogenous deposition from the environment likely enriches inert hair tissue with mercury following both the spring and autumn moult.

Key words: Apodemus flavicollis, *Apodemus sylvaticus*, mercury, Principal Component Analysis, exogenous deposition, food

Introduction

Mercury (Hg) is not a biogenic element and is highly toxic to organisms in all its forms and compounds. The presence of the contaminant in the environment, particularly in the soil, however, does not necessarily mean that it is available for plant bodies, and the total concentration of the contaminant in the soil is not the same as that available for plants (Rogival *et al.* 2007). Available forms of Hg bind to organic components in the upper horizons of the soil. The overall availability of soil Hg for plants is low, and the concentration of this element in plant organs rarely exceeds the concentration of the surrounding environment. Hg accumulates in the roots, which effectively stabilize its movement, and thus its transport into above-ground parts is prevented (Patra and Sharma 2000). Fungi, unlike plants, can accumulate Hg to a greater extent in the fruiting bodies, especially in the hymenium on the underside of the cap (Kavčič *et al.* 2019). Another crucial factor that participates in determination of the overall concentration of Hg in the plant is foliar uptake directly from the atmosphere (Jiskra *et al.* 2018). The plant absorbs elemental mercury through its leaves along with other gases, and it is photo reduced to another form, which is more persistent in plant organs and is not so easily degradable. During the autumn, Hg deposited in fallen leaves gets into the leaf litter, where it undergoes mineralization and enters the humus layer of soil (Jiskra *et al.* 2018).

In animal bodies, mercury (especially organic form methylmercury) tends to persist in tissues after consumption of contaminated food, caused by biomagnification in the food chain. This phenomenon is well observed in marine (Cardellicchio *et al.* 2002), as well as in terrestrial (Komov *et al.* 2017) ecosystems. Predators at the top of the food chain are exposed to elevated levels of mercury contamination (Mierle *et al.* 2000). Small rodents, which are a source of food for predators, can be an intermediate step transferring contaminants to higher positions in the trophic chain (Gerstenberger *et al.* 2006).

Among tissues, most Hg is accumulated in skin derivatives, such as mammalian hair (Mierle *et al.* 2000, Evans *et al.* 2016) and bird feathers (Dietz *et al.* 2009). Up to 70% of total body burden of Hg can be concentrated in hair (Bearhop *et al.* 2000) and there is up to 250 times more contaminant in hair than in blood (Dietz *et al.* 2009). As animals have a relatively larger surface area given their small dimensions, their fur volume is also proportionally larger compared to body weight. Hart (1956) discusses the effectiveness and limitation of thermoregulatory properties of fur in small and large animals, but little is known about detoxication potential or the possibility of removing contaminants contained in blood. If a mammal is capable of displacing a contaminant from their blood into the newly growing coat, growing of the greater mass of the new coat in small animals may lead to more efficient detoxification than in large animals, whose coat accounts for a smaller proportion of total body weight. Excretion of Hg into feathers and the subsequent decline of concentrations in organs and blood as a result of feather growth has been described in many bird species (e.g. Stewart *et al.* 1994, Caldwell *et al.* 1999, Bearhop *et al.* 2000, Condon and Cristol 2009, Kopec *et al.* 2018, Renedo *et al.* 2018, Janiga and Haas 2019, Albert *et al.* 2021). An effective detoxication mechanism in birds can displace 70-93% of MeHg from the total body load into feathers (Rimmer *et al.* 2005). The assumption is that this efficiency is due to the large relative weight of feathers in relation to total body weight. However, knowledge of a similar detoxification capability in mammals is limited.

Binding of mercury to keratin in skin derivatives can be an effective adaptation or detoxification mechanism, in which the contaminant is deposited in inert tissues, and thus, more sensitive organs are protected. In birds, Hg is isolated from other tissues and binds to disulphide linkages in feather keratin (Dietz *et al.* 2009). The circulation of Hg in blood and its translocation to tissues is more complex, as birds actively excrete Hg into the feathers during moulting, and then shed those feathers. Once Hg is stored in the feather keratin, further translocation to other parts of the body are no longer possible, as the feather is a metabolically inactive tissue that loses contact with circulation through keratinization (Renedo *et al.* 2018). However, moulting in birds takes place differently from in mammals, as this exchange of feathers does not take place during regular intervals throughout the year, but occurs at different intensities throughout. During periods when feather replacement does not take place, the concentration of Hg in blood rises because excretion is suspended. Thus, during the intermoult period, the only alternative for avian metabolism of Hg is storage in other organ tissues.

Mammals, unlike most birds, have an advantage in that they shed their fur twice per year. New hairs are created next to ones waiting to be shed. Right after the new hair is formed, the hair follicle becomes inactive and is not activated until the next moult (Johnson 1972). The finished hair is a structure of inert cells, thus, it cannot be repaired after damage, and a complete replacement is the only option (Beltran *et al.* 2018). In terms of the annual cycle, the greatest attention is paid to the seasonal changes of summer coat to winter coat (autumn moulting) and winter coat to summer coat (spring moulting).

Material and Methods

Site characteristics and sample collection

Mice samples were collected at the site in the cadastral area of the municipalities of Považská Bystrica, part Považská Teplá, and Plevník-Drienové (N49.15365° E18.47638°, and altitude range of 314-425 m a.s.l.). The vegetation cover in the area consists largely of mixed temperate forests in various succession stages. Extensive small-area timber logging takes place at the locality.

At the site, high concentrations of mercury were not expected. The low-polluted location allows the investigation of the processes caused only by naturally changing seasonal conditions without the influence of anthropogenic pollution.

Concentration data was processed during this examination of seasonal changes in mercury concentrations and the influence of morphometrics. Animals were trapped throughout the year between December 2020 and January 2022. It was not initially intended to analyze internal organs, as the purpose of the study was to collect samples of hair and blood from living animals and release them after sampling. Animals were live-trapped using baited Sherman traps. However, despite the effort to minimalize animal mortality by providing thermal insulation and checking traps as soon as possible, some animals were found deceased in the traps. The carcasses were stored in a freezing box at -20 °C for necropsies. Among all trapped animals, complete data on all five organs (blood, hair, liver, brain, kidney) was obtained from 102 dead mice. These animals are reported herein. No animals were intentionally killed, most of this deceased cohort died overnight in traps or passed unexpectedly during blood collection while narcotized by Isoflurane. Where possible, sex, age (juvenile/subadult/adult), and morphometric data were obtained as well.

Sample preparation and laboratory analysis

Samples were analyzed in a dry state. Blood was dried at room temperature as a drop on Petri dish. Hair was analyzed without any pre-treatment and was not washed. Dissected organs were dried for 24 hours in a laboratory Incubator IF 160 Plus (Memmert, Germany) at 50 °C. The FAN was set to 20%. The weight of each sample was determined by a KERN 770 balance (KERN, Germany). Concentration of Total Hg was detected by 2-cell DMA-80 (Milestone, Italy) with nickel boats. The temperature settings were as followed: temperature for combustion 650 °C, for catalyst 615 °C, cuvette temperature 125 °C. The Certified standard reference material Beef liver NCS ZC 7001 (CHNACIS, China) was used to ensure the accuracy of the measurement.

Sample preparation and laboratory analysis

Multivariate Principal component analysis was used to identify factors influencing relationships between variables, (i.e., Hg concentrations in blood, liver, brain, kidney and hair). The total variance of five principal components (PC) was calculated. Factor coordinates of cases were then used as a new variable. Subsequently, the correlation between morphometric parameters (weight and body length) and factor coordinates was assessed.

The difference in factor coordinates between males and females and between age categories was also investigated. Two age categories were distinguished, adults and immatures (including all juveniles and subadults). Normal distribution was evaluated by the Shapiro-Wilk test. Because the groups did not have a normal distribution, comparisons between two independent groups were made using a non-parametric test (the Mann-Whitney U test).

Based on seasonality, 6 seasonal categories were created (February, March - early spring; April, May, June – spring/summer; July, August - summer; September, October - autumn; November - late autumn; December, January - winter). Before comparing the effect of seasonality, homogeneity of variances was evaluated by Levene's test. When variances did not differ, ANOVA was applied to assess the seasonal differences. In cases of significant variance of Levene's test (different variances), an alternative Welch F test for unequal variances was used. Different couples were detected by Tukey HSD (honestly significant difference) test.

The threshold for significance of correlations and for two/multiple sample tests was $p \leq 0.05$. The Statistica 7.0 software was applied to calculate the factor coordinates of variables and cases and to detect correlations. Other analyses (Shapiro-Wilk normality test, Levene's test for homogeneity of variances, ANOVA and Welch F test, Mann-Whitney U test, Tukey HSD test) were calculated by PAST4.03.

Results

Full data of mercury (Hg) concentrations in blood, hair, liver, brain and kidney were obtained from 102 mice, and all adults and immatures were included together. These data were used to determine the effect of principal components that influence the concentration of Hg. The coordinates of the principal components based on correlations are presented in Table 1.

PC 1 is a unilinear vector, affecting mercury intoxication of all organs investigated, but acting on individual organs with different intensities. It is least pronounced in the blood and most pronounced in the kidney and liver. This means that if the Hg concentration in the blood increases 3.486-fold under the influence of this factor, the Hg concentration in the kidneys increases 8.515-fold at the same time, and in the liver it rises to 8.962-fold. PC 1 contributes the most to the total variance, resulting from the synchronous and antagonistic effects of all five factors, and represents 45.1% of the total variance.

PC 2 acts independently of the first factor and determines the toxification level in blood. It acts on the blood and liver in a negative direction, and on hair, brain and kidneys in a positive direction. This means that the factor causes an increase in blood and liver concentrations while decreasing brain, kidney and hair concentrations, and vice versa; if blood and liver concentrations decrease, brain, hair and kidney concentrations increase. It accounts for 19.2% of the total variability.

PC 3 affects hair, which is the only variable on which it acts in a negative direction.

PC 4 reflects Hg deposition in the brain, without significant involvement of other organs.

PC 5 acts with nearly equal intensity on the liver and kidneys, but antagonistically.

The effect of morphometric variables, sex, age, and season on the principal components

The effect of morphometric variables, including sex, age, and season on the five strongest principal components was tested. Results (p-values) are presented in Table 2.

The absence of a significant correlation between the coordinates of all five factors and body weight data indicates that one of the factors influencing mercury contamination in mice is not body weight. However, there was a significant negative correlation ($r = -0.40$) between body length and the coordinates of PC 3. This means that PC 3 causes higher concentrations in the brain and lower concentrations in hair in smaller mice and causes lower concentrations in the brain and higher concentrations in hair in larger mice.

An interaction between the PCA factors and sex was not demonstrated, nor was the effect of age. No differences between sexes or age groups were detected.

Variable	**PC 1**	**PC 2**	**PC 3**	**PC 4**	**PC 5**
Blood	-0.3486	-0.8903	0.0508	0.2852	-0.0443
Hair	-0.5346	0.1403	-0.7927	0.2570	0.0091
Liver	-0.8962	-0.0173	0.1453	-0.2446	0.3400
Brain	-0.5653	0.3830	0.4097	0.6021	-0.0587
Kidney	-0.8515	0.0403	0.0520	-0.4204	-0.3065
% Total variance	45.1	19.2	16.5	14.9	4.3

Table 1. Factor coordinates of the variables, based on correlations, and percent of variance associated with the components.

Variable	**PC 1**	**PC 2**	**PC 3**	**PC 4**	**PC 5**	**test**
Weight	0.7429	0.5188	0.2599	0.8389	0.7225	Correlation
Body length	0.1461	0.6491	0.0238	0.9283	0.3421	Correlation
Sex	0.8413	0.5434	0.4392	0.3842	0.5073	M-W
Age	0.3406	0.9594	0,5426	0.9157	0.1345	M-W
Season	0.7743	0.0104*	0.3508	0.0128*	0.0464	ANOVA

Table 2. p-values of relationships between variables and coordinates of cases on factors. * refers to the use of Welch F test in cases of unequal variances. Values in bold refer to significant correlations/differences.

PC 1 coordinates of samples showed no seasonal variation (Fig. 1), and no significantly different seasonal pairs were detected.

Comparison of coordinates of PC 2 across seasonal periods (Fig. 2) indicated that animals are generally intoxicated by mercury in equal amounts throughout the year, except for summer and late autumn. The high PC 2 values of coordinates of samples in the summer contribute to low blood Hg intoxication values in the summer, and the low factor coordinate values in the late autumn result in elevated blood Hg concentrations during this period. High values of factor coordinates of cases during summer were significantly different from the values obtained for late autumn.

The seasonal pattern of the effect of PC 3 (Fig. 3) shows high values of coordinates in autumn and early spring, resulting in low values of Hg contamination in hair. In both following seasons (spring/summer, late autumn) there is a sharp decline, which means that contamination levels in hair are increasing.

PC 4 acts primarily on Hg concentrations in the brain in a positive direction and in the kidneys in a negative direction. Low values for these factors in early spring and spring/summer periods (Fig. 4) account for low levels of brain Hg intoxication during this period. In summer there is a sharp increase in concentration, caused by PC 4. In the other periods there is a slight decrease.

PC 5 causes elevated levels of contamination in kidneys and low concentrations in the liver during early spring and causes high levels of contamination in the liver and low levels of mercury accumulation in the kidneys during other seasons (Fig. 5).

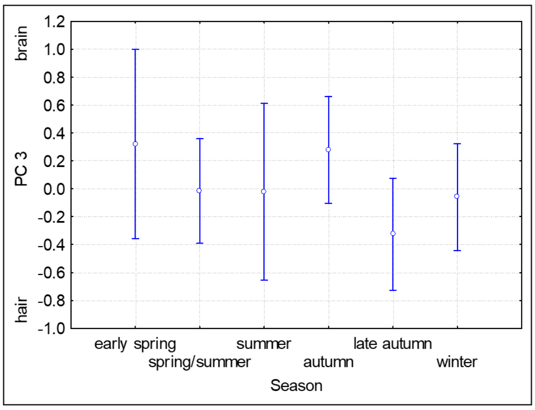

Fig. 3. Factor coordinates of PC 3 in different seasons. Vertical bars denote 95% confidence intervals. There are no statistically different couples (Tukey HSD test).

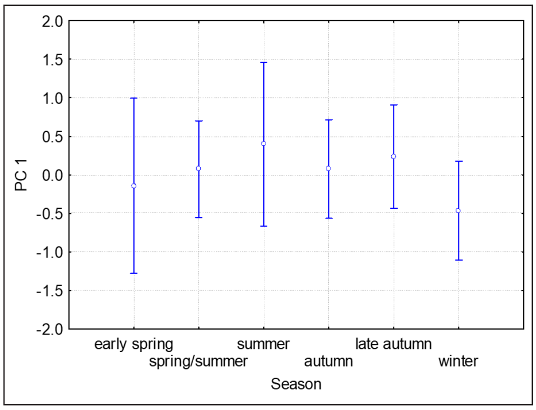

Fig. 1. Factor coordinates of PC 1 in different seasons. Vertical bars denote 95% confidence intervals. There are no statistically different couples (Tukey HSD test).

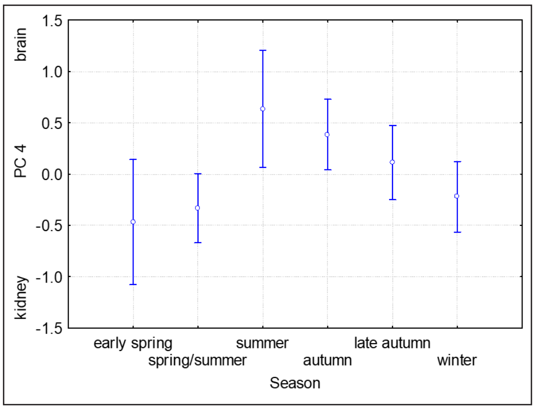

Fig. 4. Factor coordinates of PC 4 in different seasons. Vertical bars denote 95% confidence intervals. Statistically different couple: spring/summer-autumn (Tukey HSD test). See also Table 2.

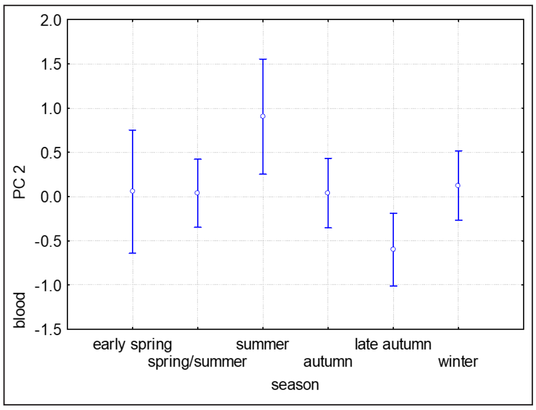

Fig. 2. Factor coordinates of samples (PC 2) in different seasons. Vertical bars denote 95% confidence intervals. Statistically different couple: summer-late autumn (Tukey HSD test). See also Table 2.

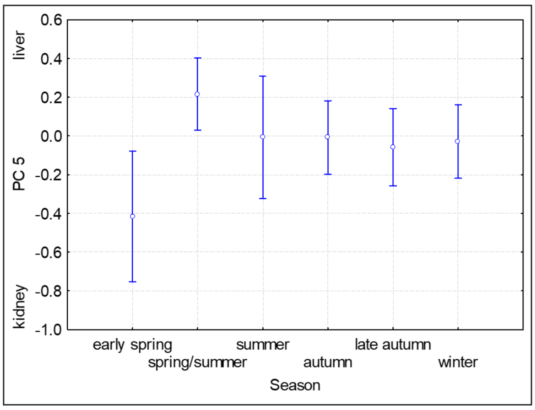

Fig. 5. Factor coordinates of PC 5 in different seasons. Vertical bars denote 95% confidence intervals. Statistically different couple: early spring-spring/summer (Tukey HSD test).

Discussion

Principal component 1

PC 1 acts on all variables in a negative direction. No significant correlation with morphometrics or differences between sex, age or seasonal groups were detected. Therefore, this component cannot be explained exogenously or endogenously (age, sex). This factor affects Hg concentrations in the liver and kidneys, organs where Hg and other contaminants are commonly deposited (Dainowski *et al.* 2015, Antonova *et al.* 2017).

Principal component 2

PC 2 indicates absorption of mercury into blood, regardless of age or sex. The seasonal pattern of this negatively acting factor indicates the lowest values of factor coordinates in late autumn. When comparing the seasonal changes in blood Hg concentrations, it is evident that the highest values of Hg in blood were measured during spring and autumn (Fig. 6). The opposite trend is observed in the summer – Hg values in blood were the lowest. This factor mostly explains low Hg levels in blood during summer months and increase in autumn. However, the body reacts to this sudden influx, because in winter, the amount of Hg in blood decreases, and the high variability observed in autumn months also decreases.

Hg concentrations in blood reflect a short-term state (Yates *et al.* 2014), and unstable and fluctuating levels of Hg in blood are influenced by recent dietary Hg uptake. Because of this, PC 2 may be related to food consumption. Wood mice and yellow-necked mice are considered omnivores. They have a diverse diet with a prevalence of seeds, if their habitat and the season allow (Watts 1968, Green 1979, Montgomery and Montgomery 1990, Zubaid and Gorman 1991, Gorman *et al.* 1993, Rogers and Gorman 1995, Abt and Bock 1998). However, when living in a habitat with a lack seed in production, they can compensate for this deficiency by consuming other plant food (Rogers and Gorman 1995) or animal food in higher quantities (Zubaid and Gorman 1991, Gorman *et al.* 1993). Depending on seasonal availability, mice can adapt to a temporary deficiency of their main food source in their usual woodland habitat. Tree seeds are predominant in the diet of *Apodemus* mice in the autumn and early winter, due to seed abundance and availability. Fewer seeds were found in the stomach contents of mice during spring and early summer. At this time of year, seed resources from the previous autumn begin to be run out, and vegetation has not yet produced additional seeds (Montgomery and Montgomery 1990). Thus when mice lack this main food component, they seek an alternative and consume more animal food. A diet of animal origin is more prevalent in spring and early summer, and is predominated by larvae, and adult insects (Montgomery and Montgomery 1990) such as beetles. Diptera and Lepidoptera, predominate, followed by less abundant centipedes and sporadically molluscs. Mice may also scavenge vertebrate flesh. In the spring, when tree dwelling caterpillars leave the trees to pupate on the ground, those larvae can serve as a reliable source of food for mice (Watts 1968).

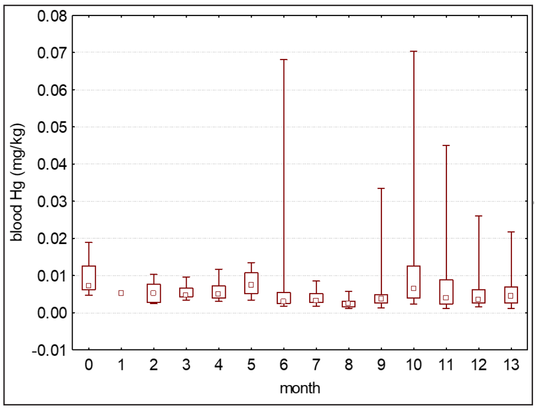

Fig. 6. Concentrations of Hg in mouse blood within season. 0th month is December 2020, 1-12 are January-December 2021 and 13th is January 2022 (Square: Median; Box: 25%-75% Percentiles; Whisker: Min-Max).

Because of the biomagnification of mercury in the food chain, we would expect this high consumption of meat to yield high Hg levels in blood during spring and summer. Therefore, the abundance of a preferred food source (tree seeds) in autumn assumes a decrease of animal food intake and a decrease in blood Hg levels. However, the results we observed yielded the opposite. The mice contain the lowest concentrations of mercury in summer.

One explanation is that more contaminated food is consumed in autumn due to higher moisture and humidity. It is generally accepted that in aquatic environments, animal bodies are more burdened by Hg, because the aquatic environment is more capable of methylation of inorganic Hg (Jitaru and Adams 2004), resulting in persistence of MeHg in animal bodies. In semiaquatic habitats such as floodplains or marshes (Peterson *et al.* 2021), higher Hg levels were found in meso-predator animals whose home range contains wetter areas. Humidity and moisture can increase Hg levels in animal tissues spatially and temporally. Higher concentrations of Hg were found in snow vole (*Chionomys nivalis*) tails during months with more precipitation (Martinková *et al.* 2019), however, in alpine ecosystems, where snow voles were collected, spring was a wetter period than autumn. The climate of the temperate mid-altitude region of Považská Bystrica (including the sampling site) is characterized by hot and dry summers (own observation), which could theoretically explain low summer Hg values.

Another explanation is mycophagy. Unlike plants, which do not accumulate Hg from the soil in high concentrations (Tomiyasu *et al.* 2005), fungi are able to accumulate Hg from the soil at several times higher concentrations than is in the soil (Alonso *et al.* 2000, Falandysz *et al.* 2003). According to Watts (1968), *Apodemus* mice eat the most fungi in the summer (15%) and November (7%). According to Hansson (1971), mosses and fungi were evenly distributed in mouse food throughout the year. The published studies do not refer to an increased fungi consumption in the autumn season, so mycophagy does not seem to be a sufficient explanation for increased Hg concentrations during the autumn months. But the abundance of mushrooms can be site-specific, and fruiting bodies of edible mushrooms were abundant in some trapping places during autumn 2021 (own observations).

A third explanation is the impact of proteins from an animal-based diet. It was found that laboratory mice fed by a low-protein diet had more Hg in their organs than mice in the control group, fed by an average-protein diet. Additionally, the first group of mice excreted less Hg through urine, though faecal excretion was the same in both groups (Adachi *et al.* 1992). This finding indicates that quantity of protein in diet may have an important effect on animal health and Hg storage in the body and excretion. Animal food can therefore cause two antagonistic effects, higher accumulation of contaminants due to biomagnification, as well as increased excretion from the body.

Finally, seasonal changes in gut microbiota may have an influence on Hg concentrations. Intestinal microbes are an effective barrier preventing MeHg from entering the bloodstream from the intestine (Lapanje *et al.* 2008) as MeHg demethylation has a decisive effect on Hg excretion rates (Rowland *et al.* 1984). As mice can temporarily adapt to different available food sources, there can be observable shifts in gut microbial communities (Maurice *et al.* 2015), depending on type of currently favorable food (plant/animal). It was found that cultures of Lactobacillus isolated from rat intestine can degrade MeHg into volatile Hg0 (Li *et al.* 2019), which is absorbed by the gastrointestinal tract in negligible quantities (Gochfeld 2003). Lactobacillus microbes are more prevalent in microbiota of *A. sylvaticus* during spring but are less prevalent in autumn (Maurice *et al.* 2015). The presence of demethylating intestine microbes in spring can theoretically decrease the accumulation of Hg originating from animal food. A question that emerges, is whether the presence of demethylating microbes is an evolutionary adaptation against higher Hg contamination originating from more contaminated food sources. However, the trade-off does not allow the same defensive mechanism during seasons when less contaminated food items are abundant and consumed. As such, Hg from less contaminated food accumulates in a season when the body is less protected by demethylating microbes.

Principal component 3

PC 3 reflected high contamination levels in hair during late autumn. The antagonistic relationship between PC3 and the amount of mercury in hair and other organs (especially the brain), implies a decrease of mercury in soft tissues during the period of increase in hair. This may imply synchronisation of mercury displacement from organs during seasonal coat exchange.

Given that mammalian hair is an inert tissue (Beltran *et al.* 2018) there should be no changes in the amount of Hg present in hair during the existence of the whole coat, and a possible change would only occur if the whole coat is replaced by a new one (i.e., shedding). However, during the season, there were slight changes observed (Fig. 7). In winter, and at the onset of spring, Hg concentration values were low, but between the end of spring and throughout summer, a gradual increase in concentration was observed. In October, there was a decrease followed by a subsequent increase.

Peterson *et al.* (2021) noted the opposite situation with decreasing Hg concentrations in hair of animals that were captured twice during the season between winter and early summer. This finding is explained by the abrasion of the guard hairs when the distal part of the hair is lost. In this area of hair, Hg is most concentrated, and its concentration decreases toward the base of the hair. There are two hypotheses that explain why most the concentrated amount of Hg is found in the distal end of the hair. One of them is endogenous theory. During hair growth, the distal end of the hair forms first, when the contaminant in blood is in the highest concentration, so that the largest proportion of the contaminant is concentrated in the newly formed hair. As hair continues to grow, the concentration of Hg in the blood decreases as it is stored and deposited into the emerging keratin structures. Therefore, the basal section no longer contains the high concentrations that were present at the initiation of hair growth (Peterson *et al.* 2021). Sobańska (2005) explains this through an exogenous theory, and claims that the distal part of the hair is in the contact with the environment for a longer time period, and thus absorbs the most Hg from the environment.

The observed increase in Hg concentration during summer and late autumn supports both the endogenous and exogenous hypotheses of hair Hg deposition. The newly formed coat contains only endogenously deposited mercury, metabolically degraded from the blood. High autumn Hg levels in hair are to some extent caused by high blood Hg levels during this season (see PC2), and after the direct contact with the environment, hair is enriched with exogenous mercury.

PC 3 is more influential during early summer and late autumn, in moulting and post-moulting periods, whereas later, its effect is hindered by

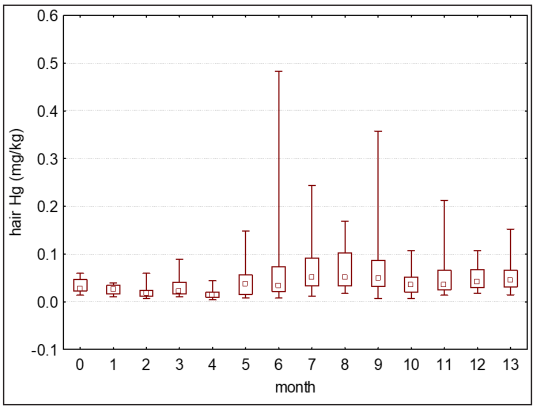

Fig. 7. Concentrations of Hg in mouse hair within season. 0th month is December 2020, 1-12 are January-December 2021 and 13th is January 2022 (Square: Median; Box: 25%-75% Percentiles; Whisker: Min-Max).

the carrying capacity of hair. Small microparticles can accumulate by adhesion or under keratin scales in hair, but this increase in concentration is limited. Additionally, there is the possibility of hair abrasion (Peterson *et al.* 2021), whereby hair sheds some of the concentrated exogenous and endogenous Hg, and the increase in Hg concentration caused by deposition from the environment is slowed. If an abrasion is present, it may represent the participation of both antagonistic factors (exogenous deposition and abrasion), which were not recognized by PCA. The PCA likely combined them into a single factor. Deposition from the environment has a stronger effect in the initial phases of fur duration, and abrasion, occurring later in the cycle, mitigates the effect of exogenous enrichment. Continual decrease in hair Hg concentrations can indicate that abrasion has a stronger effect than exogenous deposition during the months of this decline. An increase in factor coordinates between late autumn and summer and the repetition of this trend during the other half of the year indicates initial rapid toxification of hair and a subsequent slow degradation. In the pre-moulting and moulting seasons, the influence of exogenous deposition is minimized by the exhausted carrying capacity of hair to accumulate exogenous Hg and by subsequent fur exchange cycle.

The hair in this experiment was not washed, while Peterson *et al.* (2021) cleaned sample hair. Thus, there is no clear and precise data on exact concentrations found directly in the hair, though the seasonal pattern provides information about the concentration ratio among months. Sobańska (2005) compares concentrations in washed and unwashed wild boar hair, where washed hair was cleaned using deionized this water. A very strong correlation ($r = 0.99$; $p < 0.05$) reflects the effectiveness of this method, but does not show the delta in how much Hg is deposited from the environment and withstands cleaning. Hair sampling throughout the season did not show significant differences in the concentration of washed and unwashed hair of a wild boar kept in the zoo, although there was a slight indication of changes.

The theory of exogenous deposition of Hg in hair also corresponds to the findings of Gerstenberger *et al.* (2006). Their research was not focused on seasonality, but on a comparison of rodent species living in desert habitats. It has been found that rodents dwelling in burrows have higher Hg concentrations in their hair than species living in above-ground niches, even in the case of animals that have been captured in the same or similar habitat. Liver concentrations were not impacted by whether the animal lived in a burrow or on the surface. *A. flavicollis* and *A. sylvaticus* dig their own burrows, or occupy burrows created by moles (own observation). Gerstenberger *et al.* (2006) also argued that leaf litter prevents direct contact with soil. On the other hand, plant tissues, especially roots and leaves, may also contain trace amounts of Hg. However, their origin is different. Little Hg enters the plant body through the root system. Concentrations in the roots do not reach the same concentrations as in the surrounding soil (Tomiyasu *et al.* 2005), and Hg travels into the above-ground organs of plants in even smaller quantities, as the root system stabilizes absorbed Hg from soil. However, it is also possible to find Hg in the leaves. The main source of Hg in the leaves is either soil vapor (Patra and Sharma 2000) or atmospheric Hg transported from a remote source (Jiskra *et al.* 2018) that enters the humus layer and the soil by litterfall. As low concentrations in all animal tissues indicate an unpolluted site, significant deposition from the atmosphere is not expected, so soil is likely the main source of Hg at the trapping site. Therefore, the hypothesis that soil contact may also affect and increase Hg concentrations in mammalian hair, and be, in addition to bioaccumulation in the food chain, another decisive factor influencing Hg concentration, seems to be based on truth. It is therefore likely that high initial concentration in the newly formed coat, mainly collected endogenously, may be additionally enriched with exogenous mercury from the soil, and thus, the total amount of Hg increases as the fur persists. Small microparticles of soil or dust can accumulate under cuticular hair scales, representing the significant effect of exogenous deposition, which supplements more stable endogenous deposition.

There was also a negative correlation of the factor with body length (Table 2). This negative correlation might result in more Hg in the brain during autumn and early spring in shorter animals, and simultaneously in more Hg in hair during spring/summer and late autumn in longer animals.

Principal component 4

PC 4 acts primarily on the brain in a positive direction. When comparing the seasonal effects of the component, the coordinates of samples of the factor reach the highest numbers in the summer, then decrease in every other season until they reach a minimum during the early spring. Therefore PC4 has the strongest effect in the summer, followed by a lower effect in autumn and late autumn. This factor is the main cause of unexpected high Hg concentration values in the brain during July and August (Fig. 8).

An explanation may be that Hg in the brain originates from inorganic elementary mercury found

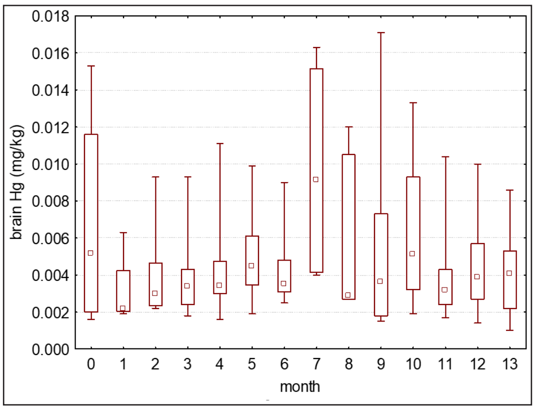

Fig. 8. Concentrations of Hg in mouse brain within season. 0th month is December 2020, 1-12 are January-December 2021 and 13th is January 2022 (Square: Median; Box: 25%-75% Percentiles; Whisker: Min-Max).

in soil. Hg in its elementary form enters the body primarily through inhalation (Gochfeld 2003). It undergoes oxidation in the lungs, but some fraction remains unoxidized and enters the brain through the blood-brain barrier (Warfvinge *et al.* 1992, Chételat *et al.* 2020). Physiologically, increased brain Hg accumulation after exposure to elementary Hg is related to its solubility in lipids, and subsequent oxidation in the brain into its ionic form, Hg^{2+} (Aschner and Aschner 1990). It is likely that elevated temperatures and low rainfall in summer (own observation) caused increased soil dustiness. As burrow-dwelling animals, mice are in direct contact with the soil, which may have increased inhalation of dust containing mercury in its elementary form.

Principal component 5

PC 5 affects primarily the liver and kidneys, organs where mercury accumulates to the greatest extent. In contrast to PC 1, the strongest acting factor, this factor has an antagonistic effect on both organs. Both organs play a role in detoxification, but there are differences in the form of accumulated mercury present in each organ. While kidneys are the target organ for inorganic Hg deposition (Pokorny and Ribarič-Lasnik 2002), the liver mainly exhibits concentrations of the organic methylated form (Kalisinska *et al.* 2021). Therefore, the detoxication process in both organs is likely not co-dependent.

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TECHNIQUES AND STRATEGIES

A case study on the use of multivariate techniques in phytogeographic analysis of bryophytes in the Veľká Fatra Mountains

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Abstract. Classical phytogeographic analysis focuses on the analysis of areal types, floristic elements, vertical distribution of species, ecology, and threats. The focus of this study is the use of alternative approaches in phytogeographic analysis. The results of multidimensional analysis proved to be effective, revealing previously undiscovered relationships. For example, a common feature of species with Holarctic to North American distribution is the predominance of the (sub)boreal areal type, while a common feature of species with the European to African areal type is the predominance of the (sub)oceanic areal type.

Key words: phytogeographic analysis, bryophytes, distribution, areal types, multivariate analysis, Veľká Fatra Mountains

Introduction

460 taxa of bryophytes (99 liverworts and 361 mosses) were counted within Veľká Fatra National Park (Slovakia). They inhabit a wide range of habitats, including rocks, forests and scrubs, bare soil, tree bark, stumps, roots, rotting wood, meadows, pastures, fens, raised bogs, waterways, and other anthropogenic biotopes. Some species are characterized by a wide range of vertical distribution while others are bound to a narrower range of altitudes.

Phytogeography as a scientific discipline deals with the principles of the spread of vegetation, and is dedicated to the study of plant environments and their relationship to a habitat. It takes into account historical, climatic and pedological conditions, contributes to the protection of a specific location, identifies its characteristics, reveals vulnerable elements and the direction of conservation interventions. Phytogeography records the presence of areal types, floristical elements, and the occurrence of dealpine or thermophilic species.

The goal of the presented study is the use of multidimensional techniques that have not been used in phytogeographical analysis to-date. The study illustrates the possibility of an alternative use of data on horizontal distribution of bryophyte species for the study of their relationship to the habitat and also their preference for areal types. Multidimensional techniques offer the possibility of using other predictors for the study of the relationship between the plant environment and the habitat, (e.g., pedological, climatic, phytocenological and other parameters).

Material and Methods

Phytogeographical analysis is based on the works of Düll (1994a; 1994b) and Düll and Meinunger (1989). For the purpose of vertical differentiation, processed species were included in altitude levels: planar (average altitude 150 m a.s.l.), colline (average altitude 225 m a.s.l.), submontane (average altitude 750 m a.s.l.), montane (average altitude 1200 m a.s.l.), subalpine (average altitude 1650 m a.s.l.), alpine (average altitude 2000 m a.s.l.). These parameters are guided by literary data and previous records. In the case of occurrence of species in several vegetation stages, the average altitude was decisive for classifying the species in the vertical level. In the case of the alpine vegetation stage, this region included species that also descend to lower altitudes, but are optimum in the alpine stage, e.g., *Orthotrichum alpestre, Hylocomiastrum pyrenaicum, Tortula hoppeana* and others.

When tested by the detrended method, the length of the first gradient in the log report was 2.38, and we have available predictors that represent data on the horizontal distribution of bryophyte species and also their preference for types of areas. Taking into account the above, the linear method (RDA) (Lepš and Šmilauer 1999; Ter Braak and Šmilauer 2002) was used for ordination analysis. As distribution of data did not meet the criteria of a normal distribution (Gauss probability distribution), we subjected the data to a logarithmic transformation. Processing marginal vegetation stages, the vertical range is considerably reduced, therefore we used indirect unimodal correspondence analysis (CA).

In order to clarify the correlation structure, we used factor analysis (Kubíková *et al.* 2013), the result of the factor analysis are groups of variables (factor loadings) that explain the correlation structure. The advantage of this multivariate statistical method is the possibility of factor coordinate rotation, so that a distinct correlation structure is created.

The nomenclature of mosses follows Mišíková *et al.* (2020), while liverwort follows Mišíková *et al.* (2021).

Results and Discussion

97 species of liverworts and 348 species of mosses recorded in Veľká Fatra were processed.

I. Ordination analysis

We used the direct linear method (RDA) to analyze the relationship between the areal types, vertical levels and types of horizontal distribution of liverworts and mosses.

Liverworts

The input species data set of the ordination diagram illustrating the relationship between areal types (Fig. 1) and vertical levels consists of the numbers of liverwort species in the types of horizontal distribution represented in the vertical levels (Table 1). The number of liverwort species in the types of horizontal distribution represented in the areal types were used as environmental variables (Table 2).

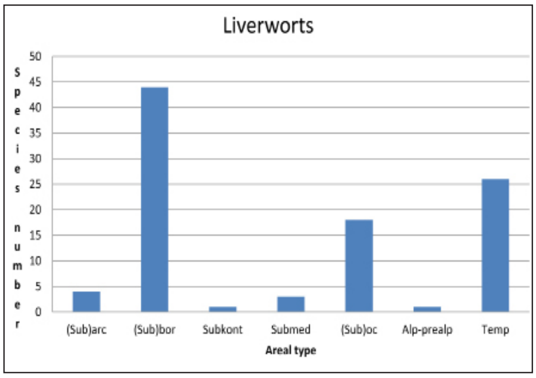

Fig. 1. Liverworts, species number in areal types.

Ordinal axis 1 explains 78.2% of the variance (Fig. 2), this axis is positively correlated, particularly for (sub)boreal (r = 0.8677), (sub)oceanic (r = 0.7995) and temperate (r = 0.7857) areal types. Holarctic species are predominantly presented and the species-environment correlation is 0.882. Ordinal axis 2 explains 3.9% of the variance, and this axis is negatively correlated with subcontinental (r = -0.1533) and alpine-pre-alpine (r = -0.2349) areal types. In Euro-Siberian and North American species, the species-environmental correlation is 0.118. While the temperate, (sub)boreal and (sub) oceanic areal types prefer species of the colline to montane vegetation level, the subcontinental and alpine-prealpine areal type often prefer the species of the subalpine to alpine vegetation level (Fig. 2) (*Scapania helvetica*). In addition to a high number of liverwort species with a (sub)boreal areal type and a distribution center in Northern Europe (e.g., *Neoorthocaulis attenuatus, Conocephalum conicum, Mylia anomala*). We recorded a high number of temperate liverwort species (e.g., *Marchantia polymorpha, Pellia epiphylla, Plagiochila asplenioides*), including species with a distribution center in the temperate zone. Some temperate species occur in the lowest areas of the territory, (e.g., *Riccia cavernosa, R. fluitans* in the planar to colline level (Fig. 2, upper left hand corner, the area consists of Eurasia, Africa, North America). Vertically higher, up to the submontane level, some temperate species occur (e.g., *Cephaloziella rubella, Cephaloziella*

Vertical levels	*altitude (m a.s.l.)	a	b	c	d	e	f	g	h	i	j	k	Sum
Plan-collin	200	1	0	0	0	0	0	1	1	0	0	0	3
Collin-submont	600	0	0	0	0	0	0	0	1	0	0	7	8
Submont-mont	950	1	0	3	3	2	2	1	5	3	1	32	53
Mont-subalp	1400	0	0	1	0	2	1	0	5	3	0	20	32
Subalp-alp	1900	0	1	0	0	0	0	0	0	0	0	0	1

* Altitude refers to the interface of the respective vertical levels.

Table 1. Numbers of liverworts in types of horizontal distribution presented in vertical levels. Ordinal values: a – cosmopol; b – eur; c – eur-afr; d – eur-afr-n.am; e – euras; f – euras-afr; g – eur-as-afr-n.am; h – eur-as-n.am; i – eur-n.am; j – eurosib-n.am; k – holarc.

Areal type	a	b	c	d	e	f	g	h	i	j	k	Sum
(Sub)arc	0	0	0	0	0	0	1	1	0	0	2	4
(Sub)bor	0	0	0	0	0	1	4	5	1	3	30	44
Subcont	0	0	0	0	0	0	0	0	0	1	0	1
Submed	0	0	0	0	0	1	1	0	0	0	1	3
(Sub)oc	0	0	1	2	2	1	3	1	0	0	8	18
Alp-prealp	0	1	0	0	0	0	0	0	0	0	0	1
Temp	2	0	0	1	1	2	4	0	1	0	15	26

Table 2. Numbers of liverworts in types of horizontal distribution presented in areal types. Ordinal values: a – cosmopol; b – eur; c – eur-afr; d – eur-afr-n.am; e – euras; f – euras-afr; g – eur-as-afr-n.am; h – eur-as-n.am; i – eur-n.am; j – eurosib-n.am; k – holarc.

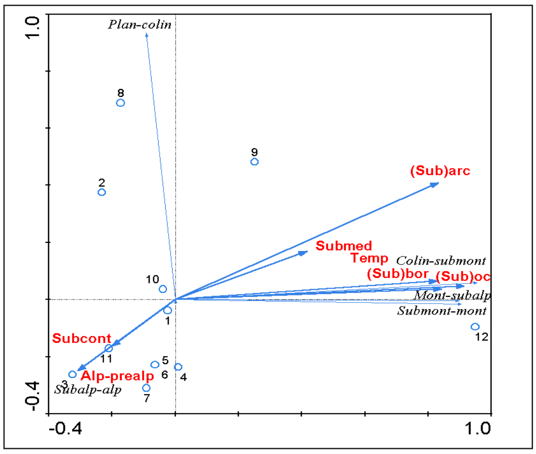

Fig. 2. RDA, liverworts, triplot, relationship of areal types, vertical levels and types of horizontal distribution. a – cosmopol; b – eur; c – eur-afr; d – eur-afr-n.am; e – euras; f – euras-afr; g – eur-as-afr-n.am; h – eur-as-n.am; i – eur-n.am; j – eurosib-n.am; k – holarc.

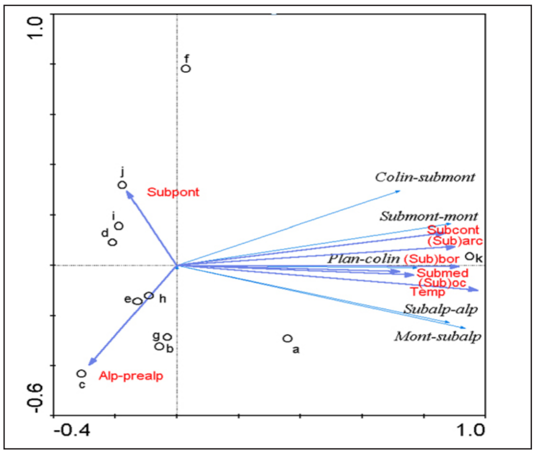

Fig. 3. RDA, moss species, triplot, relationship of areal types, vertical levels and types of horizontal distribution. a – subkosm; b – circpol; c – eur; d – eur-afr; e – eur-afr-n.am; f – euras; g – eur-as-afr; h – eur-as-afr-n.am; i – eur-as-n.am; j – eur-n.am; k – holarc.

**sullivantii* (Fig. 2, right hand section). There are numerous species with a suboceanic areal type, and they often occupy wetter habitats (e.g., *Fuscocephaloziopsis connivens, Scapania aspera, Trichocolea tomentella*). Several suboceanic species have a wide areal in Europe, Africa, North America, or Asia, (e.g., *Pedinophyllum interruptum, Calypogeia suecica* and others). Sub(boreal) and temperate species often have Holarctic distribution. We recorded four subarctic liverwort species, two of which are representatives of the genus *Scapania* (*Scapania apiculata, S. scandica*).

Mosses

The input species data set of the diagram illustrating the relationship between areal types and vertical levels (Fig. 3) are the numbers of moss species in the types of horizontal distribution presented in vertical levels (Table 3). As environmental variables, the numbers of moss species in the types of horizontal distribution presented in the areal types were used (Table 4).

Ordinal axis 1 explains 74.9% of the variance, (Fig. 3). (Sub)boreal (r = 0.8651), subcontinental

Vertical levels	*altitude (m a.s.l.)	a	b	c	d	e	f	g	h	i	j	k	Sum
Plan-collin	200	1	0	0	0	0	0	1	0	1	1	4	7
Collin-submont	600	2	0	1	1	4	6	1	1	5	1	29	51
Submont-mont	950	29	4	0	2	5	13	3	3	8	4	127	198
Mont-subalp	1400	12	2	1	0	2	1	1	1	1	0	45	66
Subalp-alp	1900	2	0	0	0	2	0	2	1	0	0	19	26

* Altitude refers to the interface of the respective vertical levels

Table 3. Numbers of moss species in types of horizontal distribution presented in vertical levels. Ordinal values: a – subcosm; b – circpol; c – eur; d – eur-afr; e – eur-afr-n.am; f – euras; g – eur-as-afr; h – eur-as-afr-n.am; i – eur-as-n.am; j – eur-n.am; k – holarc.

Areal type	a	b	c	d	e	f	g	h	i	j	k	Sum
(Sub)arc	2	1	1	0	0	3	0	2	0	1	25	35
(Sub)bor	12	4	1	0	1	2	5	0	0	2	114	141
Subcont	0	0	0	0	0	3	3	1	0	0	13	20
Submed	1	1	0	0	1	3	7	1	0	0	8	22
(Sub)oc	0	1	1	2	1	2	6	8	1	1	18	41
Subpont	0	0	0	0	0	0	0	0	0	1	0	1
Alp-prealp	0	0	1	0	0	0	0	0	0	0	0	1
Temp	23	0	0	0	0	1	6	3	0	1	53	87

Table 4. Number of moss species in types of horizontal distribution presented in arealtypes. Ordinal values: a – subcosm; b – circpol; c – eur; d – eur-afr; e – eur-afr-n.am; f – euras; g – eur-as-afr; h – eur-as-afr-n.am; i – eur-as-n.am; j eur-n.am; k – holarc.

(r = 0.8213), subarctic (r = 0.8524) and (sub)oceanic (r = 0.7250) areal types, are positively correlated with this axis. Holarctic species are predominantly presented, preferring the planar level to the colline level. The species-environment correlation is 0.882. Ordinal axis 2 explains 5.0% of the variance. On the 2nd axis, the subpontic areal type is slightly positively correlated (r = 0.2351) with European to North American species, and the alpine-prealpine areal type is negatively correlated (r = -0.3185) with predominantly Eurasian, African and North American species. The species-environmental correlation is 0.802.

Similarly to liverworts, the most abundant (sub)boreal areal type and temperate areal type for mosses were recorded (Fig. 4). Species of (sub) boreal areal type have an optimum distribution in Northern Europe. In the study area, they are often found in the montane to subalpine vegetation level. Temperate species have a center of distribution in the temperate zone. Both (sub)boreal and temperate species have the most frequent Holarctic distribution (Fig. 3). In the Sub-mediterranean element, we noted the presence of rare thermophilic species, (e.g., *Pottiopsis caespitosa* and *Entosthodon muhlenbergii*). The vertical diversity of the territory lends it to a relatively high number of species with a subarctic areal type, and a preference for colder habitats (e.g., *Molendoa sendtneriana, Roaldia revoluta*). Some of these species prefer the highest locations of the studied area, (e.g., *Dicranum spadiceum, Lescuraea plicata, Stegonia latifolia,* and *Syntrichia norvegic*).

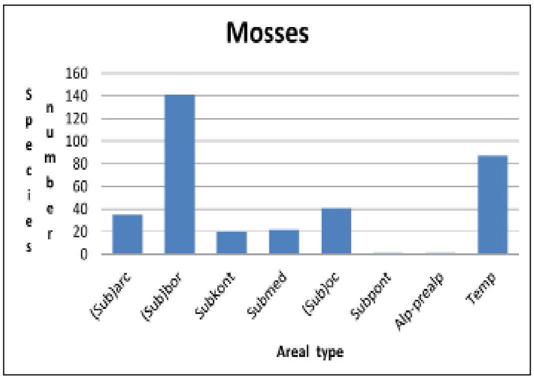

Fig. 4. Mosses, species number in areal types.

Phytogeographical analysis of bryophytes of marginal vegetation levels (lowest and highest locations of Veľká Fatra Mountains)

All bryophyte species presented in the Veľká Fatra orographic unit are analyzed, with a total of 340 species. Liverworts (*Hepaticopsida*) and mosses (*Muscopsida*) are distinguished. When analyzing the bryophytes from marginal vegetation levels, due to the limited number of species, these taxa are evaluated together. We omitted the variable "altitude" from the data matrix, because by focusing on a narrow vertical range, this parameter is considerably reduced. We used unimodal indirect correspondence analysis (CA).

Bryophytes with the occurrence center in the lowest levels of Veľká Fatra Mountains

In the analysis, we included a total of 30 species with a focus of occurrence in Slovakia in the planar to colline vegetation level. The vertical optimum for the occurrence of the selected species in the conditions of Veľká Fatra Mountains is at an altitude of approximately 400 to 650 m a.s.l. Some species with an optimum occurrence in the planar to colline vegetation level were also included (Table 5). In this case the limestone or dolomite substrate allows them to penetrate to higher altitudes (Table 6, Fig. 5). For example, the moss species *Grimmia tergestina* has an optimum occurrence in the colline level, but the moss was recorded above Plavecké Podhradie Settlement (300 m a.s.l.), on Pohanská hora Hill near Plavecký Mikuláš Settlement (300 m a.s.l.), on Súľovské skály Rocks (400 m a.s.l.), on the Silická planina Plain (400-600 m a.s.l.) as well as in other locations (Šmarda 1948). In the conditions of Veľká Fatra Mountains, we also recorded moss at significantly higher altitudes (Ostredok, 1520 m a.s.l.). The occurrence of the moss species *Trichostomum crispulum* in Veľká Fatra Mountains is recorded on the Súľovské skaly Rocks (380 m a.s.l.), in Manínska tiesňava Ravine (650 m a.s.l.), at the mouth of Suchá Belá Gorge (550 m a.s.l.), at the Kláštorisko Resort (600 m a.s.l.) (Šmarda 1961a) and in the Prielom Dunajca Gorge (450 m a.s.l.) (Blackburn *et al.* 1997). However, in the Veľká Fatra Mountains the moss also penetrated to altitudes of more than 1300 m a.s.l. (Haľamova kopa Mt, 1340 m a.s.l.; Tlstá Mt, 1370 m a.s.l.). The moss species *Pterygoneurum ovatum* is found in Slovakia on loess substrata in the planar to colline level, (e.g., near the villages of Veľké Zálužice (180 m a.s.l.), Bučany (140 m a.s.l.), Kamenica nad Hronom (300 m a.s.l.), near Trnava Settlement (140 m a.s.l.) (Peciar 1970). In the Veľká Fatra Mountains, this moss was recorded at an altitude of 950 m a.s.l. on Drienok Hill. The liverwort *Cephaloziella *sullivantii* was recorded in the Veľká Fatra Mountains at an altitude of 1000 m a.s.l. on the Kútnikov kopec Hill near Ľubochna Settlement (Duda and Váňa 1974). In Slovakia, this liverwort was also recorded at a significantly lower altitude, near Zemianske Podhradie Settlement at an altitude of 248 m a.s.l. m (Šmarda 1961b).

Ordinal axis 1 (Eigenvalue 0.393) explains more than 63% of the variance. The temperate areal type positively correlates with this axis, and sub-cosmopolitan, Holarctic, European to North American species as well. Ordinal axis 2 (Eigenvalue 0.168) explains 27% of the variance, and correlates positively with the subcontinental, (sub)oceanic areal types as well as some Eurasian and African species. In the lowest locations of the Veľká Fatra Mountains, the most numerous species are Holarctic (15 species, 46.8%) – includig *Anomodon viticulosus, Callicladium haldanianum, Drepanocladus aduncus, Leskea polycarpa* and others. Thermophilic species thrive at these altitudes, while the largest number of species have a temperate areal type: *Cephaloziella *sullivantii, Riccia cavernosa, Riccia fluitans - Aloina rigida, Brachythecium mildeanum, Dicranum fulvum, Pterygoneurum ovatum, Tortula acaulon, T. caucasica, T. lindbergii. Entosthodon*

Species	Horizontal distribution	Areal types	Vertical levels
Calypogeia fissa	eur-w.as-afr-n.am	suboc	planar-submont
*Cephaloziella *sullivantii*	eur-n.am	temp	planar-submont
Riccia cavernosa	eurosib-afr(m)-n.am	temp	planar-collin
Riccia fluitans	cosm	temp	planar-collin
Aloina rigida	holarc(-bip)	temp	planar-submont
Anomodon viticulosus	holarc	temp	(planar) collin-submont
Brachythecium mildeanum	holarc	temp	(planar) collin-submont
Brachythecium laetum	eur-w.as-n.afr-n.s.am	subcont	collin-submont
Callicladium haldanianum	dj-holarc	subcont	planar-submont
Didymodon vinealis	dj-holarc	submed	planar-collin
Ditrichum pusillum	holarc	temp	planar-submont
Drepanocladus aduncus	subcosm	temp	planar-submont
Entosthodon fascicularis	eur-n.afr-n.am	suboc	planar, collin (submont)
Entosthodon muhlenbergii	eur-w.e.as-afr-n.c.am	submed	collin
Hygroamblystegium varium	holarc(-bip)	temp	planar-submont
Grimmia tergestina	eur-as-afr	submed	collin
Isothecium myosuroides	dj-holarc	oc-mont	collin-submont
Leskea polycarpa	holarc	temp	planar-submont
Mnium hornum	dj-eur-w.e.as-afr-n.am	suboc	planar-submont
Oxyrrhynchium schleicheri	eurosib-w.as-afr(m)	submed	planar-submont
Oxyrrhynchium speciosum	eur-w.e.as-afr-n.am	temp	planar-submont
Plasteurhynchium striatulum	eur-w.as-afr	submed	collin-submont
Pottiopsis caespitosa	eur-w.as-n.afr	submed	collin
Pterygoneurum ovatum	dj-holarc(-bip?)	temp	planar-submont
Racomitrium heterostichum	eur-w.as-n.am-afr(mac)	suboc	planar-submont
Tortula acaulon	holarc(-bip)	temp	planar-submont
Tortula lindbergii	dj-holarc	temp	planar-submont
Thuidium tamariscinum	dj-holarc(-bip)	suboc	planar-submont
Trichostomum crispulum	eur-w.as-afr	suboc	planar/collin
Weissia condensa	dj-holarc	submed	collin-mont

Table 5. List of selected species of the lowest levels of the Veľká Fatra Mountains.

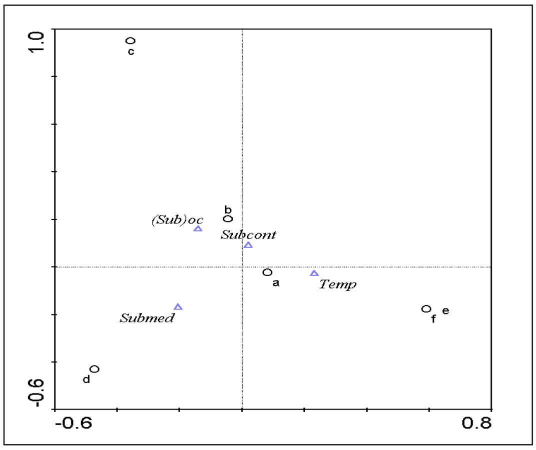

Fig. 5. Correspondence analysis (CA). Ordination of bryophytes of the lowest levels of the Veľká Fatra Mountains, relation of areal types and types of horizontal distribution. Types of horizontal distribution: a – holarc; b – eur-w.e.as-afr-n.am; c – eur-n.afr-n.am; d – eur-as-afr; e – eur-n.am; f – (sub)cosm.

Areal type	a	b	c	d	e	f
Subcont	1	1	0	0	0	0
Submed	2	1	0	4	0	0
(Sub)oc	2	3	1	1	0	0
Temp	9	2	0	0	1	2

Table 6. Numbers of bryophytes of the lowest level of the Veľká Fatra Mountains in the types of horizontal distribution. Types of horizontal distribution: a – holarc; b – eur-w.e.as-afr-n.am; c – eur-n.afr-n.am; d – eur-as-afr; e – eur-n.am; f – (sub)cosm.

muhlenbergii and *Pottiopsis caespitosa* have a sub-Mediterranean areal type, *Brachythecium laetum* has a sub-continental areal type and *Entosthodon fascicularis* has a suboceanic areal type. Temperate species make up 43.75% of the inventory of planar to submontane species. Species in the lowest vegetation zones are mostly bound to sunlit limestone rocks, though *Tortula acaulon* and *T. lindbergii* prefer soil covered rocks, *Dicranum fulvum* tolerates shaded limestone, and *Potiopsis caespitosa* occupies moist, limestone terraces.

Bryophytes with the occurrence center in the highest levels of Veľká Fatra Mountains

The highest point of Veľká Fatra is Ostredok, at 1595 m a.s.l. In the list of 20 selected species (Table 7), we have also included several species of bryophytes. In Slovakia, these have a distribution center in the alpine level, only descending down to lower vegetation levels on an exceptional basis. For example, *Mnium thomsonii* is a relatively abundant species in the Veľká Fatra Mountains in crevices and on limestone rock terraces, especially at the highest altitudes, but it descends down to 500 m a.s.l. *Plagiobryum demissum* is also a common species at the highest levels. Šmarda (1948) recorded this moss in the Necpalská dolina Valley at an altitude of only 1000 m a.s.l. The liverwort *Mesoptychia bantriensis* is a species widespread in higher altitudes, especially on limestone and dolomite rocks, but it also descends down to lower altitudes. Kochjarová *et al.* (2010) report the occurrence of liverwort in the Čertova brána Strait at an altitude of 630 m a.s.l. (Table 8, Fig. 6).

Ordinal axis 1 (Eigenvalue 1.0000) explains more than 62% of the variance. Alpine and pre-alpine areal types and the European species (*Scapania helvetica*) are positively correlated with this axis. Ordinal axis 2 (Eigenvalue 0.3259) explains more than 25% of the variance. (Sub)boreal species (moss *Mnium thomsonii*, liverworts *Bazzania tricrenata* and

Areal type	a	b	c	d	e	f
(Sub)arc	9	1	1	1	0	0
Arc-alp	2	1	0	0	0	0
(Sub)bor	2	0	0	0	0	1
(Sub)oc	1	0	0	0	0	0
Alp-prealp	0	0	0	0	1	0

Table 8. Numbers of bryophytes of the highest level of the Veľká Fatra Mountains in the types of horizontal distribution. Types of horizontal distribution: a – holarc; b – euras; c – circpol; d – eur-n.am; e – eur; f – eurosib-n.am.

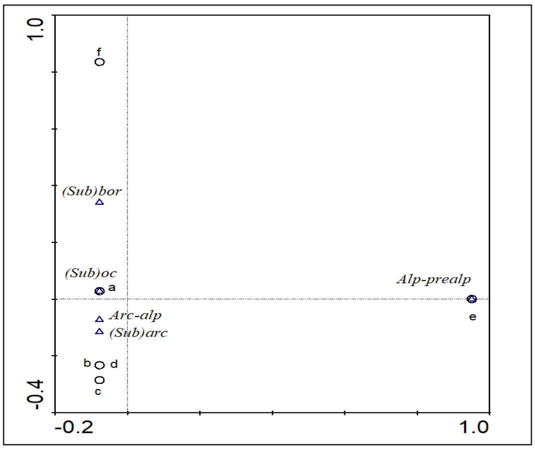

Fig. 6. Correspondence Analysis (CA). Ordination of bryophytes of the highest level of the Veľká Fatra Mountains, relation of areal types and types of horizontal distribution. Types of horizontal distribution: a – holarc; b – euras; c – circpol; d – eur-n.am; e – eur; f – eurosib-n.am.

Species	Horizontal distribution	Areal types	Vertical levels
Bazzania tricrenata	holarc	(sub)bor	mont-alp
Scapania helvetica	eur	alp-prealp	mont-alp
Mesoptychia bantriensis	eurosib-n.am	(sub)bor	mont-alp
Brachythecium erythrorrhizon	holarc	(sub)arc	(subalp) alp
Bryum archangelicum	euras	arc-alp	alp
Campylium bambergeri	circpo	(sub)arc	(subalp)alp
Dicranum elongatum	holarc	(sub)arc	(subalp)alp
Dicranum spadiceum	holarc	(sub)arc	(subalp)alp
Hylocomiastrum pyrenaicum	holarc	(sub)arc	(subalp)alp
Lescuraea plicata	eur-n.am.	(sub)arc	mont-alp
Mnium thomsonii	holarc	(sub)bor	mont-alp
Orthotrichum alpestre	holarc	(sub)arc	(subalp)alp
*Orthotrichum *fuscum*	euras	(sub)arc	(subalp)alp
Plagiobryum demisum	holarc	arc-alp	mont-alp
Plagiobryum zieri	holarc	(sub)arc	alp
Pseudostereodon procerrimus	holarc	(sub)oc	(subalp)alp
Roaldia revoluta	holarc	(sub)arc	(subalp)alp
Stereodon hamulosus	holarc	(sub)arc	(subalp)alp
Stegonia latifolia	holarc	arc-alp	alp
Tortula hoppeana	holarc	(sub)arc	(subalp)alp

Table 7. List of selected species of the highest levels of the Veľká Fatra Mountains.

Mesoptychia bantriensis) are positively correlated with this axis. (Sub)arctic species (*Dicranum spadiceum*, *Campylium bambergeri* and others) and some arctic-alpine species are negatively correlated with this axis (e.g., *Bryum archangelicum*). These species have circumpolar or European to North American distribution.

Bryophytes, which find their optimum occurrence at the alpine level. In the Veľká Fatra Mountains they often occupy limestone rocks, or rocky walls at altitudes from 1350 m a.s.l. (*Bryum archangelicum*, *Stereodon hamulosus*, *Roaldia revoluta*, *Stegonia latifolia*, *Orthotrichum *fuscum*). *Brachythecium erythrorrhizon* occupies moss-covered debris, *Dicranum spadiceum* occurs in grassy places up to an altitude of 1550 m a.s.l. *Orthotrichum alpestre* is an epiphyte, and was recorded at an altitude of 1300 m a.s.l.. The liverwort *Scapania helvetica* was recorded at the top of Majerova skala Rock (1283 m a.s.l., Boros *et al.* 1960; Šmarda 1961c). These are mostly species of the (sub)arctic areal type, of Eurasian and Holarctic species. *Scapania helvetica* has an alpine-prealpine areal type and is a European species.

II. Factor analysis

When determining the number of factors to extract for factor analysis, we opted for principal component analysis as the most frequently used method. Eigenvalues: PC1 8.057; PC2 6.352; PC3 3.885; PC4 2.359. An eigenvalue >1 belongs to the first four components and they explain 93.9% of the variance. We used the Varimax raw rotation because it transforms the factor loadings so that the variance of their values is maximal. This type of rotation offers the most meaningful interpretation. The factor loadings are shown in Table 9, and the ordination of types of horizontal distribution is depicted in Fig. 7.

Factor 1 explains 36.6% of the variance. In both liverworts (horizontal distribution types h, j, k) and mosses (horizontal distribution types m, v, z), this factor correlates primarily with European to North American and Holarctic species (Table 9). A common feature is the predominance of the (sub)boreal type of the areal (Fig. 7, bottom right hand corner).

Factor 2 explains 28.9% of the variance. In both liverworts (horizontal distribution types c, d, e) and mosses (horizontal distribution types o, t, u), this factor correlates with European to African (North American) species (Table 9). A common feature is the predominance of the (sub)oceanic areal type (Fig. 7, upper left hand corner).

Factor 3 explains 17.7% of the variance. The structure of factor loadings is mainly determined by liverworts (horizontal distribution types a, f, i, Table 9), and a common feature is the predominance of the temperate areal type.

Factor 4 explains 10.7% of the variance, the negative correlation of the European and Eurasian type of horizontal distribution is striking.

The graph of the ordination of types of horizontal distribution (Fig. 7) presents the correlation of the type of horizontal distribution of liverworts "f" (Eurasia, Africa) with the type of horizontal distribution of mosses "s" (Europe, Asia, Africa) (middle part of the graph), with the exception of the type of horizontal distribution, a common feature is also the prevailing temperate areal type.

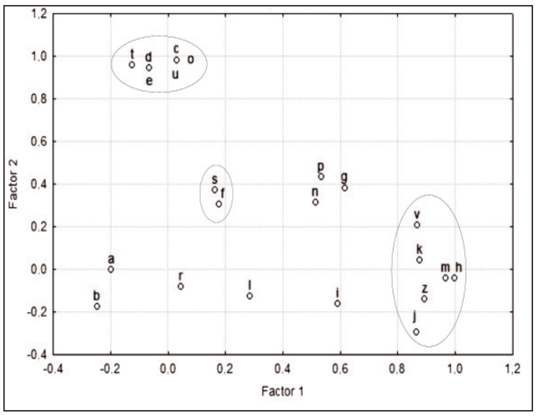

Fig. 7. Bryophytes, ordination of types of horizontal distribution, factor 1 vs. factor 2. Types of horizontal distribution: a – cosmopol; b – eur; c – eur-afr; d – eur-afr-n.am; e – euras; f – euras-afr; g – eur-as-afr-n.am; h – eur-as-n.am; i – eur-n.am; j – eurosib-n.am; k – holarc; l – subcosm; m – circpol; n – eur; o – eur-afr; p – eur-afr-n.am; r – euras; s – eur-as-afr; t – eur-as-afr-n.am; u – eur-as-n.am; v – eur-n.am; z – holarc.

Var.	Factor 1	Factor 2	Factor 3	Factor 4
a	-0.198	-0.002	**0.970**	-0.054
b	-0.246	-0.175	-0.212	**-0.826**
c	0.031	**0.983**	-0.158	0.030
d	-0.065	**0.943**	0.314	0.003
e	-0.065	**0.943**	0.314	0.003
f	0.179	0.304	**0.851**	0.280
g	0.616	0.383	0.675	0.102
h	**0.998**	-0.041	-0.047	0.007
i	0.592	-0.160	**0.787**	-0.028
j	**0.865**	-0.295	-0.029	0.117
k	**0.876**	0.041	0.472	0.014
l	0.286	-0.128	**0.944**	-0.025
m	**0.967**	-0.043	-0.106	0.170
n	0.514	0.313	-0.439	-0.611
o	0.031	**0.983**	-0.158	0.030
p	0,533	0.433	-0.168	0.473
r	0,047	-0.081	-0.390	**0.831**
s	0,165	0.373	0.462	0.672
t	-0,124	**0.959**	0.095	0.112
u	0,031	**0.983**	-0.158	0.030
v	**0.868**	0.206	0.303	-0.037
z	**0.895**	-0.140	0.406	0.061
Expl. Var.	6.548	6.493	5.037	2.577

Table 9. Factor loadings (Varimax raw; Extraction: Principal components). Types of horizontal distribution: a - k Liverworts: a – cosmopol; b – eur; c – eur-afr; d – eur-afr-n.am; e – euras; f – euras-afr; g – eur-as-afr-n.am; h – eur-as-n.am; i – eur-n.am; j – eurosib-n.am; k – holarc. l - z Mosses: l – subcosm; m – circpol; n – eur; o – eur-afr; p – eur-afr-n.am; r – euras; s – eur-as-afr; t – eur-as-afr-n.am; u – eur-as-n.am; v – eur-n.am; z – holarc.

Conclusion

Classical phytogeographical analysis focuses on the analysis of the areal types of the evaluated species, their abundance, vertical distribution, ecology, the presence of Dealpine species and threats. This is how we focused the floristic-phytogeographic characteristics of the bryophytes of the Veľká Fatra Mountains tin the monographic study of the Nature of the Veľká Fatra Mountains (Šoltés *et al.* 2008).

In the presented study, we used the linear method and entered the data of the horizontal distribution of bryophyte species into the ordination as predictors. The following redundancy analysis and factor analysis revealed new associations that were obscured by the usual approach, such as the preference of liverwort species of European to Europe-Siberian distribution in the alpine-prealpine areal type. In the case of mosses, the alpine-prealpine areal type is preferred by species with a distribution in Europe and Africa, and the subpontic areal type is preferred by species with a distribution Europe to North America. Within the lowest levels of the Veľká Fatra Mountains, species of (sub)oceanic and subcontinental areal types occupy a wide range (Europe, Asia, Africa, America), while the temperate areal type is preferred by species of Holarctic distribution. In the highest levels of the Veľká Fatra Mountains, the (sub) arctic areal type is the most common. In terms of horizontal distribution, they are of Eurasian and Holarctic species.

The analysis of factor loadings provided remarkable results. The common feature of species with North American and Holarctic distribution is the predominance of the (sub)boreal areal type, whereas the common feature of species with the European to African (North American) distribution is the predominance of the (sub) oceanic areal type.

When using climatic, pedological, orographic, phytocenological or ecological data as predictors, an alternative analysis from other points of view may also arise.

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REPORTS

Micro-mammalia monitoring in the Western Carpathians, The Grapa Nature Reserve

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Abstract. This report summarizes the results of small terrestrial mammal monitoring that took place at Grapa National Reserve during the spring and autumn seasons in 2022. The reserve is a fragment of primeval forest with well-preserved native forest structures. During a total of 11 days of trapping, individuals of 5 rodent species were captured – *Myodes glareolus*, *Apodemus flavicollis*, *Apodemus sylvaticus*, *Mus musculus*, *Muscardinus avellanarius*.

Key words: micro-mammalia, zoological monitoring, Grapa National Reserve, Tatra Mountains

Characteristics of the site

Sample capture took place within the Grapa nature reserve. The reserve has an area of 408,600 m^2 and is near the cadastral village of Tatranská Javorina. The reserve was established in 1991 by The Decree of the Slovak Commission for the Environment on state nature reserves and protected sites in Tatra National Park. Slovak legislation, through Law 543/2002 on nature and landscape protection, provides five levels of territorial protection. The extent of restrictions increases with increasing degrees of protection. The fifth, most stringent level of protection is in place for the Grapa nature reserve. Within the reserve, the subject of these measures is a young (40-80 years) forest stand, that is significant in phytocenological and florogenetic terms. A mosaic of the most diverse forest communities can be found here, as well as trees older than 100 years in smaller quantities. The reserve is populated by the typical fir-spruce community of the Western Carpathians, on a base of Hutian claystone formation and a predominance of sandstones (SAŽP 2021; ŠGÚDŠ 2017). The structure of the primeval forest is favourable, and there is large amount of deadwood of varying degrees of decay (www.pralesy.sk 2022). The locality is minimally altered by human activity. Historically, there has been a prevalence of logging in the cadastral area of Tatranská Javorina (Marhefka *et al.* 2021), leading to structural changes in forest stands. The shortcoming of this approach is the change to wood composition due to selective harvesting of beech, maple, and yew. Both beech and maple are currently being extensively regenerated, while spruce is being less-extensively regenerated (www.pralesy.sk 2022).

The presumed species of small mammals present in the reserve prior to our study were *Myodes glareolus* (Rodentia: Cricetidae), *Apodemus sylvaticus* (Rodentia: Muridae), and *Microtus subterraneus* (Rodentia: Cricetidae).

Research methodology

This study was conducted in the Grapa national reserve to observe what micro-mammalia are present at this location. Sherman traps were placed in three parallel lines. In the lower line there were 52 traps, with 45 in middle line, and 28 in upper line, placed approximately 15 m apart from each other. Traps were filled with straw and feed, such as apples, pork meat, and rodent mix.

The first trapping exercise took place in the spring of 2022 (April 5-7). Trap checking was conducted every five hours during the day and every three hours during the night and early morning (3:00, 6:00, 9:00, 14:00, 19:00 and 24:00) by groups of two people. Any trapped animals were carefully put it inside a plastic bag for species determination and then released at the same location. If it was not possible to determine species in the dark, animals were placed in a cotton bag and transported to the laboratory for more accurate identification. Species were identified through determination keys or the professional expertise of professor RNDr. Marián Janiga, CSc. Afterward, animals were released at the capture site during the next trap check.

We repeated the sampling exercise during the autumn of 2022 (October 11-14, 18-21). During the first week, traps were placed in approximately the same transects as during the spring trapping season; during the second week, the first line was moved approximately 50 m parallel from the location of the third line in the previous trapping. We placed the same number of traps in each line. Sherman traps were used, as well as 15 wooden traps, and 18 wire traps with a different trigger mechanism. The traps were checked every 4-5 hours. We collected samples of hair for future toxicological research. This fur also served to identify any individuals that had been previously captured.

Conclusion

During spring sampling, 20 small mammal individuals were trapped – 17 bank vole (*Myodes glareolus*), and 3 individuals of wood mouse (*Apodemus sylvaticus*). The small number of trapped individuals was likely due to an unexpected snowfall before trapping (Table 1).

In autumn, in contrast to spring, Muridae predominated. 17 individuals of the genus *Apodemus* were trapped, as well as one house mouse (*Mus musculus*) (Rodentia: Muridae) was found. Only a single bank vole was found, and the presence of one hazel dormouse (*Muscardinus avellanarius*) (Rodentia: Gliridae) was also confirmed (Table 1).

Absence of the order Insectivora among trapped individuals was surprising, because occurrence of the family Soricidae was expected. Our previous experience with sampling of small terrestrial mammals indicated that shrews are more commonly trapped in winter months. Additionally, due to the large number of traps, a higher number of captured individuals was expected. During previous trapping events for toxicological research, the trapping success rate (i.e., the number of individuals divided by the number of traps) in October was up to 40%. A systematic sampling would be required to determine whether this low trapping success rate is a consequence of the generally low population density at the site, or a consequence of advancing climate change and increased temperature and drought (Dhawan *et al.* 2018; Selås *et al.* 2019), and the associated decline in food or increasing prevalence of parasites.

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Family	Name	Quantity	Season
Cricetidae	*Myodes glareolus*	17	Spring
		1	Autumn
Muridae	*Apodemus sylvaticus*	3	Spring
	Apodemus sp.	17	Autumn
	Mus musculus	1	Autumn
Gliridae	*Muscardinus avellanarius*	1	Autumn

Table 1. Summary of small terrestrial mammal individuals found in traps.

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REPORTS

Short-term zoological monitoring in Zhongar-Alatau State National Nature Park with a focusing on the avifauna of the Osinovaya site

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Abstract. For long-term environmental and ecological research, it is essential to consider all components of the environment, including abiotic factors. Zhongar-Alatau State National Park is an area with unique habitats and a high biodiversity and is therefore a suitable area for new research on fauna. The aim of this monitoring research was to obtain information on the species diversity of small terrestrial mammals and avifauna in the Osinovaya site. The captured individuals were species identified and the skin integument and blood samples obtained will be used in further separate studies. In this report we provide an overview of the species monitored.

Key words: zoological monitoring, Zhongar-Alatau, birds, micro-mammalia

Characteristics of the site

Zhongar-Alatau State National Park is located on the northern slopes of the Dzungarian Alatau, a mountain range situated between the Tian Shan in the south and the Altai Mountains to the north. It was established in 2010 to protect the natural mountain landscapes, unique ecology, and the historical and aesthetic values of the Dzungarian Alatau, an isolated, glacier-covered mountain range in Kazakhstan on the southeastern border with China. The park territory belongs to the Tian Shan Mountain Steppe and Meadow Ecoregion - WWF ID PA 1019 (Carpenter and Pereladova 2022), which is characterized by its isolation and high biodiversity due to its altitudinal zones ranging from steppes to alpine meadows to glaciers, and as a transition zone between boreal, steppe and desert geographic areas. There is sufficient precipitation at mid-elevations to sustain forests (Carpenter and Pereladova 2022). Although the forests are predominantly pine and spruce, there are significant stands of wild fruit trees in the park. Approximately 1% of the park´s area is forested with Sievers apple (*Malus sieversii*), the ancestor of all cultivated apple varieties in the world (Dzhangaliev 2003; IUCN 2015). Sievers apple plantations are located at an altitude of 900-1800 m a.s.l. and present in separate, spaced, fields with an area ranging from several hundred square meters to several tens of hectares (Bakhtaulova *et al.* 2015).

The territory of the Zhongar Biosphere Reserve belongs to the Central Asian Subregion, Montana-Asia Province, Dzungar-Tian Shan District, Dzungar Region, according to the zoogeographic subdivision.

The fauna of Zhongar Biosphere Reserve includes two species of bony fish, two to four species of amphibians, 8 species of reptiles, at least 238 species of birds, and 54 species of mammals. During the 2015 survey, 575 insect species from 6 orders, 48 families, and genera were identified (www.kazmap.kz 2016).

Research methodology

The research was conducted over three days in mid-September 2022 at the Osinovaya site, (N45.40526°; E80.40581°; 1207 m a.s.l.), which is located in an apple forest. The aim of the research was to record the species distribution of birds and small ground mammals living at the site and to determine the potential for further research opportunities and activities.

Birds were captured in the ornithological nets which were checked regularly every two hours. After release from the nets, each individual was determined according field guide book (Ayé *et al.* 2012), and their basic morphological data (weight, length of tarsometatarsus, tail length, bill length) were recorded.

Small ground mammals were captured in to Sherman live traps. After species determination, basic morphological data (weight, body length, hindlimb length, tail length) were recorded.

After sampling (blood, feathers, hair), animals were immediately released at the trapping site.

Conclusion

Of the small ground mammals, *Apodemus silvaticus* (n = 20) were the dominant species. The frequency of other caught species was low: *Apodemus agrarius* (n = 1), *Microtus* sp. (n = 2), and *Crocidura suaveolens* (n = 1).

Order	Latin name	No. of captured individuals
Caprimulgiformes	*Caprimulgus europaeus*	1
Passeriformes	*Aegithalos caudatus*	10
	Curruca curruca althae	2
	Cyanistes cyanus	6
	Chloris chloris	1
	Parus major	29
	Periparus ater	7
	Phoenicurus coeruleocophila	1
	Phylloscopus collybita	1
	Phylloscopus collybita tristis	1
	Phylloscopus humei	7
	Troglodytes troglodytes	1
	Turdus atrogularis	9
	Turdus merula	1

Table 1. List of captured birds in Osinovaya.

Most of the birds belonged to the order Passerifomes (n = 76), only one bird belonged to the order Caprimulgiformes. The list and number of birds captured is given in Table 1.

To study the environmental burden of heavy metal (Hg), skin integuments as fur and tail feather were collected from the captured animals. Blood was collected from the vena brachialis of the birds and a blood smear was taken and later examined for the detection of blood parasites. The results of these investigations will be published in separate studies.

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