

Howell-Jolly bodies in peripheral blood of Yellow-necked mouse (*Apodemus flavicollis*) living in the vicinity of pulp mill industrial complex

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Abstract. The present study was focused on counting of Howell-Jolly bodies in peripheral blood of Yellow-necked mouse (*Apodemus flavicollis*), in localities Mnich and Hrboltová. The aim of the study was counting Howell-Jolly bodies per 1000 erythrocytes in peripheral blood for all 164 captured animals. Increase of Howell-Jolly bodies was recorded for 47% of Yellow-necked mice in locality Mnich and for 35% in locality Hrboltová. Amount of Howell-Jolly bodies in peripheral blood of animals between two study sites did not differ significantly, but showed tendency to increase.

Key words: Yellow-necked mouse, Howell-Jolly bodies, lead, pulp mill

Introduction

The pollution by heavy metal is a growing environmental problem in the world (Nasrabadi *et al.* 2010; Haruna *et al.* 2011; Yu *et al.* 2011; Kargar *et al.* 2012; Diviš *et al.* 2012).

Distribution of metals in the environment is a result of natural processes (volcanoes, erosion) and anthropogenic activities (industrial, agricultural processes and fossil fuel combustion) (Florea and Busselberg 2006). Human activity locally increases the concentration of heavy metals as such in soil, plants and animals (Said *et al.* 2005). Heavy metals are not degraded and therefore persist long time in the environment (Blagojević *et al.* 2012). The levels of heavy metals in individual components of environment vary (Lukačinová *et al.* 2011). Heavy metals may adversely affect human and animal health (cancer, haematological toxicity, allergy) (Dvorožňáková and Jalčová 2011). Heavy metals induce morphological, physiological and genetic changes. Most frequent pathological changes concern the blood and hematopoietic organs, digestive

tract and respiratory system (Jančová *et al.* 2004). Long-term exposure to heavy metals could result into dysregulation of cellular pathways causing subsequent toxicity (Fitsanakis and Aschner 2005). Metals and metal compounds interfere with functions of the haematopoietic system, the central nervous system, liver and kidneys (Florea and Busselberg 2006).

The metals can be categorized as essential which are presented in the living organism (such as zinc, copper, cobalt, iron, manganese, molybdenum) and non-essential (lead, cadmium, mercury) which are toxic, have unknown function in living organism (Hoffman *et al.* 2001; Marquez 2008). Essential heavy metals in living organism occur in varying amounts but excessive levels can be harmful to the organism. Long-time accumulation of non-essential toxic heavy metals such as lead, mercury, plutonium, cadmium in the body of animals and humans may cause serious illness. The uptake and distribution of essential metals is physiologically regulated, in comparison with non-essential elements (Blagojević *et al.* 2012). Toxic metals including lead, cadmium and zinc are present in all component of environment (Cimboláková *et al.* 2011). Metals are natural constituents found in rocks (Marquez 2008). They occur naturally in the earth but they are released to the environment by human activities (Çavusoglu *et al.* 2010).

Lead and cadmium are heavy metals with a wide distribution in ecosystems. They affect physiological functions in hematopoiesis and the respiratory, nervous, reproductive and excretory systems (Dvorožňáková and Jalčová 2011). People and animals may be exposed to toxic metals by drinking, eating, breathing and touching any contaminated material in the environment (Cavusoglu *et al.* 2010).

Lead is a toxic, heavy, relatively uncommon, low melting, biodegradable metal. It occurs naturally in the Earth's crust (at about 15-20 mg/kg). Levels of lead in the environment have increased over the past three centuries because of human activity. Amongst toxic heavy metals, lead in any form seems to be an environmental poison for all animals and humans (D'Souza *et al.* 2003). Humans are exposed to lead derivatives and have a daily lead intake by eating food, drinking water and by inhalation. Lead is one of the most widely studied environmental toxins (Florea and Busselberg 2006).

“Lead is absorbed through digestive and respiratory tracts and skin. After absorption into the blood, 99% of lead is bound to erythrocytes and the re-

maintaining 1 percentage stays in the plasma to be carried to other tissues" (Mugahi *et al.* 2003).

Toxic effects of lead can be considered at biochemical, subclinical, and clinical levels. Chronic exposure to lead could have consequences such as increased blood pressure, decreased fertility, nerve disorders, muscle pain and problems with memory or concentration (Tvrdá *et al.* 2011). Exposure to lead can affect synthesis of hemoglobin (causing anemia) (Kruger *et al.* 2007). Lead induces a significant increase in the incidence of Howell-Jolly (H-J) bodies (Tapisso *et al.* 2009; Mitkovska *et al.* 2012).

Cadmium is an environmental pollutant that has serious toxicity to humans and animals. Cadmium is a potent mutagenic agent (Marquez *et al.* 2005). Genotoxic effects are dependent on the exposure time. Zinc is less genotoxic than lead or cadmium. Zinc can induce the increase of micronuclei frequencies but the effects are not as high as in animals treated with lead and cadmium (Tapisso *et al.* 2009; Mitkovska *et al.* 2012).

Lead is a major environmental pollutant affecting every living organism (Okeridan *et al.* 2010). It can threaten the life of living creatures in many ways (Mugahi *et al.* 2003). Lead goes into the body by ingestion to the intestines, through the lung with inhalation, through direct injection or through the skin (Tvrdá *et al.* 2011). Lead has multiple hematological effects (microcytosis and hypochromy of red blood cells as a consequence of disturbed haem synthesis) (Liu, Goyer, R. A. and Waalkers, P. 1996; Topashka-Ancheva 2012). Lead is known to have toxic effects on the erythropoietic system that are associated with the development of anaemia (Slowjeiko 1990). Lead has influence on activities of several enzymes involved in haem biosynthesis. This is how lead changes hematological system. Anemia caused by lead toxicity may lead to the decrease of red blood cell survival because the membrane has become more fragile (Mugahi *et al.* 2003).

Some organisms provide valuable information on the chemical composition of the environment through their ability to concentrate toxins from the environment in their tissues. Small terrestrial mammals are suitable for studying the effects of environmental pollution because they achieve high abundance and their migration capability is limited (Dvorožňáková and Jalčová 2011; Jančová *et al.* 2004).

Small mammals as bioindicators

The number of studies concerning heavy metals bioaccumulation in small mammals is rising from year to year (Sheffield *et al.* 2001; Sánchez-Chardi *et al.* 2007; Blagojević *et al.* 2012). Small mammals have good ability to accumulate a wide spectrum of pollutants present in the ecosystem such as heavy metals, pesticides, radionuclides (Ieradi *et al.* 1998; Mitkovska *et al.* 2012). They have specific biological reactions (changes of hematological indices) (Topashka - Ancheva 2012). The transfer of metals from the environment to terrestrial mammals depends on several abiotic and biotic factors (season, temperature, diet, age, sex, exposure time, pollutant concentrations in the exposure area) (Lopes *et al.* 2002).

Small mammals are very useful monitors for studying the heavy metal contamination in polluted areas (Talmage and Walton 1991;

Gdula-Argasinska *et al.* 2004; Mitkovska *et al.* 2012) because they are evolutionally close to humans (Michailova *et al.* 2010). They are characterized by large populations, short generation times, and they have a territory of limited range and are good for studies based on capture-recapture methods (Leslie *et al.* 1953; Martin and Coughtrey 1982; Michailova *et al.* 2010). Another advantage of using small mammals as bioindicators is their small body size. Small body size of mice is connected with their high metabolic rate and thus their intensity of exposure is greater than in large mammals with slower metabolic rate (Sheffield *et al.* 2001; Gdula-Argasinska *et al.* 2004). The concentration of heavy metals in small mammals is dependent on exposure time, pollutant concentrations in the exposure area, season, age, gender, diet and physiological status of the animals (Lopes *et al.* 2002; Martiniaková *et al.* 2010; Blagojević *et al.* 2012).

The ingestion of contaminated food by rodents is the primary source of heavy metals in their body. The second source is inhalation of heavy metals (Ma 1994; Sheffield *et al.* 2001; Gdula-Arhasinska *et al.* 2004). Accumulation of heavy metals is different among species, based on their diet. The highest metal levels are bioaccumulated by insectivores (for example shrews). Their food is comprised mostly of earthworms which are known to have strongly accumulated metals (Roodbergen *et al.* 2008). The accumulation of toxic elements (such as lead and cadmium) decreases in the following sequence: insectivorous (shrews) > omnivorous (mice) > herbivorous (voles) (Metcheva *et al.* 1996, 1998).

Kostial *et al.* (1978) showed that the early neonatal age is a critical period for metal accumulation in rats. The concentration of heavy metals in soft tissues (liver, kidney) of small mammal population decreases with age (Lopes *et al.* 2002; Sánchez-Chardi and Nadal 2007; Blagojević 2012). It is explained by high uptakes of food and the higher metabolic rate of juveniles animals (Blagojević 2012).

A biomarker is any biological response to a chemical demonstrating a departure from the normal status of the intact organism. Biomarkers supply information for measuring possible harmful effects in the environment (Peakall and McBee 2001; Kruger *et al.* 2007).

A. flavicollis is one of the commonest woodland rodents in Europe (Marsh 1999). It is one of the most dominant species in Slovakia (Martiniakova 2010). Degrassi *et al.* (1999) suggest a higher suitability of the genus *Apodemus* in terrestrial studies of environmental pollution by toxic metals (Mitkovska *et al.* 2012).

A. flavicollis fulfils the criteria of a good bioindicator: it is abundant, easy to catch, easy to manipulate, has populations that are large and it is in contact with contaminated soil and ingests contaminated food throughout its life cycle.

Howell-Jolly bodies (micronuclei)

„Micronuclei are thought to be the result of chromosome breakage (or other anomalies occurring during mitosis) that result in the retention of small fragments of chromatin or whole chromosomes in polychromatic erythrocytes after expulsion of the nucleus in the processes of red blood cell maturation“ (Marques 2006).

Hematological parameters can be suitable indicators of the condition, the physiological status and the state of immunological resistance of animals. Red blood cell parameters reflect the ability of blood to carry oxygen (Wolk and Kozłowski 1989). A micronucleus test to detect environmental mutagens on wild living rodents has been used since 1978 (Materiy and Maslova 1987; Degrasi *et al.* 1999; Chassovnikarova *et al.* 2010). The first to use micronuclei as a marker for cytogenetic damage were Evans *et al.* (Evans *et al.* 1959). Since then many researchers have focused on counting micronuclei in the erythrocytes of small mammals, for example in Bulgaria, Spain, Czech Republic (Sawicka-Kapustova *et al.* 1987; Metcheva and Topashka-Ancheva 2003). Significant correlations between heavy metal contamination and presence of H-J bodies in erythrocytes were detected in rodents living near polluted areas (Degrasi *et al.* 1999; Tapisso *et al.* 2009). Metcheva and Topashka-Ancheva (2003) in two international projects (Bulgarian-French, Bulgarian-Swiss) found correlations between heavy metal contents and the percentage of pathological erythrocytes (H-J bodies, basophilic granulation, morphological disturbances) in small mammals. H-J bodies were present in all captured species (mainly *Myodes glareolus* and *A. flavicollis*) from polluted area.

Micronuclei are small, extranuclear bodies originating either from chromosome fragments or whole chromosomes formed during mitosis when an entire chromosome fails to migrate in one of two daughter nuclei (Degrasi *et al.* 1999). Micronucleus is formed when a chromosome (or chromosomal fragment) is not attached correctly to the spindle during mitosis (Durling 2008) (see Fig. 1).

Micronucleus tests

To test experimentally occurrence of H-J bodies, study by Marquez *et al.* (2005) on Algerian mice (*Mus spretus*) was made. Mice were contaminated by intraperitoneal injections with 5 or 10 doses of cadmium acetate, lead acetate and zinc acetate and their combinations (Cd + Zn, Pb + Zn, Pb + Cd). The increase of the micronucleus frequency was observed in all single metal treated groups in the sequence: Cd, Pb and Zn. Pb + Cd groups showed the highest frequency of micronuclei in comparison to other groups.

In Bulgaria, four different species of adult rodents were compared: Yellow-necked mouse,

Bank vole, Pine vole and Snow vole were collected in Rila mountains at different altitudes (Mala Tzerkva - 1200 m, Maliovitza - 1800 m, Moussala - 2925 m). Blood samples were taken from the sublingual vein during anaesthesia. The number of micronuclei in erythrocytes per 1000 cells of each blood smear was counted. H-J bodies were present in all monitored species. The results showed genetic damage in the rodents from the study area (mainly from Mala Tzerkva). The size and shape of erythrocytes were not changed (Metcheva *et al.* 1995). Animals were also dissected. Lead (with highest concentration) and cadmium in inner organs of rodents was present in Mala Tzerkva and Moussala.

The Bank vole, Pine vole, Yellow-necked mouse and *Sorex araneus* were collected in 1995 in the region of Beli Iskar (Bulgaria, altitude 1800 m). Snap traps were used and designated for stomach analyses were done on the trapped animals. The caught animals were classified by a trophic chain: herbivorous (Bank vole, Pine vole), omnivorous (Yellow-necked mouse), carnivorous (*S. araneus*). For the Yellow-necked mouse most usable food are seeds (75% from the quantity) and the green vegetable parts comprised only 10% of diet. The rest of food was animal food or unidentified (Metcheva *et al.* 1996). The study showed higher accumulation of lead (in kidney and spleen) in the body of Yellow-necked mouse (omnivorous) and highest accumulation of lead and cadmium in the body of Common shrew. The value of toxic metals in inner organs of Pine and Bank vole (herbivorous) was insignificant. The study showed differences between the food of rodent species and concentration of the toxic elements in the bodies of herbivorous, omnivorous and insectivorous. Blood samples were taken from the sublingual vein. The results showed no changes in shape, size and morphology of erythrocytes. Micronucleus or basophilic granulation was not found. Only in the Bank vole polychromatophilic erythrocytes was found.

Monitoring of small mammals was carried out during the years 1994-1995 in two international projects in two impact regions of pollution (National park Rila Mountain, Rhodops, National park Vithosa Mountain). The animals were dissected and blood for a blood smear was taken. Bioaccumulation of toxic elements in inner organs of all caught species showed that *A. flavicollis* is the greatest bioindicator for determination of lead. *Microtus arvalis* is the second best with bioaccumulation of lead in the body. H-J bodies and basophilic granulation were calcu-

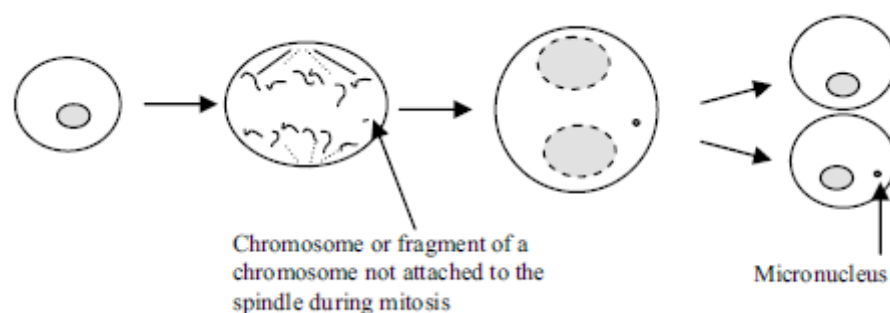


Fig. 1. Formation of micronucleus (After Durling 2008).

lated in erythrocytes. *A. sylvaticus* from the polluted region presents a high percentage of basophilic granulation. Micronuclei were observed in all species (*M. glareolus* - 100%, *A. flavicollis* - 75%, *Ch. nivalis* - 75%) from the polluted area Malla Tzerkva (Metcheva and Topashka-Ancheva 2003). There is the evidence for a correlation between pathological changes (H-J bodies, basophilic granulation) and heavy metal accumulation in small mammals (Metcheva and Topashka-Ancheva 2003).

In Czech Republic, wild rodents *A. flavicollis* and *M. glareolus* were collected in a polluted area in the town of Litvinov (petrochemical industries, thermal power plants - higher concentration of SO₂, NO_x, dusty aerosols) and in control site in northern Bohemia (Filipov). Soil samples were collected and analysed for content of heavy metals in both areas (Litvinov - higher contents of Cd and Pb were observed). The micronuclei in peripheral blood test showed increased frequency of micronuclei in *A. flavicollis* from Litvinov, in comparison to Filipov. *C. glareolus* had micronuclei in higher concentrations but those in *A. flavicollis* their amount was significantly higher. Ieradi *et al.* (2003) showed that the presence of micronuclei frequencies in erythrocytes of *A. flavicollis* is a good marker of environmental pollution. The difference between frequencies of micronuclei is dependent on the age of animals. Adult mice have a small increase of micronuclei in peripheral blood like subadults (Ieradi *et al.* 2003). He showed that the presence of micronuclei is not dependent on the sex of animals. There were no significant differences in micronucleus frequency between sexes of animals in either study species.

In a study at the University of Agriculture, Nigeria (Okeradin *et al.* 2010) 25 adult male of Wistar rats were divided into five groups (A - E). Control group A was given distilled water. Groups B, C, D and E ingested graded doses of lead during fourteen days. The blood lead concentration was significantly higher for B, C, D and E groups in comparison to the control group. The count of red blood cells decreased. The dosage of lead exposure increased (Okediran *et al.* 2010). H-J bodies and basophilic stippling were present in groups D and E. There is a connection between lead poisoning and influences on hematological parameters on rats: presence of H-J bodies, basophilic stippling, anemia, reticulocytosis and decreased hemoglobin concentration (Okediran *et al.* 2010).

A similar study was done by Mugahi *et al.* (2003), study was conducted using 25 adult males of Wistar rats divided into three groups (A - control group, B and C group intaken lead acetate). The results were very comparable to the results of the Nigerian study. Hematological study showed the presence of H-J bodies, basophilic stippling in groups given lead acetate and a decrease in red blood cells and increase in monocytes and thrombocytosis were observed.

Aims of the study

Free-living rodents are often used for monitoring of environmental pollution. The goals of present study were (1) counting the number of H-J bodies in peripheral blood of *A. flavicollis* trapped in the polluted area Ružomberok (on the Mních peak) compared to Hrboltová; (2) comparison of the number of micronuclei in the blood of captured animals

by season and (3) comparison of the number of micronuclei in the peripheral blood of different sexes of mice from polluted areas.

Material and Methods

Pulp mill factory complex is situated in Ružomberok city. The history of the company started in 1880. Until today, the factory made headway and their continual modernization caused decreases in released pollutants (Brnušáková *et al.* 2014, www.mondigroup.com 2015). The area of study covers the peak Mních (657 m) in Ružomberok, situated 2 km north-east of the pulp mill complex. The climate in this region is characterized by wet summers and mild winters. The vegetation of the study area is dominated by coniferous forest but there are also deciduous trees and meadow habitat with herbs and grasses.

The study species, *A. flavicollis* belongs to the subgenus *Sylvaemus* common in woodlands. *A. flavicollis* is a non-protected species of rodent (Wolk and Kozłowski 1989; Ondříková *et al.* 2010). It usually lives in older forest stands in mixed deciduous forests, and coniferous and deciduous forests with rocky areas (Flowerdew 1985; Degrassi *et al.* 1999). It is primarily a forest-dwelling species (Montgomery 1979). It is a good climber. Yellow-necked mice have climbed 23 metres high in Poland (Borowski 1962). The species often look for food by climbing up trees, using the long tail to hold themselves. The populations dynamics of Yellow-necked mice is characterized by regular annual cycles and irregular prolonged fluctuations (Flowerdew 1985; Horvath *et al.* 2008).

Yellow-necked mice are omnivorous. They have a variable diet, including animal food (Metcheva *et al.* 1998). The most common food is seeds. Metcheva *et al.* (1996) listed the food of Yellow-necked mouse as consisting of 75% seeds with a green vegetable part of 10%. The food is a base for intake of heavy metals to the body of the animal. To present the heavy metal and toxic element utilization through the trophic chains, it is necessary to know the food spectrum of the animals (Beltcheva *et al.* 1998).

The Yellow-necked mouse is characterized by seasonal and multiannual fluctuations in numbers (Adamczewska 1961; Wolk and Kozłowski 1989). Wójcik (1993) in his study (Poland) on fluctuating population of Yellow-necked mice came to the conclusion that the population density of *A. flavicollis* is more stable during spring season. High fluctuations were during autumn season. Species of *Apodemus* are known to be able to travel over one kilometer in short periods (Montgomery 1997). *A. flavicollis* is adaptable to multiple habitats (Bujalska and Grum 2005; Balčiauskienė 2009). They migrate mainly in summer season and in winter season stay in one place (Balčiauskienė 2009). Their ranges are from 1-10/ha but may be up to 50/ha (Macdonald and Tattersall 2001). Males are characterised by larger home ranges than females (Horvath *et al.* 2008). The mating system is characterized as typical polygamy (Wójcik 1993). Mature males have individual territories which they defend against another males. Several females may live on a single male territory (Wójcik 1993).

Yellow-necked mice start breeding in late winter or early spring (February or March)

(Balčiauskienė 2009). The reproductive period lasts from February or March till October. Reproduction is also possible in winter months, if they have enough food (Flowerdew 1985, Balčiauskienė 2009). The Yellow-necked mice were collected from September 2011 to March 2015 using Sherman live-traps. Sherman traps are one of the most used trap methods for sampling small mammals (Torre *et al.* 2010). The traps were baited with oatmeal, fresh apples, and walnuts and sometimes with a commercial seeds for rodents. Hay and dry grass from a store were used as bedding material added to the traps (during cold months). Between 60 and 80 Sherman traps were set up in the three monitoring fields before the sundown. Traps were divided into squares or in some places in lines, and set up approximately 10 meters apart from each other. The places with set traps were noted on a paper map. Sherman live traps were placed in a random fashion underneath cover (fallen branches, shrubs, mosses) (Janiga *et al.* 2012). Traps were checked early every morning (a short time after sunrise) during two consecutive days. During cold snowy months the traps were checked every 12 hours.

Animals were collected early in the morning. The traps without the animals were left in the original place for another night. Captured animals were transported alive in the traps to a laboratory two kilometers away.

Sample preparation

Each captured individual was identified to species. Taxonomic affiliation of caught individuals was assessed using the book *Rodents and Insectivores of Slovakia* (Baláž *et al.* 2010).

The captured animals were classified into four groups: winter (not breeding season, from December to February), first litter (from March to April), second litter (from May to August) and the third litter (from September to November). Each captured individual was weighed using a spring scale (100 g). Parts of the body of captured animals were measured: length of the body, length of tail, ear and length of hind foot (Janiga *et al.* 2012). Gender of animals was determined. The genitals of female rodents are situated closer than male genitals. In subadult animals, there are distinguishable small nipples of female and abdominal testes in males (Balčiauskienė *et al.* 2009).

After the measurement and division by sexes, part of the tail was clipped (app. 0,03 cm) from each captured animal. Blood was taken from the tail vein (*vena caudalis*) or by injection puncture from leg vein (*vena saphena*).

A drop of blood or more small drops of blood were placed on the clean, grease-free slide. Using another slide at a 45° angle, it was made a thin smear. Two slides from each animal were prepared. Every slide with blood smear was marked with date of actual day, capture locality and number species. The blood smears were left to dry in air for two hours and placed in a box with stand for slides.

After measurement and taking of the blood sample, animals were released at the point of capture. In the laboratory, the smears were stained with a combination of stains (May-Grünwald and Giemsa-Romanowski) using the modification meth-

od based on Pappenheim (Lucas and Jamroz 1961). The blood smears were put in a stand with concentrate May-Grünwald stain, for 3 minutes. After that, for 1 minute the blood smear was washed off in distilled water. Blood smears were given to the third cassette with dilute Giemsa-Romanowski stain (1:9), for 15 minutes. After that process the blood smear was washed off with water and left to air dry at room temperature (Doubek 2003). Stained blood smears were numerically evaluated with the use of immersion oil under 1000 times magnification using a meandering movement of the slides. All slides were scored two times by two persons to avoid interobserver variability. The number of H-J bodies in peripheral blood per 1000 cells for each animals was calculated.

Statistics

The PC program Microsoft Excel was used for creation of a data matrix. The spreadsheet includes the following data: mark of the animals, day, month and year of capturing animals, name of the species, locality of trapping (Hrboltová, Mnich), coordination of trapping, trap number, age, and sex of animals, animal weight, body length, tail length, ear length, foot length, and weather condition. The last two columns are focused on H-J bodies (count of H-J bodies and value of H-J bodies classified according to three categories). We categorized animals according to a number of H-J bodies into three groups. The first group with 0-3 H-J bodies per 1000 erythrocytes (number 1), the second group with 4-9 H-J bodies per 1000 erythrocytes (number 2) and the third group with 10 and more H-J bodies per 1000 erythrocytes (number 3). The data were copied from Microsoft Excel to the PC program STATISTICA 8.0. This program was used for statistical analysis. The data were standardized and then the correlation matrix was created. The principal component analysis was used to compare the amount of H-J bodies between different groups. The Tukey method (Tukey's test) was used for multiple comparisons. One-way analysis of variance (ANOVA) was used to determine any significant differences between the means of two or more independent groups. We tested 11 factors. The data are expressed as mean ± standard deviation (SD). P-values less than 0.05 were considered statistically significant.

Results

Occurrence of micronuclei

141 samples of peripheral blood from *A. flavicollis* were evaluated in the present study (100 from Mnich, 41 from Hrboltová).

The animals were classified into three groups (called values in the table) based on the amount of H-J bodies in their peripheral blood (see Fig. 2):

- value 1: 0-3 H-J bodies per 1000 erythrocytes
- value 2: 4-9 H-J bodies per 1000 erythrocytes
- value 3: 10 and more H-J bodies per 1000 erythrocytes

Morphology of *A. flavicollis* and H-J bodies in the blood

The morphology was studied by using principal components analysis. The component eigenvec-

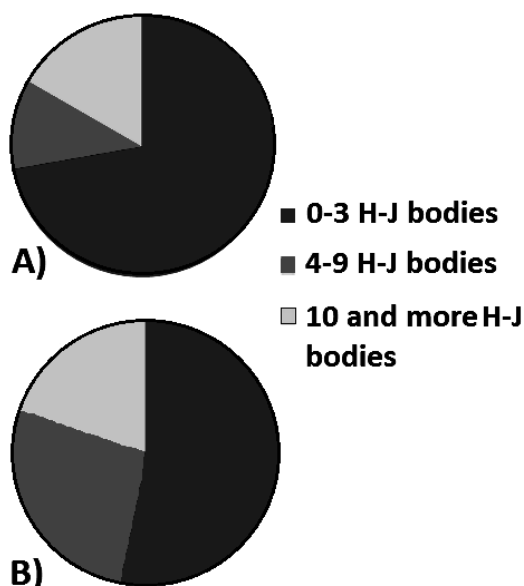


Fig. 2. Values of Howell-Jolly bodies in peripheral blood of yellow-necked mouse from A) locality Mnich, B) locality Hrboltová.

tors are presented in Table 1. The factor 1 indicates the variability in the body size of mice. The second factor mainly denotes the variation in the amount of H-B cells. The third factor refers to the variation of body length and the fourth one is more or less bipolar vector describing the contrast between the increase in the body weight and decrease in the foot length and vice versa.

Litters - generations of mice in different seasons

The animals were classified into four groups (see Fig. 3): W - winter period - no breeding season (December to February). FL - first litter period - from March to April, SL - from May to August, TL - from September to November. Although there is a tendency that winter animals contain less H-J bodies in their blood than animals from subsequent litters, the difference was not statistically significant (Fig. 3). The amount of H-J bodies does not differ according to the time of capture. The result was not statistically significant. But the scores of third principal component (factor 3) show that the differences between winter and other animals

Eigen vectors of correlation matrix				
Variable	Factor 1	Factor 2	Factor 3	Factor 4
Weight	-0.644	-0.079	-0.090	-0.755
Body length	-0.486	0.115	0.810	0.306
Foot length	-0.532	-0.435	-0.468	0.556
H - J bodies	0.254	-0.889	0.342	-0.165
Variability	41%	25%	21%	13.5%

Table 1. The eigenvectors of first four principal components.

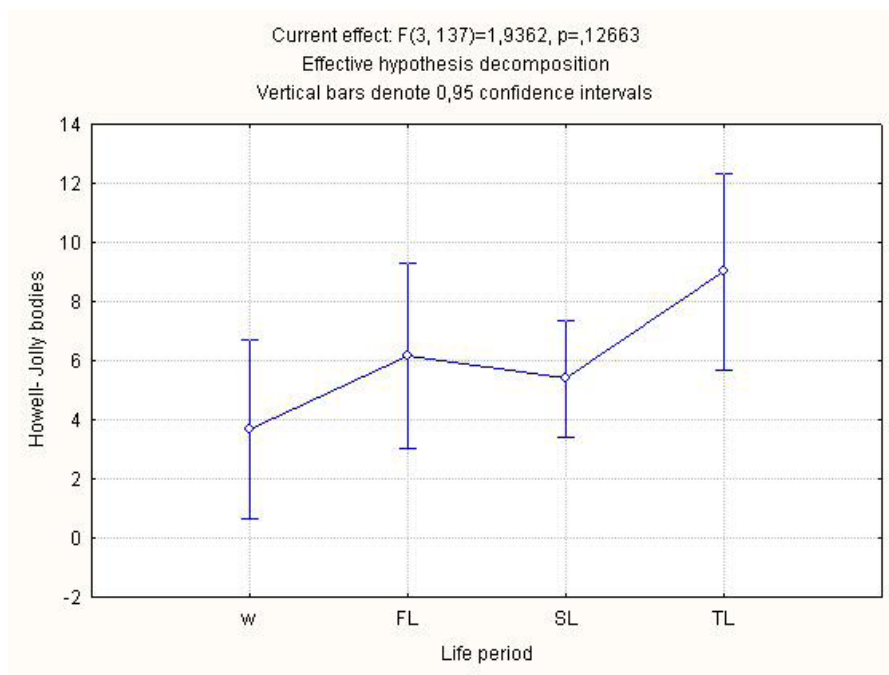


Fig. 3. Number of Howell-Jolly bodies per 1000 erythrocytes in the peripheral blood of Yellow-necked mouse in different seasons. W - winter period - no breeding season (December to February). FL - first litter period (March to April), SL - second litter period (May to August), TL - third litter period (September to November).

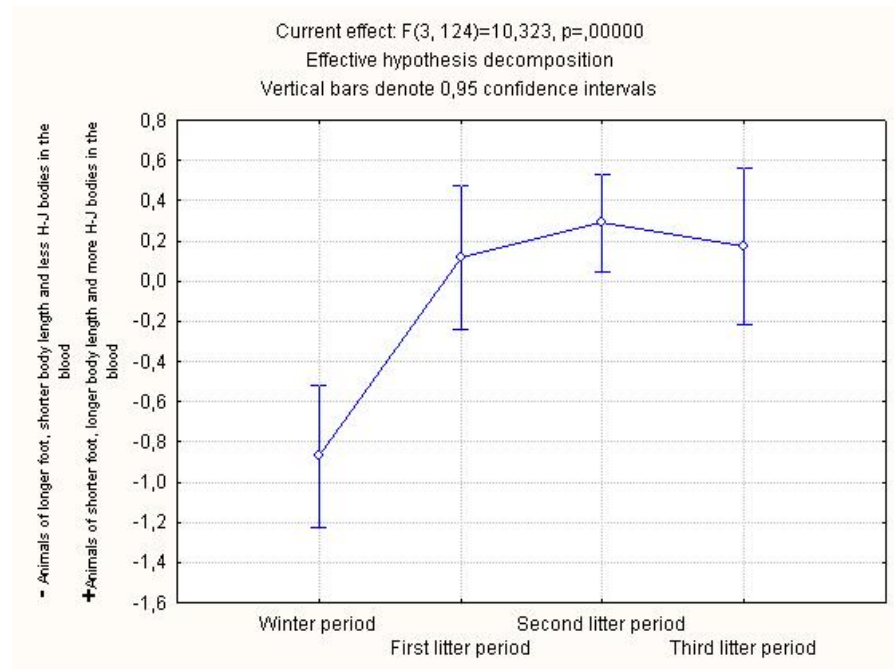


Fig. 4. Winter animals of relatively long foot and short body (adults) contained less H-J bodies than mice from the subsequent litters with relatively long body and short foot (animals from breeding period), means of component scores of factor 3 (Table 1) - W (n = 26), FL (n = 25), SL (n = 56), TL (n = 21). Group W significantly differs from the three litter groups.

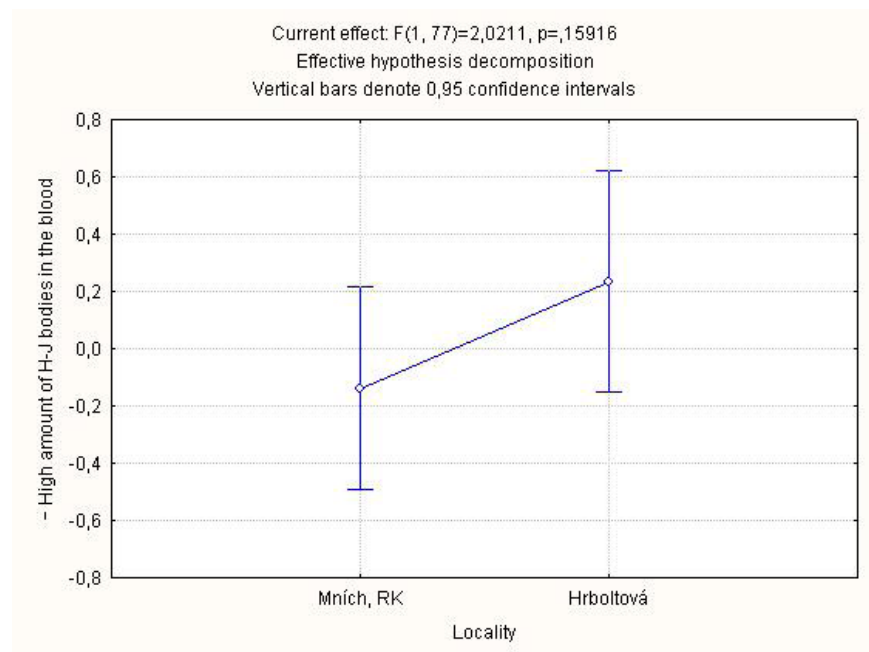


Fig. 5. Comparison of principal components scores of factor 2 (Table 1) between Mních and Hrboltová. Low numbers indicate more H-J bodies in the blood of animals.

were highly significant (Fig. 4). Animals in breeding season (FL, SL, TL) have a longer body length but relatively shorter foot length. These mice tended to have more H-J bodies in their peripheral blood than winter animals. Animals with relatively shorter body length but relatively longer foot length have had fewer H-J bodies in their peripheral blood than mice from breeding period. In winter, the adults were mainly caught, such adults which were able to survive the winter (animals of relatively longer foot). But the difference between winter and „litter“ animals is

probably not related to the age or sex of mice. Testing the original data as well as principal component scores, we found no significant differences in the number of H-J bodies between sexes or between yearlings and adults. This suggests that during the breeding period the animals are more sensitive to diseases than in winter. The worst conditions are probably in autumn.

Experimental study area

The comparison of component scores (factor 2) rep-

Month	Day	Year	Weight (g)	Sex	BL (mm)	TL (mm)	EL (mm)	FL (mm)	H-J B	Value
November	15	2011	20.5	♀	100	52	11	17	1	1
January	26	2012	16.0	♀	88	53	10	15	1	1
March	21	2012	24.0	♂	102	45	8	16	0	1
April	26	2012	27.0	♀	60	—	13	15	2	1
July	26	2012	26.5	♂	99	42	13	17	2	1
October	23	2012	27.5	♀	118	55	13	18	2	1
January	29	2015	25	♂	82	325	13	18	4	2
January	29	2015	22	♀	71	—	11	19	6	2
March	9	2015	23	♂	91	22	9	16	0	1
March	10	2015	23	♀	86	36	10	12	2	1
March	10	2015	21	♂	74	37	11	16	1	1
March	11	2015	19	♂	85	36	93	16	6	2
March	11	2015	21	♂	75	37	78	18	1	1
March	11	2015	24	♀	85	39	86	15	1	1

Table 2. Howell-Jolly bodies in peripheral blood of *Myodes glareolus* from locality Mních.

Month	Day	Year	Weight (g)	Sex	BL (mm)	TL (mm)	EL (mm)	FL (mm)	H- J B	Value
May	24	2012	21.5	♀	98	52	13	17	1	1
May	24	2012	36.5	♀	111	50	13	17	0	1
June	15	2012	18	♀	85	75	7	18	6	2
June	15	2012	26	♀	93	47	—	18	6	2
June	15	2012	18	♂	94	48	13	19	0	1
June	15	2012	20	♂	96	45	11	17	2	1
July	27	2012	31	♀	102	—	13	17	4	2
July	27	2012	25,5	♂	106	48	11	16	1	1

Table 3. Howell-Jolly bodies in peripheral blood of *Myodes glareolus* from locality Hrboltová.

representing the variation in the number of H-J bodies in the peripheral blood of animals from Mních and control area Hrboltová showed no significant differences between the groups (Fig. 5). There is a visible tendency that animals from Mních have more H-J bodies in their blood than mice from control area but the difference was not significant.

Myodes glareolus

For Mních, 14 samples of peripheral blood from *Myodes glareolus* were also evaluated. For Hrboltová - 8 samples (Tables 2 and 3). This species has significantly less H-J bodies in peripheral blood than *A. flavicollis*.

Discussion

Wild Yellow-necked mice (*A. flavicollis*) were chosen as bioindicators of heavy metal pollution. This species has been widely used as a bioindicator in environmental studies performed in Bulgaria, Spain, Czech Republic, North America and Slovakia (Sawicka-Kapustova *et al.* 1987; Talmage and Walton 1991; Metcheva *et al.* 1993; Metcheva and Topashka-

Ancheva 2003; Blagojević *et al.* 2012). Degraasi *et al.* (1999) suggest a high suitability of the genus *Apodemus* in terrestrial studies on environmental pollution.

We collected 114 animals from Mních (closely to paper mill complex) and 50 animals from Hrboltová from September 2011 to March 2015 (Yellow-necked mouse represented 85% of all animals, the rest *M. glareolus*). The animals were classified by the age into the three groups: juveniles, subadults and adults. They were also divided into seasonal groups (see above). Yellow-necked mice (*A. flavicollis*) have successive pregnancies from February to October producing litters of 2-11 young (Baláž and Ambros 2010). Some females born at the beginning of spring are able to have a litter by the end of season (Janeau and Aulagnier 1997). Reproduction is possible also in winter months, if they have enough food (Flowerdew 1985; Balčiauskienė 2009).

164 blood smears of captured animals were evaluated. The number of H-J bodies in peripheral blood per 1000 cells for each animal was calculated. The animals were classified into three categories: category 1 (0-3 H-J bodies per 1000 erythrocytes); category 2 (4-9 H-J bodies per 1000 erythrocytes) and category 3 (10 and

more H-J bodies per 1000 erythrocytes). According to Materly and Maslova (1978) and the first group (0-3) was considered a normal healthy animals with no increased micronuclei. The second and the third group were considered to be the individuals with increased amount of H-J bodies. Number of mice of the second or third groups was evidently higher in Mnich than in Hrboltová. The animals did not significantly differ in the "intensity of polluted blood" between the two areas but there were more "polluted" animals in the Mnich area than in control site Hrboltová. The amount of H-J bodies significantly increases in spring - summer - autumn period but it is not dependent on age or sex of mice. This suggests that in breeding period there are more members of mouse population which are at risk of becoming infected. More animals were susceptible to blood induced damage in Mnich than in control Hrboltová area.

The results showed no differences between the amount of H-J bodies in peripheral blood of males and females. Chassovnikarova *et al.* (2010) in National Biomonitoring Program of Bulgaria also found no significant differences in micronuclei frequencies between male and female captured in non-polluted area and in group from polluted region. The same results were published by Ieradi *et al.* (2003). Surprisingly, we did not find differences in the number of micronuclei between the age groups, between young and adults. Our outputs differ from the results published e.g. by Ieradi *et al.* (2003), they found the age differences in the number of micronuclei. Our results from Mnich probably reflect intensive environmental pollution from spring to autumn so the difference between age groups is minimized.

In the present study, 24 animals of species *M. glareolus* were also captured, and their blood smears evaluated. This species had significantly less H-J bodies in peripheral blood than *A. flavicollis*. The count of H-J bodies in the animal peripheral blood was approximately the same.

The number of H-J bodies in the blood of animals is an important indicator to detect the genetic damage induced by chemicals or radiation (Çavusoglu *et al.* 2010).

Free living small mammals are not in permanent contact with polluted area. They have a wide range of dispersion. Yellow-necked mouse is characterized by seasonal and multiannual fluctuations in numbers (Adamczewska 1961; Wolk and Kozłowski 1989). High traffic may influence the increase in the number of micronuclei in erythrocytes (Ieradi *et al.* 1996). Both Mnich and Hrboltová are situated along a heavily trafficked road, and probably this may be a reason that there were not statistically significant differences in the amount of micronuclei between the two localities.

In Bulgarian research, *A. flavicollis* was presented as a suitable species for biomonitoring studies using the frequencies of micronuclei (Metcheva *et al.* 1993; Metcheva *et al.* 1996, Chassovnikarova *et al.* 2010; Mitkovska *et al.* 2012). Differences between sensitivity of rodent species were found (*M. arvalis*, *M. macedonicus*, *A. flavicollis*). The best bioindicator of pollution with a significantly higher increase of micronuclei was species *A. flavicollis* (Chassovnikarova *et al.* 2010). In Czech Republic, Ieradi *et al.* (2003) showed presence of micronucleus frequencies in erythrocytes

of *A. flavicollis* that is a good marker of environmental pollution. The micronucleus test showed significantly higher frequency of H-J bodies in polluted area with coal industry in comparison to a non-polluted site. Another one monitoring species *M. glareolus* showed no significant increase of micronuclei.

Chassovnikarova *et al.* (2010) in National Biomonitoring Program of Bulgaria proved no significant differences in micronuclei frequencies between male and female captured in non-polluted area and in group from polluted region. They used micronucleus test of wild rodents for detection environmental pollution. The study area covered two regions in Bulgaria: area with high environmental polymetal contamination – Asenovgrad and unpolluted Rhozen region situated in Western Rhodopes. Asenovgrad is one of the most polluted regions in Bulgaria. It is area of the lead-zinc smelting factory, contaminated by polymetal dust emissions of lead, cadmium and microaggregates of zinc.

12 adult animals of this rodent species: Yellow-necked mouse (*A. flavicollis*), Algerian mouse (*M. spretus*) and common vole (*M. arvalis*) were collected in areas exposed to heavy metal pollution. The same number and rodent species were trapped from the background region-Rhozen. T - test analysis did not show the differences in micronucleus frequencies between sexes of animals in the same study areas.

Micronucleus frequencies of the three rodent species collected in unpolluted Rhozen region did not differ among the rodent species. The results of counting micronuclei in peripheral erythrocytes of wild rodents lived in polluted area - Asenovgrad, showed differences in sensitivity among the collected three species as bioindicators. Micronucleus frequency in *A. flavicollis* was 0.45%, *M. arvalis* - 0.075% and *M. macedonicus* 0.025%. There was no difference between *M. arvalis* and *M. macedonius*. *M. arvalis* and *M. macedonius* are different from *A. flavicollis*. Micronucleus frequencies were elevated almost five-fold in *A. flavicollis* and three-fold in *M. arvalis* from the polluted region as compared with study objects from unpolluted region Rhozen. The result of this study showed us that the analysis of micronuclei is a very useful method how to monitor environmental contamination in small mammals, mainly in species of *A. flavicollis*.

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