ABSTRACT: Since the mountains often represent a barrier for the pollutants in many regions, the concentrations of toxic elements in the tissues of alpine animals may remain relatively high and do not decrease in the current times. To estimate heavy metal contamination of alpine ecosystems Snow voles (Chionomys nivalis) are very useful monitors. They are small, easy to catch, have a territory of limited range, fairly short life span and they are closely adjusted to their environment.

The voles were monthly bait-trapped in the West Tatras, the Western Carpathians, Slovakia. The local population was studied at the Brestová mountain chain (49°13’29.43”N; 19°40’46.07”E, 1902 m.a.s.l.). The animals were trapped in September 2009 and from May to November 2010. The global sampling yielded a total of 50 samples (trapped and retrapped individuals). The lead concentrations in the tail vertebrae and the number of micronuclei in peripheral blood were examined.

The Pb levels in voles were exceptionally high in overwintering mature animals (16.1 μg g⁻¹ dry weight in average) in comparison to young immatures (4.3 μg g⁻¹). Females had significantly higher concentrations of Pb in their bones (13 μg g⁻¹) than males (7.3 μg g⁻¹). Snow vole adults caught in the spring exhibited significantly higher micronuclei frequencies in peripheral blood than immature ones trapped in summer or fall. Given that Pb is bioaccumulated in the diet of voles, this study showed that feeding on winter diet (mosses, lichens) could constitute a major pathway for the entry of Pb into food chain of alpine habitats. The usefulness of Snow voles as biomonitors of environmental contamination in alpine ecosystems was highly recognized.

KEY WORDS: Snow vole, atmospheric lead, alpine habitats, Tatra Mountains

1. INTRODUCTION

Worldwide, industrial and transportation activity releases trace metals into the atmosphere, polluting ecosystems at the local, regional, and global scale. Although lead is referred as one of the few elements which can be transported with the air dust, as the component of airborne particulate matter, for longer distances (Krüger 1996, Ackermann and Hanrahan 1999), results from several European regions suggest that deposition of trace metals occurs within 200 km from the source (De Caritat et al. 1997, Reimann et al. 1997). From this point of view, the West Tatras Mts. are well suited for studying the metal distribution of airborne pollutants as they combine two important centres for potential heavy metal emissions, North Moravia in Czech Republic and Malopolska district in Southern Po-

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SEASONAL EFFECTS OF LEAD UPTAKE BY SNOW VOLE CHIONOMYS NIVALIS (MARTINS, 1842) IN WEST TATRA MTS.: BONE METAL CONCENTRATIONS AND HEMATOLOGICAL INDICES
The annual mean lead concentration in 2009 ranged from 0.012 to 0.034 μg m⁻³ in Ostrava and Karviná area (North Moravia, Statistical environmental yearbook of the Czech republic 2010) and 0.039 μg m⁻³ in Krakow (Małopolska, Environment 2010). The value of standardized average annual concentration of PM10 in 2009 ranged from 25.6 to 53.2 μg m⁻³ in Ostrava and Karviná area (North Moravia, Statistical environmental yearbook of the Czech republic 2010) and in Krakow agglomeration it was 60.7 μg m⁻³ (Małopolska, Environment 2010). “With regards to pollution by suspended particles, the most serious situation remains in the Moravskoslezský region (the Ostrava and Karviná area). This is because there are other significant sources of suspended particles in this area, namely metallurgical and the fuel processing industries, in addition to transport and local sources, which are the main emission sources of suspended particles in other regions. Another factor that contributes to high local concentrations in this area is regional transfer from sources in Poland (the highly industrialised Katowice area)” (Statistical environmental yearbook of the Czech Republic 2010). Although the most important anthropogenic source of Pb to the global atmosphere remains vehicle emissions, the regional and local impacts from stationary sources may be significant and tend to increase (Shotyk and Le Roux 2005). “Traffic-related emissions may make a substantial contribution to the concentration of suspended particulates in areas close to traffic. Some agroindustrial processes and road traffic represent additional anthropogenic sources of mostly coarse particulate emissions. The largest stationary sources of particulate emissions include fossil-fuel-based thermal power plants, metallurgical processes, and cement manufacturing” (Ackermann and Hanrahan 1999). After the atmospheric transfer, the lead is accumulated to the soils and soil dust today remains an important component of the elevated Pb flux (Shotyk et al. 1998).

Since the Tatras represent a barrier for the pollutants in central Europe, the concentration of lead in the tissue of alpine vertebrates remains relatively high and exhibits no decrease at the moment. For example, the samples of lead in the chamois bones (16 μg g⁻¹) and its diet (17–18 μg g⁻¹) indicate that the West Carpathians are one of the most polluted mountains by lead in Europe (Mirek 1996, Janiga et al. 1998).

Several ecological, physiological and practical arguments support the use of small mammals in pollution biomonitoring and hazard assessment. Small mammals are often considered to represent an intermediate stage between low and high trophic levels, since they feed on herbs, fruits and invertebrates and on the other hand they constitute an important element in the diet of carnivorous birds and mammals. Moreover, they participate actively in soil bioturbation and take part in different subsystems due to their wide species variation in trophic types, which include herbivorous grazers as well as carnivorous predators of soil invertebrates (Metcheva et al. 2003). The physiological argument supporting the use of small mammals as bioindicators of exposure to environmental pollutants is related to their small body size. Due to a high metabolic rate their degree of exposure may be expected to be greater than in large mammals, which have a slower metabolic rate (Sheffield et al. 2001).

The Snow vole Chionomys nivalis well meets the criteria of good bioindicator. In comparison to many other species of alpine mammals, it is abundant and easily caught; it is exposed to metals mainly by ingestion of contaminated food or soil, and it is accessible for both population investigation and experimental research (Belcheva et al. 1998). The species differ from that of other vole species, owing to their different social organization; mainly during overwintering periods. The wintering animals are active, partly migrating and surviving the winter with the elevated levels of intraspecific aggression and spatial isolation (Luque-Larena et al. 2002a). It is a species with very low turnover: young usually do not reproduce in their year of birth, females produce in general two litters per season (spring and summer–autumn), litter size is usually low and survival is high (Yoccoz and Ims 1999).

In this study, we quantified the lead concentrations in the bones of Snow voles inhabiting the alpine areas of the West Tatra Mountains, the Western Carpathians, and examined the seasonal bioaccumulation pattern...
in this species. The effects of age and sex were also measured and connections between lead bioaccumulation and hematological markers were explained in order to understand the variation found in these parameters. Finally, we discussed the cycle of atmospheric lead in the mountains and the suitability of small rodents as bioindicators.

2. MATERIALS AND METHODS

2.1. Sample collection

We studied a local population of Snow voles at the Brestová mountain chain (49°13’29.43”N, 19°40’46.07”E, 1902 m.a.s.l.), the West Carpathians, Slovakia. We regularly sampled *C. nivalis* in September 2009 and from May to November 2010. The study area included three sites of alpine meadows mixed with rocky fields. This research was a part of a complex research on studying the atmospheric lead cycle in the alpine environment. Two study sites comprised of approximately 2 ha (south Brestová, 1a. 49°13’27.17”N; 19°40’40.19”E, 1847 m.a.s.l. and 1b. 49°13’23.10”N; 19°40’42.89”E, 1824 m.a.s.l) and 1 ha (north Brestová, 49°13’32.89”N; 9°40’44.43”E, 1839 m.a.s.l.) of rocky environment. In the three monitoring fields, the Sherman traps were released monthly, divided into squares (south) or lines (north) and were set up approximately 10 m apart from one another. Between 96 and 100 traps were controlled per day. Coordinates within the grid were noted. Traps were baited with fresh apples and commercial seeds for rodents. Dry grass was added to the traps as bedding material mainly during cold months. Traps were checked for two or three consecutive days every twelve hours, at dawn and dusk. The global sampling yielded a total of 50 samples (trapped and re-trapped individuals). In each captured individual, part (app. 3 mm) of the tail was clipped one or more times if an animal was retrapped. Sex determination was based on recognition of external genitalia and verified by PCR. 42 samples (of mature and immature animals) were finally used to examine difference in the content of lead between females and males. The animals were sorted by weight, body length, tail length and hind foot length (Baláž and Ambros 2010). Weight was taken using a spring scale (100 g Pesola). After the measurement the animals were immediately released at the point of capture. We clearly defined three age-classes of *C. nivalis* for the analysis: young immatures (born and caught in summer or autumn), old immatures (born in spring–summer, caught in autumn) and mature (overwinters) (Le Louarn and Janeau 1975, Mukhacheva and Bezél 1995). In this study, we followed the guidelines set out by the Slovak Republic Nature Protection Law and the ethical principles for treatment of animals in behavioral research defined by the International Association for the Study of Animal Behaviour.

2.2. Hematological indices and PCR sex determination

The peripheral blood was collected from the tail vein of all animals. Then, a drop of blood was smeared on a clean slide, air-dried, fixed in methanol and stained with May-Gruenwald and Giemsa solutions. Other blood was absorbed on the paper tampon, air dried and left for later PCR analysis. The stained smears were scanned under 1000× magnification. In twenty six representative samples of the three age groups, micronucleus frequency was scored on 10 000 erythrocytes.

DNA was extracted from blood using QIAamp DNA Mini Kit (Qiagen) in accordance with standard procedure for DNA isolation from dried blood spot. Sex was determined according to Brya and Konečný (2003) using two sets of primers. First set of primers, HMG-SRY for and HMG-SRY rev, amplify a 202 bp fragment of the SRY-HMG box in males and the second, ZFY/ZFX for and ZFY/ZFX rev, amplify a 447/445 bp fragment of the Zfy/Zfx gene for both (males and females). PCR was performed in master mix of total volume 12.5 μl as follows: 1.5 mM MgCl₂, 0.2 μM of each primers, 200 μM dNTPs and 1.5 U Go Taq DNA polymerase (Promega). An initial denaturing step at 95°C for 5 min was followed by 30 cycles of 95°C for 1 min, 50°C for 1 min and 72°C for 1 min. A final extension of 72°C for 5 min completed the program. PCR products were visualised on 3% agarose gel.
2.3. Estimation of Pb content

Approximately 0.0020–0.0374 g of a dried sample was digested with 2 ml of water, 4 ml of concentrated HNO₃ (Merck, Darmstadt, Germany) and 1 ml of H₂O₂ (Slavus, Bratislava, Slovakia) by using the microwave oven Mars Xpress (CEM Corporation, Matthews, USA). Decomposition temperature was 140°C, ramp time 15 minutes and hold time 13 minutes. After mineralization solution was diluted to 6–10 ml with deionized water.

Pb contents were determined by electrothermal atomic absorption spectroscopy (AAS Perkin Elmer 1100B, Norwalk, Connecticut, USA) equipped with deuterium background correction and HGA 700 graphite furnace with automated sampler AS-70. The instrumental conditions were: wavelength 283.3 nm; slit 0.7 nm; lamp current 10 mA.

The temperature programme was as follows: Drying 1: 70/10/10; Drying 2:150/2/60; Pyrolysis: 800/15/30; Atomization: 1800/0/3; Cleaning: 2500/0/3 (temperature (°C)/ramp time (s)/hold time (s)).

To prepare calibration solutions, aliquots were taken from a stock standard solution 1000 mg l⁻¹ of Pb (Merck, Darmstadt, Germany). The calibration range was 5–20 μg l⁻¹. The matrix modifier NH₄H₂PO₄ (0.2 mg) was used by the determination of Pb.

The results were evaluated from calibration curve, the accuracy of the determination was proved by the standard addition technique.

2.4. Statistics

In case that the data were normally distributed the results were statistically compared using one way ANOVA and Tukey’s test at the 95% confidence level (P <0.05). In case that lead content values showed a highly skewed distribution a non-parametric approach to the analysis of the data was necessary (Esselink et al. 1995). The significance of differences between groups of voles was tested in the Mann-Whitney rank sum test. When P <0.05, the data were considered significantly different. All statistical analyses were performed with Statistica 8 software for Windows. Groups of animals with different amount of micronuclei were compared by c² test.

3. RESULTS

Significantly higher concentrations of lead were detected in voles collected immediately after the winter season than in animals caught in summer or autumn (Table 1). The bioaccumulation of lead in C. nivalis bones showed clear seasonal differences. From summer to autumn, any pattern of age-dependent increase of lead in the re-trapped animals was not detected (Table 2). The female voles showed higher mean lead concentrations in the tail bones than males. Although females are more numerous than males in the spring, the different way of lead accumulation between sexes did not interact with seasonal effects of metal absorption In all seasons, females accumulated the lead into their bones more intensively than males (Two-way ANOVA on 42 samples, F of factor interactions = 1.61, P = 0.21). Micronuclei in erythrocytes in peripheral blood smears were more often observed in animals from early spring than from summer or autumn period (Table 3).

Table 1. Mean (SE) tail bone lead concentrations (μg g⁻¹ dry weight) in Snow voles, divided according to age and sex. The West Tatras, 2009–2010. Means with different letter indexes in a column are significantly different by Tukey multiple range test (P <0.05).

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th>Pb mean (SE)</th>
<th>One-way ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Generation:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overwinters – matures</td>
<td>21</td>
<td>16.11⁺ (1.22)</td>
<td></td>
</tr>
<tr>
<td>Young immatures (born and caught in summer or autumn)</td>
<td>14</td>
<td>4.34⁺ (1.49)</td>
<td>F = 17.09, P = 0.0001</td>
</tr>
<tr>
<td>Old immatures (born in spring–summer, caught in autumn)</td>
<td>15</td>
<td>7.76⁺ (1.44)</td>
<td></td>
</tr>
<tr>
<td>Sex:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td>16</td>
<td>13.07⁺ (1.74)</td>
<td>F = 7.04</td>
</tr>
<tr>
<td>Males</td>
<td>26</td>
<td>7.33⁺ (1.36)</td>
<td>P = 0.01</td>
</tr>
</tbody>
</table>
4. DISCUSSION

Our findings indicate that transboundary fluxes and lead wet deposition in winter are still an important source of metal pollution in the Western Carpathians. As well as in chamois, the metal may bioaccumulate and be biomagnified along the food-chain. In Rila Mountains, 87% of summer diet of Snow vole contents is composed from *Taraxacum officinale* Weber (25%), *Poa alpina* L. (49%) and *Nardus stricta* L. (13%). The rest of the stomach content is composed from mosses and lichens. Although *Taraxacum, Poa* and *Nardus* contained relatively high amounts of lead (8, 12 and 81 mg g⁻¹ in average, respectively), mosses and lichens may also significantly influence the uptake of lead in winter (Belcheva et al. 1998, Metcheva et al. 2008). 

Sivertsen et al. (1995) published the data on metal contamination in reindeer, moose and domestic sheep. Reindeer generally take up more elements from atmospheric deposition than moose or sheep. This is probably due to the high levels of lichens in their diet. In general, mosses and lichens are very suitable organisms for biological monitoring of air pollution due to their specific physiological features which easily enable to measure the heavy metal deposition (Zechmeister 1995, Šoltész 1998). Bryophytes have no roots, but instead rely exclusively upon atmospheric inputs for nutrient elements. Lichens can be used for the same purpose, but because they grow on a rock substrate, more care might be needed to separate atmospheric from lithogenic inputs (Shotyk and Le Roux 2005).

The vole’s ecological niche is represented by petricolic soils. Below this type of soil the thermal range is constant and permits the presence of the Snow vole in habitats with cold temperatures (Nappi 2002). Leconte (1983) considered only two seasons in Snow voles: the first from November to May (temperature around 0°C) and the second from May to November. More or less stenothermal conditions are very important factors determining the species mountain distribution. This may contribute to reduced costs of thermoregulation. The animals display almost identical energy budgets for winter and summer (Bienkowski and Marszalek 1974). In winter, the presence of snow cover affects the survival of many nonhibernating animals. The lack of snow cover in a cold environment (e.g. arctic) usually leads to large multiannual fluctuations in small rodent population numbers (Janau and Aulagnier 1997). The Snow vole is indeed not known to exhibit large fluctuations. The absence of cycles in alpine regions is an indirect consequence of selection in stable environments (Yoccoz and Ims 1999). In this habitat, the sexual activity starts in early spring when Snow voles are still under the snow cover (Kocianová-Adamcová et al. 2006) and their density is relatively low. In the Bank voles, Mukhacheva and Bezel (1995) found that high amounts of lead may influence the survivorship of wintering animals and consequently may contribute to the selection in a population.

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th>Pb mean (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caught</td>
<td>18</td>
<td>12.87 (1.81)</td>
</tr>
<tr>
<td>Retrapped</td>
<td>14</td>
<td>11.42 (2.05)</td>
</tr>
</tbody>
</table>

Table 2. Comparison of mean (SE) tail bone lead concentrations (mg g⁻¹ dry weight) between animals caught in spring or summer and those of them which were retrapped in summer–autumn season. No significant difference was found between the groups (Mann-Whitney rank sum test at P < 0.05) The West Tatras, 2009–2010.

<table>
<thead>
<tr>
<th>No. of micronuclei per 10 000 erythrocytes</th>
<th>Matures</th>
<th>Older immatures</th>
<th>Younger immatures</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>1–7</td>
<td>7</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>8 and more</td>
<td>4</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3. Number of micronuclei in matures and immature Snow voles (c² = 17.7, P = 0.001). The West Tatras, 2009–2010.
Our results revealed an increase of lead with age in winter, the increase did not continue in overwintered adults in summer and autumn. This variation is in accordance with results reported for Bank voles (Mukhacheva and Bezel 1995). Although not expected, the summer hepatic lead increase (Metcheva et al. 1995, Belcheva et al. 1998, Metcheva and Topashka-Ancheva 2003) in Snow voles may be attributed to the differences between hard and soft tissues accumulation. This bioaccumulation in hepatic tissue is often assumed as a stable detoxification mechanism to prevent toxic effects and does not show a clear bioaccumulation pattern with age (Metcheva et al. 2008). The observation contrasts with results from bones from the same species. This discordant result could be attributed to the differences between hard and soft tissues bioaccumulation and/or by differences in exposure levels or chemical forms of Pb. The half-life of Pb in soft tissues is in the order of days or weeks and the level of this element indicates a relatively recent exposure, whereas the main Pb body burden in mammals is the bones, where it is stored in a lower toxic form (Sánchez-Chardi et al. 2007b).

When compared with seasons, sex remains of less importance in bioaccumulation patterns. Lead bioaccumulation in mammals is related to reproduction and hormonal status. During pregnancy and/or lactation females may mobilized metals and transfer them across the placenta to the foetus or via milk to sucklings. Therefore, a priori a reduction in the concentration of toxic metals in females might be expected; however, contradictory results have been reported (Stansley and Roscoe 1996, Lopes et al. 2002). This is probably because females depone calcium to the embryo. Subsequently the lead substitutes for calcium in the hydroxyapatite crystal lattice with the hydroxyapatite crystal of the bones or teeth remaining intact and functional although altered chemically. Its deposition within the skeleton has been regarded as a protective feature to reduce soft tissue Pb levels (Ronis et al. 2001). The high concentrations of lead in female Snow voles may be due to nutritional status as well as to differences in dietary intake and uptake of metals (cf. Stansley and Roscoe 1996, Lopes et al. 2002). During their life the females are probably more exposed to environmental lead than males. Population sex ratios in these rodents vary seasonally, with the old females being more numerous over winter and spring (Baláž and Ambros 2010). More females may survive two winters than males (Reinhard 2008) and on the other hand some females born at the beginning of summer are able to have a litter by the end of the season, males are usually sexually active only next summer (Janaeu and Aulagnier 1997). Overwintered females normally produce two litters of one to four young. The gestation is about 20 days, the new-born weights range from three to four grams, what may be more than one third of the female body weight. So during the time when the snow melts, at the beginning of the breeding period, the metabolic rate of females is very intensive. The first emergence of immatures from the burrows is usually observed when they are two to three weeks old (Janaeu and Aulagnier 1997). In winter, males are less aggressive than females but the intensity of aggression displayed during interactions is higher in males than in females. Low degree of social tolerance suggests that individuals are spatially associated during overwintering periods (Luque-Larena et al. 2002c). Over the breeding season, sex ratio and home range of sexes changes (Kocianová-Adamcová et al. 2004). Reproductive females use zones of large rocks suggesting a pattern of site attachment related to reproductive tasks. A similar pattern of site tenacity was also observed in juveniles (Luque-Larena et al. 2002b). It agrees with the social organization model proposed by Le Louarn and Janaeu (1975), wherein the young remain within the home range of their mothers until the end of the reproductive period. Since offspring only disperse as adults, this might imply that dispersal is considered to be under the selection. The presence of the mother at the natal site does not cause dispersal of female offspring either (Le Galliard et al. 2007), suggesting mother-daughter cooperation at the natal site (Reinhard 2008). In contrast, adult male voles are generally more mobile than females (Nieder and Bocchini 1994, Luque-Larena et al. 2002b, Reinhard 2008). In winter, they usually spend more time in investigative behaviour than females (Luque-Larena et al. 2002c).
During the breeding season, adult females defend exclusive territories (Lecalouarn and Janeau 1975) and the spatial association of reproductive males trying to monopolize several females may produce a less discriminative use of local habitats. Although both males and females are socially intolerant, the different patterns observed between the sexes may underlie different strategies of food site selection. At the end of breeding period, voles become solitary and more nomadic along rocky structures (Lecalouarn and Janeau 1975). Snow melting period in the alpine zone is connected to the beginning of the vole breeding season. At this time, females are more conservative in selection of different food sites than males what may lead to the speculation that environment may induce differential uptake of metals between sexes.

Currently, approaches used to evaluate the consequences of environmental contamination involve the assessment of genotoxic damages and other cell-detrimental effects on organisms (Tanzarella et al. 2001). Micro nuclei, derived from chromatid, chromosome fragments, or lagging chromosomes provide an idea on the clastogenic potential of any agent and have been often used to evaluate somatic cell damage (Marques et al. 2006). Snow vole adults caught in the spring exhibited significantly higher micronuclei frequencies in peripheral blood than immature trapped in summer or fall. This result is in agreement with other studies on small mammals, reporting an association of micronuclei frequencies and metal concentrations (e.g. Ieradi et al. 1996, Topashka-Ancheva et al. 2003, Metcheva et al. 2008).

The lead concentrations observed in the alpine animals probably do not reach toxic levels for many rare species, but they are often higher than the no-observable-adverse-effects-level in vertebrates and may indicate a considerable environmental pollution (Janiga 2001). Scarce information is available about food-chain transfer and uptake and tissue concentration (Janiga 1999, 2002). In fact, the assessment of environmental pollution effects in wild biota has been a vital challenge for ecotoxicologists in spite of evident difficulties caused by constantly changing Alpine environments and high intra-specific variability (Janiga 2008).

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