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Seasonal and areal accumulation of heavy metals by algae and cyanobacteria in Javorinka mountain stream

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Abstract. In this study we have compared concentrations of accumulated elements in algae and cyanobacteria from different localities over several years and throughout the seasons. We collected chrysophyceae Hydrurus foetidus and cyanobacteria Oscillatoria sp. in Javorinka mountain stream over three years (2019 - 2021). A comparison of the concentrations collected from two localities showed that Ba, Fe, Mn and Ti are being accumulated at almost double the rate at the site with lower stream velocity. Depending on the year, we have observed a significant increase in Ca concentrations. We also examined the trend of intense uptake of these elements by algae and cyanobacteria during the summer season, due to the growth of reproductive organs during this season. Analysis was performed using the Kruskal-Wallis test (p < 0.05). It has been confirmed that algae and cyanobacteria can be used as bioindicators of spatial and seasonal pollution and element concentration variations.

Key words: algae, cyanobacteria, accumulation of elements, seasonality, mountain stream

Introduction

The biological value of algae has been known since the mid-19th century. Its greatest importance is as a bio-indicator. Russian scientists have studied the saprobity of algae; the physiological and biochemical characteristics which allow it to live in an environment that contains organic matter, with a certain degree of pollution. Their study was focused on organic pollution in stream and river environments. This system was altered, modified, and expanded over the years by several authors. Because saprobity is defined by the intensity of heterotrophic activity, all photoautotrophic species were finally excluded from the saprobic system when the inorganic nutrient load in rivers became increasingly problematic. During the process of studying saprobity in running water, the trophic classification system was developed for lakes. It is

based on inorganic nutrients which are gained from catchments. In both cases, there is a belief that the presence, absence, or abundance of certain species can reflect the current biochemical, physical, and general state of the environment in which these organisms were located. Such organisms are referred to as bio-indicators. Their specificity is the low ecological valence for living conditions and their specific stress symptoms, which respond to changes in the environment. Thanks to these manifestations of an organism, indicators are not only their presence, absence, but also biomass or growth of algae. Such species are a very reliable indicator of natural pollution. The indicator species then becomes a 'bio-sensor'. During its lifespan, organic and inorganic elements from the environment accumulate, and reflect the level of substances in the environment and exposure to organisms. Such species are labelled as 'bio-accumulators' which can be particularly useful when concentrating very low levels of a substance (Dokulil 2003).

Freshwater algae provide two main types of information about water quality:

- Long-term information; the status quo. In the case of a temperate lake, for example, detection of an intense summer bloom of the colonial bluegreen algae *Microcystis* is indicative of a preexisting high-nutrient (eutrophic) status.
- Short-term information; environmental change. In another lake environment, detection of a change in subsequent years from low to high blue-green dominance (with increased algal biomass) may indicate a change in eutrophic status. This may be an adverse transition (possibly caused by human activity) that requires changes in management practice and lake restoration.

In the context of change, bioindicators can thus serve as early-warning markers of the health status of an aquatic system (Bellinger and Sigee 2015).

Lethal concentration of zinc in algae have been determined to be 30 μ g/L. Productivity of several studied algae (green algae (*Ankistrodesmus* and *Scenedesmus*), one diatom (*Navicula*), and natural phytoplankton) at this concentration was reduced by 59, 55, 49, and 52 per cent respectively. At 17 μ g/L there was a 37 % reduction in primary production, and 3 μ g/L of zinc, there was an 80 % reduction in nitrogen fixation. Zinc is often found in labile or in free ionic form even though Cu, Cd and Pb are in strongly bound compounds. More than 80 % of zinc in water is in free (labile) form (Wong and Chau 1990). Based on the algae species, toxic levels of chromium can range between 20 μ g/L to 10,000

2 J. Tuchvňa μ g/L. It should be considered that these results are also a function of several additional conditions such as pH, temperature and salinity. The most common symptoms of toxicity were inhibition of growth and photosynthesis inhibition. Toxic concentrations for *Cladophora glomerata* were 250 μ g/L, *Thalassiosira pseudonana* < 20 μ g/L, and *Skeletonema costatum* at 980 μ g/L (Nriagu and Nieboer 1988). When the chromium concentration changed it caused a shift in community dominance. When algae was exposed to wastewater with a level of chromium between 1 - 40 mg/L only *Oscillatoria* sp. were present (Nriagu and Nieboer 1988).

The metal sorption ability of algae varies greatly from species to species and even among strains of a single species for any given metal. Each type of heavy metal also uniquely impacts the sorption abilities of different species of algae (Mehta and Gaur 2001).

By studying different species (Codium vermilara, Spirogyra insignis, Asparagpsis armata, Chondrus crispus, Ascophyllum nodosum and Fucus spiralis) from three main algae groups; green algae (Chlorophyta), red algae (Rhodophyta), and brown algae (Chromophyta), we developed a sequence of metal uptake decrease, as follows: $Pb > Cu \ge Cd > Ni >$ Zn. This sequence has held true for all studied species. Corroborating to this claim, we hypothesize that the binding of metal to active sites of the cell wall is related to their inner metal properties, such as ionic radii and electronegativity of atoms (Romera *et al* 2006).

On the other hand, brown algae achieved significantly better results with higher uptake capacity. At worst, brown algae reacted two times better than other species, as likely due to alginate groups, brown algae anchors the metal to its biomass. The sequence obtained as a function of the type of algae was: brown > red > green. In conclusion, besides the type of algae, or the physical and chemical properties of the environment, metal uptake is largely dependent on the type of metal to which the algae is exposed (Romera *et al.* 2006).

The cell wall of green algae likely contains two adjacent carboxylic groups to share the bond with one metal cation. Therefore, it shows lower levels of metal recovery. The reason why some biomasses show high affinity for a given metal and low sorption capacity, or vice versa, may be related to the degree of affinity of a specific biomass for each metal (Romera *et al.* 2006).

Many individuals of the chlorella species have excellent sorption capacity, in some cases up to 714 mg·g⁻¹ when exposed to Cu²⁺. Generally, its capacity is around 100 mg·g⁻¹ (Zeraatkar et al. 2016). In a study of chlorella vulgaris that contained concentrations of 2.5 ppm, samples were able to remove between 69 % and 80 % of Ni (II) and Cu (II) cations. When the concentration was increased to 10 ppm, the metal removal rate was reduced to 37 % and 42 % (Mehta and Gaur 2001). Sargassum sp., as saltwater algae, has a maximum sorption of 72,5 mg·g⁻¹. Sargassum sp. absorb more heavy metal elements and their compounds. This is due to a high alginate content, along with other brown algae. For example, the alginate content of Sargassum fluitans is 45 % of its dry weight (Mehta and Gaur 2001).

In general, Chlorophyta, commonly known as green algae, has significant accumulating abili-

ties, especially the *Cladophora* species; one of the best bio-indicators in water environments for nutrient and heavy metal pollution. They generate the majority of algal biomass in some water bodies (Chmielewská and Medveď 2001). Using *Cladophora* completely removed arsenic from drinking water (Salama *et al.* 2019).

Using the cyanobacteria Gloeothece sp., we observed its high susceptibility to Cu2+. Metal concentrations of 1.0 mg $Cu^{2+}.l^{\text{-}1}$ led to cell death. Whereas when using Pb2+, similar effects were achieved using a concentration of 40 or 50 mg.l⁻¹. A bi-metal system, where cyanobacteria were exposed to both $Cu^{\scriptscriptstyle 2+}$ and $Pb^{\scriptscriptstyle 2+}\!\!\!\!$, caused decrease in removal of each metal. $Cu^{\scriptscriptstyle 2+}$ removal decreased by 47.2 % and $Pb^{\scriptscriptstyle 2+}$ removal decreased by 13.8 % (Pereira et al. 2011). In cyanobacteria, copper is a micronutrient, functioning as a cofactor and required for structural and catalytic properties of enzymes. It is also a cofactor of the electron transport protein plastocyanin and other proteins associated with thylakoids, which imposes an extra complexity to copper homeostasis in these organisms compared with non-photosynthetic prokaryotes. However, exposure to excessive amounts of copper ions, may lead to cell destruction, similarly to all photosynthetic organisms (Pereira et al. 2011).

Accumulation of metals is due to adsorption onto the cell surface (wall, membrane, or external polysaccharides) and binding to cytoplasmic ligands, phytochelatins and metallothioneins, as well as other intracellular molecules. Through electron microscopy and X-ray energy dispersive analysis, studies have been carried out on the localization of heavy metal ions. On the algal cell wall there are many functional groups, such as, hydroxyl (OH), phosphoryl (PO₃O₂), amino (NH₂), carboxyl (COOH), sulphydryl (SH), etc., which confer a negative charge to the cell surface. Metal ions are absorbed, because generally they exist in water in cationic form (Mehta and Gaur 2001).

The distribution of functional groups varies among algae, therefore, biosorption is different. Metal sorption depends on these groups and if they are present in a given cell. Their presence is dependent on cell wall components, (e.g., peptidoglycan, teichouronic acid, teichoic acids, polysaccharides, and proteins). Conversely, cyanobacteria are dependent on typical carboxylic groups which exist on peptidoglycan, consisting of linear chains of N-acetylglucosamine and β 1, 4-N-acetylmuramic acid with peptide chains. Lipopolysaccharides, lipids, and membrane proteins are also important for sorption. Some studies prove that the carboxyl groups are the primary location for metal binding in cyanobacteria (bluegreen algae). Most of the species of this group has a capsule, which tends to have an anionic nature, thus giving them a very high affinity towards metal ions. This could result in significant removal of heavy metals in waste waters (Mehta and Gaur 2001).

In green algae contain significant carboxyl and sulphate groups. The percentage of protein content in cell walls on which these group are attached varies from 10 - 70 %. As previously mentioned, in brown algae the main mean of metal biosorption are alginate groups. Alginate is commonly found in cell wall membranes, where through the sorption is metal binding site (Mehta and Gaur 2001). Accumulation of heavy metals by algae and cyanobacteria Participation of carboxyl groups in adsorption of heavy meatal such as Cu, Cd and Pb has been demonstrated on cyanobacterial cell walls, as well as in green and brown algae (*Macrocystis pyrifera*, *Kjellmaniella crassiforia* and *Undaria pinnatifida*). A study of carboxyl groups presented great success in binding Au and bivalent metal ions. Research has even shown binding of Cd and Pb ions, to small binding on sulphate groups. Good sorption of heavy metals by carboxyl groups has been very well demonstrated, even by fungi and higher plants (Mehta and Gaur 2001).

Other groups exhibit a lesser extent of binding compared to carboxyl group. Non-living sargassum has demonstrated binding of Co on carbonyl groups. The amino group plays an important role in the binding of Au in *chlorella* (Mehta and Gaur 2001).

The above discussion leads to the generalization that carboxyl groups of cell wall polysacharides play a predominant role in heavy metal sorption by algae and cyanobacteria. The other functional groups, such as sulfonate and amino, play a relatively minor role in metal sorption (Mehta and Gaur 2001).

Binding mechanisms. The process of biosorption includes a number of mechanisms, like ion exchange, complexation, electrostatic attraction and microprecipitation. The most significant of these has been proved to be ion exchange. It was observed in Spirulina platensis, that during the binding of Cu²⁺, $Pb^{\scriptscriptstyle 2+}\!\!\!,~Zn^{\scriptscriptstyle 2+}\!\!\!,~Ni^{\scriptscriptstyle 2+}$ and $Cr^{\scriptscriptstyle 3+}$ protons were liberated from biomass, which leads us to the conclusion of ion exchange. We also observed the release of Cu²⁺ and Ca²⁺ by algal biomass while binding Zn²⁺ and Ni²⁺ ions. All these studies suggest that the exchange of metal ions with surface bound protons or cations play a significant role. in heavy metal sorption, though this varies among algal species. We reported the maximum ion exchange capacity (in term of Na+/H+ exchange) of five algal species ranging from 41 – 825 μ eg g⁻¹ dry weight (Mehta and Gaur 2001). Large variability in ion exchange may be due to cell composition, meaning unicellular algae has a higher surface/volume ratio than filamentous forms, and therefore a higher ion exchange capacity. This capacity can be increased by increasing the pH level. However, this mechanism is not the only one and it can be combined with others. In a study of Sargassum vulgaris it was reported that Cd binding involved chelation, while he binding of Pb consisted of ion exchange and chelation. In the adsorption of Ni and Zn on Chaetophora elegans it was observed that electrostatic attraction and covalent binding played a role. In view of the complexity of the composition of algal surfaces, it is possible that various mechanisms operate simultaneously to varying degrees depending on algal species and environmental conditions (Mehta and Gaur 2001). If they are not attached to cell wall, metal ions may get inside the cell and bind to intracellular components or precipitate. In algae cells metals can be detoxified. Their activity is impacted by biological macromolecules and enzymes that are being accumulated in polyphosphate bodies and in intracellular metal binding proteins. In Ankistrodesmus falcatus, Sn has been part of about 85 % of cellular polysaccharide fraction, and 15 % in the protein fraction. In some eukaryotic algae, metal solutions were observed within the vacuoles (Mehta and Gaur 2001).

Factors of binding. Results may be affected by several factors, including concentration of metal and biomass, pH, temperature, contact time, presence of competing ions and the metabolic stage of the organism (Mehta and Gaur 2001).

Concentration of metal. Initial concentration of metal ions in the solution is a significant factor of biosorption ability. Biosorption initially increases as the initial concentration of metal ion increases up to the optional concentration. It was reported, that in dry biomass of Scenedesmus obliqus, a 5-fold increase in initial concentrations of ${\rm Zn}^{\scriptscriptstyle 2+}$ (from 10 to 50 ppm) boosted the metal ion sorption from 19 to 209.6 mg Zn^{2+} /g. Based on that, the biosorption capacity is increased, but the removal yields of the metal ion are reduced. Therefore, the higher the metal ion concentration, the lower the efficiency and removal yield would be. In another study, Chlorella vulgaris in concentrations of 2.5 ppm were able to remove 69 % and 80 % of $\rm Ni^{2+}$ and $\rm Cu^{2+}$ cations. When the concentration was increased up to 10 ppm, the metal removal rate was reduced to 37 % and 42 %(Zeraatkar et al. 2016). Getting to know proper concentration is a necessity for efficient growth of algae. Higher rates can inhibit growth or lead to cell and cell structure destruction. For some algae, heavy metals can be beneficial and enhance their growth, while to the others, heavy metal may be toxic at all concentrations. In Cladophora fracta, continual increase of lead and cadmium exposure resulted in total chlorophyll content loss, reduced number of chloroplasts, and disintegrated cell walls responsible for cell death and reduced cell growth. Algae have better sorption abilities at lower concentration, because the presence of any excessive toxicity in algal bodies is harmful and can lead to cell destruction (Zeraatkar et al. 2016).

pH level. The majority of studies show that biosorption of metal ions is highly dependent on the pH of the solution. Functional groups on cell wall membranes do not have same features at every pH, and therefore it was important to deduce the optional pH for heavy metal removal. The sorption of Cr6+ and Cd on Padina sp. and Sargassum sp. and Cs sorption on Padina australis was optimal at pH 2. In a study of Durvillaea potatorum, very little sorption of Cu was observed at a pH below 2, but this sorption increased with a rise in pH. They found maximum Cu sorption between pH 3 and 4, and a plateau was reached at around pH 5. There are numerous studies showing increased metal sorption with increasing pH of the solution. It was suggested that the optimum pH for Cu and Cd sorption by Laminaria japonica and Sargassum kjellmanianum lies between 4 and 5, while the maximum Cr^{3+} sorption capacity of Sargassum sp. was observed at pH 4. The majority of metal binding groups on cell walls are acidic (e.g., carboxyl), and their availability is pH dependent. In acidic pH they generate a charged surface, and since the metal ions are at most charged positively, there is increase in their biosorption. However, at extremely acidic pH (< 2), metal sorption decreased. In general, for metal binding, an acidic pH is more efficient, although this pH level varies (1 - 7) (Mehta and Gaur 2001). It has been clearly shown that algae prefer acidic or slightly acidic environments. However, this prefer-

ence is also dependent on which metal is bonded. The optimum sorption pH of cadmium, nickel and zinc was 6. For copper, the value ranged between 4 and 5, while optimal pH for the sorption of lead was between 3 and 5, based on the type of biomass. The pH value rose between brown algae, through red algae to green algae. Within higher pH values the metal uptake capacity did not show significant differences (Romera *et al.* 2006).

Temperature. Temperature affects each algae species differently based on the type of metal to which they are exposed. It is known that metal sorption capacity is a function of temperature. There are several reasons why increasing temperature causes an increase in metal ion biosorption. Increased temperature results in an increased number of active metal binding sites with an increased tendency. Additionally, reduction in mass transfer resistance in the diffusion layer is caused by a reduction of the thickness of the diffusion boundary layer around the adsorbent groups. Along with temperature changes, a complex formation constant that assists metal binding also changes. On the other hand, some studies confirmed higher rate with lower temperature, while others confirmed no significant effect of indicative temperature on biosorption ability. Similarly, several studies have determined temperature-linked changes in metal ion uptake by living algal cells (Zeraatkar et al. 2016). These reported a slight increase in cation sorption by powdered seaweed biomass with an increase in temperature from 4 to 55°C. Similarly, was recorded increased Ni²⁺ biosorption by dried biomass of Chlorella vulgaris with an increase of temperature from 15 °C (48.1 mg/g) to 45 °C (60.2 mg/g). This suggests that heavy metal biosorption is most likely an endothermic process, though it has also been suggested that the effect of temperature on sorption is not as certain as that of pH (Mehta and Gaur 2001).

Time. Heavy metal ion biosorption is highly dependent on contact time. Biosorption occurs in two stages. First, for algal biomass, metal ions are passively adsorbed into cell membranes, and biosorption of metal ions occurs rapidly within the first minute. Second, for live algae, active biosorption occurs as the algal cell slowly uptakes the HM ions (Salama *et al.* 2019). Biosorption of uranium by non-living *Chlorella vulgaris* achieved more than 90 % dissolved uranium adsorb during the first 5 min. In other studies, with biomass of *Chlamydomunas reinhardtii*, microalgae rapidly ad-

sorbed free ions biosorption and equilibrium was achieved in 60 min. All these examples show that biosorption of heavy metal ions is passive and occurs relatively rapidly. Higher rates of accumulation occurred with less contact time. In separately harvested *Cladophora fracta*, after 2, 4, 6, and 8 days of Cd and Hg exposure it was found that while the algal growth rate decreased over time, a greater biosorption capacity was obtained in older cultures. These results suggest that while passive heavy metal biosorption commences swiftly in the first moments of contact, a greater level of heavy metal bio removal can be achieved with longer contact times using living algae (Zeraatkar *et al.* 2016).

Our main objective in this work was to follow the annual cycles of some heavy metal pollutants and their abundance in aquatic ecosystems. Salt as well as freshwater algae have been used in the past for bioremediation of heavy metals and the results of these studies have been valuable. We sought to determine how the Javorinka stream changes over the course of the year and which elements fluctuate over time. Secondly, we examined what types of algae live in the aquatic foothills, whether they increase or decrease in volume, and whether biosorption capacity changes through the seasons.

Material and Methods

Sample collection

Samples are collected from attached algae in the mountain stream Javorinka. Two collection sections were chosen based on the findings of previous studies.

The first section is a segment of river situated between two villages, Tatranská Javorina and Podspády (Fig. 1). It is about 3 km long. The altitude range is between 930 - 970 m a.s.l The stream bed consists of large and small granitic boulders and occasionally limestone. Water in this section is fast moving and rough, with many small, but deep lagoons that serve as an excellent habitat for fish and other aquatic organisms. Due to its dynamic nature, algae coverage of the rocks is sparse and for the most part of the year only microscopic or foliose algae grow. This section is marked as upper stream.

The second section is situated near the village of Vojtasová (N 49.292550° , E 20.169038°). In this section, the stream is significantly milder and slower. Depth is consistent over the length of



Fig. 1. Profile of downstream section (Photo: J. Tuchyňa 2021).



Fig. 2. Rough nature of upstream (Photo: J. Tuchyňa, 2021).

Accumulation of heavy metals by algae and cyanobacteria the river. Altitude here varies between 850 - 865 m a.s.l. Algae growth occurs at a higher rate and tends to contain macroscopic algae. This section is marked as downstream (Fig. 2).

Sample preparation and laboratory analyses

Following sample collection, all invertebrates and large soil particles are removed. Afterwards they are left to dry naturally prior to further processing. In next step we crushed dried samples into fine dust using a cryo-mill (which mills each sample for 40 seconds). Following this, we measured element concentrations using a ED-XRF spectrometer DELTA (Olympus, Innov-x Systems, USA). In approximately 13 minutes results are available. For data analysis, Statistica 8 (StatSoft, USA) software was used. The comparison of individual elements conducted using the nonparametric Kruskal-Wallis test, because variances were highly variable between groups. Values with p < 0.05 were considered to be statistically significant. The potential synergic effect of elements was evaluated by principal component analysis (PCA), which is widely used in ecotoxicological studies. It is a variable reduction technique that maximizes the amount of variance accounted for in the observed variables by a smaller group of variables called components or factors.

From all the elements we chose 3 model elements with which we will work more extensively – chromium, zinc, and lead (Cr, Zn, Pb).

During our work we also had to learn from scientific literature, where we mainly focused on algae biology and their processes throughout their lifespan. The algae species were identified with the professional assistance of Mgr. Alica Hindáková, PhD.

Studied species

Hydnuns foetidus (Fig. 3) is a cold-water golden alga (Chrysophyceae) found in fast-flowing rivers mainly during periods of snowmelt. *Hydnunus* is a rheophile, preferring swiftly flowing water. It is also a psychrophile, dependent on low temperature. *Hydnunus* appears to be of specific importance for the early emerging aquatic larvae of chironomids. *Hydnunus* thrives well under seasonal climatic conditions, where there is snow in the winter, cold meltwater, and moderate summer temperatures. Its early emergence during snowmelt makes it visually prominent (Klaveness 2019).

Diatoma ehrenbergii (Fig. 4) are a species of the class bacillariophyceae. It is almost always present in fibers of *Hydrurus foetidus*.

Valves are narrow and elongate becoming lanceolate in smaller valves. Apices are broadly rounded sub-rostrate to capitate. Costae are mostly primary, and number 9 - 15 in 10 μ m. Secondary and tertiary costae are commonly present. Frustules are attached to the substratum or joined in zig-zag colonies by mucilage (Kalina and Váňa 2010).

Oscillatoria sp. (Fig. 5). Cyanobacteria can easily handle sudden physical and chemical alterations of light, salinity, temperature, and nutrient composition. These organisms are very efficient for sewage water treatment, remediation of aquatic and terrestrial habitats, chemical industries, industrial



Fig. 3. Hydrurus foetidus (Photo: J. Tuchyňa, 2021).



Fig. 4. Diatoma Ehrenbergii (Photo: J. Tuchyňa, 2021).



Fig. 5. Oscillatoria sp. (Photo: J. Tuchyňa, 2021).

effluents, etc. In addition, organic solutions, nitrate and phosphate compounds, carbon dioxide or alkalinity are important factors in determining the distribution of cyanobacteria (Dubey *et al.* 2011).

Five different cyanobacteria groups were studied (*Oscillatoria* sp., *Synechococcus* sp., *Nodularia* sp., *Nostoc* sp. and *Cyanothece* sp.). All of them were superior degraders and removers of pollutants. They were monitored in 2, 4 and 7 day periods. During the 2^{nd} day, the majority of samples (with the exception of one) were between 92 % and 99.7 % effective at removing pollutants. At their fastest rate, these species could remove almost 99 % of present pollutants while showing high resistance against toxicity (Dubey *et al.* 2011).

Results

In this study we conducted several tests to better understand the seasonality of accumulation in this stream. All our tests could register some changes in the aquatic environment; therefore, we can say that algae and cyanobacteria are suitable for ecotoxicological studies.

There is a trend of higher concentrations at the downstream location compared to the upstream location. This was demonstrated by almost all elements which can be influenced by different dynamics of each part of the stream as well as other physical properties of the stream. Out of all the studied elements, four in particular were notable because of their significant variation between the two localities (Figs. 6 and 7). These significant elements were detected by the KW-H test with a p-value of < 0,05. At the downstream location, mean values were almost double of those upstream. Those elements were Ti, Mn, Fe and Ba.

Changes year to year (Table 1, Fig. 8) between most of the elements where either insignificant, or slight.

Regarding seasonal accumulation, most of the elements exhibited the highest concentrations in summer (Figs. 9 - 17). With the exception of Sr, Ca, and S, every other element showed a significant increase in concentration during summer. In case of Fe (Fig. 11) the rise was almost double the spring values. On the other hand, standard deviation was also relatively high, which could attribute this increase to flash floods and resulting transport of elements and their compounds. Granite bedrock is covered in Fe (OH)₂ and due to flash floods, it is released and adsorbed by algae and cyanobacteria.

During the summer season the level of water in Javorinka stream is generally low and the density is high. Elements are much more concentrated than in any other season.

Altogether we did three univariate statistics and one multivariable statistic (Table 2). We compared how they reacted together throughout the study and if we could find any patterns or interdependency. We chose 5 of 13 factors with a variance greater than 3 %. In the first pattern we can see mutual growth in S, K, Ti, Cr, Mn, Fe, Zn, Rb, Zr, Cd, Ba and Pb. Its variance is 51 % (the highest observed). The second factor is a function where the mutual decrease of S and K affects growth in Sr.

| | Year | Mean \pm SD (n) | KW-H | р |
|----|------|-------------------------|------|-------|
| S | 2019 | 8,636 ± 15,883.5 (10) | | |
| | 2020 | 3,931 ± 4,243 (25) | 0.48 | 0.79 |
| | 2021 | 3,182 ± 1,479 (24) | | |
| Cl | 2019 | 750.77 ± 717.22 (9) | | |
| | 2020 | 494.8 ± 325.82 (15) | 0.58 | 0.74 |
| | 2021 | 403.27 ± 134.69 (16) | | |
| K | 2019 | 13,271 ± 15,006 (10) | | |
| | 2020 | 12,366 ± 10,963 (25) | 0.26 | 0.88 |
| | 2021 | 11,263 ± 6,523 (24) | | |
| Ca | 2019 | 17,845 ± 13,968 (10) | | |
| | 2020 | 34,660 ± 28,282 (25) | 9.68 | 0.008 |
| | 2021 | 54,636 ± 42,408 (24) | | |
| Ti | 2019 | 1,034 ± 932 (9) | | |
| | 2020 | $1,654 \pm 1,481$ (24) | 2.16 | 0.34 |
| | 2021 | 1,823 ± 1,422 (24) | | |
| Cr | 2019 | 153 ± 127.97 (10) | | |
| | 2020 | 108.6 ± 57.39 (25) | 2.45 | 0.29 |
| | 2021 | 157.59 ± 120.76 (24) | | |
| Mn | 2019 | 394.9 ± 328.43 (10) | | |
| | 2020 | 317.96 ± 324.19 (25) | 0.69 | 0.71 |
| | 2021 | 304 ± 243.18 (24) | | |
| Fe | 2019 | $10,034 \pm 8,101$ (10) | | |
| | 2020 | 16,084±16,636 (25) | 1.88 | 0.39 |
| | 2021 | 17,792 ± 16,932 (24) | | |
| Cu | 2019 | 18.5 ± 8.31 (6) | | |
| | 2020 | 20.38 ± 5.88 (9) | 1.31 | 0.52 |
| | 2021 | 24 ± 13.54 (9) | | |
| Zn | 2019 | 63.6 ± 47.59 (10) | | |
| | 2020 | 71.76 ± 48.16 (25) | 0.99 | 0.61 |
| | 2021 | 69.45 ± 41.33 (24) | | |
| Rb | 2019 | 20.61 ± 10.37 (10) | | |
| | 2020 | 32.228 ± 29.71 (25) | 0.84 | 0.66 |
| | 2021 | 28.42 ± 22.21 (24) | | |
| Sr | 2019 | 88.35 ± 70.93 (10) | | |
| | 2020 | 105.572 ± 62.37 (25) | 2.22 | 0.33 |
| | 2021 | 121.58 ± 69.01 (24) | | |
| Zr | 2019 | 89.36 ± 94.76 (9) | | |
| | 2020 | 116.26 ± 101.47 (24) | 1.24 | 0.54 |
| | 2021 | 118.60 ± 78.61 (24) | | |
| Ba | 2019 | 1,218 ± 2,189 (10) | | |
| | 2020 | 363.33 ± 265.35 (24) | 0.87 | 0.65 |
| | 2021 | 391.83 ± 236.87 (24) | | |
| Pb | 2019 | 17.2 ± 5.73 (10) | | |
| | 2020 | 20.77 ± 6.99 (25) | 3.94 | 0.14 |
| | 2021 | 23.75 ± 9.87 (24) | | |

Table 1. Mean values concentrations (\pm SD) of the measured elements in algae from mountain stream Javorinka during the individual years of the research. Mean concentration values are given in ppm.(KW-H - Kruskal-Wallis test, significant differences are in bold p < 0.05).

Accumulation of heavy metals by algae and cyanobacteria



Fig. 6. Mean (\pm 95 c. limits) concentrations of barium in dependence on sample locations. The levels were significantly higher downstream than upstream.



Fig. 7. Mean $(\pm 95 \text{ c. limits})$ concentrations of iron in dependence on sample locations. The levels were significantly higher downstream than upstream.

| | Factor 1 | Factor 2 | Factor 3 | Factor 4 | Factor 5 |
|------------------|-------------|-------------|-------------|-------------|-------------|
| S | 0.245 | 0.800 | 0.458 | 0.076 | -0.117 |
| K | -0.627 | 0.568 | 0.476 | 0.058 | 0.111 |
| Ti | -0.941 | -0.074 | 0.007 | 0.162 | -0.095 |
| Cr | -0.673 | 0.153 | -0.123 | -0.515 | -0.308 |
| Mn | -0.808 | 0.222 | -0.200 | 0.137 | 0.064 |
| Fe | -0.972 | 0.094 | -0.070 | 0.044 | 0.039 |
| Zn | -0.718 | 0.219 | -0.466 | 0.076 | 0.303 |
| Rb | -0.875 | 0.007 | 0.154 | 0.183 | 0.292 |
| Sr | -0.288 | -0.574 | 0.643 | -0.296 | 0.175 |
| Zr | -0.742 | -0.428 | 0.251 | 0.299 | -0.072 |
| Ва | -0.629 | -0.139 | -0.076 | -0.367 | 0.291 |
| Pb | -0.680 | -0.266 | -0.005 | 0.296 | -0.519 |
| Variance in % | 51.5 | 13.3 | 9.4 | 7.1 | 5.9 |

In the third factor, decrease in S, K and Sr results in the growth of Zn level. In the fourth factor, Cr and Ba grow together. The last factor was mainly described by mutual growth of Pb and Cr with an antagonistic effect on Ba and Rb.



Fig. 8. Mean (\pm 95 c. limits) concentrations of calcium depended on year. The levels were significantly higher by each year.



Fig. 9. Mean $(\pm$ 95 c. limits) concentrations of barium depended on season of collection.



Fig. 10. Mean (\pm 95 c. limits) concentrations of chromium depended on season of collection.

Other factors were not seasonally dependent. Factor 2 of a mutual decrease of S and K, while Sr values growth was not affected by seasonality (KW-H (3,54) = 0.5, p = 0.91). Factor 3, which described an accumulation of Zn while S, K and Sr decreased was not affected by seasonality (KW-H (3,54) = 6.18, p = 0.27). Factor 4 of mutual accumulation Cr and Ba did not depend on seasonality (KW-H (3,54) = 3.89, p = 0.27). Factor 5 of accumulation Pb and Cr while antagonistically decreasing Rb and Ba was not affected on seasonality (KW-H (3,54) = 0.82, p = 0.85). Only

- J. Tuchyňa



Fig. 11. Mean (\pm 95 c. limits) concentrations of iron depended on season of collection.



Fig. 12. Mean (\pm 95 c. limits) concentrations of potassium depended on season of collection.



Fig. 13. Mean (\pm 95 c. limits) concentrations of manganese depended on season of collection.

factor 1 showed any dependence on any of variables, namely that the summer season is a period of intense uptake of elements (Fig. 19).

Discussion

Many elements exhibited variation between years. Some elements tended to decreased (e.g., S, Cl, K, ...) while others increased (e.g., Ca, Ti, Pb, ...). Notable changes throughout the years were found only in Ca (KW-H = 9.68, p = 0.0079). The concentration of Ca in the stream experienced constant



Fig. 14. Mean (\pm 95 c. limits) concentrations of lead depended on season of collection.



Fig. 15. Mean (\pm 95 c. limits) concentrations of rubidium depended on season of collection.



Fig. 16. Mean (\pm 95 c. limits) concentrations of titanium depended on season of collection.

growth. Grounds for this trend are likely the flash floods, which expose the bedrock and constantly weather the rocks. These trends could also be a direct consequence of global warming and more extreme changes in weather.

Occasional elements

Although we have measured many elements, not all were detectible in tests. Some of them were unable to be detected at all, due to their very low concentration. Among these were Co, Ag and Hg. Accumulation by algae showed a high affinity toAccumulation of heavy metals by algae and cyanobacteria

9



Fig. 17. Mean (\pm 95 c. limits) concentrations of zinc depended on season of collection



Fig. 18. Mean (\pm 95 c. limits) concentrations of zircon depended on season of collection.



Fig. 19. Mean (\pm 95 c. limits) of principal component rates on factor 1 mutual concentration of K, Ti, Cr, Mn, Fe, Zn, Rb, Zr, Ba, Pb highly depended on seasons. The elements accumulated in algae mainly in summer (KW-H (3,54) = 15.8, p = 0.0013).

wards Hg by log K' MS (mean conditional stability constant of M-sites of algae) with a value of 18.0 \pm 0.3 (Vasconcelos and Leal 2001). Based on this we can assume that mercury short-term concentrations in Javorinka stream are not high. Other elements were found in some samples, but certainly not often. Some of them were studied more extensively but most remained unused. P, Se, Mo, Sn, Sb were only found in a few samples and were not used in any of the tests. Ni and As, as well as Cu and Cl were detected in more samples, but not in sufficient amounts for all our tests.

There was some seasonality associated with phosphorus, with the highest values in algae in summer and spring (Francoeur *et al.* 1999). The detectable values were mostly found between June to August, though phosphorus was also present in May and September. We obtained more values from the downstream location due to the slower stream.

Nickel has its highest accumulation in algae in autumn months (Haritonidis and Malea 1995; Villares *et al.* 2002). The most common were values present during late summer and autumn months, from July to November. However, the most significant results occurred between August and October.

Astatine was detected throughout the year in relative equality. It was more abundant in July and August, but this might be attributable to the higher density of the stream. Astatine is a radioactive and highly unstable element with a minute natural abundance (Al-Adilah *et al* 2022; Sturges and Barrie 1988), therefore its values are often more-or-less random.

Selenium was found only between December and March. Organic selenium in water is at its peak in late summer, but total selenium exhibits maximum concentrations in winter (Sherrard *et al.* 2004). We were able only to detect selenium during winter months, due to the significant seasonal growth during that period.

Molybdenum was only present in summer, between June and August, presumably due to a higher density of elements in the stream during this period. We only found tin 5 times out of 59 samples, and most often in January samples.

Antimony follows the same cycle patterns as selenium (Pelachaty *et al.* 2004) and most of the measurable values were detected in late autumn, winter, and early spring, (October to March). Neither Cu nor Cl had any visible seasonal pattern in their accumulation.

Univariate statistics

Concentration of elements in relation to the location. We tested 17 elements and their mean concentration depended on the locality of sample collection (downstream and upstream). In general, uptake of elements was higher at the downstream location, with the exception of Zn and Ni. Four elements (Ba, Fe, Mn, Ti) exhibited significant variation between localities. Their concentration nearly doubled at the downstream location compared to the upstream location. Standard deviation varied among all 17 elements. Some had higher deviation at the downstream location, while others had higher deviation at the upstream location.

Uptake of barium is related to its concentration in the environment; the higher the concentration the higher the uptake. *Oscillatoria* sp. were able to remove 10 % of Ba in the medium. Cell size or pH of the cultures did not affect this accumulation (Fisher *et al.* 1991). Brown and green algae have many alginate groups with a high affinity to barium. Accumulation takes place through ion exchange. This process occurs rapidly at first and then takes time to reach its equilibrium (Fontao *et al.* 2020). Barium has a minimal affinity toward minerals (Dong *et al.* 2003) that leave its compounds unattached. Additionally, natural levels of Ba are the result of bleaching and eroding of sedimentary rocks (Fontao *et al.* 2020).

Higher uptake of Ba at the downstream location was presumably caused primarily by differences in physical properties of the stream, lower velocity and by bleaching of limestone and slates. With lower velocity and higher abundance of Ba in the water, there was more time and enough element for algal cell walls to react with and reach equilibrium of biosorption.

Algae accumulate iron mainly in Fe²⁺ form, the concentration of which is controlled by the solubility of FeCO₃, Fe (OH)₂ and FeS. Most of it is connected to the cell wall (Mahasneh 1988). Sources of iron in surface waters are primarily from bedrock and soil, (i.e., granite and granodiorite) (Erel *et al.* 1990; Romanescu *et al.* 2016).

The granite bedrock of Javorinka river may contribute to the higher concentration of iron at the downstream location. Continual weathering of these rocks creates higher concentrations at lower localities.

Accumulation of manganese is mostly processed by the cell wall, where it is absorbed by alginate structures. It is an element present in the Earth's core and is naturally occurring in the environment. Anthropic sources include fertilizers, mining, metal manufacturing industries (Kaonga et al. 2008), and municipal sewage (Moore 2012). When algae is exposed to Mn pollution it can exhibit higher values depending on velocity of the stream. In seasons where stream had lower velocity, its values increased up to $4.203 \text{ (ppm)} \pm 0.805$. During seasons with higher velocity at both locations, its concentration was only 1.438 (ppm) \pm 0.196 (Kaonga et al. 2008). Other studies, such as Scott et al. (2002), came to the conclusion that, in lower localities less manganese was accumulated in the stream than in higher localities. This is most likely due to plant uptake. Manganese accumulation takes place in two phases, rapid and slow. Its rapid phase can absorb up to 25 % of accumulated metal (Garnham et al. 1992).

Titanium, similarly to other metals, transfers within aquatic environment in oxides form, which has a high sedimentary rate (Kulacki *et al.* 2012). Therefore at lower velocity, it begins to sediment at higher rates (Lin *et al.* 2015). Similarly to other metals, titanium uptake undergoes 2 phases, rapid and slow (Kulacki *et al.* 2012). Most of the elements did not differ based on whether the algae were collected in higher located and faster moving parts of the stream, or at a lower and slower locality. Variation was only detected in Ba, Fe, Mn and Ti. This could also be related to lower velocity, varying transfer processes in aquatic environment, and different physical properties of water in lower lying areas.

Concentration of elements between years.

Calcium was the only element that showed significant growth over the year. Calcium is dependent on bedrock and parental geology. Weathering of the soil and rocks helps maintain natural levels of calcium in the environment (Ohta *et al.* 2018). In July of 2018, the High Tatra experienced an extensive flood which resulted in a great decrease in both nutrients and sediments in water environments (Hrivnáková et al. 2020). According to Meurant (2012), the concentration of calcium does not decrease immediately after a flood decreases, contrary to other elements. Flooding does not affected its cycles significantly. Calcium concentration mainly affects plant uptake, discharge, and bedrock. According to Munn and Meyer (1990), the lowest uptake of calcium occurred in gravelly soils, whereas high uptake of calcium occurred at rock outcrops during early spring. Higher flushing rates through the sediments may result in a less stable and less productive epibenthic community, resulting in a lower nutrient demand.

Our results suggest that higher concentration of calcium in the stream is likely due to the massive flood that occurred in 2018. Flood exposed and accumulated bedrock results in flushing calcium through the stream. Continual erosion of the riverside and its sediments exacerbates this phenomenon and the concentration of calcium in the stream grows.

Almost all of elements (S, K, Ca, Ti, Cr, Mn, Fe, Zn, Rb, Sr, Zr, Ba and Pb) that we studied using this test had significant seasonality variance (p < 0.05), except S, Ca and Sr. Other seasonal patterns were noted, but note were as distinct as the first. S and Ca were the only elements to experience a peak during autumn, while the rest of elements were recorded at their peak during summer.

Other studies (Skriptsova 2016; Imbs et al. 2009; Villbrandt et al. 1991) proved that the highest uptake of elements in water streams occurs during summer or early autumn. In summer, season algae acquire mature reproductive organs. Intensive growth and development of reproductive structures causes an intense uptake of nutrients (Skriptsova 2016). Gross primary production in summer months ranged from 5.8 to 11.3 g O_2/m^2 per day during the summer and algal respiration from 1.9 to 2.7 g O_2/m^2 per day. Photosynthesis/respiration ratios depending on month were ranging from 1.59 in December to 5.04in May (Stockner 1968). Physical characteristics, such as temperature and irradiation affect these processes to a significant extent. Warmer water during summer months, and higher irradiance increase the tendency of the cell wall structures that absorb metals (Zeraatkar et al. 2016). According to our results 7, out of 10 elements were metals, which confirm their higher affinity for the algae cell wall during summer.

In terms of space-time these critical phenomena for heavy metal accumulation in algae and cyanobacteria seem to occur during the summer season. During this time of intense growth, they are absorbing elements (K, Ti, Cr, Mn, Fe, Zn, Rb, Zr, Ba and Pb) and nutrients from their surroundings, including both biogenic as well as non-biogenic elements (Pb). In summer, higher contamination of water and the necessity of element uptake for the growth of reproductive structures occur.

The smallest variance in values was observed during winter. Many elements are stored in snow and ice in the mountains. Thus, the composition of water in colder months is more stable and there is no disturbance present. Accumulation of heavy metals by algae and cyanobacteria Synergic accumulation depended on season

The results clearly indicate significant variance based on season, mainly during summer. Synergic effects proved a notable increase in accumulation of K, Ti, Cr, Mn, Fe, Zn, Rb, Zr, Ba and Pb during the season. An increased tendency of cell wall structures (Zeraatkar *et al.* 2016) and the need for growth of algae and their reproductive organs enhances metal uptake during this period (Skriptsova 2016).

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References

- Al-Adilah, H., Feiters, M.C., Carpenter, L.J., Kumari, P., Carrano, C.J., Al-Bader, D. and Küpper, F.C. 2022: Halogens in seaweeds: Biological and environmental significance. *Phycology*, **2**: 132-171.
- Bellinger, G.E. and Sigee, D.C. 2015: Algea as bioindicators. Freshwater algea: Identification, Enumeration and Use as Bioindicators. John Wiley & Sons.
- Dokulil, M.T. 2003: Algae as ecological bio-indicators. In: Trace Metals and other Contaminants in the Environment, Volume 6, Bioindicators & Biomonitors, Principles, Concepts and Applications, (eds. B.A. Markert, A.M. Breure and H.G. Zechmeister), pp. 285-327. Elsevier.
- Dong, D., Li, Y., Zhang, J. and Hua, X. 2003: Comparison of the adsorption of lead, cadmium, copper, zinc and barium to freshwater surface coatings. *Chemosphere*, **51**: 369-373.
- Dubey, S.K., Dubey, J., Mehra, S., Tiwari, P. and Bishwas, A.J. 2011: Potential use of cyanobacterial species in bioremediation of industrial effluents. *Afr. J. Biotechnol.*, **10**: 1125-1132.
- Erel, Y., Morgan, J.J. and Patterson, C.C. 1991: Natural levels of lead and cadmium in a remote mountain stream. *Geochim. Cosmochim. Acta*, 55: 707-719.
- Fisher, N.S., Guillard, R.R.L. and Bankston, D.C. 1991: The accumulation of barium by marine phytoplankton grown in culture. J. Mar. Res., **49**: 339-354.
- Fontao, N.C., Hackbarth, F.V., Mayer, D.A., Mazur, L.P., de Souza, A.A.U., Vilar, V.J.P., Guelli, U. and de Souza, S.M.A. 2020: A step forward on mathematical modeling of barium removal from aqueous solutions using seaweeds as natural cation exchangers: batch and fixed-bed systems. J. Chem. Eng., 401: 6-10.
- Francoeur, S.N., Biggs, B.J., Smith, R.A. and Lowe, R.L. 1999: Nutrient limitation of algal biomass accrual in streams: seasonal patterns and a comparison of methods. J. North Am. Benthol. Soc., 18: 242-260.
- Garnham, G., Codd, G. and Gadd, G. 1992: Kinetics of uptake and intracellular location of cobalt, manganese and zinc in the estuarine green alga *Chlorella salina*. *App. Microbiol. Biotechnol.*, **37**: 270-276.
- Haritonidis, S. and Malea, P. 1995: Seasonal and local variation of Cr, Ni and Co concentrations in *Ulva rigida* C. Agardh and *Enteromorpha linza* (Linnaeus) from Thermaikos Gulf, Greece. *Environ. Poll.*, **89**: 319-327.

- Hrivnáková, K., Janiga, M. and Pogányová, A. 2020: Effects of flooding on the physical and chemical water composition of the alpine lake Kolové pleso (High Tatra, West Carpathians). *Oecologia Montana*, **29**: 23-27.
- Chmielewská, E. and Medved, J. 2001: Bioaccumulation of heavy metals by green algae *Cladophora glomerata* in a refinery sewage lagoon. *Croat. Chem. Acta*, **74**: 135-145.
- Imbs, T.I., Shevchenko, N.M., Sukhoverkhov, S.V., Semenova, T.L., Skriptsova, A.V. and Zvyagintseva, T.N. 2009: Seasonal variations of the composition and structural characteristics of polysaccharides from the brown alga *Costaria costata*. *Chem. Nat. Compd.*, **45**: 786-791.
- Kalina, T. and Váňa, J. 2005: Heterokontophyta. In: Sinice, řasy, houby, mechorosty a podobné organismy v současné biologii. Karolinum, Praha.
- Kaonga, C.C., Chiotha, S.S., Monjerezi, M., Fabiano, E. and Henry, E.M. 2008: Levels of cadmium, manganese and lead in water and algae; *Spirogyra aequinoctialis*. *Int. J. Environ. Sci. Tech.*, **5**: 471-478.
- Klaveness, D. 2019: *Hydrurus foetidus* (Chrysophyceae): an update and request for observations. *Algae*, **34**: 1-5.
- Kulacki, K.J., Cardinale, B.J., Keller, A.A., Bier, R. and Dickson, H. 2012: How do stream organisms respond to, and influence, the concentration of titanium dioxide nanoparticles? A mesocosm study with algae and herbivores. *Environ. Toxicol. Chem.*, **31**: 2414-2422.
- Lin, M., Tseng, Y.H. and Huang, C.P. 2015: Interactions between nano-TiO₂ particles and algal cells at moderate particle concentration. *Front. Chem. Sci. Eng.*, **9**: 242-257.
- Mahasneh, I.A. 1988: Iron accumulation by blue-green algae from saline environments. PhD. Thesis, Durham University, UK, 19-22.
- Mehta, S.K. and Gaur, J.P. 2005: Use of algae for removing heavy metal ions from wastewater: progress and prospects. *Crit. Rev. Biotechnol.*, **25**: 113-152.
- Meurant, G. 2012: Flooding and Plant Growth. Academic Press, London, UK.
- Moore, J.W. 2012: Inorganic contaminants of surface water: research and monitoring priorities. Springer-Verlag, New York.
- Munn, N.L. and Meyer, J.L. 1990: Habitat-specific solute retention in two small streams: An intersite comparison. *Ecol.*, **71**: 2069-2082.
- Nriagu, J.O. and Nieboer, E. 1988: Chromium in the natural and human environments. John Wiley & Sons, Ontario, Canada.
- Ohta, T., Shin, K.C., Saitoh, Y., Nakano, T. and Hiura, T. 2018: The effects of differences in vegetation on calcium dynamics in headwater streams. *Ecosystems*, 21: 1390-1403.
- Pelachaty, M., Pelachata, A., Niedzielski, P., Siepak, M. and Sobczyński, T. 2004: Analysis of the spatial and seasonal variability of inorganic species of arsenic, antimony and selenium in a Shallow Lake subjected to moderate anthropopressure. *Pol. J. Environ. Stud.*, **13**: 185-190.
- Pereira, S., Micheletti, E., Zille, A., Santos, A., Moradas-Ferreira, P., Tamagnini, P. and De Philippis, R. 2011: Using extracellular polymeric substance (EPS) - producing cyanobacteria for the bioremediation of heavy metals: do cations compete for the EPS functional groups and accumulate inside the cell. *Microbiology*, **157**: 451-458.
- Romanescu, G., Miftode, D., Pintilie, A.M., Stoleriu, C.C. and Sandu, I. 2016: Water quality analysis in mountain freshwater: Poiana Uzului Reservoir in the Eastern Carpathians. *Rev. Chim.*, **67**: 2318-2326.
- Romera, E., González, F., Ballester, A., Blázquez, M.L. and Munoz, J.A. 2006: Comparative study of biosorption of heavy metals using different types of algae. *Bioresour. Technol.*, **98**: 3344-3353.
- Salama, E., Roh, H., Dev, S., Khan, M.A., Abou-Shanab, R.A.I., Chang, S.W. and Jeon, B. 2019: Algae as a green technology for heavy metals removal from various wastewater. World J. Microbiol. Biotechnol., 35: 1-19.
- Scott, D.T., McKnight, D.M., Voelker, B.M. and Hrncir, D.C. 2002: Redox processes controlling manganese

fate and transport in a mountain stream. *Environ. Sci. Technol.*, **36**: 453-459.

- Sherrard, J.C., Hunter, K.A. and Boyd, P.W. 2004: Selenium speciation in subantarctic and subtropical waters east of New Zealand: trends and temporal variations. *Deep-Sea Res. I: Oceanogr. Res. Pap.*, **51**: 491-506.
- Skriptsova, A.V. 2016: Seasonal variations in the fucoidan content of brown algae from Peter the Great Bay, Sea of Japan. *Russ. J. Mar. Biol.*, **42**: 351-356.
- Stockner, J.G. 1968: Algal growth and primary productivity in a thermal stream. J. Fish. Res. Board Can., 25: 2037-2058.
- Sturges, W.T. and Barrie, L.A. 1988: Chlorine, bromine and iodine in arctic aerosols. *Atmos. Environ.*, **22**: 1179-1194. Vasconcelos, M.T.S.D. and Leal, M.F.C. 2001: Seasonal

variability in the kinetics of Cu, Pb, Cd and Hg accu-

mulation by macroalgae. Mar. Chem., 74: 65-85.

- Villares, R., Puente, X. and Carballeira, A. 2002: Seasonal variation and background levels of heavy metals in two green seaweeds. *Environ. Poll.*, **119**: 79-90.
- Villbrandt, M., Krumbein, W.E. and Stal, L.J. 1991: Diurnal and seasonal variations of nitrogen fixation and photosynthesis in cyanobacterial mats. *Plant and Soil*, **137**: 13-16.
- Wong, P.T.S. and Chau, Y.K. 1990: Zinc toxicity to freshwater algae. *Toxicity Assessment*, **5**: 167-177.
- Zeraatkar, A.K., Ahmadzadeh, H., Talebi, A.F., Moheimani, N.R. and McHenry, M.P. 2016: Potential use of algae for heavy metal bioremediation, a critical review. *J. Environ. Manage.*, **181**: 817-831.

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Biology of alpine accentor (*Prunella collaris*) VII. Mountain tourism, climbing and hiking – a cause of drastic synanthropy in alpine accentors in the last 200 years

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Abstract. This study briefly describes the history of synanthropy in a high-altitude bird species, *Prunella collaris.* A list of food items that synanthropic individuals seek in garbage in the mountains is presented. Possible influences on behavioural changes and physiology of this protected species are discussed. The large number of synanthropic individuals of this species is evidence of how the development of alpine tourism in Europe over the last 200 years has seriously affected the life of alpine fauna.

Key words: Prunella collaris, bird synanthrophy, hiking and climbing

Introduction

The family Prunellidae is a relatively old family of songbirds. The group appears to split from outgroup families approximately 14.8 million years ago, in the middle Miocene (Drovetski et al. 2013). The divergence of the large Prunella collaris and Prunella himalayana group and other small accentors occurred during the late Miocene, approximately 7.31 million years ago according to Drovetskii et al. (2013) or 9.1 million years ago according to Liu et al. (2017). Ancestral area reconstructions indicate a Himalayan region origin breeding north of the Qinghai-Tibet Plateau. Later, new areas were facilitated during glacial periods, when suitable accentor habitats expanded west to east, and then subsequently northward from the Plateau and the great Asian mountain ranges (Liu et al. 2017). P. collaris and P. himalayana diverged from each other approximately 3 - 4 million years ago during the late Pliocene, in either the Himalayan region or the central Palearctic (Drovetski et al. 2013; Liu et al. 2017). The sympatric species in the Sino-Himalayan Mountains have divergent habitat preferences, with P. collaris residing at higher altitudes than the others, in alpine habitats. Most speciation events in accentors occured before the Pleistocene, but climatic oscillations during that period may have

substantially modified their distribution. Liu *et al.* (2017) suggest that colonization of new areas by accentors was facilitated during glacial periods, when they expanded away from the great Asian mountain ranges across low-elevation areas, followed by the fragmentation of suitable habitats during interglacial periods. In the period 115,000 BP - 10,000 BP, the alpine accentor was already widespread in Euroasia, as well as in Europe, with a number of isolated populations in different mountain ranges, late Pleistocene fossils also exist from the lowlands between mountainous regions (Tyrberg 1991).

Recent studies show that alpine accentors can detect human activity in winter very guickly (Janiga 2021), therefore, it may be that the first associations between ancient humans and these birds occurred in Central Asia as early as 200 to 300 thousand years ago, at the time of the Denisovans. The age of Central Asian Denisovan fossils is estimated at 280,000 vears (Jeenbaev 2007). Denisovans were likely adapted to life in the high mountains, where alpine accentors also lived. Living at high altitudes is hard. The air there carries less oxygen, making it harder to breathe and causing a host of problems; but modern inhabitants of Tibet have adapted. Many studies have linked their altitude adaptation to several genes including EPAS1; part of the system that helps the body react to low levels of oxygen. Huerta-Sánchez et al. (2014) discovered that genetic adaptation in modern Tibetans has its roots in Denisovans and that gene flow from this extinct group is found to have had important consequences. Alpine accentors may also have come into contact with Heidelberg or Neanderthal man in Europe. For example, many Würmian fossil records of alpine accentors are from SW Europe and link the populations in the Pyrenees, Massif Central and the Alps. Some records also exist from Greece (Tyrberg 1991).

Following the arrival of modern humans as many as 50,000 years ago (Svoboda 2014; Soukup 2015), most of the early relationships between larger birds and humans were ones of predation. In the case of small bird species, however, the relation was more of a commensalism, with birds visiting human settlements mainly in winter. Especially in the Gravettien, human populations became more and more settled with sufficient food sources in the mammoth steppe. After the onset of the Neolithic – Agricultural Revolution, significant changes also occurred in the biology of many wild bird species, including alpine accentors.

Humans began to impact the original biota in a number of ways, including: the institution of agri-

14 *M. Janiga* culture, storing of foodstuffs, modification of surface of terrestrial ecosystems (including wetlands, lakes and rivers), use of fire, fragmentation of wild nature, and the introduction of invasive or alien organisms. In late autumn, winter or early spring, some alpine accentors are commonly found close to farms, and they exploit feeding opportunities offered by this association with agriculture (Schiferli et al. 1980; Del Hoyo et al. 2005), such as feeding in barns, close to livestock, or nearby mountain chalets (Fig. 1). The muscular gizzard and crop that characterize acentors are adaptations to cope with their seeddominated autumn and winter diet. The process of digestion of seeds is further assisted by the consumption of grid. Therefore, some alpine accentor individuals are commonly found close to human habitation in winter. In this period of intense medieval pastoralism, birds undoubtedly found food in the vicinity of haylofts or stables sheltering sheep, cows or horses. Such phenomena persist in suitable conditions today, and accentors are often seen at horse farms, where they will search for and collect seeds and grain from horse manure (Géroudet 1957; Dyrcz 1976; Šťastný and Hudec 2011). Millet or wheat, for example, has been found in the stomach of birds caught in winter (Kovshar 1966).

A radical change in the relationship of alpine accentors to humans has occurred in Eurasia over the last 200 - 300 years with the development of alpine tourism, which is seriously impacting breeding sites of this species. In the past, a chalet in the alpine area was mainly used by shepherds as a shelter for themselves and their flocks. Herders often visited the mountains to pasture their livestock and store milk and butter. This was often the practice with herders in all Eurasia. During the winter months, chalets were usually left unattended because herders would take their livestock and products to the flatlands. As time went on, outdoor enthusiasts started traveling to these mountains for holidays and the herders gave out their homes as shelter. Then as more people started visiting the alpine for holidays, the herders discovered the market for these vacationers (Fig. 1). More so, this was also largely the advent of mountain travel, leading to a boom in tourism. The most important buildings of this type in the High and Low Tatras were built in the 19th and 20th centuries: 1841 - Skalnatá chata,

1883 – Ťatliakova útulňa, 1893 – touristic path in Veľká Studená dolina (Great Cold Valley), 1895 -Chata pri Zelenom plese, 1899 – Téryho chata, 1932 - Chata Plesnivec, 1933 - Chata Rysy. In 1920, one of today's most popular valleys, Žiarska dolina, was almost unvisited, with the mountain cottage there completed in 1939. In 1936 work began on the cableway to Skalnaté pleso and in 1949 the cableway reached one of the highest summits of the Tatras, Lomnicky Peak. In 1964 the cableway transported 270,000 people. In 1949 - 1950 the construction of the cableway to Chopok summit began the development of the largest ski resort in Jasná. The history of the development of alpine summer and winter resorts in the Alps dates back about 100 years earlier than in the Carpathians, and a boom in the construction of mountain resorts in the Tian Shan mountains is currently underway. More recently, the market for chalets in Eurasia is even stronger than that of hotels, and one can often rent luxurious private accommodation with many amenities. Chairlifts, cable cars and these accommodations allow many more tourists to visit high mountain valleys or accesses mountain peaks. However, as a result a huge amount of biological waste and litter is left behind. The impact of tourism greatly affects the lifestyle of alpine accentor, as these birds scavenge this waste throughout the year (Géroudet 1957; Praz 1976; Martí et al. 1988), often congregating close to winter centres and hotels (Hudec 1983; Cramp 1988; Heer and Fraenkel 1999) or around skilift stations during winter (Dick and Holupirek 1978; Janiga 2020). The birds can also be found looking for food left by hikers on the tops of mountains or in camps for climbers during the summer months (Martín-Vivaldi et al. 1995) (Fig. 2). Sometimes relatively few sites (mountain huts and refuges) may highly influence the life of accentors in several valleys (Janiga 1998). Accentors regularly and actively forage on food leftovers of hikers (Delestrade 1995; Henry 2011). As accentors often consume cakes, fat or bread on mountain tops, feeding on rubbish can be expected to have an adverse effect on their general body condition (Dyrcz and Janiga 1997). The aim of this study is to provide a summary of the food that these birds feed on in human-influenced environments. The findings come from both the summer and winter seasons.



Fig. 1. Switzerland. Left For many alpine accentors, seasonal movement to lower elevations introduces them to more heavily vegetated habitats and often brings them into cultivated areas, where they are commonly associated with mountain villages and livestock. Right: As more people started visiting the alpine, herders discovered the market for these vacationers, and birds discovered new sources of diet (e.g., garbage left in the wake of tourists) (Photo: M. Janiga, 2013).

15 Synanthropy in alpine accentors



Fig. 2. Left: Racek Mountain Camp, Ala Archa National Park, Kyrgyzstan. Alpine accentors feed on biological waste. The waste was stored in barrels behind the mountain hut. Right. Rozsutec summit, The Malá Fatra National Park, the West Carpathians, Slovakia. A young alpine accentor feeds on bread. In an undisturbed environment, the parents feed the chicks with insects; after flying out of the nest, the fledglings learn to hunt insects and thus to be predators. If the parents feed them bread, the animals' foraging behaviour changes, potentially resulting in lower survival rates. The physiology of individuals is also affected by changing nutrition based on this artificial diet (Photo: M. Janiga, 2012 - left, 2017 - right).

Material and Methods

As noted in many of our previous studies, alpine accentors are the subject of long-term research at the Institute of High Mountain Biology. The first data on this bird species was systematically collected in 1984, and various types of research have continued to the present day. In addition to systematic data collection, we have had many opportunities to observe many other phenomena in the life of this species. The results herein are the summary of longterm observations of accentor feeding patterns on garbage near mountain summits and near mountain chalets, hotels and huts. The data mainly originate from the West Carpathians, but observations from the Alps, Pyrenees, Apennines, Tian Shan, Balkan mountain range, and Krkonoše are also included. The observations date from 1984 to the present.

Results

An overview of the diet of alpine accentors offered by people is presented in Table 1. Alpine accentors usually arrive at their wintering grounds in November, and if they find places without snow, they forage for grass seed. They seek anthropogenic food sources particularly in times of snowfall, frost, snowstorms, or heavy snow cover. In cases of bad weather, one individual can eat up to 7.5 g of poppy seeds or 3 g of bread in the morning. Of these anthropogenic foods, birds prefer poppy seeds to bread, bread to biscuits, and biscuits to cheese. In the case of poppies, they make approximately 40 to 60 pecks per minute. During sunny winter days, birds actively hunt for insects (flies) under the roofs or along the windows of hotels. In March, they visibly switch to foraging for animal food.

Discussion

Organisms that cohabit with humans may be called synanthropic. Many species leave habitats modifieds by human activity. Some experienced populations decline, and a few have been extirpated. However, a good number of other species of birds accommodated to habitat modification. The existence of such synanthropic is no secret, but the degree to which the birds of a continental avifauna use habitats modified by humans has not been historically and comprehensively evaluated until recently. Species of early ecological succession and those known to be habitat generalists are likely to be synanthropic in at least part of their distribution. A large proportion of Eurasian birds could likely be designated as synanthropic, a distinction probably owing to a longer period of exposure to land use by humans. The enormous number of feeding associations of birds with other taxa, including primates, suggests the possibility that, in the past, birds foraged with some of our human and pre-human ancestors (see Introduction). Thus, evolution has played a role in bird-human feeding associations and the associations of birds with humans might be an example of very ancient symbiosis (Haeming et al. 2015). The alpine accentor is no exception; its winter occurrence has long been observed on castles (Ferianc 1979), roads (Boehme 1926), or in towns (Schmidt 1985).

However, fundamental changes in the life of alpine accentors and other mountain bird species occurred following the Industrial Revolution with the start of construction of hotels and restaurants beginning to occur in the alpine. Cable cars and chairlifts allowed ever greater concentrations of people on summits. The result is huge amounts of litter at mountaineering campsites, on high mountain peaks or at mountain huts and hotels. Birds frequently visit these sites, particularly outdoor serving areas to feed on scraps and leftovers (Heer 1998; Janiga 2020). Such summer and autumn food supplementation could have deleterious impacts. It may result in an unbalanced diet and consequently to changes in phenotypic structures of breeding populations (Jones and Reynolds 2008; Robb et al 2008: Plummer et al 2013a). The food list in Table 1 confirms that the alpine accentors seek out bread scraps (Fig. 3), chocolate and sweet biscuits, sausage, bacon, and butter. A large body of scientific literature, as well as the experience of songbird breeders, warns that these food sources are not healthy for wildlife. They can cause disease, mouth and throat injuries, and even death. Songbirds must be very ef-

| 16 M. Janiga | Food |
|------------------------|-------------------------|
| | Breads, rolls a buns |

| Food | Jan | Feb | Mar | Apr | Мау | June | July | Aug | Sep | Oct | Nov | Dec | Sum |
|------------------------------------|-----|-----|-----|-----|-----|------|------|-----|-----|-----|-----|-----|-----|
| Breads, rolls and buns | 9 | 7 | 3 | 1 | 2 | | 4 | 5 | 8 | 3 | 4 | 16 | 62 |
| Chocolate and sweet biscuits | 6 | 14 | 4 | 1 | | | | 1 | | 1 | | 7 | 34 |
| Sausage, salami, bacon | | 2 | 2 | | 1 | | 2 | 1 | | | | | 8 |
| Butter, bread and butter | | 3 | 5 | 1 | 1 | | | | | | | 2 | 12 |
| Poppy seed rolls, poppy | | 16 | | | | | | | | | 4 | 13 | 33 |
| Rice, hotel garbage | | | 7 | | | | | | | | | | 7 |
| Cheese, milk products | | | | | | | | | | | | 2 | 2 |
| Synathropic insect on hotels | | 7 | 23 | 1 | | | | | | | 2 | | 33 |
| Melted grassy spots near hotels | 1 | 6 | 5 | 6 | | | | | | | 2 | 2 | 22 |

Table. 1. Anthropogenic food of alpine accentors. The numbers indicate the number of times a particular bird was observed feeding on a given food source. They depend on the possibility of daily field visits as well as the possibility of food identification. Numbers therefore characterize qualitative food sources; they do not represent seasonal changes in foraging. Identified food sources in the months November to April come from the winter ski resort of Malinô Brdo, observations from May to October come from mountain peaks and chalets. Data on the capture of synanthropic insects by birds and on the change of their diet in places with melted snow and exposed grass come only from the Malinô Brdo ski resort. To identify the problem of synanthropy in alpine accentors, observations must be multiplied by the huge number of days and the number of individuals that visit feeding sites and consume anthropogenic food every day in summer, autumn or winter.



Fig. 3. Left: A female alpine accentor collecting bread leftovers after tourists on Malolučniak summit, High Tatras, Polish – Slovakian border. The female was bringing bread to her young. When young wild animals learn to depend on humans for food, they become less experienced in foraging and consequently less likely to survive. Right: Furthermore, wild birds that are accustomed to food provided by humans commonly lose their fear of people (Photo: M. Janiga, 2021).

ficient eaters due to rapid metabolism and the small size of their stomachs. For example, bread robs birds of the opportunity to take in the true nourishment their bodies require to survive. Bread has zero nutrition for wild birds. It is heavily processed and contains chemicals and preservatives that are not suitable for wild birds. Bread contains very little protein, calcium, phosphorus, or other vitamins and trace minerals (Burt et al 2021), which birds need to develop muscles and feathers. As a carbohydrate-rich food, these bread products do not contain sufficient fat, and while it will provide momentary satiety, does not provide much nutrition (Burt et al. 2021). This is a deadly combination of factors, considering how many calories birds need to eat in order to survive. The same is true for similar bread-like products such as buns, bagels, crackers, chips, pretzels, cookies, cereal, and donuts. For a small bird, this can lead to tragedy very quickly.

It can freeze to death overnight, with its stomach full of bread. Foods such as chips, cookies, and crackers are reported as terrible food choices for birds because they are full of trans fats and crammed full of sugar and salt. Bacon drippings are another dangerous diet to offer wild birds. Bacon is full of nitrates, salt and other additives which are very harmful to wild birds' health. Fresh milk products or chocolate are also not a good food for wild birds because birds are lactose intolerant. Small chocolate residues can be fatal to a bird, due to the theobromine and caffeine.

They cause a bird digestive distress as well and heart and breathing difficulties. These snippets of information from veterinary literature and guides for songbird enthusiasts are just a very brief overview of what anthropogenic food can do to birds in the high mountains. Moreover, feeding a bird can change its normal behaviour and cause it to lose Synanthropy in alpine accentors

its natural fear of humans and pets (Fischer and Miller 2015). A food-conditioned animal is likely to approach other people looking for food (Clark et al. 2015). Birds can become dependent on artificial food sources, less able to survive on their own (Plummer et al. 2013b) and pass important survival skills onto their young (Fig.3).

Humans are influencing food supply for birds directly by providing feeders, but also indirectly via waste treatment and through the creation of urban spaces at the expense of natural habitats (Chace and Walsh 2006). In this study, the important findings on the effects of food supplementation on alpine accentor ecology were highlighted. It can be estimated that between 10 and 30 percent of the alpine accentor population may be fed in this way during the summer and autumn months in the Tatra mountains. Perhaps this phenomenon may be corrected only by different feeding preferences among individual birds (Janiga and Novotná 2006). It is known that some accentors visit places with plenty of anthropogenic food frequently, while others do so only sporadically (Heer 1998). However, many young birds can be observed on summits collecting food from humans (Fig. 2). The distance they must travel to do so are likely farther than previously imagined. This indicates that the effects of supplementary feeding on alpine acccentors are likely to extend far beyond just one mountain valley. Thus, learning and information transfer between generations can be a serious phenomenon that threatens the population stability of this protected species in the high mountains (Janiga 1998). As incidental supplementary provisioning of wildlife is hugely prevalent and increasing in these habitats, this may have large and widespread ramifications for biodiversity conservation in many mountain national parks, and we urge caution upon policy makers in national parks to ensure hygiene and enforce maintenance of tourist destinations in affected areas.

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References

- Arntzen, J.W., Goudie, I.B.J., Halley, J., Jehle, R. and Boehme, L.B. 1926: The birds of North Ossetia and Ingoushia (with the adjoining regions). Bulletin Scientifique de l'Institut de l'Exploration Regional du Causase du Nord, 1: 1-274.
- Burt, S.A., Vos, C.J., Buijs, J.A. and Corbee, R.B. 2021: Nutritional implications of feeding free-living birds in public urban areas. J. Anim. Physiol Anim. Nutr., 105: 385-393. Chace, J.F. and Walsh, J.J. 2006: Urban effects on native
- avifauna: a review. Landsc. Urban Plan., 74: 46-69.
- Clark, D., Whitney, J.J., MacKenzie, K.G., Koenen, K.K.G. and DeStefano, S. 2015: Assessing gull abundance and food availability in urban parking lots. Hum.-Wildl. Interact., 9: Article 7.
- Cramp, S. (ed.) 1988: Handbook of the birds of Europe the Middle East and North Africa. The birds of the Western Palearctic. Volume V. Tyrant Flycatchers to Thrushes. Oxford University Press, Oxford, New York.

Del Hoyo, J., Elliott, A. and Christie, D. (eds.) 2005: Hand-

book of the birds of the world. Volume 10. Cuckooshrikes to Thrushes. Lynx Editions, Barcelona.

- Delestrade, A. 1995: Impact of human activity on foraging flocks and populations of the alpine chough Pyrrhocorax graculus. Avocetta, 19: 189-193.
- Dick, W. and Holupirek, H. 1978: Über die Alpenbraunelle auf dem Territorium der DDR. Falke, 25: 308-312.
- Drovetski, S.V., Semenov, G., Drovetskaya, S.S., Fadeev, I.V. Reďkin, Y.A. and Voelker, G. 2013: Geographic mode of speciation in a mountain specialist avian family endemic to the Palearctic. Ecol. Evol., 3: 1518-1528. Dyrcz, A. 1976: Contributions to the biology of Alpine Ac-
- centor. Notatki Ornitologiczne, 17: 79-92.
- Dyrcz, A. and Janiga, M. 1997: Alpine Accentor Prunella collaris. In: The EBCC atlas of European breedingbirds: their distribution and abundance (eds. E.J.M. Hagemeijer and M.J. Blair), pp. 510. T & AD Poyser, London.
- Ferianc, O. 1979: Vtáky Slovenska 2. Veda, Bratislava. Fischer, J.D. and Miller, J.R. 2015: Direct and indirect effects of anthropologic bird food on population dynamics of a songbird. Acta Oecol., 69: 46-51.
- Géroudet, P. 1957: L' Accenteur alpin. In: Les Passereaux III, pp. 49-54. Delachaux et Niestlé, Neuchätel, Swiss. Haemig, P.D., Sjöstedt de Luna, S., Blank, H. and Lundqvist,
- H. 2015: Ecology and phylogeny of birds foraging at outdoor restaurants in Sweden. Biodivers. Data J., 3: e6360.
- Heer, L. 1998: The polygynandrous mating system of the Alpine Accentor Prunella collaris, indivdual reproductive tactics, breeding adaptations on high mountain conditions and winter ecology. PhD thesis, Institute of Zoology, University of Bern.
- Heer, L. and Fraenkl, A.C. 1999: Zur Verbreitung, sozialen Organisation, Raum- und Habitatnutzung der Alpenbraunelle Prunella collaris in Winter. Orn. Beob., 96: 25-36.
- Henry, P.-Y. 2011: Differential migration in the polygynandrous Alpine Accentor Prunella collaris. Bird Study, 58: 160-170.
- Hudec, K. (ed.) 1983: Fauna ČSSR. 23. Ptáci Aves. Díl III/1. Academia, Praha.
- Huerta-Sánchez, E., Jin, X., Asan, Bianba, Z., Peter, B.M., Vinckenbosch, N., Liang, Y., Yi, X., He, M., Somel, M., Ni, P., Wang, B., Ou, X., Huasang, Luosang, J., Cuo, Z.X.P., Li, K., Gao, G., Yin, Y., Wang, W., Zhang, X., Xu, X., Yang, H., Li, Y., Wang, J., Wang, J. and Nielsen, R. 2014: Altitude adaptation in Tibetans caused by introgression of Denisovan-like DNA. Nature. 512: 194-197.
- Janiga, M. 1998: Diet, the reason of the first steps of synanthropy in high altitude birds. In: Proceedings of the VII International Congress of Ecology, 19-25 July 1998, (eds. A. Farina, J. Kennedy and V. Bossú), pp. 206. Firenze, Italy.
- Janiga, M. 2020: The biology of the Alpine accentor Prunella collaris. IV. Maintenance activities and their clusters from late autumn to early spring. 20 year observation. Oecologia Montana, 29: 28-38.
- Janiga, M. 2021: Biology of alpine accentor (Prunella collaris) VI. Interspecific relationships among alpine accentors and other species of birds during winter. Oecologia Montana, 30: 85-99.
- Janiga, M. and Novotná, M. 2006: Feeding preferences and foraging behaviour in the Alpine Accentor Prunella collaris. Ornis Fenn., 83: 170-180.
- Jeenbaev J.J. (ed.) 2007: Problems of mountainous countries (by the example of Kyrgyzstan). Threats and challenges of modern times. National Academy of Sciences of the Kyrgyz Republic, Bishkek.
- Jones, D.N. and Reynolds, S.J. 2008: Feeding birds in our towns and cities: a global research opportunity. J. Avian Biol., 39: 265-271.
- Kovshar, A.F. 1966: [Birds of Talas Alatau.] Kainar, Alma Ata (in Russian).
- Liu, B., Alström, P., Olsson, U., Fjeldsa, J., Quan, Q., Roselaar, C.S., Saitoh, T., Yao, C.-t., Hao, Y., Wang, W., Qu, Y. and Lei, F. 2017: Explosive radiation and spatial expansion across the cold environments of the Old World in an avian family. Ecol. Evol., 7: 6346-6357.

- Martí, R., Gómez-Manzaneque, A. and Perales, J.A. 1988: Invernada del acentor alpino (*Prunella collaris* Scop.) en los altos niveles de la Sierra de Gredos (Sistema Central). Ardeola, **35**: 143-146.
- Martín-Vivaldi, M., Marín, J.M. and Villar, M. 1995: Seleccion de habitat, tamaño de bando y movimientos locales del accentor alpino (*Prunella collaris*) en Sierra Nevada (se de España). *Ardeola*, **42**: 11-30.
- Plummer, K.E., Bearhop, S., Leech, D.I., Chamberlain, D.E. and Blount, J.D. 2013a: Fat provisioning in winter impairs egg production during the following spring: a landscape-scale study of blue tits. J. Anim. Ecol., 82: 673-682.
- Plummer, K.E., Bearhop, S., Leech, D.I., Chamberlain, D.E. and Blount, J.D. 2013b: Winter food provisioning reduces future breeding performance in a wild bird. *Sci. Rep.*, **3**: 1-6.
- Praz, J.C. 1976: Notes sur l, Accenteur alpin *Prunella collaris* dans le Val d' Hérens (Valais). *Nos Oiseaux*, **33**: 257-264.

- Robb, G.N., McDonald, R.A., Chamberlain, D.E. and Bearhop, S. 2008: Food for thought: supplementary feeding as a driver of ecological change in avian populations. *Front. Ecol. Environ.*, 6: 476-484.
- Schifferli, A., Géroudet, P. and Winkler, R. 1980: Verbreitungsatlas der Brutvögel der Schweiz/Atlas des oiseaux nicheurs de Suisse. Schweizerische Vogelwarte/ Station ornithologique suisse, Sempach.
- Schmidt, E. 1985: The Alpine Accentor (*Prunella collaris*) in Hungary. Aquilla, 92: 105-111.
- Šťastný, K. and Hudec, K. (eds.) 2011: Ptáci Aves. III/1. Academia, Praha.
- Svoboda, J.A. 2014: Předkové. Evoluce člověka. Academia, Praha.
- Soukup, V. 2015: Prehistorie rodu Homo. Karolinum, Praha.
- Tyrberg, T. 1991: Arctic, montane and steppe birds as glacial relicts in the Western Palearctic. *Orn. Verh.*, **25**: 29-49.

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Variation in the accumulation of chemical elements in the bones of chamois during their two-year exposure in the field

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Abstract. The accumulation of chemical elements in the bones of Tatra chamois (Rupicapra rupicapra tatrica) in Tatranská Javorina in the area of the Belianske Tatras were studied. Over a two year period, bones taken from the same site were sampled four times. During each sampling, chemical elements were measured, and then the bones were put back in their original position. The site was at an altitude of 1000 m a.s.l. We investigated the effect of time on the concentration of elements in the bones of deceased chamois. It is clear from our research that the elements behave differently over time. It can be said that if bone samples are taken directly from the field, the results may differ due to element exchanges, mainly between bones, precipitation water and air. Elements like P, Cr, Mn, Fe, Zn, Rb, Sr, Mo, Sb, Ba, and Pb tended to increase or do not change concentrations in the bones over time, while concentrations of Ca, K, Cl decreased.

Key words: bones, chamois, Tatras, elements, measurement, XRF spectrometry

Introduction

Elements can be stored in the bones of an animal throughout its life (Ballová *et al.* 2019). Bone of a dead animal is a material that can absorb trace elements from the surrounding soil and act as a funnel for these trace elements. However, it appears that bone content does not always match soil content (for some elements), suggesting that bone may be affected by factors other than soil, such as moisture, porosity, pH, reactive conditions, climate and time (Krajcarz 2019). Therefore, the information obtained will not be reliably usable in other conditions in which the experiment was performed.

The concentration of elements in the bones of dead wild ruminants reflects the environmental

conditions in which these animals lived, as well as post-mortem changes in the levels of the elements. It has been shown that the content of heavy metals increases with altitude and heavy metals can have adverse effects on animal health. We therefore consider ruminants living in alpine ecosystems to be a good indicator of environmental contamination by heavy metals from the atmosphere (Ballová *et al.* 2019). Increased pollution in our environment has exposed flora and fauna to the harmful effects of toxic substances (Wavita 2018) and this pollution can also be seen in the study of bones from dead individuals. Heavy metals that can be observed in bones include: lead, mercury, arsenic, cadmium, nickel, or chromium.

This study focuses on assessing the impact of the natural external environment over a relatively short time (approximately 2.5 years) on the concentration of elements in the bones of dead Tatra chamois (Rupicapra rupicapra tatrica). The influence of the environment on the concentrations of elements in the bones can manifest itself. for example, as a chemical change in the main component of the bone; trace metal sorption; and adhesion of fine dirt particles to the bone surface (López-Costas et al. 2016). Furthermore, a large number of microcracks are likely to increase bone fragility, which provides space for the accumulation of elements (Jans et al. 2002). When the bones are warm, the dry environment affects them almost immediately. Exposure to a temperature-controlled environment results in dramatic changes in the internal composition of the elements in the bones, but much more slowly than is shown for bones exposed to warmer conditions (Karr and Outram 2015).

Material and Methods

Study area

The experiment was carried out using freshly prepared bones (ribs and pelvic bones) from Tatra chamois (*Rupicapra rupicapra tatrica*), which died in the winter of 2017/2018 and were stored in freezers at a temperature of -70° C. The site where the bones were stored is located on the land belonging to the Institute of Hight Mountain Biology (IHMB), at an altitude of 1000 m a.s.l within an alder (*Alnus*) forest. It is a place affected by different conditions and

Breceli. S. M. Janiga, Z. Kompišová Ballová & G. Chovancová

the climate is closest to the conditions of a deciduous forest, though it is comprised of mountain alder floodplain forests with gray alder (Alnus incana). This type of floodplain forest occurs along mountain streams and at springs up to 1200 m a.s.l. Throughout our research, all the bones were placed at the same location, at the coordinates: N 49.266262°, $E 20.141956^{\circ}$. The samples were placed at the site during the summer (Fig. 1a) and winter seasons (Fig. 1b). Bones were labeled using a sterile bandage to allow precipitation and climatic conditions to affect it. All bones were placed in a mesh to protect them from predators.

Sample preparation and laboratory analyses

Samples from Tatra chamois (n = 30) used for this study came from naturally deceased Tatra chamois found in their natural habitat (1600 - 2100 m a.s.l.) during the winter season (2017/2018). Animals were recovered by the staff of the State Forests of Tatra National Park. We obtained bone samples by dissecting these dead chamois. Subsequently, we removed the pelvis and rib bones from each individual. After dissecting the chamois bones, we cleaned off as much soft tissue as possible. The chamois bone samples included 30 pelvic bones and 30 rib bones, so that together there were 60 bone samples. At the beginning of the experiment, we cut a small piece of each bone sample using a hand saw. The remains of the bones were placed in mesh in the alder forest, where they were affected by environmental conditions. At certain intervals from October 2019 to December 2021 (October 2019, May 2020, June 2021, December 2021), we examined the next piece of each bone. We obtained a total of 4 samples from each bone during the 2.5 years of the experiment. These bone pieces were dried in a laboratory Incubator IF 160 Plus (Memmert, Germany) at 80 °C for 8 hours.

Subsequently, these dried samples were ground using a cryomill (Retsch, Germany) at a frequency of 30 oscillations per second for 50 seconds, to a powder. Powder samples were continuously analyzed immediately after sawing for elemental concentrations using a hand-held XRF spectrophotometer Delta (Olympus, Innov-x Systems, USA). Measurements run in closed Delta XRF Portable WorkStation. Samples were analyzed in plastic cuvettes with plastic foil at the bottom for 240 s in three 80 s intervals, from which the average was calculated. A spectrometer was calibrated for bones by using certificated reference Bone Meal standard (SRM 1486, Maryland). The detection limits were determined continuously for each measurement and for each element by software using Compton Normalization method.

Statistical analyses

The results processed by the XRF spectrometer were stored in an Excel spreadsheet. Subsequently, statistical analyses were performed with Statistica 12 software for Windows (Stat Soft CR, Prague, Czech Republic). Elements that had many values below the detection limit of the XRF spectrophotometer were excluded from statistical analyses: sulfur, titanium, cobaltum, niccolum, cuprum, arsenicum, selenium, zirconium, argentum, cadmium, stannum, hydrargyrum. Selected elements that we used in statistical analyzes were: P, Cl, K, Ca, Cr, Mn, Fe, Zn, Rb, Sr, Mo, Sb, Ba, Pb. Because the distribution of observed levels of elements between groups was non-normal according to the Shapiro-Wilk test, we used non-parametric tests. Differences in elemental concentrations in the two bone types (pelvis and rib) between different months of exposure were compared using the non-parametric Kruskal-Wallis test. Elemental concentrations were also compared between rib and pelvic bones using the Kruskal-Wallis test. As the concentration of elements in the pelvic and rib bones did not differ for most ele-





Fig. 1. a) Place of storage of samples in summer. b) Place of storage of samples in winter (Photo: S. Brecelj, 2019/2020).

21

Elements accumulations in chamois bones during field exposure ments other than Fe, both bone types were used in one cluster to investigate the differences in the concentration levels of elements in different sexes and in juvenile and adult individuals. These differences were analyzed using the least squares average method.

Results

Phosphorus. The level of phosphorus in the pelvis and ribs ranged from 40,000 to $80,000 \ \mu$ g/g. Its amount increased after exposure of the bone to the outdoor environment from 8 to 19 months. Phosphorus began to decline slightly 26 months after the bone was placed in the external environment. This means that phosphorus levels increased after the first and second winter periods (Fig. 2).

Chlorine. The amount of chlorine reaches a standard lower level immediately after the first winter and then did not change significantly for the remainder of the observation period (Fig. 3).

Potassium behaves in the same way as chlorine in both the ribs and the pelvis (Fig. 4). It is very likely that it leached out of the bones in the external environment in the form of salts (KCl). of calcium in the pelvis after the first winter with a subsequent increase following the second winter. At the end of 26 months, calcium was leached from the pelvis due to environmental conditions. Calcium in the ribs did not decrease after the first winter or after the second winter. Similarly to the pelvis, the calcium content increased after 19 months and then decreased after 26 months. The trend is not identical but in the time horizon after 19 months, it is similar to the variability of phosphorus in bones.

Calcium. Figure 5 shows a decrease in the amount

Chromium. After the first winter, the concentration of chromium in the ribs increased, but the concentration of chromium in the pelvic bones remained unchanged throughout the experiment (Fig. 6).

Manganese is also a biogenic element. The concentrations of this element in the ribs increased significantly during the experiment. There was also a slight increase in Mn in the pelvic bones, but this was not significant (Fig. 7).

Iron. The concentrations of iron did not change significantly for either type of bone (Fig. 8).

Zinc concentrations increased significantly in the pelvic and rib bones during the experiment (Fig. 9). The measured values ranged from 79 to $5604 \ \mu g/g$.



Fig. 2. Mean concentrations of phosphorus in the bones of Tatra chamois. Amount of phosphorus significantly increased in both types of bones from 8 to 19 months after the exposure (pelvis: KW-H (3,67) = 27.4, p = 0.00000, rib: KW-H (3,85) = 24.5, p = 0.00002).



Fig. 3. Mean chlorine concentrations in chamois bones. The amount of chlorine decreased in both bone types from 0 to 8 months after exposure (pelvis: KW-H (3,62) = 50.9, p = 0.0000, rib: KW-H (3,77) = 66.1, p = 0.0000).



Fig. 4. Mean potassium concentrations in chamois bones. The amount of potassium decreased in both bone types from 0 to 8 months after exposure (pelvis: KW-H (3,66) = 27.9, p = 0.0000, Rib: KW-H (3,84) = 20.2, p = 0.0002).



Fig. 5. Mean calcium concentrations in chamois bones (pelvis: KW-H (3,67) = 14.0, p = 0.0029, rib: KW-H (3,87) = 7.6, p = 0.0556).

22 S. Brecelj, M. Janiga, Z. Kompišová Ballová & G. Chovancová



Fig. 6. Mean chromium concentrations in chamois bones (pelvis: KW-H (3,67) = 6.3, p = 0.0958, rib: KW-H (3,84) = 32.1, p = 0.0000).



Fig. 8. Mean iron concentrations in chamois bones (pelvis: KW-H (3,67) = 8.5, p = 0.0368, rib: KW-H (3,84) = 5.3, p = 0.1499).

Rubidium. The course of accumulation of rubidium levels is different for the pelvic bones and for the rib bones as a whole. In the pelvic bones, the level of rubidium was lower at the beginning of our study than at the end at 26 months. In the rib bones, the rubidium level was higher at the beginning than at the end of the experiment (Fig. 10).

Strontium. Changes in strontium levels were not significant in the ribs or pelvic bones (Fig. 11).

Molybdenum. Molybdenum levels increased over time in both types of bone. In the pelvic bones, the level of molybdenum first decreased slightly (within 8 months) and then rose until the 26^{th} month, when it exceeded the original values. Similarly on the rib bones, the molybdenum levels dropped slightly until the 19^{th} month and then rose by the 26^{th} month (Fig. 12).

Antimony. Changes in antimony levels were not significant in the ribs or pelvic bones in different measured periods (Fig. 13).

Barium. The concentration of barium in the rib bone increased after 8^{th} and then after 26^{th} month. However, in the pelvis, changes in the concentration of this element were not significant (Fig. 14).

Lead. The concentration of lead rose significantly for two years, with a visible curved line. This means that the amount of lead in the bones constantly increased over time (Fig. 15).



Fig. 7. Mean manganese concentrations in chamois bones (pelvis: KW-H (3,84) = 7.2, p = 0.0658, rib: KW-H (3,84) = 17.3, p = 0.0006).



Fig. 9. Mean concentrations of zinc in the bones of chamois. The amount of zinc significantly increased in both types of bones from 0 to 26 months after the exposure (pelvis: KW-H (3,67) = 45.4, p = 0.0000, rib: KW-H (3,84) = 49.1, p = 0.0000).

Elements in the bones of dead chamois

Levels of element concentration in the pelvic and rib bones of chamois were not significant in most cases (Table 1). Higher levels of iron were measured in the rib bones compared to the pelvis.

Interesting differences were found between juveniles and adults older than 2 years, where the juveniles were compared before the first winter of survival. Juveniles had the same calcium content as adults (Table 1). However, they had also more biogenic elements in their bones (P, Fe, Zn) compared to adults. In addition, they had higher levels of metals such as Sr and Sb.

After excluding juveniles from the measured set, concentration of elements did not differ between sexes (Table 1).

Discussion

We found that the phosphorus in the bones of the Tatra chamois had values from 40,000 to 80,000 μ g/g. Hancock *et al.* (1989) have phosphorus data in their work ranging from 180,000 to 191,000 μ g/g in human archeological bones. Further, Zaichick *et al.* (2009) have values of 123,000 and 122,730 μ g/g in their work about rib bones in healthy humans. Farswan and Elements accumulations in chamois bones during field exposure



Fig. 10. Mean rubidium concentrations in chamois bones (pelvis: KW-H (3,67) = 16.3, p = 0.0010, rib: KW-H (3,84) = 28.9, p = 0.00000).



Fig. 11. Mean strontium concentrations in chamois bones (pelvis: KW-H (3,67) = 2.9, p = 0.4007, rib: KW-H (3,84) = 3.7, p = 0.3015).



Fig. 12. Mean molybdenum concentrations in chamois bones (pelvis: KW-H (3,51) = 7.1, p = 0.0681, rib: KW-H (3,75) = 16.5, p = 0.0009).

Nautiyal (1997) state that the phosphorus value in mountain soils can be from about 400 to 1400 μ g/g. Phosphorus is a strontium-related element and can enter the bones after death through snails that feed on these bones because there is up to 10,000 μ g/g of phosphorus in the slug mucus (Greistorfer *et al.* 2017).

Average chlorine in our bone samples ranged from approximately 100 to 4000 μ g/g. Kilburn *et al.* (2021) report a value of chlorine of about 200 μ g/g in archaeological human skeletal remains. In work



Fig. 13. Mean antimony concentrations in chamois bones. The amount of antimony did not differ among different periods of exposure. (pelvis: KW-H (2,9) = 2.2, p = 0.3316, rib: KW-H (3,19) = 2.7, p = 0.4428).



Fig. 14. Mean barium concentrations in chamois bones after different time of exposure of bones in the field (pelvis: KW-H (3,62) = 3.3, p = 0.3462, rib: KW-H (3,81) = 7.9, p = 0.0478).



Fig. 15. Mean lead concentrations in chamois bones (pelvis: KW-H (3,4) = 6.7, p = 0.0832, rib: KW-H (3,62) = 13.7, p = 0.0033).

by Blondiaux *et al.* (1992), on bones from the 6th century, case data are reported as: compact bone 213 μ g/g; trebacular bone 465 μ g/g; and new bone 373 μ g/g. Chlorine values of human archeological bones are reported as follows: 118.6 μ g/g, 127.7 μ g/g, 254.9 μ g/g, 683 μ g/g and 934 μ g/g (Edward 1990). Chlorine is an element that is absorbed by bone mainly during the life of the animal. Following death, or environmental exposure, the bones quickly lose chlorine to almost zero values (Piga *et al.* 2013).

24 S. Brecelj, M. Janiga, Z. Kom pišová Ballová & G. Chovancová

| Element | | Rb | Ca | Р | К | Cr | Mn | Fe |
|---------|-----------|---------------------------|-----------------------------------|-----------------------------|--------------------------|-------------------------|-----------------------------|-----------------------------|
| | Pelvis | 24.8 ± 6.8 (29) a | 266,147.2 ± 13,933.8 (29) a | 3167.8 ± 465.7 (29) a | 39.8 ± 8.4 (29) a | 30.6 ± 5.2 (29) a | 412.9 ± 101.9 (29) a | 138.4 ± 12.2 (29) a |
| | Ribs | 31.9 ± 7.1 (27) a | 235,778.0 ± 14,440.7 (27) a | 3323.0 ± 482.6 (27) a | 36.5 ± 8.7 (27) a | 35.4 ± 5.4 (27) a | 637.3 ± 105.6 (27) a | 179.4 ± 12.6 (27) b |
| Sample | Males | 21.4 ± 6.2 (32) a | 244,049.1 ± 13 649.4 (32) a | 3516.9 ± 447.1 (32) a | 35.9 ± 8.3 (32) a | 36.9 ± 5.1 (32) a | 554.0 ± 102.3 (32) a | 160.1 ± 12.3 (32) a |
| type | Females | 35.0 ± 8.0 (19) a | 260,346.6 ± 17,713.8 (19) a | 3049.8 ± 580.2 (19) a | 45.3 ± 10.8 (32)a | 27.2 ± 6.6 (19) a | 483.5 ± 132.8 (19) a | 155.5 ± 16.0 (19) a |
| | Adults | 31.6 ± 6.7 (32) a | 243,266.0 ± 13,550.0 (32) a | 2493.6 ± 342.4 (32) a | 31.4 ± 4.6 (32) a | 26.2 ± 2.0 (32) a | 372.7 ± 60.4 (32) a | 138.1 ± 9.8 (32) a |
| | Juveniles | 24.3 ± 9.4 (16) a | 242,675.3 ± 19,162.6 (16) a | 4122.3 ± 484.2 (16) b | 37.9 ± 6.5 (16) a | 32.4 ± 2.7 (16) a | 576.1 ± 85.5 (16) a | 186.6 ± 13.9 (16) b |
| Element | | Zn | Sr | Мо | Sb | Ba | Pb | Cl |
| | Pelvis | 182.9 ±13.4 (29) a | 4.2 ± 0.4 (23) a | 16.8 ± 1.6 (6) a | 104.0 ± 8.1 (27) a | 9.9 ± 1.0 (20) a | 2424.5 ± 606.0 (29) a | 2424.5 ± 606.0 (29) a |
| | Ribs | 182.4 ± 14.0 (27) a | 5.1 ± 0.5 (20) a | 15.5 ± 1.6 (6) a | 105.3 ± 8.4 (25) a | 12.0 ± 1.0 (22) a | 3680.9 ± 628.1 (27) a | 3680.9 ± 628.1 (27) a |
| Sample | Males | 179.3 ± 12.8 (32) a | 4.5 ± 0.4 (27) a | 16.2 ± 1.3 (10) a | 99.7 ± 8.0 (29) a | 11.4 ± 1.0 (23) a | 3253.3 ± 613.2 (32) a | 3253.3 ± 613.2 (32) a |
| type | Females | 191.5 ± 16.6 (19) a | 5.0 ± 0.6 (13) a | 16.0 ± 2.9 (2) a | 108.3 ± 9.9 (19) a | 10.5 ± 1.3 (14) a | 2882.7 ± 795.8 (19) a | 2882.6 ± 795.8 (19) a |
| | Adults | 176.7 ± 10.5 (32) a | 3.8 ± 0.3 (24) a | 14.9 ± 0.7 (8) a | 87.3 ± 5.1 (32) a | 9.5 ± 0.9 (21) a | 2539.3 ± 214.3 (32) a | 2539.3 ± 214.3 (32) a |
| | Juveniles | 236.9 ±14.8 (16) b | 5.7 ± 0.5 (12) b | 16.0 ± 1.1 (3) a | 118.1 ± 7.2 (16) b | 11.8 ± 1.0 (15) a | 2767.6 ± 303.1 (16) a | 2767.6 ± 303.1 (16) a |

Table 1. Least square means with standard errors of element concentrations ($\mu g/g$) in the bones of dead Tatra chamois (One-way ANOVA, different letters in a column denote the significant differences between groups, p < 0.05, number of samples is in the parentheses).

In our results, mean potassium in chamois bones has values of about 3000 μ g/g at the first measurement and later drops to almost zero. Work by Blondiaux et al. (1992), states that the bones from the 5^{th} and 6th century case contain compact bone values of 255 μ g/g, trebacular bone values of 270 μ g/g and per new bone of 1155 μ g/g. Values of rib bone of healthy humans are 412 and 427 µg/g (Zaichick et al. 2009). Potassium in the human skeleton is 810 µg/g (Brätter 1977). According to Yamagata (1962), in human tissues such as bones, potassium values increase from 0.98 to $20.0 \mu g/g$. It is probably stored in the bones of animals mainly during their lives and after death, when their bones are exposed to external environmental influences, potassium levels drop rapidly to almost zero (Follis et al. 1942).

In our results, average calcium in chamois bones was found at values between 270,000 μ g/g and 190,000 μ g/g. According to Hancock *et al.* (1989), values of 396,000, 400,000 and 402,000 μ g/g calcium were found in human archeological bones.

Zaichick et al. (2009), in turn reported calcium values of 265,800 and 267,300 $\mu g/g$ in rib bones of healthy humans and Blondiaux et al. (1992) report compact bone values of 30, trabecular bone values of 31, and new bone values of 32 μ g/g in bones from the 5^{th} and 6^{th} century. Calcium values were from 200,000 to 240,000 µg/g in human archeological bones (Edward 1990). Calcium is an element that occurs naturally in animals and is an important part of bones. This element is important to bone strength. Calcium is leached from the bone after death. According to Vass (2001), calcium concentration can be used to determine the length of time since death because it is leached from the bone at a rate determined primarily by temperature and exposure to moisture. This is corroborated by our results, where the level of calcium was lower in warmer months than in the colder months.

Chromium increased more significantly in rib bones. In the pelvic bones, changes in chromium levels are less noticeable. Bones as a sorbent can Elements accumulations in chamois bones during field exposure absorb chromium well from aqueous solutions (Chojnacka 2005). Average manganese in our bone samples reached a maximum value of 60 μ g/g. Blodiaux *et al.* (1992) recorded manganese values of 3.4, 65 and 252 μ g/g, in their samples from the 5th and 6th century, which are quite consistent with our work. Conversely, other studies, (Zaichick *et al.* 2009) showed manganese values in human rib bones was as high as 1000 to 1020 μ g/g. Kilburn *et al.* (2021) recorded manganese values of 1000 μ g/g in archaeological human skeletal remains.

In our results, mean iron reached a level of 1000 μ g/g in chamois bones. Iron levels in archaeological human skeletal remains were recorded at a maximum of 30,000 μ g/g, or conversely, Kilburn *et al.* (2021) recorded valuesa of 1000 μ g/g. In Zaicshick *et al.* (2009) iron values in rib bone of healthy humans were 92,000 and 99,000 μ g/g.

In our results, maximum average zinc concentrations were measured at 1500 μ g/g. Zaichick *et al.* (2009) recorded zinc values in rib bones of healthy humans at concentrations of 143,000 and 147,000 μ g/g. Work by Blondiaux *et al.* (1992) reported zinc values in archeological bones at 82 μ g/g for compact bone, 128 μ g/g for trebacular bone and 350 μ g/g for new bone. Zinc was almost identical in the pelvic and rib bones. Although there were some differences, they were less significant than in other elements. It appears that zinc was only present in small amounts in the bones and concentrations increased over time. This increase may be due to diffusion from the soil, as this element occurs in the environment due to zinc smelters (Wierzbicka and Pielichowska 2004).

Rubidium in chamois bones was measured at values 60 μ g/g and less. Yamagata (1962) records rubidium values in human tissues such as bones at 4.9 μ g/g, 5.4 μ g/g, 9.7 μ g/g and 10.5 μ g/g (the work contained more bones numbered as a type). Thus, rubidium is already present in bone tissue, but in small quantities (Yamagata 1962). Rubidium is normally found in animal and human bones, and Yamagata (1962) found 7.6 μ g/g of rubidium in human bone ash.

Based on our results, strontium exists in bones at values around 200 µg/g. Zaichick *et al.* (2009) recordss values in rib bone of healthy humans at 264,000 and 251,000 µg/g. Blondiaux *et al.* (1992) write that strontium values in bones from the 5th and 6th century are 220 for compact bone, 185 for trabecular bone and 257 µg/g for new bone. Furthermore, Edward (1990) mentions values in human archeological bones such as 85.7 µg/g, 90.1 µg/g, 90.7 µg/g, 99.3 µg/g, 100.2 µg/g. This may be due to food intake, even in the case of plant foods (Price 2002).

In our results, molybdenum has the highest average value of approximately 9 μ g/g in bones. Hidiroglou *et al.* (1982) studies fresh long bones of the left thoracic limb, and reported molybdenum values averaging 1.66 μ g/g, 1.69 μ g/g and 1.74 μ g/g. Other molybdenum values are also mentioned, which are: 46, 48 and 49 μ g/g (Hidiroglou *et al.* 1982).

Antimony is an element found in the bones that highest average values are 20 μ g/g. Another study (Heydorn 1967) reported antimony values for hair in men, namely: 0.153 μ g/g. Antimony levels in the bones, as with many other elements, generally increased. High levels of antimony, which were measured at the first measurement, can be taken up into the bones through food or from the atmosphere

(Nixon 1969). Antimony concentration generally increased, but this was mainly observed in the rib bones. Measurements for pelvic bones at 26 months were unavailable. Bones (such as teeth) absorb less antimony than other parts of the body (such as soft tissues) (Friedrich *et al.* 2012).

Barium has the highest average value in bones, with concentrations above 150 μ g/g. According to Zaichick *et al.* (2009), the barium value in rib bones of healthy humans is 270,000 μ g/g and Brätter (1977) mentions a barium value in the human skeleton of 71 μ g/g. Again, according to Kilburn *et al.* (2021) the barium value in archaeological human skeletal remains is around 200 μ g/g. The level of barium in the bones generally increased, as with other elements. As with other elements, the levels in the pelvis and rib bones do not match at all. Barium is an element that is most likely to be absorbed from the soil (Carvalho *et al.* 2004), which can also be seen in the graph.

Manganese is an element that generally does not have high uptake in ruminants (Ballová *et al.* 2019), which can also be seen in the results.

The highest average lead was shown by our data as just below 20 μ g/g. According to Brätter (1977), lead values in human skeleton reach 3.43 μ g/g. Lead levels have generally risen sharply. Lead levels were higher in the rib bones than in the pelvic bones. Lead can also enter the environment due to road pollution (Minoranskij 1990). According to Ballová *et al* (2019), lead and zinc contamination in the High Tatras is prevalent. Pollution or contamination by these elements is most likely caused due to the influence of the coal and mining industries. The sources of these pollutants do not neccessarily have to be in close proximity to the High Tatras, but can travel long distances (e.g., Moravia in the Czech Republic).

The mountains are a good identifier of air pollution concentrations (Ba, Mn, Pb, Sr, Zn) in the bones and teeth of wild ruminants from the West Carpathians and the Tian-Shan Mountains, as evidenced by Ballová *et al.* (2019). These high pollution concentrations likely have had a significant effect on element concentrations in chamois bones. Further, bones, as calcified tissues, are good bioindicators of long-term accumulation of elements. Pollution in the mountains can also be related to emissions from transport, which means that large cities are also significant polluters (Ballová *et al.* 2019).

Air pollution from transport emissions is mainly a source of lead and barium. According to Krajcarz (2019). the most important lithological features of the soil in terms of chemical changes in metal concentrations in bone are the content of organic matter and sediment moisture. Both factors acting together are necessary to significantly change the chemical composition of bones. Further, other elements such as sulfur, titanium, cobaltum, niccolum, cuprum, arsenicum, selenium, zirconium, argentum, cadmium, stannum, hydrargyrum, had poorly measured levels in chamois bones (Rupicapra rupicapra tatrica). Therefore, due to their non-quantified levels, they were not included in the processing of data on the synergistic behavior of the elements, even though these elements are sufficiently interesting from a research point of view.

Calcium is leached from the bone after death, as confirmed by Vass (2001). Calcium is used as

S. Breceli. M. Janiga, Z. Kompišová Ballová & G. Chovancová

an element to determine the length of time since death because it is leached from the bone at a rate determined primarily by temperature and moisture exposure. Zinc was present in the bones only in small amounts and increased over time. This increase may be due to diffusion from the soil, as this element occurs in the environment due to zinc smelting (Wierzbicka and Pielichowska 2004). According to Ballová et al. (2019), environmental contamination by lead as well as zinc is strong in the High Tatras. Pollution is most likely caused by the coal and mining industries.

Furthermore, bones as calcified tissues are good bioindicators of long-term accumulation of elements. Pollution in the mountains can be related to emissions from transport, which means that even large cities are significant polluters (Ballová et al. 2019). Air pollution from transport emissions consists mainly of the elements lead and barium. Elements such as sulfur, titanium, cobalt, nickel, copper, arsenic, selenium, zirconium, silver, cadmium, tin, hydrargyrum had low levels in chamois (Rupicapra rupicapra tatrica) bones. If bone samples are taken directly from the field to compare concentrations between mountain ranges, results may differ due to these element exchanges mainly between bones, soil and air. A duration of 26 months is enough to change the chemical composition of the bone to a detectable level, if environmental conditions are suitable (Krajcarz 2019).

References

- Ballová, Z., Janiga, M. and Hančinský, R. 2019: Comparison of element concentrations (Ba, Mn, Pb, Sr, Zn) in the bones and teeth of wild ruminants from the West Carpathians and the Tian-Shan Mountains as indicators of air pollution. Atmosphere, 10: 64.
- Blondiaux, J., Baud, C.A., Boscher-Barré, N., Dardenne, C., Deschamps, N., Trocellier, P. and Buchet, L. 1992: Trace elements in palaeopathology: quantitative analysis of a case of hypertrophic osteoarthropathy by instrumental neutron activation analysis. Int. J. Osteoarchaeol., 2: 241-244.
- Brätter, P., Gawlik, D., Lausch, J. and Rösick, U. 1977: On the distribution of trace elements in human skeletons. J. Radioanal. Chem., 37: 393-403.
- Carvalho, M.L., Marques, A.F., Lima, M.T. and Reus, U. 2004: Trace elements distribution and post-mortem intake in human bones from Middle Age by total reflection X-ray fluorescence. Spectrochimica Acta Part B: Atomic Spectroscopy, 59: 1251-1257.
- Chojnacka, K. 2005: Equilibrium and kinetic modelling of chromium (III) sorption by animal bones. Chemosphere 59: 315-320
- Edward, J. 1990: Ion exchange behavior of fresh human bone. J. Radioanal. Nuc. Chem., 144: 317-322.
- Farswan, Y. S., and Nautiyal, V. 1997: Investigation of phosphorus enrichment in the burial soil of Kumaun, midcentral Himalaya, India. J. Archaeol. Sci., 24: 251-258.
- Follis, Jr, R.H., Orent-Keiles, E. and McCollum, E.V. 1942: The production of cardiac and renal lesions in rats by a diet extremely deficient in potassium. Am. J. Pathol, 18: 29-39.
- Friedrich, K., Vieira, F.A., Porrozzi, R., Marchevsky, R.S., Miekeley, N., Grimaldi Jr, G. and Paumgartten, F.J. 2012: Disposition of antimony in rhesus monkeys infected with

Leishmania braziliensis and treated with meglumine antimoniate. J. Toxicol. Environ. Health A, 75: 63-75.

- Greistorfer, S., Klepal, W., Cyran, N., Gugumuck, A., Rudoll, L., Suppan, J. and von Byern, J. 2017: Snail mucus - glandular origin and composition in Helix pomatia. Zoology, 122: 126-138.
- Hancock, R.G.V., Grynpas, M.D. and Pritzker, K.P.H. 1989: The abuse of bone analyses for archaeological dietary studies. Archaeometry, 31: 169-179.
- Heydorn, K. 1967: Improvement of accuracy by multiple carrier addition followed by re-irradiation yield determination in a simple method for the determination of arsenic in biological material. In: Proceedings of the Symposium on Nuclear Activation Techniques in the Life Sciences, pp. 179-187. I.A.E.A., Vienna.
- Hidiroglou, M., Morris, G. and Ivan, M. 1982: Chemical composition of sheep bones as influenced by molybdenum supplementation. J. Dairy Sci., 65: 619-624.
- Jans, M.M.E., Kars, H., Nielsen-Marsh, C.M., Smith, C.I., Nord, A.G., Arthur, P. and Earl, N. 2002: In situ preservation of archaeological bone: a histological study within a multidisciplinary approach. Archaeometry, 44: 343-352.
- Karr, L.P., and Outram, A.K. 2015: Bone degradation and environment: understanding, assessing and conducting archaeological experiments using modern animal bones. Int. J. Osteoarchaeol., 25: 201-212.
- Kilburn, N.N., Gowland, R.L., Halldórsdóttir, H.H., Williams, R., and Thompson, T.J. 2021: Assessing pathological conditions in archaeological bone using portable X-ray fluorescence (pXRF). J. Archaeol. Sci. Rep., 37: 102980.
- Krajcarz, M.T. 2019: Alteration of the metal content in animal bones after 2.5-year experimental exposure to sediments. Archaeol. Anthropol. Sci., 11: 361-372.
- López-Costas, O., Lantes-Suárez, Ó. and Cortizas, A.M. 2016: Chemical compositional changes in archaeological human bones due to diagenesis: Type of bone vs soil environment. J. Archaeol. Sci., 67: 43-51.
- Minoranskij, V.A., Biedron, J. and Wojciechowski, W. 1990: Some notes on car transport pollutants in relation to the occurrence of lead, zinc and cadmium in bodies of insects. Acta Biol. Siles., 16: 235-243.
- Nixon, G.S., Smith, H. and Livingston, H.D. 1969: Trace elements in human tooth enamel. In: Proceedings of the Symposium on Nuclear Activation Techniques in the Life Sciences, pp. 455-462. I.A.E.A., Vienna.
- Piga, G., Solinas, G., Thompson, T.J.U., Brunetti, A., Malgosa, A. and Enzo, S. 2013: Is X-ray diffraction able to distinguish between animal and human bones? J. Archaeol. Sci., 40: 778-785.
- Price, T.D., Burton, J.H. and Bentley, R.A. 2002: The characterization of biologically available strontium isotope ratios for the study of prehistoric migration. Archaeometry, **44**: 117-135.
- Vass. A.A. 2001: Beyond the grave-understanding human decomposition. Microbiol. Today, 28: 190-193.
- Wavita, Z. 2018: Ťažké kovy vážne ohrozujú naše zdravie. https://www.klubzdravia.sk/a/1318 tazke-Online: kovy-vazne-ohrozuju-nase-zdravie (retrieved: 1.1.2022).
- Wierzbicka, M. and Pielichowska, M. 2004: Adaptation of Biscutella laevigata L, a metal hyperaccumulator, to growth on a zinc-lead waste heap in southern Poland: I: differences between waste-heap and mountain populations. Chemosphere, 54: 1663-1674.
- Yamagata, N. 1962: The concentration of common cesium and rubidium in human body. J. Radiat. Res., 3: 9-30.
- Zaichick, V., Zaichick, S., Karandashev, V. and Nosenko, S. 2009: The effect of age and gender on Al, B, Ba, Ca, Cu, Fe, K, Li, Mg, Mn, Na, P, S, Sr, V, and Zn contents in rib bone of healthy humans. Biol. Trace Elem. Res., **129** 107-115

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The impact of forest management on the microclimate - a case from the Belianske Tatry, Slovakia

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Abstract. Temperature is a crucial driver of ecosystem dynamics in forests. Thermal changes within forests can be highly variable and are dependent on multiple factors, such as forest type, soil composition, edge orientation, slope, humidity and climate conditions. In this study we focused on temperature measurement of forest stands using a thermal camera, in three different localities within the Belianske Tatras. These localities were selected based on slope orientation and differentiated by vegetation type. The main aim of the study was to determine the surface temperature of the forest area in terms of seasonal and diurnal/night temporal changes. Field measurements were carried out continuously for two years. The results suggested significant temperature differences between the measured localities and also showed in which locations the most heat accumulates, the influence of slope orientation on tree growth, and the response of vegetation to seasonal changes. If the forest is significantly damaged, it cannot function properly to mitigate large temperature fluctuations. The forest must inhabit a particular footprint and utilize the correct management strategy for proper growth and regeneration in order to maintain a mild microclimate. From an ecological perspective, it preferable to leave old mixed forests as intervention free as possible. Intervention may include logging, planting of monocultures, or drainage of slopes with the help of heavy machinery, and contributes to long-standing problems that persist for decades. Belianske Tatras, as an eastern locality with unique dolomites and rare vegetation, fully captures the nature of the Tatras and is a prime example of the microclimate within this region. This microclimate is crucial to this mountain complex for the prevention of periphery drought, whereby water resources will be lost. Additionally, within this study, thermal imaging proved to be a suitable tool to understand ecosystem response to temperature extremes This technique still has its flaws, and must be fine-tuned to fully determine temperature, thermal conductivity, specific heat and emissivity within these environments.

Key words: Belianske Tatry, forested areas, daily and seasonal time scales, climatic conditions, forest temperature

Introduction

Forests are an important part of our lives, providing beauty, recreation, and an ecosystem that helps us to combat climate change, but deforestation is still a widespread issue worldwide. Deforestation is not limited to rainforests alone; approximately 3.7 million hectares of forest in Europe has been damaged by insects, fires and human activity. An additional threat to this ecosystem is forest fragmentation (Ledig et al. 1992), which occurs due to the continuous development of cities and related infrastructure. Every day we lose an area of forest equal to double the area of the High Tatras due to deforestation. Forests produce oxygen, which is requisite for life; they capture and filter water; provide sustenance and shelter for fauna and flora; and propagate medicinal substances. As the human population grows every year, more and more food crops need to be grown. Agriculture causes 80 % of deforestation. When forests are clear-cut to create more land for growing crops, especially in rainforests during the dry season, the soil becomes poorly nourished, dry, and infertile, leading to a loss in value. Nutrients are often supplemented with chemical sprays that are unnatural to the environment, or "cutting and burning" is relied on as an agricultural technique. This land is fertile, but only for a short period, and farmers guickly abandon fallow land for new areas.

Fortunately, in many countries, emissions are falling as a result of the introduction of new laws and policies, such as fuel standards, stricter building regulations and emission limits for power plants. Also contributing to this are developments in technology and lifestyle trends, and increasingly incorporating the principles of sustainable use of resources, partly in response to the growing interest of governments, business and the general public about the threat of social unrest and conflict, and from worsening environmental problems (Gallopin et al. 1997). Many countries signed the Paris Agreement in December 2015 and committed themselves to better policies to combat climate change. This change will be reflected in investment in cleaner energy sources, such as wind turbines, solar panels, hydropower and power plants that burn natural gas instead of coal. Countries also set carbon taxes

28 D. Surovčík & M. Kompiš on emissions, which could have a profound impact on and convince industries to start using cleaner energy sources faster. However, in Slovakia, use of forest resources is largely an issue of economy, often due to the necessity for construction of new human dwellings, tourism and transport. Increasing deforestation in national parks, nature reserves, and protected landscape areas continue to occur due to state support and fundraising. Harvested wood is also used in house construction, paper production and heating. In addition, the loss of such habitats in Slovakia also causes significant negative effects, including: a reduction in biodiversity, contribution to global warming, interruption of water cycles, soil erosion, increase in greenhouse gases and, last but not least, increasing the temperature of the forest and surrounding soils. This last factor was the focus of this study, wherein we sought to evaluate the extent to which this temperature increase has impacted forests and their surrounding soils. The main aim of the study was to obtain data on forest temperature at selected localities, and to find out how the forest temperature fluctuated depending on seasonal changes, daily changes, and depending on how much the given soil substrates overheat to loss of vegetation.

Material and Methods

Study area

We carried out research on measurement of forest temperatures in the Belianske Tatras, which comprise the northeast part of the Tatra Mountains (in North Central Slovakia). We wanted to focus on slopes oriented in different direction, therefore we chose three localities that identify the forest temperature due to the slope's orientation to sun. Localities are affected by various local climate factors.

We chose the first locality L1 Pod Muráňom (measurement from point - N 49.24965°, E 20.15829°) in the Javorová valley (Fig. 1). The slope is south-west oriented, and is located at an altitude of 1084 m a.s.l. This locality was divided into three positions for a better overview of the temperature at different heights with different vegetation composition. The first position is located between 55 and 168 m from the point of measurement, at an altitude of 1100 - 1200 m a.s.l. The first position is composed of young spruce forest that is not exposed to severe weather conditions and is close to the meadow ecosystem. The second position is located between 168 and 461 m from the point of measurement, at an altitude of 1200 - 1300 m a.s.l. The second position is a forest following a natural disturbance. It consists of a mixed stand in which natural disorders occur. It is mainly affected by stronger winds and larger temperature fluctuations due to higher altitudes. At this level of the slope, there is a natural regeneration of the forest, which is composed of nutrients from the previous stand of trees. Restoration in this area began with a young deciduous forest of beech, maple and rowan. The third position is located between 461 and 636 m from the point of measurement, at an altitude of 1300 - 1450 m a.s.l. This position is characterized as an old

spruce forest without current human intervention. It withstands the greatest natural conditions and forms the basic floor at this altitude. There are individuals of old age who will later provide a good basis for future generations of young tree species.

We chose the second locality L2 Čierna valley (measurement from point - N 49.24158°, E 20.30701°), near the village Tatranská Kotlina (Fig. 2). This slope is south-east oriented, and is located at an altitude of 793 m a.s.l. In this case, we measured two positions. The first position is located between 60 and 105 m from the point of measurement, at altitude. This position is characterized as a clear-cut forest area. It does not contain any wood residues or trunks. It consists only of soil or low grassland. This area is fully exposed to the sun in the summer and frost in the winter as it is not shielded by any geographical features and has no other protection. The second position is located between 50 and 70 m from the point of measurement, at altitude. This position consists of Norway spruce monoculture and is fully forested with spruce trees that are tall and slender. However, they are exposed from the bottom and have no lower growth below them. The crowns have grown up completely at the ends due to dense growth.

We chose the third locality L3 Kôň (measurement from point - N 49.263752°, E 20.141879°), in the village Tatranská Javorina (Fig. 3). This slope is northwest oriented, and is located at an altitude of 950 m a.s.l. The slope is located on a limestone subsoil.



Fig. 1. Photo from locality L1 pod Muráňom. The image show slope with the relevant tree positions (Photo: D. Surovčík, 2021).



Fig. 2. Photo from locality L2, Čierna valley, Tatranská Kotlina. A preserved fragment of the forest in Čierna Valley, divided into two positions (Photo: D. Surovčík, 2021).

Impact of forest management on the microclimate The intervening element during the measurement is the river Javorinka, which flows through the bottom of the hill. This locality was divided into three positions. The first position is located between 70 to 100 m from the point of measurement. This position is comprised of beech clear-cut, along with natural regeneration of other trees. The second position is located between 100 and 134 m from the point of measurement. This position is a fully clearcut forest area with lonely beeches that are quite bright. The third position is located between 134 and 145 m from the point of measurement. This position is characterised by young mixed forest with a predominance of beech trees.

Fig. 3. Photo from locality L3 Kôň. The locality is characterized by solitary beeches and low, young vegetation (Photo: D. Surovčík, 2021).

Field experiments

Measurements took place between November 2019 and December 2021. Field measurements took place every month, and day/night intervals were recorded in a regular ratio 5 times (morning, afternoon, evening, night, the next morning). They were measured using a thermal imager testo 882 (Testo, USA). Thermal infrared imaging is a powerful tool for ecological studies, providing the possibility of accurate, continuous, real-time measurement of vegetation temperatures. Thermal cameras are analogous to monochrome digital cameras; each pixel in the sensor records a digital number that represents the light intensity it receives. Parameters: Spectral range: 8 - 14 μ m; Detector type: 320 x 240 pixels; Thermal sensitivity: 50 mK; Temperature range: -20 - 100 °C.

Three images were recorded for each measurement. Each image was processed by the Testo analysis program (Figs. 4-7) using the true environmental parameters measured at the time the image was recorded. This produces the true temperature value for each pixel. Thermal interference from the atmosphere and surroundings can dramatically affect the recorded temperature of forest area. Therefore, we tried to adhere to following parameters: Emissivity: 0.94; Distance: 55 - 636 m; Relative humidity: 0 - 100 % rF; Reflected object temperature: 50 - 100 \degree C.



Fig. 4. Image analysis program, Testo IRSoft Software Version 3 (Photo: D. Surovčík, 2021).



Fig. 5. Evaluated image with average temperature from locality L1 Pod Muráňom, Belianske Tatras 28. January 2021. (Photo: D. Surovčík, 2021).



Fig. 6. Locality L2, Čierna valley from date 23. February 2021 (Photo: D. Surovčík, 2021).



Fig. 7. Locality L3 Kôň, with the Square function displayed, Tatranská Javorina, 23. February 2021 (Photo: D. Surovčík, 2021).

30D. Surovčík& M. Kompiš

Corrections caused by thermal interference are important, but the quality of image data should be considered before applying any corrections. Careful checking of imaging conditions, parameter ranges, and general accuracy is more important than ultraaccurate measurements of meteorological conditions.

All three measured localities were within the same mountain range (Belianske Tatry). For this reason, we have taken into account the direct and indirect factors that affect forest temperature. The first of these factors is the type of forest. Measuring deciduous and coniferous forests requires an understanding of their inherent differences. While deciduous forests manage water better, and are less dependent (it can withstand larger periods of drought) (like in the case of L1 Pod Muráňom position 2, L3 Kôň all three position), coniferous forests are scavengers (in the case of L1 Pod Muráňom position 1 and 3; L2 Čierna Valley position 2). Additionally, it is colder beneath deciduous trees due to the fact that they have larger leaves and thus shade a larger area of land below. Coniferous forests have smaller needles, which are more likely to withstand winter periods. The second of these factors is soil type. L1 Pod Muráňom, L2 Čierna Valley and L3 Kôň are located on a limestone subsoil. In these mountain localities soils often occur on slopes where there is a high risk of erosion. As a result, there is a lot of semi-natural vegetation in these areas. This contributes to faster drainage and drying of slopes where vegetation is lacking. This is especially the case in the locality of L2 Čierna Valley, where natural vegetation is removed or missing. Slope orientation is another factor influencing forest temperature. L1 Pod Muráňom has a south-west orientation, while L2 Čierna Valley has a south-east orientation, and L3 Kôň has a north-west orientation. The warmest slopes are those with the greatest sun exposure; thus, southern slopes were selected. Meteorological conditions have significant impact on the ability to utilize thermal imaging. Measurements may be distorted by direct sunlight, rain (precipitation) and high humidity, including fog. We attempted to select optimal conditions for measurement (e.g., partial sunlight or cloud). Altitude and slope inclination have an impact on measurement as well. Given that the selected localities are in mountainous terrain, we took into account altitude, slope inclination and land cover. Air temperature also had a significant effect on measurements, and was included in the binding conditions for our measurements.

Preparation and analysis of images

Measurement took place five times per day in intervals of six hours. Previous images were designed based on the same shooting time. There are 830 complete observations with 12 variables in the dataset. The main variables for data evaluation were the type of locality, seasonality, minimal and maximal day/night temperature and type of forest area. Statistical analysis was processed in the environment of programming language "R" version 4.1.2. in the user interface RStudio 2021.09.2 Build+382 "Ghost Orchid" Release.

Results

Temperature of the studied forests from seasonality point of view

The highest median monthly temperature was recorded in all localities in June, and the lowest (depending on the locality) in March (L1 Pod Muráňom), January (L2 Čierna Valley) or December (L3 Kôň) (Figs. 8 and 9). Measured annual temperature at locality L2 Čierna Valley at the "clear-cut forest area" experienced a large range of temperature variation (Fig. 8). We can infer that this unshielded clear-cut area over-heats faster and more significantly on sunny days than the forest position comprised of "Norway spruce monoculture" in the same locality.

Figure 10 shows an evident rise in temperature in the morning and afternoon in some more stable positions like "old spruce forest" and "natural disturbance-based mixed forest", and a drop in temperature at these positions during the evening and night.

For position "clear-cut forest area", the average temperature reached the largest range of variation (it changed the most during the day and within the year). This is likely related to the lower water content in this type of forest area, which would dampen temperature fluctuations when the temperature changes occur. The smallest temperature fluctuations were recorded in the position "old spruce forest", whose humidity can likely cope with temperature fluctuations.

Discussion

We learned that there is high variability of forest temperatures in different localities. In our case, L1 Pod Muráňom, L2 Čierna Valley and L3 Kôň. The Belianske Tatras are part of the Eastern Tatras, and the sun tends to warm this range the most as it rises in the morning. The habitats that occur here are diverse, depending on the type of soil and geological bedrock, but are mostly comprised of spruce forests. We have found that deforested areas like locality L2 with position "clear-cut forest areas" on hillsides are overheating due to increased forest management, when compared to forests left without intervention, like the position "natural disturbance-based mixed forest" in locality L1 (Fig. 8). Temperate forests show moderate cooling in summer and moderate warming in winter with net cooling annually. The cooling or warming effects are mainly influenced by rainfall and snow (Li et al. 2015). The forest stands (deciduous and mixed, coniferous) are surfaces that cool the landscape due to evapotranspiration, which is reflected in low surface temperatures (Hesslerová et al. 2018).

In our measurement localities we also observed a significant variation due to seasonality. During summer, the most warm days were observed, particularly during the afternoon. In addition, the hottest temperatures at locality L2 were recorded in the morning (Figs. 8 and 9). The habitat in locality L2 was severely disturbed and sufficiently grafted to such an extent that the overall respiratory activity was disturbed and generally drained. The forest in the position "norway spruce monoculture" could Impact of forest management on the microclimate



Fig. 8. Boxplot of average monthly temperature of monitored localities by forest types. Locality: L1 Pod Muráňom; L2 Čierna Valley; L3 Kôň. The graph shows the presence of a considerable number of extreme values, which are marked with black dots.







Fig. 10. Interaction plot of section of the day and the square temperature.

32 D. Surovčík & M. Kompiš not retain moisture that fell several times a month, yet there was a precipitation deficit. The trees lacked middle and lower levels of vegetation in this position, and this effect was further enhanced. The opposite effect occurred in winter, when ambient temperatures dropped deeper below zero. In this case, the vegetation acted as an insulating layer for the bare roots of the trees and protected them from the cold. In the position "clear-cut forest area", only smaller shrubs and weeds were observed. According to Hesslerová et al. (2018), an increase in surface temperature is apparent in an area where the forest canopy layer died in the order of 2 - 4 °C. High surface temperature in the harvested forest area indicates production of sensible heat instead of evapotranspiration and cooling like in the case of position "clear-cut forest area".

Comparatively, in the position "old spruce forest" in locality L1, the temperature was completely different throughout the year (Figs. 8 and 9). The forest in this position was complete without serious intervention by anthropogenic activity, it retained moisture, was larger in size and was comprised of a variety of ages of vegetation. A major advantage such as stand density helped to balance the temperature effect, keep the same moisture in the summer and protect it from frost. In the position "natural disturbance-based mixed forest", dead trees, young beech, pioneer trees, rowan trees, and shrubs acted like sponges and supplied the habitat with the necessary water to survive the warm months. The large variability of microclimates in forest undergrowth is one of the most important legacies following natural disturbances (e.g., deadwood, diverse species and age composition) (Swanson et al. 2011). In winter, this position also showed only small temperature variations. We found that if the forest is of a mixed nature, and also consists of deciduous trees, it helps to better cope with moisture than a monocultural forest. Deciduous trees can manage the obtained water much more than coniferous forests (Wohlleben 2015). Trees like beech, maple and birch are also able to provide water to the surrounding environment and strengthen the entire ecosystem. The Belianske Tatras also have interesting soil conditions, with a composition of igneous rocks of granitoids and crystalline shales. The casing consists mainly of limestone, dolomite and clayey shales. This area does not have rich nutrient soils and is very often overheated through the earthenware base.

Our findings suggest that a forest without human intervention is far better able to tolerate different temperature fluctuations. The larger the forest ecosystem in terms of area, the more its microclimate and its internal functioning as a whole are affected. If a forest is extremely disturbed, fragmented or logged, it struggles to recover. This claim is also supported by the results which clearly show the differences between winter and summer seasons, daily and night time differences and their large temperature fluctuations. It is clear from our research that the highest temperatures in the areas occurred in June and July. Clear-cut areas displayed the highest values (Figs. 8 and 9), with temperatures ranging on average from 18 - 22 °C. However, it is worth mentioning that gusty temperatures were able to climb up to 38 degrees locally. Soil and air temperature are influenced by plant cover and the extremes within seasons and in day and night-time air temperatures are a function of transpiring vegetation height in hot and dry days and seasonal periods (Tesař *et al.* 2006).

The idea that harvested forests are less equipped to adapt is also supported by findings from the winter, that clearly demonstrate the same result. The average winter temperature was between -8 to -10 °C, but occasionally dipped to -12 (Fig. 8). Other forest areas, such as young mixed forests, tolerated such temperatures somewhat better. However, old coniferous forests based on natural disturbance were the areas that performed best in terms of their microclimate conditions. Soil saturation provides evaporation which in turn cools the forest area. This reflects the general observation that soil respiration rates decrease in saturated soils (Kucera and Kirkham 1971) and very dry soils.

These types of forests also fared better winter where vegetation prevented severe frost from penetrating the ecosystem and causing significant frost dieback of seedlings. Spruce forests and their monocultures are more susceptible to mortality caused by changes in soil temperature. The results of a Swedish study on soil warming (Bergh and Linder 1999; Majdi and Öhrvika 2004) led to the assumption that shallow roots determine the length of the growing season and that root mortality increases with increasing soil temperature. Closer analysis of the results revealed how temperature varies during the day at our studied localities.

In two localities (L1 Pod Muráňom, L3 Kôň) we found the same temperature pattern, namely that the lowest temperature was measured early in the morning when the air was still cool from the night, and gradually began to warm up during the day (Fig. 10). At that time, the sun is usually further from the centre and the accumulated heat is held in the canopy of trees or the sun-charged parts of holly trees. The crucial factor in these results is the elevation of the two localities (L1 Pod Muráňom and L2 Čierna Valley). As the locality Pod Muráňom has a higher altitude, it is colder compared to Čierna Valley (Fig. 8). Where the forest was left undisturbed, whether it was old or young, the air was constantly warming up along with the forest temperature. In this ideal case, heat is accumulated in the forest ecosystem, and as the forest is not in thermal stress, it functions properly (photosynthesis takes place). Transpiration is the plant-regulated evaporation of water from leaves or needles. Transpiration is only switched on when a plant is at risk of overheating due to ambient heat - from absorbed solar radiation and/or from warm air (Liu et al. 2016).

If the temperature of the plant drops - due to heat being consumed for evaporation and/or heat being radiated to cooler air, transpiration is switched off. It is thus a control with negative feedback. It is characterised by the fact that the control maintains the maximum temperature of the plant so that it stays within a narrow range of approximately 23 to 27 °C, with an optimum value of 25 °C (Šír *et al.* 2003). The L2 Čierna Valley in Tatranská Kotlina, however, behaved oppositely. There is a predominance of monoculture forest and clear-cut

Impact of forest management on the microclimate areas, where the temperature of the forest rises faster on sunny days than the air would be able to compensate for. The slope absorbs a greater amount of heat, causing greater evaporation, and the landscape dehydrates faster, resulting in vegetation mortality (Hesslerová *et al.* 2018). The air only later equalizes the temperature of the forest and this marks a non-constant curve. The areas studied are evenly matched in ambient temperatures and strive to maintain the same warming throughout the day. This equilibrium is also due to direct sunlight, which is evenly dispersed in the dense vegetation and thus warms the air equally.

Deciduous, coniferous and mixed forests respond differently to climate and temperature changes through the canopy level, leaves, branches and roots in the soil. The presence of a canopy of trees or litter layer on the soil surface may limit the flux of heat transfer from the atmosphere to the soil during the day, particularly in summer. However, at night, or on cool and cloudy days, a canopy of trees or litter layer may limit the upward trajectory of thermal radiation from the soil to the atmosphere (Paul *et al.* 2004).

Hutchison and Matt (1977) found a decrease in diffuse radiation transmission under cloudy skies. Compared to clear days in hardwood stands, demonstrating the effect of scattered direct radiation (Hutchison and Matt 1976). If the area is open, a greater amount of radiation falls and is immediately absorbed by the surface. If this area is disturbed by any activity, it is all the more vulnerable. Everything that receives the sun's rays heats up. The darker the object, the greater the response. However, nature is more complex and different principles apply. According to the interactions of forest temperatures, we can confirm that the first heat wave had its peak in February, then decreased gently and started to increase again at the end of April until June when it peaked (Figs. 8 and 9). Indirectly, we can argue that summer temperature seasons are becoming both more intense and longer lasting compared to winter seasons. When water was non-limiting, warm and even very warm summers had no negative effect on the canopy of mountain forests exposed to heat waves. The summer water balance partially expands the green period in late summer and early fall (Corona-Lozada et al. 2019).

Forest areas responded about the same in the measured mountainous localities with a few exceptions. Some areas have warmed up so much that they have prevented the growth of a new generation of forest. These are forests areas with no vegetation (bare-root areas). The land surface temperature regimes that affect these areas may be subject to changes in the form of altered energy balance, evapotranspiration and precipitation (Culf *et al.* 1996). Deforestation can accelerate climate warming, both directly as a result of the surface and indirectly as a result of increased greenhouse gases. It is also one of the main causes of biodiversity loss worldwide (Cusack *et al.* 2016), including in our measured localities.

Looking at the progression of the months more closely we can analyse the temperature variation during the day. We know that under ideal

conditions, the sun is strongest, and thus emits the most light rays between morning to afternoon, and this is exactly what can be observed in our measurements (Fig. 10). The temperature at the localities rises between morning and afternoon, and the areas that are not shaded or have no vegetation cover are the ones that overheat the most. Calmer lower temperatures occur in the evening and at night. Various scientific studies indicate that temperatures during the day are higher than normal and therefore many forest areas are drying out. Plant regeneration and growth are also affected (Frost 1992), as tree mortality (Ruth et al. 1953; Gratkowski 1956) and ecosystem processes such as productivity and decomposition are strongly influenced by human encroachment.

Removing tree canopies through harvesting may result in increasing surface and air temperatures and in decreasing humidity. Soil compaction and rut formation by heavy machinery leads to increasing surface flow, enhanced erosion, and accelerated run-off generation in managed areas (Beudert et al. 2018). Microclimatic conditions and temperature changes are manifested differently in clear-cut areas compared to those where natural disturbance took place. Our results in two positions, ("clear-cut forest area" in the locality L2 and "natural disturbance-based mixed forest" in the locality L1) present exactly this scenario (Fig. 8). Dead trunks following natural disturbance provide shade and limit wind speed, thus maintaining microclimatic variability in their surroundings and underlying soils (Kopáček et al. 2020). Stands regenerating after disturbance in unmanaged areas have a heterogeneous structure due to the predominant association of seedlings with specific microhabitats (Bače et al. 2015).

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References

- Bače, R., Svoboda, M., Janda, P., Morrissey, R.C., Wild, J., Clear, J.L., Čada, V. and Donato, D.C. 2015: Legacy of pre-disturbance spatial pattern determines early structural diversity following severe disturbance in montane spruce forests. *PLoS One*, **10**: 139-214.
- Bergh, J. and Linder, S. 1999: Effects of soil warming during spring on photosynthetic recovery in boreal Norway spruce stands. *Glob. Change Biol.*, 5: 245-253.
- Beudert, B., Bernsteinová, J., Premier, J. and Bässler, C. 2018: Natural disturbance by bark beetle offsets climate change effects on streamflow in headwater catchments of the Bohemian Forest. *Silva Gabreta*, **24**: 21-45.
- Corona-Lozada, M.C., Morin, S. and Choler, P. 2019: Drought offsets the positive effect of summer heat waves on the canopy greenness of mountain grasslands. Agr. Forest Meteorol., 276: 107-617.
- Culf, A.D., Esteves, J.L., Filho, A.O.M. and Da Rocha, H.R. 1996: Radiation, temperature and humidity over forest and pasture in Amazonia. *Amazonian Deforestation and Climate*, **5**: 175-192.
- Cusack, D.F., Karpman, J., Ashdown, D., Cao, O., Ciochina, M., Halterman, S., Lydon, S. and Neupane, A. 2016:

D. Surovčík & M. Kompiš

34

A Global change effects on humid tropical forests: Evidence for biogeochemical and biodiversity shifts at an ecosystem scale. *Rev. Geophys.*, **54**: 523-610.

- Frost, E.J. 1992: The effects of forest-clearcut edges on the structure and composition of oldgrowth mixed conifer stands in the western Klamath mountains. Doctoral dissertation, Humboldt State University, CA, USA.
- Gallopin, G.C., Hammond, A., Raskin, P. and Swart, R. 1997: Branch points: global scenarios and human choice. SEI, Stockholm, Sweden.
- Gratkowski, H.J. 1956: Wind throw around staggered settings in old-growth Douglas-fir. *Forest Sci.*, **2**: 60-74.
- Hesslerová, P., Huryna, H., Pokorný, J. and Procházka, J. 2018: The effect of forest disturbance on landscape temperature. *Ecol. Eng.*, **120**: 345-354.
- Hutchison, B.A. and Matt, D.R. 1976: Beam enrichment of diffuse radiation in a deciduous forest. Agr. Meteorol., 17: 93-110.
- Hutchison, B.A. and Matt, D.R. 1977: The distribution of solar radiation within a deciduous forest. *Ecol. Monogr.*, 47: 185-207.
- Kopáček, J., Bače, R., Hejzlar, J., Kaňa, J., Kučera, T., Matějka, K., Porcal, P. and Turek, J. 2020: Changes in microclimate and hydrology in an unmanaged mountain forest catchment after insect-induced tree dieback. *Sci. Total Environ.*, **720**: 137-518.
- Kucera, C.L. and Kirkham, D.L. 1971: Soil respiration studies in tallgrass prairie in Missouri. *Ecology*, **52**: 912-915.
- Ledig, F.T. 1992: Human impacts on genetic diversity in forest ecosystems. *Oikos*, 63:87-108.
- Li, Y., Zhao, M., Motesharrei, S., Mu, O., Kalnay, E. and Li, S. 2015: Local cooling and warming effects of forests based on satellite observations. *Nat. commun.*, **6**: 1-8.

Seasonal shift in climatic limiting factors on tree transpiration: Evidence from sap flow observations at alpine treelines in southeast Tibet. *Front. Plant Sci.*, **7**: 10-18.

- Majdi, H. and Öhrvik, J. 2004: Interactive effects of soil warming and fertilization on root production, mortality, and longevity in a Norway spruce stand in Northern Sweden. *Glob. Change Biol.*, **10**: 182-188.
- Paul, K.I., Polglase, P.J., Smethurst, P.J., O'Connell, A.M., Carlyle, C.J. and Khanna, P.K. 2004: Soil temperature under forests: a simple model for predicting soil temperature under a range of forest types. *Agr. Forest Meteorol.*, **121**: 167-182.
- Ruth, R.H., Forest, P.N. and Yoder, R.A. 1953: Reducing wind damage in the forests of the Oregon Coast Range. U.S. Department of Agriculture, Portland, Oregon, USA.
- Swanson, M.E., Franklin, J.F., Beschta, R.L., Crisafulli, C.M., DellaSala, D.A., Hutto, R.L., Lindenmayer, D.B. and Swanson, F.J. 2011: The forgotten stage of forest succession: earlysuccessional ecosystems on forest sites. Front. Ecol. Environ., 9: 117-125.
- Šír, M., Tesař, M., Lichner, L. and Syrovátka, O. 2002: Klimatická změna a transpirace rostlin. In: Extrémní hydrologické jevy v povodí (eds. A. Patera, J. Váška, and A. Jakubíková), pp. 151-156. Fakulta stavební ČVUT a Česká vědeckotechnická vodohospodářská společnost, Praha, Czech republic.
- Tesař, M., Šír, M., Lichner, L. and Zelenková, E. 2006: Influence of vegetation cover on thermal regime of mountainous catchments. *Biologia*, **61**: 311-314.
- Wohlleben, P. 2015: Das geheime Leben der Bäume: was sie fühlen, wie sie kommunizieren-die Entdeckung einer verborgenen Welt. Ludwig Verlang, München, Germany.

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Liu, X., Nie, Y., Luo, T., Yu, J., Shen, W. and Zhang, L. 2016:

Variability in ventral spot patterns of frogs of the species *Bombina variegata* from different localities in Slovakia

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Abstract. The work is focused on the study of ventral spotting of the yellow-bellied toad (Bombina variegata) depending on different localities. The individuals analysed in this study come from four localities (Rosina, Žilina, Spišská Magura and Bukovské vrchy), which are guite spatially separated. The research was based on photographs of the abdominal side of individuals captured in 2013, 2018, 2020 and 2021. Evaluation of ventral spot patterns was performed using Image-Pro Plus 6.0 software. The resulting factors, determined by the statistical method of PCA (principal components analysis), were correlated with individual morphometry of individuals, sex, location of capture. Subadults had a more yellow abdomen and less variable spots. Spatial spot analysis confirmed that individual populations of frogs differed and showed differences in colouration and abdominal spots in the compared localities. Individuals from Rosina were darker, black spots were more variable in shape, their number was higher, and yellow prevailed in the B/Y ratio. Individuals from Dubeň were darker, with a strong circularity and a lower number of black spots, and a yellow colour predominated in the B/Y ratio. Individuals from Bukovské vrchv were darker, smaller in body size, with more variable and a greater quantity of black spots. Black predominated in the B/Y ratio. Individuals from Spišská Magura were lighter, with a more pronounced circularity of black spots, a high number of black spots, and black colour predominated in the B/Y ratio. We also evaluated microsatellite variation and ecomorphological differences in B. variegata from the locality Bukovské vrchy (Carpathian primeval beech forests, Poloniny National Park). We amplified 5 microsatellite loci, of which 4 were used to perform further fragmentation analysis. The number of alleles per locus ranged from 1 to 9. The observed heterozygosity ranged from 0 to 0.925 and expected heterozygosity from 0 to 0.846. Deviation from the Hardy-Weinberg equilibrium was not detected.

Key words: Bombina variegata, spotting - pattern mapping, biotope, locality, microsatellites

Introduction

Camouflage through background colour matching has been considered a primary force driving the evolution of colour changing ability (Kang et al. 2016). Animals use their distinctive colour mainly to protect themselves from predators. Bright carotene shades of yellow and fiery red draw attention to toxicity of an object. Striking patterns and colouring is often displayed during the mating season. Overall, colouration is also a result of natural selection, which takes into account several aspects of natural history, such as protection against solar radiation, thermoregulation, osmoregulation, nitrogen metabolism (e.g., Kobelt and Linsenmair 1986; Schmuek and Linsenmair 1988; Kaul and Shoemaker 1989; Tattersall et al. 2006). Colouration and spot pattern are important features used for traditional animal identification within taxa (Todd et al. 2005), distinguishing between populations (Costa et al. 2008), and individuals (Carafa and Biondi 2004).

Amphibians possess a diverse range of colour pattern and body markings (Hoffman and Blouin 2000). Pattern recognition is influenced by animal posture, hormonal status, injury marks, environmental influences, as well as dirt (Jørgensen and Larsen 1960; Kindermann *et al.* 2014). In anurans, it has been suggested that striking male colouration during mating time is a visual signal that supports partner recognition (Ries *et al.* 2008; Sztatecsny *et al.* 2012).

The term 'spot pattern', or 'colour pattern', refers to a mosaic of colour spots of various sizes and shapes that are arranged in a certain position relative to each other. Pattern mapping has been widely used among herpetologists in amphibian studies for several years (e.g., Donnelly et al. 1994; Arntzen et al. 2004; Moon et al. 2004; Davis and Grayson 2007). The principle of mapping is to study the colouration of individuals and compare spots, pattern distribution, their position, and connections. The aim of our study was to find out how spot patterns vary in different localities and populations. For this purpose, we used the analysis of photographs of the abdominal side of *B. variegata*. In the example population, we tried to evaluate the variability of microsatellites by selecting appropriate loci and summarized the findings of population studies of the genus Bombina based on the applicability of molecular methods in the protection of amphibians. The fire-bellied toad (B. bombina) and its sister species, the yellow-bellied toad (B. variegata), are anurans. Phylogenetically, they likely separated

A. Zakharova, E. Karaščáková, M. Haas, Z. Hrehová & M. Janiga during the Pleistocene glaciation (Szymura 1998). B. bombina is widespread throughout Central and Southern Europe (Fijarczyk et al. 2011), except in the southwestern part. It is distributed from central France, across central Germany, northern and western Switzerland, north-eastern Italy, Turkey (Bülbül et al. 2016), the Balkan region and the Carpathian Mountains (Csanády et al. 2020). In Europe, the yellow-bellied toad, Bombina variegata has an elevational range of 100 - 2100 m a.s.l. (e.g., Lác 1968; Kuzmin et al. 2019). In Slovakia, it is commonly located in the range of 250 to 1200 m a.s.l, or higher (Zwach 2013). B. variegata occurs in various water bodies, including lakes, ponds, swamps, rivers and stream pools (sometimes streams with swift currents), and springs. Its requirement for water quality is relatively low. This toad even occurs in highly polluted wetlands, where water has high concentrations of hydrogen sulphide and salts. It is the most common amphibian species inhabiting the broadest range of habitats.

Toads of the species B. variegata present brightly coloured yellow-and-black ventral patterns, which act as aposematic colouration (Di Cerbo and Biancardi 2010). Quantitative evaluation of ventral patterns (e.g., dark to yellow ratio, colour, amount, and shape of spots) are considered important diagnostic features for distinguishing between B. bombina and B. variegata (Lác 1961; Sas et al. 2005; Ghiurca and Gherghel 2007; Vörös et al. 2007; Covaciu-Marcov et al. 2009), but may also be important features within individual populations or individual characteristics (Delarze et al. 2000; Seidel et al. 2001). The method of analysing digital images through graphics programs is a relatively inexpensive and non-invasive, albeit time-consuming process undertaken by individuals (Arntzen et al. 2004; Patel and Das 2020). This method is suitable for amphibian species that have highly variable dorsal or ventral spot patterns. The advantages of spot mapping include its non-invasive nature, re-use of images, and feasibility in the field in a relatively short time. This way, on an individual basis, we can create a history of photos that allow a comprehensive examination of population size (Plăiașu et al. 2005).

The use of molecular methods in population studies allow not only knowledge of genetic diversity within a population, but also knowledge of the status of species and populations over relatively short periods of time in contrast to demographic studies, which can last up to several years (Pechmann et al. 1991). Suitable methods for evaluating the genomes of amphibian populations are analysis of highly variable genetic markers such as microsatellites, SNP markers, or the AFLP method (Storfer et al. 2009). Hypervariable genetic markers, such as microsatellites or SNP markers, are widely used in landscape genetics, mainly because of their high statistical ability to distinguish the genetic diversity of individuals, groups, or populations (Storfer et al. 2007). Another method useful in amphibian protection is real-time PCR (a method that is a variation of the standard polymerase chain reaction). Using it, we can visualize SNP markers needed to characterize population and species diversity. Microsatellites have been shown to be particularly useful for measuring gene flow and migration, for classifying individuals in the most likely population of their origin, for measuring effective population size by comparing the frequency of alleles between generations, and for detecting demographic bottlenecks in the past (Storfer *et al.* 2009).

Microsatellites represent the repetitive tandem stretches of DNA that occur in the genomes of prokaryotic and eukaryotic organisms. Within the genome, they are in regions encoding proteins but also in non-coding regions (Tóth et al. 2000). The most common are repeats of 1 - 6 base pairs with a length of 5 - 40 repeats (Selkoe and Toonen 2006). Both ends of microsatellites are bounded by adjacent regions of DNA called flanking regions. The sequences of these regions are generally identical between individuals of the same species (sometimes within related species) and based on them we can characterize specific microsatellite locus. Therefore, locus-specific primers are proposed that allow us to amplify microsatellites in a PCR process (Selkoe and Toonen 2006).

In population genetics studies, microsatellites have the potential to provide up-to-date information and estimates of migratory routes, to distinguish between migration and panmixia and can also reliably determine the relationship of individuals (Selkoe and Toonen 2006). Due to its high mutation rate, it is one of the most informative molecular markers (Hoshino *et al.* 2012). Research on microsatellites in the genus *Bombina* focuses mainly on the study of population structure, gene flow between populations, migration, and hybrid zones.

Material and Methods

Sampling

Research took place in mountainous areas of northern Slovakia in 2013, 2018, 2020, and 2021. Sample collection was conducted in Bukovské vrchy mountain (Poloniny NP; N 49.035527°; E 22.327672°) in 2013, within pools on or near forest roads in the shade of a beech forest. The second locality was the cadastre of the village Rosina in 2018; a marginal part of the forest with a temporal pond (N 49.179959°, E 18.75314°). In 2020, two sites in Spišska Magura - Lendak southwest (N 49.25590°, E 20.35353°), and Príslop northwest (N 49.2815°, E 20.226°) were chosen, with pools on forest roads. Lastly, in 2021, temporary pools in the Dubeň forest park (Žilina, N 49.234274°, E 18.753079°) were used as a locality.

The animals were caught using hand nets or directly by hands. They were measured (head width, body length, length of leg, thigh length) and weighed immediately in the field (Fig. 1). Gender was determined based on external morphological features, like horny mating calluses (nuptial) on the forearm and fingers of the forelegs and sometimes on the hind limb in males (Mikuliček and Vongrej 2005). Females were identified at the time of mating by a wider abdomen, indicating egg maturation. Individuals who did not show these signs of sexual activity were marked as subadults.

Photographs of the ventral side of each individual (Fig. 2) were taken for ventral spot analysis, with an emphasis on fixation, so that it was possible to

37 Variability in ventral spot patterns in B. variegata



Fig. 1. Measurement lines. BL - body length; HW - head width; LL - leg length (Photo: V. Ruček, 2020).

scan the largest possible area without deformations caused by gripping the frog. A tissue sample was taken from the captured individuals (by cutting a piece from the hind limb finger) for DNA extraction and subsequent analysis of microsatellites. Immediately after handling, the animals were released at the capture site.

Spot analysis

The method of quantitative pattern analysis (applied to B. variegata and B. bombina) is described by Vörös et al. (2007). Abdominal spot analysis was performed using the highly specific Image-Pro Plus 6.0 program (2D Image Analysis Software by Media cybernetics Inc.; IPro). The preparation of the photos went through several stages of editing to achieve acceptable results. In the first step, the image was corrected using Microsoft Paint (Microsoft Windows). In this step, the alignment and cropping of the image to the format required for our purposes was achieved, followed by colour adjustment for simplified manipulation in IPro, so that different shades did not interfere with the subsequent immediate analysis of the finished image. The most extensive work in Paint was hand-drawing areas (black spots) with their tannic unification for further manipulation. After this stage, the image could continue to be edited in IPro: adjusting the colour spectrum of the examined sample as well as pigment designation (designation of the specific colour of pixels - in fact it is black but passing through the adjustment filters it is white, which is referred to as a preoccupation of interest; Fig. 3). Thus, all pixels



Fig. 3. Spot analysis. Preview from IPro (Photo: A. Zakharova, 2021).



Fig. 2. Taking a picture of the ventral side of *B. variegata* in the field (Photo: A. Zakharova, 2020).

of the same colour were marked and combined into the examined spots exactly according to the manual plot of the flood spot. The program automatically extracts the necessary data from which a matrix is created for further evaluation and analysis.

Basic data were selected in IPro, including object area (total area of black spots; does not include holes), perimeter (per sum – perimeter of all black spots; per mean – average perimeter of black spots), number of black spots, and mean roundness (circularity).

From these values, other variables characterizing the abdominal pattern were calculated: per cent of black colour (ratio of total black spot area to total calculated abdominal area), per cent of yellow colour (expressed as the difference between total abdominal area and per cent of black colour), B/Y (ratio colour to yellow), RMPA (ratio of average black spot content to average black spot circumference).

DNA isolation and PCR

DNA was isolated from tissue samples using the commercially available DNeasy Blood and Tissue Kit (OIAGEN, USA). Isolation was performed using a standard protocol (Purification of Total DNA from Animal Tissues).

The isolated DNA samples were subsequently analysed in 1 % agarose gels in 1 x TBE buffer. For DNA visualization, ethidium bromide was added to the gels at a final concentration of $0.5 \ \mu g.ml^{-1}$. Electrophoresis was performed for approximately 1 hour at a potential gradient of 8 - 10 V.cm⁻¹. We observed DNA fragments under UV light. The size of the fragments was determined based on their mobility in agarose gels by comparison with a 1 kb ladder.

For testing microsatellites, we used loci amplified in *B. variegata* by Stuckas and Tiedemann (2006), and Hauswaldt *et al.* (2007). The individual loci that were amplified using the polymerase chain reaction (PCR) are listed in Table 1.

The reaction mixture contained the following reagents: ultrapure water, 1 x GoTag Flexi Buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.2 mM forward primer, 0.2 mM reverse primer, FirePol polymerase, DNA sample. In the case of locus 5F, we increased the concentration of MgCl₂ from 1.5 to 2.0 mM. The samples were then placed in a thermal cycler with a pre-set program designed according by Stuckas and Tiedemann (2006). As the reaction produced non-specific products, it was necessary to optimize the conditions of the PCR reaction - they increased annealing A. Zakharova, E. Karaščáková, M. Haas, Z. Hrehová & M. Janiga

38

| Lokus | Forward and reverse primer | \mathbf{T}_{a} | References |
|--------------|---|------------------|--------------------------------|
| Bobom B13 | For: 5´-Cy5-ATATTTCTTGCTATGTTGATG-3´ Rev: 5´-AATTGTTTAACTTATTTTATA-3´ | 46 °C | Stuckas and Tiedemann (2006) |
| Bobom F22 | For: 5' -Cy5-AGGCAAAGGATTCTGAGAATG-3' Rev: 5' -CCTTCAAAGTCGAAAAATATT-3' | 56 °C | Stuckas and Tiedemann (2006) |
| Bobom F2 | For: 5′-Cy5-AGCAGAGATGAGAGGACAGTG-3′ Rev: 5′-TCAGGGGTAGCAGATTTTCA-3′ | 60 °C | Stuckas and Tiedemann (2006) |
| Bobom F5 | For: 5´-Cy5-ATGAATTGGAAGGTAAGAACTTACACC-3´ Rev: 5´-CAAATGATACAAATCAAGTGGAATGG-3´ | 63 °C | Hauswaldt <i>et al.</i> (2007) |
| Bobom 1 A | For: 5′-Cy5-ATGTGGCTTCCATTGACCTTTGC-3′ Rev: 5′-CATGCCAAGAAGGATTGAGTCTGT-3′ | 65 °C | Hauswaldt <i>et al.</i> (2007) |

Table 1. List of primers used (forward primers fluorescently labelled with Cy-5, T_a = annealing temperature).

| Initial denaturation | 95 °C 2 min. | |
|----------------------|--|-----------|
| Denaturation | 95 °C 1 min. | |
| Annealing | Primers F2, F22: (T _a + 3) °C 1 min. Primers B13, 1A, 5F: (T _a + 5) °C 1 min. | 3 cycles |
| Extension | 72 °C 1 min. | |
| Denaturation | 95 °C 1 min. | |
| Annealing | Primers F2, F22: (T _a + 3) °C 1 min. Primers B13, 1A, 5F: (T _a + 5) °C 1 min. | 38 cycles |
| Extension | 72 °C 1 min. | |
| Final extension | 72 °C 40 min. | |

Table 2. Temperature and time profile.

temperature. The resulting temperature and time profile is shown in Table 2. After PCR, the products were visualized using electrophoresis on a 1 % agarose gel using a 50 bp DNA Step Ladder as a weight standard.

Analysis of microsatellites

Analysis of PCR products was conducted using fragment analysis performed in the GenomeLab GEXP sequencer (Beckman Coulter), which is based on the principle of capillary electrophoresis and detection of fluorescently labelled fragments by laser. One sample contained 0.16 µl of PCR product, 29.5 µl of Sample Loading Solution (SLS) and 0.16 µl of DNA Size Standard-400. Primary data obtained by fragment analysis were evaluated using the GenomeLab-Fragment Analysis software program (Beckman Coulter), which is part of the sequencer. The output data from this program were graphs showing individual alleles. For each individual, we subtracted the size of the amplified alleles from the graph, and used it in statistical analysis in Cervus 3.0.6 (Kalinowski et al. 2007) and in the construction of a phylogenetic tree. The phylogenetic tree was constructed using the distance matrix by the UPG-MA method (unweighted pair group method analysis). Jaccard's distance was used for the distance matrix. The following characteristics of genetic diversity and population differentiation were also calculated in Cervus: expected (HEXP) and observed (HOBS) heterozygosity, polymorphic information content (PIC), Hardy-Weinberg equilibrium test, allele frequency, zero allele frequency, number of alleles per locus, and number of alleles. Using Genepop 4.2.2 (Rousset 2008), we calculated the FIS inbreeding coefficient.

Statistical analysis

Statistical evaluation of ventral spotting was performed in Statistica Ver. 8 (StatSoft, USA). To determine the main component weights and percentage of variation among abdominal spot patterns, we used Principal component analysis (PCA). We used One-way ANOVA and Tukey's test HSD (honestly significant difference) at a 95 % confidence level (p < 0.05) to evaluate the impact of variables (individual predispositions, locality) and main factors.

Results

Principal components method

A total of 196 individuals of *B. variegata* were captured and documented (Rosina n = 47, Spišská Magura n = 29, Dubeň n = 20, Bukovské vrchy n = 100).

Using PCA, we have identified seven main factors (F) with the highest variation (Table 3): F1 melanism, F2 - image processing errors; F3 - animal size (head width, body length, weigh), F4 - circularity, F5 - leg length, F6 - number of dark spots, F7 - B/Y ratio. The PCA method shows that morphometric variables, in their variability, behave independently of variants that resemble melanism and spotting. Factor 2 defines irregularities in the processing of the photograph, and it is defined mainly by the total abdominal area. This characterizes the entire analysed section of the photograph, followed by the perimeter sum, and the total area of a black spot. Differences in the quality of photographs are **39** Variability in ventral spot patterns in B. variegata

| Variable | Factor 1 | Factor 2 | Factor 3 | Factor 4 | Factor 5 | Factor 6 | Factor 7 | Factor 8 |
|------------------------------|----------|----------|----------|----------|----------|----------|----------|----------|
| Dody longth | 0.20 | 0 E1 | 0.67 | 0.10 | 0.00 | 0.06 | 0.06 | 0.04 |
| body length | -0.29 | -0,51 | 0.07 | 0.10 | 0.06 | -0.00 | -0.00 | 0.04 |
| Head width | -0.20 | -0,48 | 0.75 | 0.06 | -0.13 | -0.06 | 0.03 | -0.29 |
| Hind limb /femur | 0.41 | -0.00 | 0.35 | -0.33 | -0.69 | -0.31 | -0.04 | 0.04 |
| Weight | -0.26 | -0.45 | 0.72 | 0.11 | 0.14 | 0.23 | 0.05 | 0.25 |
| Total abdominal area | -0.49 | 0.76 | 0.32 | 0.01 | 0.13 | -0.05 | 0.00 | -0.13 |
| Total area of black spots | -0.76 | 0.58 | 0.15 | 0.13 | -0.09 | -0.09 | -0.01 | 0.07 |
| Average area of black spot | -0.91 | 0.17 | 0.03 | -0.20 | -0.04 | -0.08 | -0.20 | 0.15 |
| Black colour % | -0.71 | -0.37 | -0.37 | 0.29 | -0.32 | 0.00 | 0.14 | 0.03 |
| Yellow colour % | 0.71 | 0.38 | 0.37 | -0.29 | 0.32 | 0.00 | -0.15 | -0.03 |
| Ratio B/Y | -0.65 | -0.50 | -0.33 | 0.16 | 0.01 | 0.08 | -0.34 | -0.18 |
| Number of dark patches | 0.57 | 0.42 | 0.14 | 0.26 | -0.41 | 0.41 | -0.23 | 0.06 |
| Mean roundness (circularity) | -0.58 | -0.36 | -0.09 | -0.64 | -0.11 | 0.29 | 0.04 | 0.00 |
| Perimeter mean | -0.89 | 0.04 | 0.01 | -0.38 | 0.05 | 0.08 | -0.01 | -0.03 |
| Perimeter sum | -0.54 | 0.68 | 0.22 | 0.08 | -0.17 | 0.29 | 0.18 | -0.12 |
| RMPA | -0.86 | 0.35 | 0.05 | 0.16 | 0.05 | -0.25 | -0.07 | 0.08 |
| Variance % | 39.7 | 20.5 | 14.9 | 7.1 | 6.5 | 3.9 | 2 | 1.7 |

Table 3. Factor coordinates of variables according to correlation matrix in PCA was used. The highest correlations are in bold.

caused by optical properties of the lens, sensitivity of the sensor in poor lighting conditions, ambient light conditions, image crop range, or positioning of the individual sample out of plane of sharpness. The quality of the image depends on the camera, the photographer, and both automatic and manual post-processing.

Influence of age and gender on the pattern of ventral spots

Factor 1 presents the degree of dark colouration of the abdominal side (total abdominal area, total area of black spots, perimeter sum, RMPA - ratio of black spot area to spot circumference length), represents the degree of melanism. The abdominal spot pattern in subadults is significantly lighter (fewer dark spots) and with less variable black spots (F (2,179) = 9.96, p = 0.0008) than in adults (males, females) (Fig. 4).



Fig. 4. Factor coordinates of variables at the Factor 1. Subadults show significantly lighter abdominal discolouration and lower variability of dark spots as males and females. Data in factor coordinates are given in averages and SD - standard deviation (\pm) .

Factor 3 represents size variability and clearly determines the dependence of individual size (body length, head width, weight) on age status (subadults, adult males and females) adults are significantly larger (F (2.179) = 5.69, p = 0.004). Factor 4 is related to the circularity of the spots but is independent of age and sex (F (2,179) = 2.33, p = 0.09). Factor 5 takes into account the length of the hind leg (femur). Subadults have significantly longer legs than adult males and females (F (2,179) = 8.90, p = 0.0002). The number of dark spots (Factor 6) is not significantly determined by age and sex (F (2,179) = 0.26, p = 0.77). Factor 7 is the ratio of black to yellow within the spot pattern. This ratio is also not statistically significantly conditioned by age and gender (F (2,179) = 0.17, p = 0.85).

Spatial variation in the pattern of ventral spots and local population characteristics parameters

In the spatial analysis of abdominal spotting, we excluded a group of subadults, due to significantly different types of spots. Thus, when comparing spatial variability, we focused only on the sexually mature stages.

The darker colouration of the abdominal patterns characterized by Factor 1 significantly differentiates the population of frogs from the Spišská Magura locality (Fig. 5). Individuals of other localities did not differ from each other (F (3,180) = 14.06, p = 0.00000). In localities with more melanic colouration of frogs (Rosina, Dubeň, Bukovské vrchy), they have a larger average area of black spots and at the same time a larger circumference (total perimeter) and RMPA, as irregular curvature of spots also increases their circumference.

We also evaluated the differences between localities on the basis of Factor 3, which is directly related to the morphometry of individuals (Fig. 6). It was found that in the locality Bukovske vrchy individuals were significantly smaller than in other localities (F (3.180) = 12.479, p = 0.00000). A. Zakharova, E. Karaščáková, M. Haas, Z. Hrehová & M. Janiga

40



Fig. 5. The population from Spišská Magura differed significantly between the populations of individual localities (index b). Negative values indicate individuals with greater variability and areas of black spots, positive values indicate individuals with a lighter (more yellow) abdomen and less variability of black spots.

Also, the shape of the spots defined by Factor 4 (roundness) varies between sites (Fig. 7). Individuals from the Spišská Magura and Dubeň localities had more rounded spots, (i.e., they showed less variability of spot curvature (F = (3,200) = 9.6300, p = 0.0001)).

The number of dark spots (Factor 6) was significantly lower in individuals from the Dubeň locality (F (3,200) = 7.42, p = 0.0001). The number of dark spots was higher in Rosina, Spišská Magura and Bukovské vrchy (Fig. 8).

The ratio of black and yellow (individual pattern) on the abdomen of *B. variegata* (Factor 7) also exem-



Fig. 7. Rounding of spots in individual localities. While in Rosina and Bukovksé vrchy individuals have more variable curvature of spots (index a), in Spišská Magura and Dubna the spots showed higher circularity (index b).



Fig. 8. Dubeň (index b) manifests as a locality with individuals, who have a smaller number of dark spots than other localities (index a).



Fig. 6. Differences in the size of individuals in localities. Individuals in the population of frogs from Bukovské vrchy is significantly different (index b) from other localities. A higher numerical value presents larger individuals.

plifies significant differences between localities (F (3,200) = 5.36, p = 0.001). The most similar patterns occurred in Bukovské vrchy and Spišská Magura; these individuals have a larger area of dark spots and a higher number of dark spots. Populations from Rosina and Dubeň show lighter colouration (more yellow) and fewer black spots (Fig. 9).



Fig. 9. Abdominal spot pattern conditioned by B/Y ratio and a number of dark spots in localities. Negative values describe individuals with a larger area and a higher number of dark spots), positive values represent individuals with a lighter colour (more yellow) and a smaller number of black spots. Between the localities of Bukovské vrchy and Spišská Magura (index b), frogs did not differ. Individuals from the locality of Dubeň (index a,b) do not differ from any compared group.

Genetic analysis

DNA was isolated from the samples taken in the field and this was subsequently used in analysis of microsatellites. After optimization of the PCR conditions, the individual loci were amplified.

The resulting PCR products (except for the Bob F2 locus, for which the standard was not large enough) were analysed in the sequencer by fragment analysis. From the output graphs, we read the sizes of the amplified alleles for each individual, and in the CERVUS 3.0.6 program, we calculated indicators of genetic diversity. In addition to the monomorphic locus Bob F22, the remaining analysed loci were polymorphic. Variability in ventral spot patterns in B. variegata The number of alleles per locus ranged from 1 to 9. The lowest number of alleles per locus was recorded at the locus by Bob B13, and the highest at Bob by 5F. No significant deviation from the Hardy-Weinberg equilibrium was found for the Bob B13 and Bob 1A loci. However, for the Bob F22 and Bob 5F loci, this test was not performed due to the low amount of data. The observed heterozygosity ranged from 0 to 0.92 and expected heterozygosity in the range of 0to 0.846 (Table 4). A comparison of the observed and expected heterozygosity confirmed the trend toward lower observed heterozygosity at the Bob B13 locus, which also had a higher value of the FIS inbreeding coefficient. The values of the frequency of zero alleles for individual loci are close to zero, and thus indicate the absence of zero alleles in the population. Detailed values of the basic characteristics of genetic diversity for individual alleles are shown in Table 5.

The phylogenetic tree constructed using the distance matrix by the UPGMA (unweighted pair group method) is shown in Fig. 10. It can be seen from the figure that samples 54 and 62, and samples 47 and 61 show identical individuals. This phenomenon is due to the small number of loci on which individuals have the same allele sizes. Therefore it was not possible to distinguish them from each other. In addition, the monomorphic locus Bob F22 does not give any information for comparison, as it does not change. It appears to be a locus that does not undergo mutations over time. We can also see from the tree that the group of individuals 19, 34, 37, 42, 46, 74, 98, 102 is separated from the others. This is not due to geographical separation of these individuals from others (for example, by the influence of geographical barrier), as these are individuals from different habitats. It is most likely that this discrepancy is due to the low number of loci used for analysis.

| Locus | H _{OBS} | H _{EXP} | P _{IC} | HW | F (Null) | N _A | F _{IS} |
|-----------|------------------|------------------|-----------------|----|----------|----------------|------------------------|
| Bobom B13 | 0.450 | 0.604 | 0.521 | NS | 0.1267 | 3 | 0.2571 |
| Bobom F22 | 0.000 | 0,000 | 0.000 | ND | ND | 1 | - |
| Bobom 5F | 0.925 | 0.846 | 0.816 | ND | -0.0521 | 9 | -0.0882 |
| Bobom 1A | 0.675 | 0.648 | 0.586 | NS | -0.0295 | 7 | -0.0335 |
| Primer | 0.51225 | 0.5245 | 0.4806 | - | 0.0150 | 5 | 0.0313 |

Table 4. Overview of genetic diversity characteristics for specific loci. HOBS = observed heterozygosity, HEXP = expected heterozygosity, PIC = polymorphic information content, HW = deviation from Hardy-Weinberg equilibrium (NS = not significant, ND = not done), F (Null) = frequency of zero alleles, NA = number of alleles per locus, FIS = inbreeding coefficient.

| Locus | Allela | Frequency | Homs. | Hets. | Frequency | Frek NULL |
|-----------|--------|-----------|-------|-------|-----------|-----------|
| Bobom B13 | 113 | 42 | 18 | 12 | 0.5250 | 0.4881 |
| | 119 | 26 | 10 | 8 | 0.3250 | 0.2535 |
| | 121 | 12 | 8 | 2 | 0.1500 | 0.1317 |
| Bobom F22 | 135 | 80 | 0 | 40 | 1 | ND |
| Bobom 5F | 115 | 6 | 6 | 0 | 0.0750 | 0.0778 |
| | 119 | 17 | 17 | 0 | 0.2125 | 0.2410 |
| | 123 | 20 | 16 | 2 | 0.2500 | 0.2576 |
| | 127 | 6 | 6 | 0 | 0.0750 | 0.0778 |
| | 131 | 10 | 10 | 0 | 0.1250 | 0.1336 |
| | 135 | 3 | 3 | 0 | 0.0375 | 0.0381 |
| | 139 | 2 | 2 | 0 | 0.0250 | 0.0253 |
| | 143 | 11 | 11 | 1 | 0.1625 | 0.1629 |
| | 147 | 3 | 3 | 0 | 0.0375 | 0.0381 |
| Bobom 1A | 322 | 41 | 21 | 10 | 0.5125 | 0.5249 |
| | 326 | 23 | 19 | 2 | 0.2875 | 0.3105 |
| | 330 | 1 | 1 | 0 | 0.0125 | 0.0126 |
| | 341 | 2 | 2 | 0 | 0.0250 | 0.0253 |
| | 345 | 1 | 1 | 0 | 0.0125 | 0.0126 |
| | 349 | 9 | 7 | 1 | 0.1125 | 0.1055 |
| | 353 | 3 | 3 | 0 | 0.0375 | 0.0382 |

Table 5. Overview of genetic diversity characteristics for individual alleles: Frequency = number of allele occurrences in the genotype set, Hets = number of heterozygotes for a given allele in the genotype, Homs = number of homozygotes for a given allele in the genotype, Frequency = number of allele occurrences divided by the total number of alleles, FrekNULL = allele frequency taking into account the possible presence of null alleles.

A. Zakharova, E. Karaščáková, M. Haas, Z. Hrehová & M. Janiga

42



Fig. 10. Phylogenetic tree compiled by the UPGMA method.

Discussion

The study of the belly colour pattern of Bombina species and the identification of interspecific hybrids has been of interest for more than a century. Several useful methods have been developed to analyse the abdominal patterns of two species based on similar symbols: distribution, position, size, and colour of light spots. However, without precise quantification, these features appear subjective. An example of a practical application is the results of Budapest scientists, Vörös et al. (2007), who showed that *B. variegata* has a smaller quantity of larger, and more regular spots and that the colour (RGB) composition of this species consists of higher red and green components, indicating a bright yellow colour. Conversely, B. bombina has darker, orange spots, with lower values for the red and green colour components. The results also showed that using their computer method combined with multivariate statistical analysis, it was possible to distinguish "pure" populations of B. bombina and B. variegata based on a ventral colour pattern. Features derived from the ventral colour pattern were useful for separation, except for the least variable blue component of the colour characteristics.

Analysis of individual spot patterns in B. variegata is a tool to better understand the degree of variation between individuals of a particular population. Individual identification is vital to revealing demographic patterns to understand population dynamics (Patel and Das 2020), and to improve species protection. Photo identification is a non-invasive technique, which is a clear advantage (Renet et al. 2019). Digital evaluation of spot patterns thus becomes an inexpensive and particularly non-invasive technique that can be used in studies of amphibian populations that do not undergo significant changes in spot patterns in adulthood. Yellow-bellied toads are a suitable model for studying environmental influences, due to their common availability in the territory. Their colouration and spot pattern stabilize following their first year (Novitsky et al. 2001), and they tolerate short-term manipulation very well. Barandun and Reyer (1997) report that mating was observed in individuals within one hour after handling.

Quantitative characteristics associated with the pattern on the ventral side of the body, such as the ratio of black to light areas, number, shape and relative position of spots, are considered by many authors to be the main diagnostic features distinguishing *B. bombina*, *B. variegata* and their hybrids (Lác 1961; Gollmann 1984; Plăiașu *et al.* 2005; Sas *et al.* 2005; Vörös *et al.* 2007; Covaciu-Marcov *et al.* 2009). The advantage of the abdominal spots of

Bombina individuals is that the pattern is permanent after metamorphosis (Streich et al. 1997; Gollmann and Gollmann 2012). Most changes in the pattern occur a few weeks after metamorphosis, and therefore it is not recommended to use photographs of the abdominal part taken during the period when the complete pattern has not yet been created (Gollmann and Gollmann 2011). The development of black spots, which are a characteristic individual feature of B. variegata, is also related to the ontogenetic development of colouration. Factor 1 represents variations in colour and spotting. In correlation with age, it consistently evaluates subadult individuals as lighter with less variability of black spots. However, the circularity of spots (F 4), the number of dark spots (F 6) and the ratio of B/Y colour (F 7) is not significantly conditioned by age. Thus, we can say that despite significant differences in colouration, individual differences in basic abdominal spot patterns are subject to ontogenetic development to a limited extent. This phenomenon is partly confirmed by previous studies, where individual patterns of spotting and discolouration stabilize only after the first wintering (Novitsky et al. 2001). Older animals are more characteristic of darker colours in the process of ontogenesis (Kraus and Allison 2009). It is also thought that changes in variations in colouration are hormonally conditioned during ontogenesis (Richards 1982; Hayes and Menendez 1999; Hoffman and Blouin 2000).

Juveniles commonly use cryptic dyes and mature into a striking colouration in adulthood (Bulbert et al. 2018). Thus, the cryptic colouration (camouflage) changes with age. This defence mechanism is used by organisms to mask their appearance, usually to blend in with their surroundings. Temporary pools, especially on forest or field paths, often have a cloudy sandy appearance. The light colour of subadult forms could be camouflage in this case. Younger individuals are also exposed to more frequent predator attacks due to their insufficient experience and lower mobility. Predators can be detrimental to B. variegata, and Barandun and Reyer (1997) mention that this predation is extremely dangerous in the early stages of life. The opposite defensive strategy is an aposematism, which is a striking warning colouration by which the animal warns predators that it is poisonous. The yellow colour generally discourages animals and sends a warning signal against the attack and possible poisoning. The yellow-bellied toad displays a cryptic dorsal colouration in different shades of grey and brown and it's aposematic colouring is a conspicuous ventral yellow with contrasting black spots (Kwet 2015). Bright colours can warn predators that potential prey is toxic, thus,

Variability in ventral spot patterns in B. variegata we can infer that predators are the most significant contributing factor to the development of aposematism (Rojas *et al.* 2020). However, the study of colour polymorphism in frog venom suggests instead this is the result of parental repression and sexual selection (Deegan and Engel 2019), as evidenced by a study by Jeckel *et al.* (2019) which found that *Adelphobates galactonotus* (Dendrobatidae) from two sites with different colour morphs did not differ in their toxin profile.

Amphibians often change colour during development, responding to various environmental factors. This colour variability and different melanin content have been shown to play an important role in thermoregulation (Tattersall et al. 2006), reproduction, protection against UV radiation (Garcia et al. 2004) and predation (Rudh and Qvarnström 2013). Licht and Grant (1997) hypothesized that the depleting ozone layer and increasing amount of UV-B radiation may cause increased amphibian mortality. Effective barriers to the propagation of UV-B radiation are the depth of water and its colouration (Smith and Baker 1979; Scully and Lean 1994). This is consistent with our observations that individuals have a higher black pigment content in cleaner, non-turbid water, which should transmit UV-B radiation better.

However, this hypothesis required additional research as conflicting results have characterised this area of study to-date. In some species, higher mortality was observed after exposure to UV-B radiation, but in others there was no effect (Licht and Grant 1997; Rudh and Qvarnström 2013). The advantage of darker pigmented individuals is their ability to reach a higher body temperature faster than ambient temperature (Brattstrom 1963; Duellmann and Trueb 1994; Vences et al. 2002), which in turn allows them to exhibit higher activity and more rapid growth (Lillywhite et al. 1973). Lighter colouring, on the other hand, prevents the body from overheating, but may increase the risk of being detected by predators (Tattersall et al. 2006). Our results showed that frogs found in muddy water were lighter, which may indicate that they do not need significant protection against UV-B radiation in the form of a higher melanin content, and that the predominance of a strong yellow colour may not be a disadvantage for them in avoiding predation. Thus, the observed difference in the level of pigmentation may be due to several factors. According to Stugren and Vancea (1968), the ratios between yellow and black, in B. variegata should not be considered as the result of metabolic changes influenced by the origin of the environment alone. While these different forms can also be caused by more random mutations, their frequency and distribution indicate that they are below environmental control. At present, many causes of these colour adaptations remain undetected and unexplored, while few genes have been discovered that affect the pigmentation of individuals (Rudh and Qvarnström 2013).

Variation in the pattern of ventral spots according to spatial distribution

The yellow-bellied toad belongs to a species that engages in daily activity, is less bound to water, and possesses migratory ability. It reproduces in smaller stagnant waters and periodic pools on mountain pastures, forest road tracks, flooded pits, etc. Some studies suggest that B. variegata prefers free wastewater. According to the research of Csanády et al. (2020), one of the most important factors positively influencing the fauna of *B. variegata* in temporary rainwater pools, is the volume and area of the rain pools, while the presence of shadows has a negative effect. The influence of other parameters (e.g., water temperature, biotope structure, the presence of chemical compounds or chemical properties, pH) was also significant (Csanády et al. 2020). Temporary lakes or pools on field roads are characterized by low or zero vegetation cover and high solar radiation. Research by Warren and Buttner (2008) also confirms that the probability of occurrence of the B. variegata increased in water reservoirs on bare land (rare vegetation), as a result of human activity.

In our research, we analysed ventral spotting in four populations of *B. variegata* from different areas of Slovakia. The sites were spatially distant to ensure a reduction in the genetic impact in colouration and spotting. Two localities were from the vicinity of Žilina (Rosina, Dubeň), one from Spišská Magura and one from Bukovské vrchy. All areas were large, and sampling (individuals) was not limited to a small area where all individuals could be assumed to belong to the same family line. Spatial spot analysis confirmed that individual populations of frogs differed and showed differences in colouration and abdominal spots in the compared localities. Individuals from Rosina were darker (F1), with black spots that were more variable in shape (F 4), higher in quantity (F 6), and with a prevalence of yellow in the B/Y (F 7). Individuals from Dubeň were darker (F 1), with a strong circularity (F 4), lower number of black spots (F 6), and a yellow colour predominance in the B/Y ratio (F 7). Individuals from Bukovské vrchy were darker (F 1), smaller in body size, with a greater quantity and greater variation of black spots (F 4, F 6), and a predominance of black colouration in the B/Y ratio. Individuals from Spišská Magura were brighter (F 1), with a more pronounced circularity and higher number of black spots (F 4, F 6) and a predominance of black colouration in the B/Y ratio.

Environmental conditions such as brightness, structuring of habitats, and presence or absence of vegetation have a strong impact on the conspicuousness, and thus on the transmission and detectability of, a visual signal (Endler 1992; Peters 2008). The locations in our research were diverse. In the case of Rosina, the habitat was represented by small temporary tanks, shaded by the forest or buildings. In both areas, the water was muddy and exposed to the sun only a limited time period. The Dubeň locality is characterized as a forest road sufficiently open to sunlight, but interspersed with sufficient tree-cover to provide shade; there is an abundance of grassland, and the water is muddy. Spišská Magura is characterized by temporary water pools formed on the forest road. All collection points were in the open out of the shade. In the locality of Bukovské vrchy, there are natural as well as artificially created water areas, reservoirs, and areas exposed to sunlight (Karaščáková 2014). Due to the characteristics of these sampling sites, it follows that the darker coloured individuals exhibited by Factor 1 occur in localities with lower solar ra-

A. Zakharova, E. Karaščáková, M. Haas, Z. Hrehová & M. Janiga diation. On the contrary, in the explicitly sunny site (Spišská Magura), the individuals were characterized by lighter colouration. The colour of the water may have an additional influence on this factor. In Spišská Magura, the water was quite turbid and muddy with a light brown to yellow colour, unlike other locations. We can assume that this represents an adaptation to the environment in which each frog develops.

For anurans, short-term adaptation to the environment is common. Short-term adaptation of B. variegata to environmental conditions was also confirmed in a study by Preißler et al. (2021), wherein frogs transferred to a lighter substrate were observed to lighten in colour in just one day, whereas frogs transferred to a darker substrate steadily decreased in brightness. However, it should be noted that the above study focussed on dorsal discolouration, while our study focusses on patterns of abdominal spots. Many amphibians get darker against a black background, or lighter against a white background, owing to the dispersal of melanin-containing organelles (melanosomes) or aggregation of iridophores (cells with platelets involved in structural colouration) (Preißler et al. 2021). Geographical variations in aposematism and crypsis (Mappes et al. 2005) are also common. Thus, we can assume that the amount of sunlight and the surrounding vegetation plays an important role in the formation of abdominal spot patterns and the overall colouration of B. variegata. The colouration of a particular population group may be the result of the collective evolution of colouration and toxicity (Summers and Clough 2001; Summers et al. 2003).

In general, it is not possible to determine which of the environmental factors has the greatest effect on the spot pattern. Environmental conditions also include the presence or absence of predators. The variety of colour patterns seen in animals tends to reflect different defensive strategies (Ruxton et al. 2004). In this respect, the colour and spot patterns are important. However, it is not only short-term predisposition to adapt to the environment that protects animals from negative effects of the environment. For example, ontogenetic colour change and non-reversible change in body colour occur as individuals transition between different life stages (Booth 1990; Grant 2007; Wilson et al. 2007). Therefore, unique stain patterns are often subject to more long-term development.

Environmental factors that affect anuran colour changes include the intensity of light, background colour, and temperature (Kang et al. 2016). In general, frogs are more brightly coloured in stronger light and higher temperatures (Stegen et al. 2004). The darkening of animals with age or ontogenesis is largely expressed as a transition between green and brown colour states (Kraus and Allison 2009). We can assume that in the long term, lighter individuals will be more prevalent in sunny habitats while darker individuals will be more prevalent in shady habitats. In the population, this leads to a favour for specific phenotypes that are more reflective of the environment and more protective from predators, due to natural selection. Long-term colour adaptation to local environments that evolved in response to natural selection is reported in lizards (Phyrnocephalus versicolour) (Tong et al. 2019), that exhibit geographically divergent body colourations to maximize camouflage against local backgrounds.

B. variegata is a long-lived anuran, and its maximum lifespan is between 5 and 23 years in the wild (Plytycz and Bigaj 1993; Hantzschmann et al. 2019; Di Cerbo et al. 2011), or 27 - 29 years in captivity, (in the absence of extrinsic mortality factors) (Mertens 1970; Abbühl and Durrer 1998), though there is still variability in life expectancy. Gene flow between neighbouring populations appears to be limited to less than 5 km (Hantzsmann et al 2019). Hantzsmann et al (2019) also showed that migration is limited to shorter distances in short-lived populations than in long-lived populations. Thus, life expectancy is another factor that can potentially play a role in the spread of the phenotype in a given environment, and it likely plays a role in the spread of favourable ventral spot patterns. Based on this hypothesis, we can assume that the structure and characteristics of a particular habitat play a role in the differences in spotting between populations that are more spatially separated. The population (i.e., the locality) has a significant influence on the distribution of properties of the individuals, indicating the substantial level of geographical differentiation among the populations (Radojičic et al. 2002).

The availability of food sources is also related to environmental conditions. Ogilvy *et al.* (2012) found that in some anurans, a diet fortified with carotenoids seems to have a strong impact on development and growth, but also on reproductive success and colouration of adult individuals. For instance, the colouration of the red ventral patch of *Bombina orientalis* is dependent on the supply of pigments in food and a lack of these carotenes under rearing conditions leads to a yellowish colouration (Steinicke 1976; Frost and Robinson 1984).

These results also suggest that differences such as food source variability, which vary between habitats, can lead to colour changes in frogs. Thus, the habitat may not only play an important role in the presence of the species in the locality (Csanády *et al.* 2020) but may also lead to a preference for other spot patterns that take into account environmental conditions.

Genetic diversity and population analysis

Studies dealing with genetic diversity and differentiation can not only offer basic information on ecology and evolution, but also provide background material for practical use in gene pool protection. The analysis of microsatellites and genetic diversity of B. variegata has not yet been processed. Current research on microsatellites (Stuckas and Tiedemann 2006; Hauswaldt et al. 2007) has focused on B. bombina. In our work we were able to amplify 5 and analyse 4 microsatellite loci, previously amplified in B. bombina, B. variegata and B. orientalis in the above mentioned studies. Stuckas and Tiedemann (2006) used samples from Denmark and Germany as study material and described 8 new microsatellite loci (7 of which were polymorphic) for the critically endangered *B. bombina* species. The observed heterozygosity produced values between 0.27 - 0.7 and did not differ significantly from the expected values (0.39 to 0.8), with the exception of locus Bob B13, (which we also used in our work), which yielded the largest deviation. The values for individual loci in B.

Variability in ventral spot patterns in B. variegata *variegata*, recorded by Stuckas and Tiedemann, are: Bob F22 - size 132 bp (in our results the locus was homologous with size 135 bp), Bob B13 - size 126 -134 bp (our results 113 - 121 bp).

Hauswaldt *et al.* (2007), also analysed microsatellites. They described 9 new polymorphic microsatellite loci in *B. bombina.* Expected heterozygosity ranged from 0.47 to 0.91 and no significant deviation was reported. The results for locus Bob 1A in *B. variegata* are similar to ours; 5 alleles with a size of 320 - 354 bp were observed, which was comparative to the results of our study, where 7 alleles were present with a size of 322 - 353 bp. Finally, for locus Bob 5F Hauswaldt found 5 alleles measuring between 130 - 150 bp, compared to our observation of 9 alleles with a size of 115 - 147 bp.

The results from the analysis of microsatellites in B. variegata in Bukovské vrchy (Poloniny NP) are similar to these previous works. The observed heterozygosity (except for the Bob B13 locus) was not significantly different from expected heterozygosity, nor was there any significant deviation from the Hardy-Weinberg equilibrium. The trend in the Bob B13 locus towards lower observed heterozygosity could indicate a lack of homozygotes due to inbreeding, or selection against heterozygotes due to subpopulations (Wahlund effect). According to our averaged values, the observed heterozygosity of 0.5123 did not differ significantly from the expected heterozygosity of 0.5245, and thus the Wahlund effect did not appear to have a significant impact, as the HOBS value was not demonstrably lower than the HEXP value. Since the value of the inbreeding coefficient in the case of the Bob B13 locus was slightly higher than zero than in the case of other loci, we could therefore consider inbreeding as the more probable cause of the observed phenomenon. As the observed heterozygosity is high, we can assume that the originally isolated populations were mixed, which would correspond to the determined genetic diversity of B. variegata within the monitored population presented in this study. The allele number data (Table 5) show that for the Bob 5F and 1A loci, there are one or more particularly widespread alleles, respectively, and the remaining alleles are less common. This phenomenon could indicate that the early founders of the populations had a limited number of alleles (for example due to the bottleneck effect) and the newly formed alleles had not yet succeeded or could not expand sufficiently. Genetic diversity depends not only on the number of alleles, (which was sufficient based on our observations), but also on their frequency. Because alleles were not evenly distributed, we can assume that the genetic diversity of the population is smaller. As can be seen (Fig. 10), a phylogenetic tree constructed by the UPGMA method does not have the best explanatory value, as we worked with only four loci, of which the Bob F22 locus is monomorphic.

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References

- Arntzen, J.W., Goudie I. B. J., Halley, J. and Jehle, R. 2004: Cost comparison of marking techniques in long-term population studies: PIT-tags versus pattern maps. *Amphib.-Reptil.*, **25**: 305-315.
- Barandun, J. and Reyer, H.U. 1997: Reproductive ecology of *Bombina variegata*. Development of eggs and larvae. J. Herpetol, **31**: 107-110.
- Booth, C.L. 1990: Evolutionary significance of ontogenetic colour change in animals. *Biol. J. Linn. Soc.*, 40: 125-163.
- Brattstrom B.H. 1963: A preliminary review of the thermal requirements of amphibians. *Ecol.*, **44**: 238-255.
- Bulbert, M. W., White, T. E., Saporito, R. A., and Kraus, F. 2018: Ontogenetic colourchange in *Oreophryne ezra* (Anura: Microhylidae) reflects an unusual shift from conspicuousness to crypsis but not in toxicity. *Biol. J. Linn. Soc.*, **123**: 12-20.
- Carafa, M. and Biondi, M. 2004: Application of a method for individual photographic identification during a study on *Salamandra salamandra gigliolii* in central Italy. *Ital. J. Zool.*, **2**: 181-184.
- Costa, C., Angelini, C., Scardi, M., Menesatti, P. and Utzeri, C. 2008: Using image analysis on the ventral colour pattern in *Salamandrina perspicillata* (Amphibia: Salamandridae) to discriminate among populations. *Biol. J. Linn. Soc.*, **96**: 35-43.
- Covaciu-Marcov, S.-D., Ferenti, S., Bogdan, H.-V., Groza M.I. and Bata, Z.S. 2009: On the hybrid zone between Bombina bombina and Bombina variegata in Livada Forest, northwestern Romania. Biharean Biol., 3: 5-12.
- Csanády, A., Oboňa, J. and Duranková, S. 2020: Bombina variegata (Linnaeus, 1758) in rain pools in the Carpathian Mountains (Slovakia). Acta Musei Silesiae. Scientiae Naturales, 69: 165-174.
- Davis, K. and Grayson K.L. 2007: Improving natural history research with image analysis: The relationship between skin color, sex, size and stage in adult redspotted newts (*Notophthalmus viridescens viride*scens). Herpetol. Conserv. Biol., **2**: 65-70.
- Delarze, R., Ciardo, F. and Pellet, J. 2000: Identification individuelle de crapaudssonneurs (*Bombina variegata*, Anura): application á l'estimation des populations. *Bull. Murithienne*, **118**: 83-86.
- Deegan, D.F. and Engel, N. 2019: Sexual dimorphism in the age of genomics: How, when, where. Front. Cell Dev. Biol., 7: 186.
- Di Cerbo, A.R. and Biancardi, C.M. 2010: Quantitative analysis of the ventral colour pattern within the genus Bombina. Atti VIII Congresso Nazionale Societas Herpetologica Italica. Chieti.
- Di Cerbo, A.R., Dino, M., Milesi, S. and Biancardi, C. 2011: Long term monitoring of yellowbellied toad populations in Italy. In: Abstract of the 16th European Congress of Herpetology, Luxembourg and Trier.
- Donnelly, M.A., Guyer, C., Juterbock, J.E. and Alford, R.A. 1994: Measuring and monitoring biological diversity: Standard methods for Amphibians. Smithsonian Books, Washington DC.
- Duellman, W.E. and Trueb, L. 1986: Biology of Amphibians. JHU Press, USA.
- Endler, J.A. 1992: Signals, signal conditions, and the direction of evolution. *Am. Nat.*, **139**: 125-153.
- Fijarczyk, A., Nadachowska, K., Hofman, S., Litvinchuk, S.N., Babik, W., Stuglik, M. and Szymura, J.M. 2011: Nuclear and mitochondrial phylogeography of the European fire-bellied toads *Bombina bombina* and *Bombina variegata* supports their independent histories. *Mol. Ecol.*, **20**: 3381-3398.
- Frost, S.K. and Robinson, S.J. 1984: Pigment cell differentiation in the fire-bellied toad, *Bombina orientalis*. I. Structural, chemical, and physical aspects of the adult pigment pattern. J. Morph., **179**: 229-242.
- Garcia, T.S, Stacy, J. and Sih, A. 2004: Larval salamander response to UV radiation and predation risk: color change

A. Zakharova, E. Karaščáková, M. Haas, Z. Hrehová & M. Janiga and microhabitat use. Ecol. Appl., 14: 1055-1064.

- Ghiurca, D. and Gherghel, I. 2007: Research upon the hybridization areas between *Bombina bombina* and *Bombina variegata* in the middle Siret River basin (Bacau and Neamt Counties, Romania). *Herpetologica Romanica*, **1**: 45-52.
- Gollman, B. and Gollmann, G. 2012. Die Gelbbauchunke: Von der Suhle zur Radspur. Bielefeld Laurenti-Verlag, Bielefeld.
- Gollmann, G. 1984: Allozymic and morfological variation in the hybrid zone between *Bombina bombina* and *Bombina variegata* (Anura Discoglossidae) in northeastern Austria. J. Zool. Syst. Evol., **22**: 51-64.
- Gollman, G. and Gollmann, B. 2011. Ontogenic change of colour pattern in *Bombina variegata*: implications for individual identifiaction. *Herpetol. Notes*, **4**: 333-335.
- Grant, T., Frost, D.R., Caldwell, J.P., Gagliardo, R., Haddad, C.F.B., Kok, P.J.R., Means, D.B., Noonan, B.P., Schargel, W.E. and Wheeler, W.C. 2006: Phylogenetic systematics of dart-poison frogs and their relatives (Amphibia: Athesphatanura: Dendrobatidae). *Bull. Am. Mus. Nat. Hist.*, **299**: 6-262.
- Hauswaldt, J.S., Schröder, C. and Tiedemann, R. 2007: Nine new tetranucleotide microsatellite markers for the fire-bellied toad (*Bombina bombina*). *Mol. Ecol. Notes*, **7**: 49-52.
- Hantzschmann, A.M., Gollmann, B., Gollmann, G. and Sinsch, U. 2019: The fast–slow continuum of longevity among yellow-bellied toad populations (*Bombina variegata*): intrinsic and extrinsic drivers of variation. *Peer J.*, **7**: 8-233.
- Hayes, T.B. and Menendez, K.P. 1999: The effect of sex steroids on primary and secondary sex differentiation in the sexually dichromatic reedfrog (*Hyperolius argus*: Hyperolidae) from the Arabuko Sokoke Forest of Kenya. *Gen. Comp. Endocrinol.*, **115**: 188-199.
- Hoffman, E.A. and Blouin, M.S. 2000: A review of colour and pattern polymorphisms in anurans. *Biol. J. Linn.* Soc., 70: 633-665.
- Hoshino, A.A., Bravo, J.P., Nobile, P.M. and Morelli, K.A. 2012: Microsatellites as tools for genetic diversity analysis. In: *Genetic diversity in microorganisms* (ed. M. Caliskan), pp. 149-170. InTechOpen, Croatia.
- Jeckel, A.M., Kocheff, S., Saporito, R.A., and Grant, T. 2019: Geographically separated orange and blue populations of the Amazonian poison frog *Adelphobates galactonotus* (Anura, Dendrobatidae) do not differ in alkaloid composition or palatability. *Chemoecology*, **29**: 225-234.
- Jørgensen, C.B. and Larsen, L.O. 1960: Control of colour change in amphibians. *Nature*, **186**: 641-642.
- Kang, C., Kim, Y.E. and Yikweon, J. 2016: Colour and pattern change against visually heterogeneous backgrounds in the tree frog *Hyla japonica*. Sci. Rep., 6: 1-12.
- Karaščáková, E. 2014: Variabilita mikrosatelitov a ekomorfologické rozdiely u druhu *Bombina variegata* v pôvodných karpatských bukových lesoch (NP Poloniny). Bachelor thesis, IHMB, Žilina University, Tatranská Javorina.
- Kaul, R. and Shoemaker, V.H. 1989: Control of thermoregulatory evaporation in the waterproof treefrog *Chiroman*tis xerampelina. J. Comp. Physiol., **158**: 643-649.
- Kindermann, C., Narayan, E.J. and Hero, J.M. 2014: The neuro-hormonal control of rapid dynamic skin colour change in an amphibian during amplexus. *PloS ONE*, **9**: 114-120.
- Kobelt, F. and Linsenmair, K.E. 1986: Adaptations of the reed frog *Hyperolius viridiflavus* (Amphibia, Anura, Hyperoliidae) to its arid environment. *Oecologia*, **68**: 533-541.
- Kraus, F. and Allison, A. 2009: A remarkable ontogenetic change in color pattern in a new species of *Oreophryne* (Anura: Microhylidae) from Papua New Guinea. *Copeia*, **4**: 690-697.
- Kuzmin, S., Denoël, M., Anthony, B., Andreone, F., Schmidt, B., Ogrodowczyk, A., Ogielska, M., Vogrin, M., Cogalniceanu, D., Kovács, T., Kiss, I., Puky, M., Vörös, J., Tarkhnishvili, D. and Ananjeva, N. 2009: Bombina variegata. The IUCN Red List of Threatened Species 2009: e.T54451A11148290.
- Kwet, A. 2015: Gelbbauchunke, Bergunke. Reptilien und Amphibien Europas: 250 Arten mit Verbreitungskarten.

Franckh-Kosmos Verlags-GmbH & Co., Stuttgart.

- Lác, J. 1968: Obojživelníky Amphibia. In: Stavovce Slovenska I. Ryby, obojživelníky a plazy. (eds. O. Oliva, S. Hrabě and J. Lác), pp. 231-312. Vydavateľstvo SAV, Bratislava.
- Lác, J. 1961: Rozšírenie kuncov (Bombina bombina L. a Bombina variegata L) na Slovensku ak problematike ich vuákomného kríženia. Vydaveteľstvo SAV, Bratislava.
- Licht, L.E and Grant, K.P. 1997: The effects of ultraviolet radiation on the biology of Amphibians. *Am. Zool.*, **37**: 137-145.
- Lillywhite, H.B., Licht P. and Chelgren, P. 1973: The role of behavioral thermoregulation in the growth energetics of the toad. *Bufo Boreas. Ecol.*, **54**: 375-383.
- Mappes, J., Marples, N. and Endler, J.A. 2005: The complex business of survival by aposematism. *Trends Ecol. Evol.*, **20**: 598-603.
- Mertens, R. 1970: Über die Lebensdauer einiger Amphibien und Reptilien in Gefangenschaft. *Der Zoologische Garten*, **39**: 193-209.
- Moon, B.R., Craig, S.I. and Johnson, J. 2004: Identifying individual rattlesnakes using tail pattern variation. *Herpetol. Rev.*, **35**: 154-156.
- Novitsky, R.V., Bakharev, V.A., Andersen, A.G., Adrados, L.-Ch. and Briggs, L.A. 2001: Fenetic analysis of fire-bellied frog (*Bombina bombina* L.) in Denmark and Belarus. *Vesti Nacionalnay Akademii Navuk Belarusi*, **4**: 97-100.
- Ogilvy, V., Preziosi, R.F. and Fidgett, A.L. 2012: A brighter future for frogs? The influence of carotenoids on the health, development and reproductive success of the red eye tree frog. *Anim. Conserv.*, **15**: 480-488.
- Patel, N.G. and Das, A. 2020: Shot the spots: A reliable field method for individual identification of *Amolops* formosus (Anura, Ranidae). *Herpetozoa*, **33**: 7-15.
- Pechmann, J.H.K., Scott, D.E., Semlitsch, R.D., Caldwell, J.P., Vitt, L.J. and Gibbons, J.W. 1991: Declining amphibian populations: The problem of separating human impacts from natural fluctuations. *Science*, **253**: 892-895.
- Peters, R.A. 2008: Environmental motion delays the detection of movementbased signals. *Biol. Lett.*, **4**: 2-5.
- Plăiaşu, R., Hartel, T., Băncilă, R.I. and Cogălniceanu, D. 2005: The use of digital images for the individual identification of amphibians. *Studii si Cercetari Biologie*, *Universitatea din Bacău*, **10**: 137-140.
- Plytycz, B., and Bigaj, J. 1993: Studies on the growth and longevity of the yellow-bellied toad, *Bombina variega*ta, in natural environments. *Amphib.-Rept.*, **14**: 35-44.
- Preißler, K., Rodríguez, A. and Pröhl, H. 2021: Evidence for coloration plasticity in the yellowbellied toad, *Bombina variegata. Ecol. Evol.*, **11**: 17557-17567.
- Radojičic, J.M., Cvetkovis, D.D., Tomovic, L.M., Džukic, G.V. and Kalezic, M.L. 2002: Sexual dimorphism in fire-bellied toads *Bombina* spp. from the central Balkans. *Folia Zool.*, **51**: 129-140.
- Ries, C., Spaethe, J., Sztatecsny, M., Strondl, C. and Hödl, W. 2008: Turning blue and ultraviolet: Sex-specific colour change during mating season in the Balkan Moor Frog. J. Zool., 276: 229-236.
- Renet, J., Lepretre, L., Champagnon, J. and Lambret, P. 2019: Monitoring amphibian species with complex chromatophore patterns: a non-invasive approach with an evaluation of software effectiveness and reliability. *Herpetol. J.*, **29**: 13-22.
- Richards, C.M. 1982: The alternation of chromatophore expression by sex hormones in the Kenyan reed frog, Hyperolius viridiflavus. Gen. Comp. Endocrinol., 46: 59-67.
- Rojas, D., Broom, D.M. and Orihuela, A. 2020: Effects of human-animal relationship on animal productivity and welfare. J. Anim. Behav. Biometeorol., 8: 196-205.
- Rousset, F. 2008: Genepop'007: A complete reimplementation of the Genepop software for Windows and Linux. *Mol. Ecol. Resour.*, **8**: 103-106.
- Rudh, A. and Qvarnström, A. 2013: Adaptive colouration in amphibians. *Sem. Cell Dev. Biol.*, **24**: 553-561.
- Ruxton, G.D, Sherratt, T.N. and Speed, M.P. 2004: Avoiding attack. Oxford University Press, New York.
- Sas, I., Covaciu-Marcov, S.D., Pop, M., Ile, R.-D., Szei-

Variability in ventral spot patterns in B. variegata

- bel N. and Duma, C. 2005: About a closed hybrid population between *Bombina bombina* and *Bombina variegata* from Oradea (Bihor county, Romania). North-Western J. Zool., **1**: 41-60.
- Scully, N.M. and Lean, D.R.S. 1994: The attenuation of the ultraviolet radiation in temperate lakes. Archiv für Hydrobiologie, 45: 135-144.
- Seidel, B., Yamashita, M., Choi, I. and Dittami, J. 2001: Water wave communication in the genus *Bombina* (Amphibia). Adv. Space Res., 28: 589-594.
- Selkoe, K.A. and Toonen, R.J. 2006: Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. *Ecol. Lett.*, **9**: 615-629.
- Smith, R.C. and Baker, K.S. 1979: Penetration of UV-B and biologically effective dose-rates in natural waters. *Pho*tochem. Photobiol., **29**: 311-323.
- Schmuek, R. and Linsenmair, K.E. 1988: Adaptations of the reed frog *Hyperolius viriditlavus* (Amphibia, Anura, Hyperoliidae) to its arid environment. III. Aspects of nitrogen metabolism and osmoregulation in the reed frog, *Hyperolius viriditlavus taeniatus*, with special reference to the role of iridophores. *Oecologia*, **75**: 354-361.
- Szymura, C. 1998: Origin of the yellow-bellied toad population, *Bombina variegata*, from Goritzhain in Saxony. J. Herpetol., 8: 201-205.
- Stegen, J.C., Gienger, C.M. and Sun, L. 2004: The control of color change in the Pacific tree frog, *Hyla regilla*. *Can. J. Zool.*, 82: 889-896.
- Steinicke, H. 1976: The problem of incomplete pigmentation in the rearing of *Bombina orientalis*. Salamandra, 12: 23-26.
- Sztatecsny, M., Preininger, D., Freudmann, A., Loretto, M. C., Maier, F. and Hödl, W. 2012: Don't get the blues: conspicuous nuptial colouration of male moor frogs (*Rana arvalis*) supports visual mate recognition during scramble competition in large breeding aggregations. *Behav. Ecol. Sociobiol.*, **66**: 1587-1593.
- Stuckas, H. and Tiedemann, R. 2006: Eight new microsatellite loci for the critically endangered fire-bellied toad *Bombina bombina* and their cross-species applicability among anuras. *Mol. Ecol. Notes*, 6: 150-152.
- Stugren, B. and Vancea, S. 1968: Geographic variation of the Yellow-bellied toad (*Bombina variegata*) from the Carpathian mountains of Romania and the USSR. J. Herpetol., **2**: 97-105.
- Summers, K. and Clough, M.E. 2001: The evolution of coloration and toxicity in the poison frog family (Dendrobatidae). P.N.A.S., 98: 6227-6232.

- Summers, K., Cronin, T. W. and Kennedy, T. 2003: Variation in spectral reflectance among populations of *Dendrobates pumilio*, the strawberry poison frog, in the Bocas del Toro Archipelago, Panama. J. Biogeogr., **30**: 35-53.
- Streich, W.J., Beckmann, H., Schneeweiss, N. and Jewgenow, K. 1997: Computergestützte Bildanalyse von Fleckenmustern der Rotbauchunke (Bombina bombina). Naturschutzrelevante Methoden der Feldherpetologie, 7: 93-102.
- Storfer, A., Eastman, J.M. and Spear, S.F. 2009: Moder molecular methods for amphibian conservation. *Bio-Science*, **59**: 559-571.
- Storfer, A., Murphy, M.A., Evans, J.S. and Waits L.P. 2007: Putting the landscape in landscape genetics. *Heredity*, 98: 128-142.
- Tattersall, G.J., Eterovick, P.C. and de Andrade, D.V. 2006: Tribute to R. G. Boutilier: skin colour and body temperature changes in basking *Bokermannohyla alvarengai* (Bokermann 1956). J. Exp. Biol., **209**: 1185-1196.
- Todd, P.A. Ladle, R.J. Briers, R.A. and Brunton, A., 2005: Quantifying two-dimensional dichromatic patterns using a photographic technique: case study on the shore crab (*Carcinus maenas* L.). *Ecol. Res.*, **20**: 497-501.
- Tóth, G., Gáspári, Z. and Jurka, J. 2000: Microsatellites in different eukaryotic genomes: survey and analysis. *Genome Res.*, **10**: 967-981.
- Tong, H., Li, J., Wo, Y., Shao, G., Zhao, W., Aguilar-Gómez, D. and Jin, Y. 2019: Effects of substrate color on intraspecific body color variation in the toad-headed lizard, *Phrynocephalus versicolor. Ecol. Evol.*, 9: 10253-10262.
- Vörös, J., Szalay, F. and Barabás, L. 2007: A new method for quantitative pattern analysis applied to two European Bombina species. J. Herpetol., 17: 97-103.
- Vences, M., Galan, P., Vieites, D.R, Puente, M., Oetter, K. and Wanke, S. 2002: Field body temperatures and heating rates in a montane frog population: the importance of black dorsal pattern for thermoregulation. *Annal. Zool. Fenn.*, **39**: 209-220.
- Warren, S. and Büttner, R. 2008: Active military training areas as refugia for disturbance dependent endangered insects. J. Insect Conserv., 12: 671-676.
- Wilson, D., Heinsohn, R. and Endler, J.A. 2007: The adaptive significance of ontogenetic colour change in a tropical python. *Biol. Lett.*, **3**: 40-43.
- Zwach, I. 2013: Amphibians and reptiles of the Czech Republic. 2nd edition. Grada Publishing, Praha.

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