

# Cadmium and mercury accumulation in European hare (*Lepus europaeus*): age-dependent relationships in renal and hepatic tissue

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**Abstract** A total of 63 European hares have been collected from five Serbian agricultural regions. The hares assayed were divided into four age groups (3–6 months, 12 months, 12–24 months, and 24–36 months) and investigated upon presence at cadmium (Cd) and mercury (Hg) in the kidney and liver. The positive significant correlation ( $P_s$ —Pearson's coefficient) between Cd concentrations in the kidney and liver within age group the 3–6 months was found ( $P_s=0.81$ ,  $p<0.001$ ). Differences between Cd content in the kidney in comparison to liver were significant within all presented age groups ( $p<0.001$ ). Differences between recorded Hg levels in the kidney were not significant between presented age groups ( $p>0.05$ ). Statistically significant differences were registered between Hg content in the liver of the hares aged 24–36 and 12 months ( $p<0.05$ ). There were no statistically significant correlations registered between Hg concentrations in the kidney and liver within any particular age group ( $p>0.05$ ). The strong statistically significant associations were registered between Cd and Hg content in the liver (Cd L/Hg L) in the age group 3–6 and 12–24 months (Cd L/Hg L,  $P_s=0.94$ ;  $p<0.001$  and  $P_s=0.91$ ;  $p<0.001$ , respectively). The polynomial regression model used for graphing the observed data seems to be a method for modeling the relationship between measured Cd and Hg concentrations in the liver and kidney as first approximation for bioaccumulation in hares.

**Keywords** Cadmium · Mercury · Kidney · Liver · Hare

## Introduction

Wild animals are normally exposed to heavy metals from their natural habitats. Metal levels in animal tissues can provide important data regarding the fate and bioavailability of heavy metals within natural ecosystems (Wren 1986).

Comprehensive studies of the Cd and Hg uptake in tissues have been performed for different herbivora animals (Medvedev 1999; Lazarus et al. 2008; Reglero et al. 2008; Danielson and Frank 2009; Hermoso et al. 2011; Berzas-Nevedo et al. 2012). However, its determination in a true biological context is intricate. Generally speaking, biological context is critical for understanding function in metabolic pathways. In other words, how organisms function and react to external stimuli (in this case to Cd and Hg uptake) at the molecular, cellular, tissue, or organismal level. In spite of careful analysis, errors when categorizing the animals can lead to misleading data interpretation and the validity of the investigation can be lost. Consequently, attention must be given to sampling and pre-sampling factors (Danielson and Frank 2009).

The biodynamic model of trace metal bioaccumulation allows the prediction and explanation of widely differing accumulated trace metal concentrations in organisms, combining geochemical analyses of environmental metal concentrations with the measurement of key physiological parameters for a species from the site under consideration (Rainbow 2007).

For metals, analogous methods to predict bioaccumulation in target tissue are not available or are not widely validated (Fairbrother et al. 2007). The lack of analogous models for metals is likely due, at least in part, to the high degree of specificity exhibited by the mechanisms and processes underlying bioaccumulation of metals (e.g., speciation, exposure conditions, and organism physiology) (Rainbow 2007). As a

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result, risk assessors are currently limited to using an empirical approach for assessing and predicting bioaccumulation of metals. Typically, this requires direct measurement of metal concentrations in the organism or experimentally determined parameters for use as input to bioaccumulation models (e.g., gill uptake rate, elimination rate, assimilation efficiency) (Fairbrother et al. 2007). The strong age dependency due to Cd accumulation and also Hg (in lesser extent) in herbivora animals precludes direct comparison of mean values for different groups (areas etc.) unless the age distributions are fairly equal. In reviewed papers, the age influence has been disregarded either due to small samples, where the random individual variations probably dominate, or due to similar mean ages between the groups, or due to poor age classification (for example young and adults). In our experiment, the sharper differentiation in age based upon real lifetime of hares and their population structure in nature conditions using appropriate method is supposed to give a better picture of age-dependent toxic metal accumulation in this kind of game.

The objective of the research was to improve bio-monitoring precision based on eventual differences in Cd and Hg distribution in target organs between the age groups and also registering environmental level of Cd and Hg in target tissue of European hare. The present study was also projected to analyze the bioaccumulation of Cd and Hg, using available basic statistical tool attempting to describe the relationship between registered renal and hepatic levels in European hare as a function of age and try to find regularities, if they exist, through these observations.

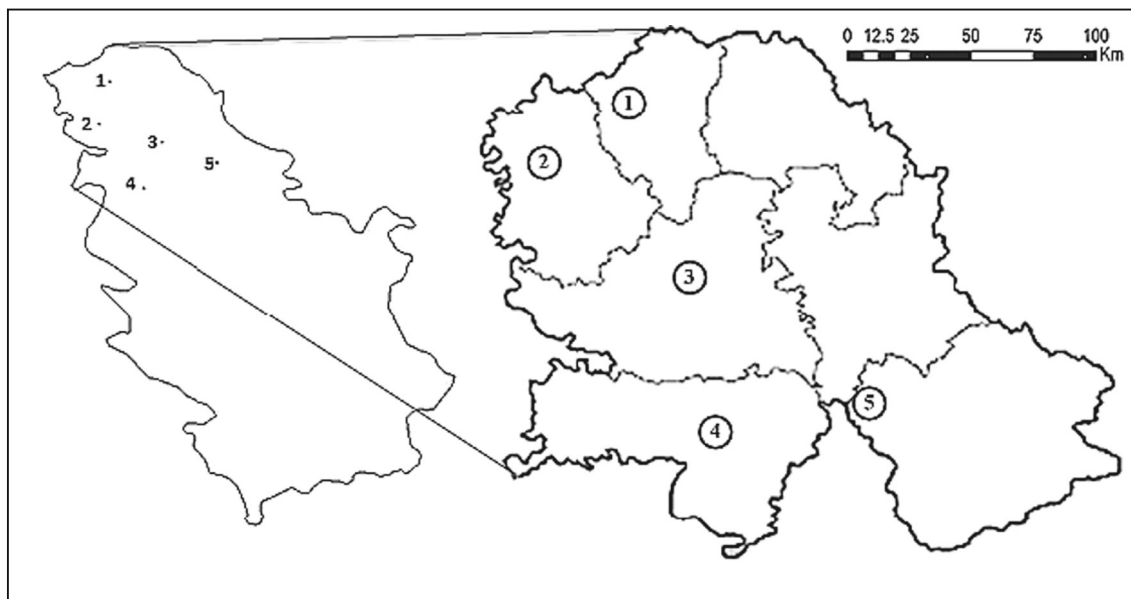
**Materials and methods**

**Sampling**

A total of 126 tissue samples (63 kidneys and 63 livers) obtained from all hunted 63 free-range hares—*Lepus europaeus*, were investigated upon Cd and Hg presence. The hares were acquired in five regions (Fig. 1) from the north province of Serbia - Vojvodina. The hares assayed were divided into four age groups and mainly inhabited mixed arable and cultivated farmland areas, in temperate, open habitats of Serbian north province Vojvodina. The hare diet in studied areas during most of the year commonly includes grazing on the young shoots of grasses and herbs as well as monocultures: agricultural crops, sunflower, soya, beans, and string beans. During the winter, diet was consisted from herbage, twigs, buds, and the bark of shrubs and young fruit trees.

**Age determination**

After extraction, eye samples were placed in marked plastic bags with zipper open/close system and immediately transported to the laboratory. The eye lenses were fixed in 5 % formalin for 72 h and then dried in a thermostat at 37 °C for 96 h, under normal pressure. After they were dried, they were weighed on a precise analytical scale (Mettler AE 200) to 1-mg precision. For further data analysis, hares were subdivided according to their age into five groups: 3–6 months old (100–200 mg), 12 months old (200–280 mg), 12–24 months old (280–310 mg), and 24–36 months old (310–370 mg).



**Fig. 1** Sampling regions

## Sample preparation

The whole liver and kidney were sampled from each animal. The liver and kidney samples were stored at  $-20\text{ }^{\circ}\text{C}$  until analyzed. Samples (1 g) after the process of homogenisation were digested with 8 ml of  $\text{HNO}_3$  (65 % v/v, Analytical grade, JT Baker, Center Valley, USA) and 2 ml of  $\text{H}_2\text{O}_2$  (30 %, Analytical grade, Kemika, Zagreb, Croatia) using the method of acid microwave digestion. Samples were destroyed in the microwave digestion unit (Milestone TC, EVISA, EU) with temperature control. The digestion program began at a potency of 1,000 W, then was ramped for 10 min to  $200\text{ }^{\circ}\text{C}$ , after which samples were held at 1,000 W and a temperature of  $180\text{ }^{\circ}\text{C}$  for 20 min. Calibrations were prepared from commercial solutions in  $\text{HNO}_3$  (0.2 %) with 1,000 mg/l of each element (JT Baker). All results are expressed on wet weight basis (w/w).

## Instrumentation

Cadmium content was determined using graphite furnace atomic absorption spectrometry (GFAAS) technique using an atomic absorption spectrophotometer-Varian SpectrAA 220, equipped with GTA 110 furnace with constant temperature zone. Cadmium was measured at 228.8 nm, LOD (0.005 mg/kg).

Mercury concentrations in samples were analyzed by hydride generation atomic absorption spectrophotometry (HG-AAS, cold vapor technique) at 253.7 nm, using Varian SpectrAA 220-atomic absorption spectrometer equipped with a Varian VGA-77 hydride generator where Hg was reduced from solution using of 30 % stannous-chloride. Calibrations were prepared from commercial solutions in  $\text{HNO}_3$  (0.2 %) with 1,000 mg/l of each element (JT Baker). Digested samples were diluted to a final volume of 50 ml with deionized water (conductivity— $0.067\text{ }\mu\text{S}/\text{cm}$ ) produced from demineralization unit—ELGA Purelab option DV 35. Blanks were processed in each batch of digestions. Detection limit of instrumental methods used for Cd and Hg determination was 0.005 mg/kg.

## Quality control

The trueness of the method was tested with the standard reference material (BCR No.186—Community Bureau of Reference) and by carrying out a recovery check using spiked (fortified) samples. The reference material data was  $\pm 10\%$  from the certified mean values. The recoveries in different sample materials were 95–97 % in the liver and 98–102 % in the kidney for cadmium; 98–101 % in the liver and 100–103 % in the kidney for mercury.

## Statistics

Statistical analysis was performed by the MINITAB software package, version 16.0. Concentrations were expressed as mean values, standard deviations, and range of minimum to maximum. Before choosing the appropriate statistical analysis, individual distribution identification was conducted to identify the native distribution (normal, lognormal, Weibull, exponential) in the first step. Box-Cox and Johnson transformation of raw data were used to follow a normal distribution. The One-way ANOVA and the post hoc HSD Tukey test were used to examine statistical differences of heavy metal concentrations in organs across groups. The significance of correlations between Cd and Hg levels were calculated using Pearson's correlation ( $P_s$ ). The differences were considered statistically significant when the  $p$  value was less than 0.05. Scatter plots with fitted regression line and best fits were used to examine and present graphical relationships between metals concentrations in hepatic and renal tissue between as well as within age groups. Box plots were used to illustrate both a measure of central tendency and variability of the data.

## Results

Concentrations of cadmium (Cd) and mercury (Hg) in the kidney and liver measured in European hare by observed age groups are presented in Table 1.

Measure of central tendency and variability of Cd and Hg concentrations in hare organs between presented age groups are graphically presented in Fig. 2.

The renal/hepatic ratios of Cd and Hg concentrations in hares by age groups are presented in Fig. 3.

Differences between Cd content in the kidney in comparison to liver were significant within all presented age groups ( $p < 0.001$ ). Significant statistical differences were registered between Cd content in the kidney and liver of hares among age groups 24–36 and 12–24 months in regard to those from 3 to 6 and 12 months ( $p < 0.001$ ). Between pairs of the above-mentioned groups, in regard to Cd concentrations measured in organs, differences were not significant ( $p > 0.05$ ).

Differences between Hg levels in the kidney among age groups were not significant within all presented age groups ( $p > 0.05$ ). Significant statistical differences were registered between Hg content in the liver of the hares age 24–36 months and 12 months ( $p < 0.05$ ). Between other groups, the differences of Hg content in the liver were not significant ( $p > 0.05$ ).

The strong positive significant correlation between Cd concentrations in the kidney and liver was found within the age 3–6 months ( $P_s = 0.81$ ,  $p < 0.001$ ). Looking at different regions where Pearson correlation for the age group 3–6 months was possible to calculate (region 1 and region 4), there

**Table 1** Cadmium and mercury content (mg/kg w/w) in the kidney and liver by age groups

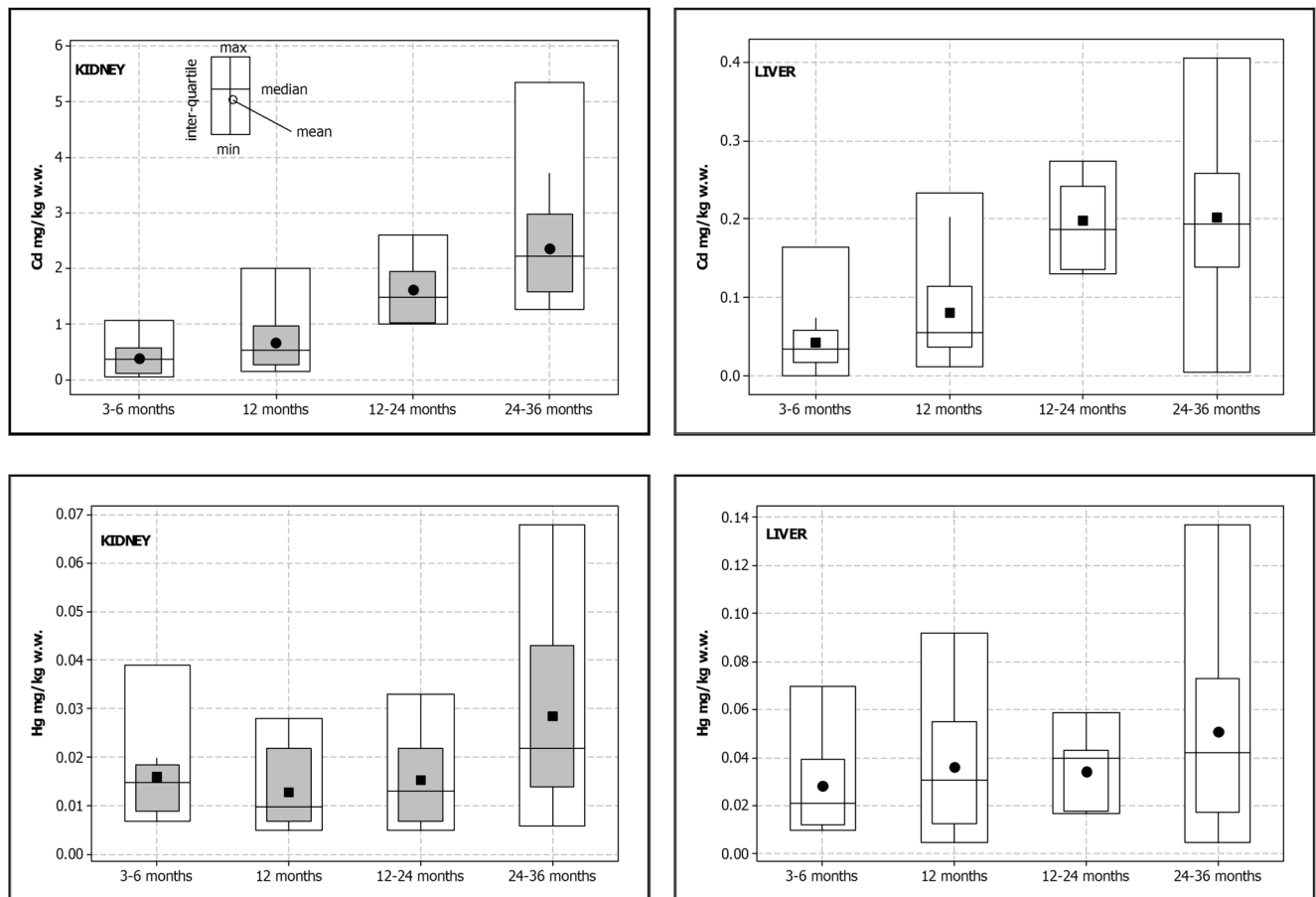
Age (months)	<i>n</i>		Cd kidney (mg/kg) w/w	Cd liver (mg/kg) w/w	Hg kidney (mg/kg) w/w	Hg liver (mg/kg) w/w
3–6	15	Mean	0.39	0.046	0.029	0.016
		St. dev.	0.27	0.039	0.020	0.010
		Range	0.06–1.07	0.01–0.16	0.010–0.070	0.007–0.039
12	16	Mean	0.67	0.081	0.036	0.013
		St. dev.	0.51	0.065	0.026	0.008
		Range	0.15–2.00	0.01–0.23	0.005–0.092	0.005–0.028
12–24	7	Mean	1.63	0.197	0.034	0.015
		St. dev.	0.57	0.054	0.016	0.010
		Range	1.03–2.61	0.13–0.27	0.017–0.059	0.005–0.033
24–36	25	Mean	2.37	0.20	0.051	0.028
		St. dev.	0.97	0.10	0.036	0.019
		Range	1.28–5.36	0.01–0.41	0.005–0.137	0.006–0.068

range: min–max

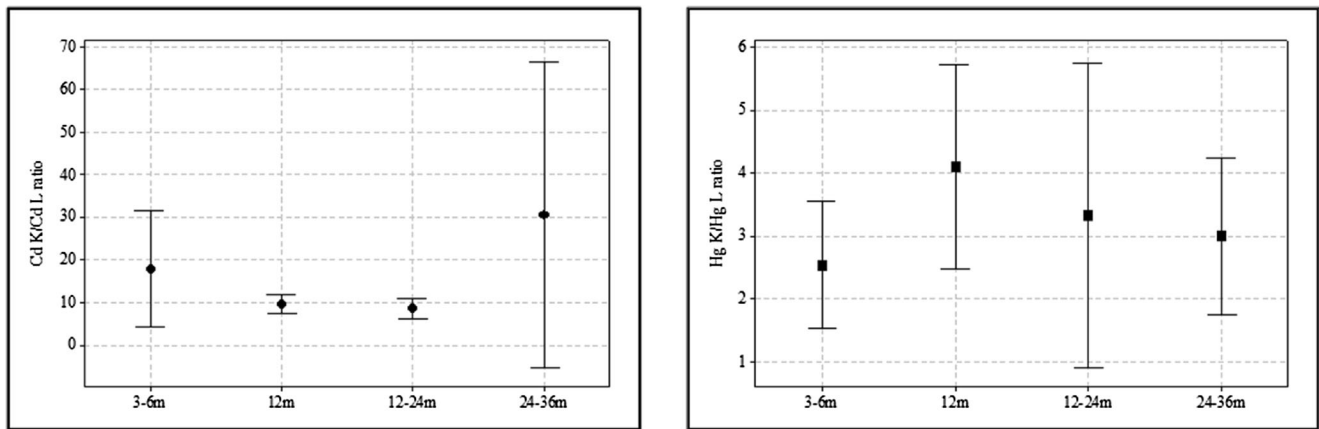
are visible strong associations between kidney and liver Cd concentrations within the age groups 3–6 months in region 1 ( $P_s=0.79, p<0.05$ ) and region 4 ( $P_s=0.94, p<0.001$ ).

Moderate positive significant statistically correlations are registered in the groups 12 months (Pearson’s coefficient:  $P_s=0.56, p<0.05$ ) and 24–36 months ( $P_s=0.60; p<0.001$ ).

In the group of hares 12–24 months, this correlation was not detected ( $P_s=0.54; p=0.28$ ). Generally speaking, the reason for absence of observations regarding this age group laying down in fact that the number of samples were minimal ( $n=7$ ) in relation to all other ( $n=15, \underline{n}=16, n=25$ ). There were registered no statistically significant correlations between Hg



**Fig. 2** Cd and Hg concentrations in the kidney and liver by age groups



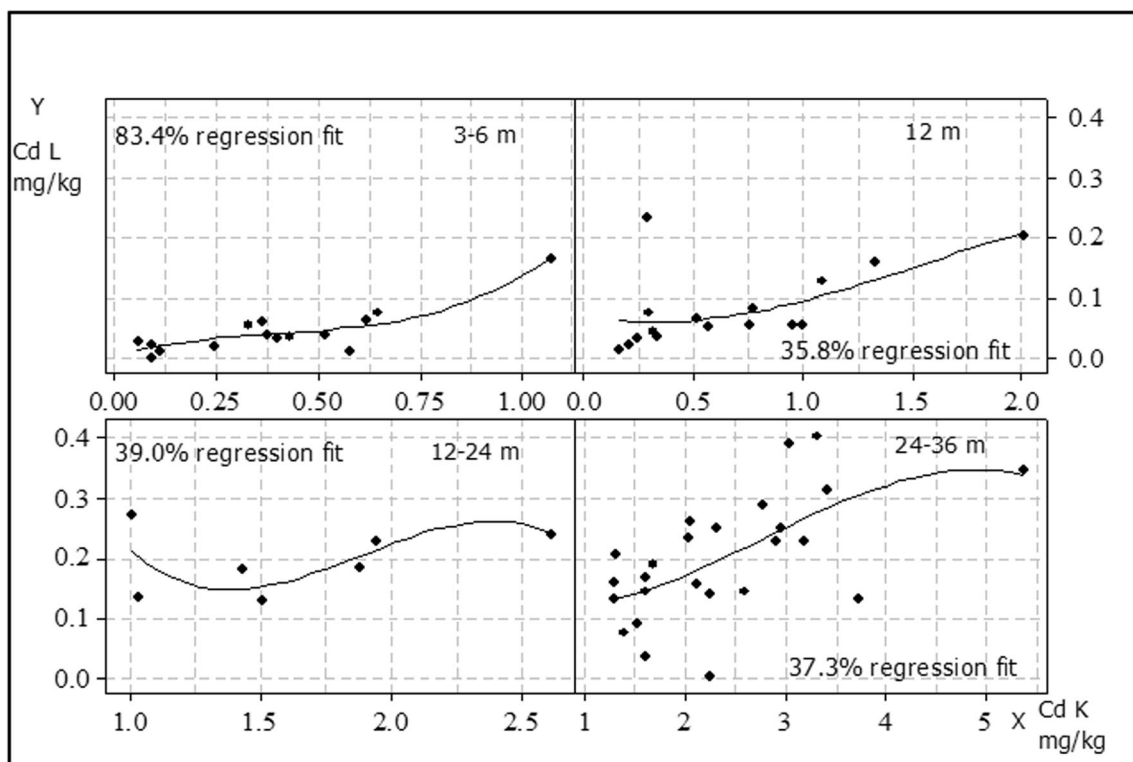
**Fig. 3** The renal/hepatic ratios of Cd and Hg concentrations

concentrations in the kidney and liver within any particular age group ( $p > 0.05$ ). Strong statistically significant associations were registered in the age group 3–6 and 12–24 months (Cd L/Hg L,  $P_s = 0.94$ ;  $p < 0.001$  and  $P_s = 0.91$ ;  $p < 0.001$ , respectively).

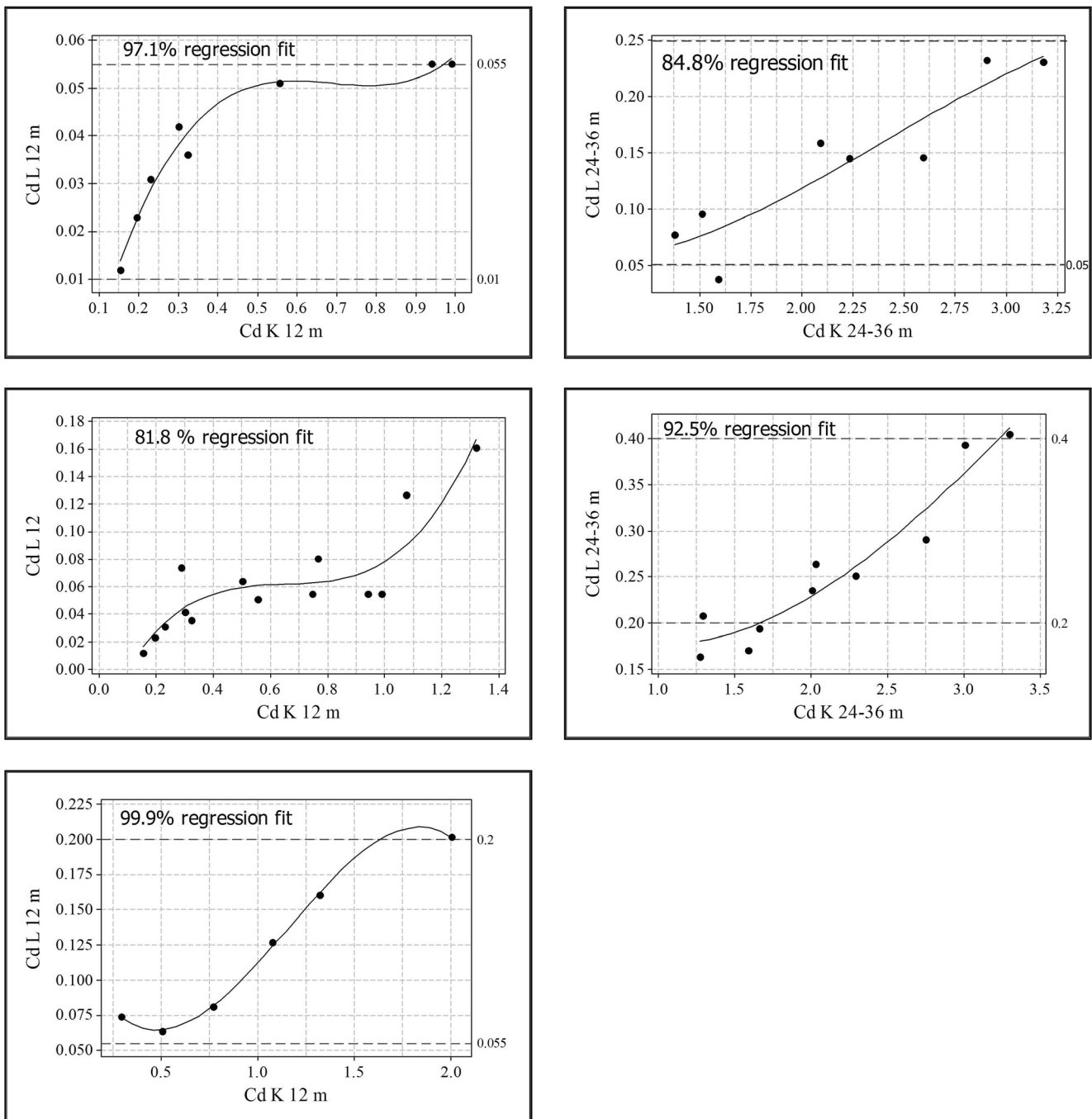
The regression models presented in Fig. 4, regarding relationship between Cd levels in the liver and kidney which include the whole samples by age groups, account for the following: 83.4 % (3–6 months), 35.8 % (12 months), 39.0 % (12–24 months), and 37.3 % (24–36 months) (95 % confidence interval). The much better regression fits (%) were obtained by graphing and observing those relationships (between measured concentrations of Cd and Hg in the liver in

relation to kidney) by including appropriate ranges as observed values within the age groups of 12 and 24–36 months (Figs. 5 and 7). For example, considering the Cd concentrations in hepatic tissue (CdL/mg/kg w/w) within age groups as an dependent variable and rising Cd concentrations in renal tissue (CdK/mg/kg w/w) as a predicting variable, the regression fits (%) were as follows: for 12 months (CdL range 0.010–0.055, 97.1 %; CdL range 0.020–0.16, 81.8 %; CdL range 0.05–0.20, 99.9 %); for 24–36 months (CdL range 0.05–0.25, 84.8 %; CdL range 0.15–0.40, 92.5 %).

In the case of Hg accumulation in hare organs (Fig. 6) including the whole samples by age groups, regression fits



**Fig. 4** Regression lines between hepatic and renal Cd concentrations within presented age groups with all data set



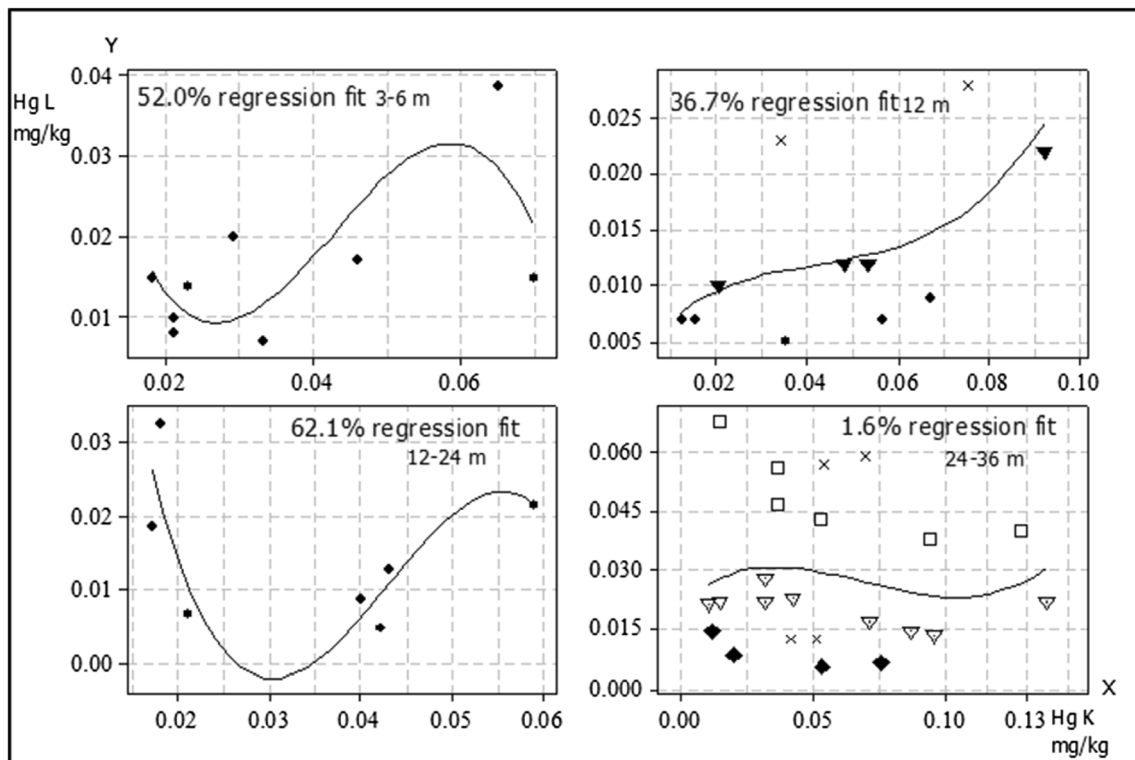
**Fig. 5** Regression lines between hepatic and renal Cd concentrations in age groups 12 months and 24–36 months with observed data set

of these models were as follows: 52.0 % (3–6 months), 36.7 % (12 months), 62.1 % (12–24 months) and 1.6 % (24–36 months). Observing the range of Hg concentrations in hepatic tissue (HgL/mg/kg *w/w*) within age groups 12 months and 24–36 months (Fig. 7) as a dependent variable and Hg renal tissue concentrations (HgK/mg/kg *w/w*) as a predicting variable, the regression fits (%) for the best subsets were for 12 months (HgL range 0.005–0.009, 97.8 %; HgL range 0.010–0.022, 100 %) and for 24–36 months (HgL range

0.05–0.015, 82.4 %; HgK range 0.015–0.025, 86.7 %; HgK range 0.038–0.047, 100 %; HgK range 0.038–0.068, 93.7 %).

**Discussion**

Bioavailability of individual metals plays a crucial role in their uptake by an organism (Jofré et al. 2013).



**Fig. 6** Regression lines between hepatic and renal Hg concentrations within presented age groups with all data set

During the past decades, accumulation of cadmium in hare organs was studied by a number of authors (Venalainen et al. 1996; Massányi et al. 2003; Eiraa et al. 2005; Kramarova et al. 2005; Myslek and Kalisinska 2006; Pedersen and Lierhagen 2006; Kolesarova et al. 2008) as well as mercury (Samek et al. 1989; Spirić et al. 2012).

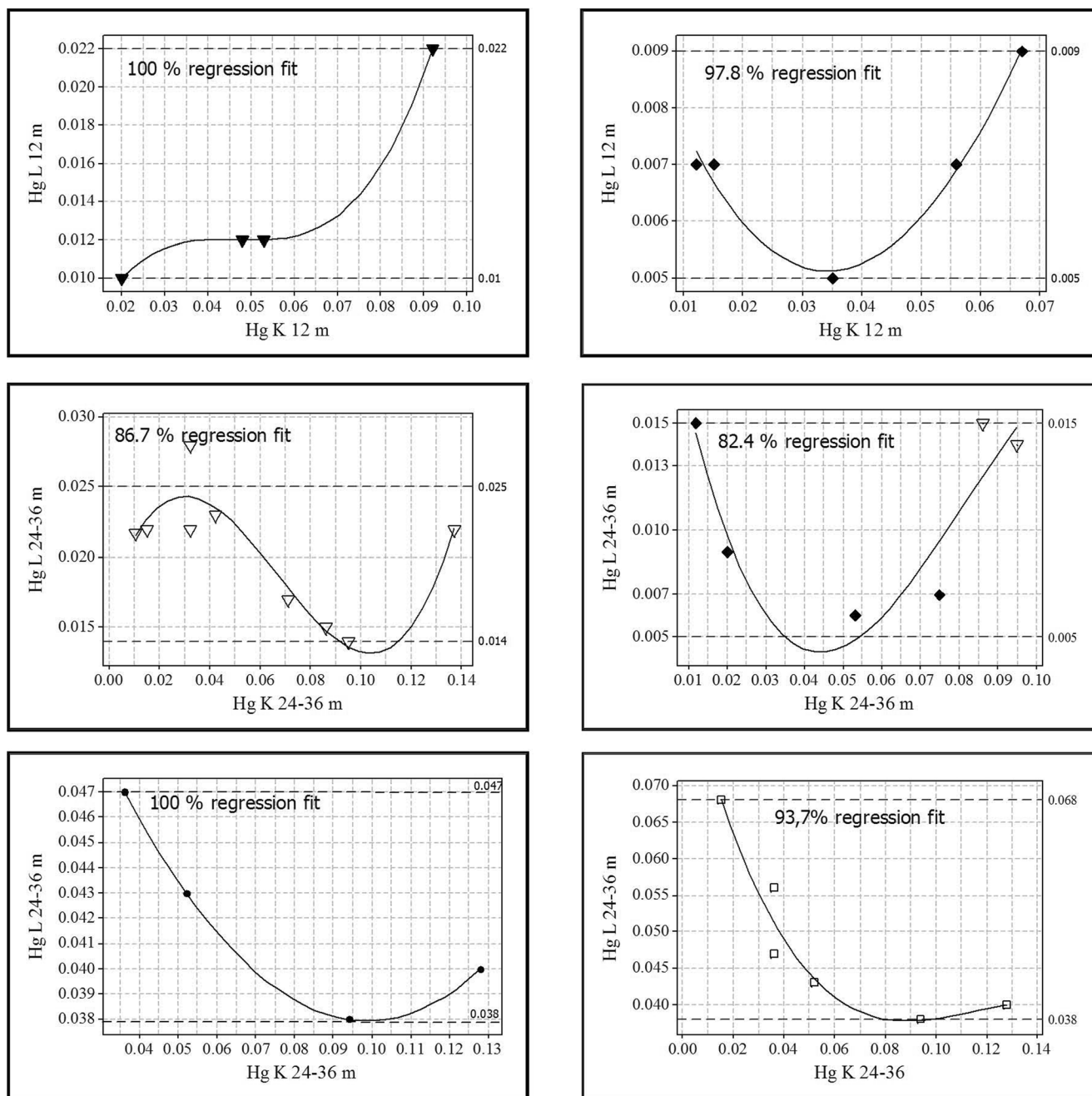
Most of these studies dealing with environmental levels in target tissues link obtained data to the existing sources of heavy metal pollution. However, the concept of using pragmatic approaches to evaluate bioaccumulation processes in mammals analyzing data from field studies is not widely established. Considering the values obtained by sampling regions, we noticed that the mean values of both the metals are probably affected by random individual variations including age structure of collected animals (especially for Cd content) and also the sample size. In principle, there has been a more or less visible increase in bioaccumulation of Cd and Hg in hare organs during subsequent stages of life in present study (Fig. 2).

Several models have been reported to describe the kinetics of cadmium and mercury in mammalian systems. Of these models, the Nordberg-Kjellström model (Kjellström and Nordberg 1978) for cadmium and Farris model (Farris et al. 1993) for methylmercury has been widely used for risk assessment.

The concentration of both elements in the kidney exceeds that in the liver following prolonged exposure (Fig. 2). The

average values of renal/hepatic ratios between Cd and Hg concentrations show remarkable difference in regard to registered levels of those elements (Fig. 3). Considering age, these differences between Cd and Hg levels in the kidney are primarily dose-dependent (Sasser and Jarboe 1977; Lehman-McKeeman and Klaassen 1987; Zalups and Lash 1990; Zalups and Barfuss 1998).

Half-lives for cadmium in the whole body of mice, rats, rabbits, and monkeys have been calculated to be from several months up to several years. Half-lives were from 20 to 50 % of the maximum life span of the animal (Nordberg et al. 1985). Because of the long half-lives and the transfer of cadmium by metallothionein (MT) from other tissues to the kidney, after long-term low-level exposure (as from dietary sources), accumulation in the kidney will occur during the major part of the hare life span. It could explain a wider range of renal/hepatic ratios regarding Cd level in eldest group of hares toward all other (Fig. 3). Also the ability of the kidney to handle metabolism of Cd is entirely different for different forms of this element originally ingested and depends also on the rate of ingestion. On the other hand, the ingestion of diet that is deficient in a particular essential element (Ca, Zn, and Fe) can enhance the accumulation and toxicity of some toxic metals (López-Alonso et al. 2004). These deficiencies may also result in an increased accumulation of Cd in the intestinal wall, liver, and kidney (Foulkes and Voner 1981; Reeves and Chaney 2008). Nevertheless, with these reservations, Cd



**Fig. 7** Regression lines between hepatic and renal Hg concentrations in age groups 12 months and 24–36 months with observed data set

analysis of hare kidney and liver is useful for monitoring, provided that individual factors are duly considered. Such factors may exert systematic influence, like age and gender, but also give rise to substantial random individual variations. The narrower the range of renal/hepatic mercury ratio in comparison to Cd in hares is evident (Fig. 3). The levels of registered average Hg concentrations in the kidney and liver are relatively comparable (Fig. 2) while looking at kidney statistics they statistically did not differ between age groups. According to Laks (2014), methyl

mercury is converted to inorganic form in the body tissue and subsequently it is deposited in the tissue for years. Excretion of unchanged methylmercury in hares predominantly occurs in the feces through biliary and in lesser extent through urinary excretion. For example, Zalups et al. (1992) demonstrated that both normal and uninephrectomized rats excreted only about 3 % of the dose of mercury in the urine by the end of the initial 7 days after the i.v. injection of a low dose (5 mg/kg) of methylmercury.



Looking at results for Hg levels in the examined hare organs by age groups (Fig. 2), it can be speculated that the binding capacity of the renal MT in hares for Hg tends to saturate with increasing doses, even at environmental exposure or ingested doses are at low level during the long-term daily intake. In this case, renal MT could play an important role in the binding Hg when it is present at low levels (Morcillo and Santamaria 1996). Other factors such as feed intake and composition, gut passage time, and gut physiology also contribute to the large inter- and intraspecies differences in bioavailability of mercury (Schlekat et al. 2005). In Austria, Reichlin et al. (2006) conducted research in regard to nutrition of hare based on samples of gastric contents in February, May, August, and November. The authors found that in autumn and winter hare's diet is dominated by crops, primarily winter wheat (51 %), and nutrients that are provided by hunters (rootnesses, sugar beets, carrots ...). In contrast, the involvement of these weeds in observed months was less than 5 %. The analysis of the gastric contents in February determined that, except the wheat, lucern and sugar beet make approximately 91 % of the total meal. In the spring diet, the gastric content of hares was dominated by field crops (83 %), primarily soybeans, while the most consumed were wild plant species, particularly after harvest of cereals in August. Katona et al. (2010) in five areas of Hungary and after a 3-year research examined the composition of the autumn diet of hares ( $n=350$ ) focusing on the stomach contents under the microscope. The authors found that the hares consumed a total of 24 plant species. From cultivated plants, dominant species in the diet of hares was wheat (*Triticum aestivum*), and in a small amount lucern (*Medicago* sp.). From other crops, they also found the presence of rape during the year by 0–3 %, while maize and sunflower at investigated time had already been harvested.

Uptake of mercury from soils by vascular plants is very limited with concentrations of Hg in plants being significantly lower than in soil where roots act as important absorption site and barrier for mercuric transport (Grigal, 2002, 2003; Millhollen et al. 2006). In contrast, the atmosphere is almost the exclusive source of mercury in vegetation (Grigal 2003; Ericksen et al. 2003, 2006; Rea et al. 2001; Millhollen et al. 2006).

Differences in the mechanisms involved in the transport and metabolism of inorganic and organic forms of mercury (in the various compartments of the body) are likely responsible for their distribution in tissues and organs (Zalups and Lash 1994). This finding in light of registered relationships between hepatic and renal Hg content within particular age groups of hare in present work (Figs. 6 and 7) shows that the bioaccumulation of Hg is not a simple linear relationship. It also appears that some aspects of hepatic function play a role in at least a component of the renal uptake and transport of Hg in hares.

Analyzing the graphs for particular age groups given in Figs. 4 and 6, generally, we observed that the more variance that is accounted for by the above regression models, the closer the data points will fall to the fitted regression line. From our observations, it seems that a cubic polynomial regression fit to a given data set. The differences in regression fits within the observed age groups and also in the accumulated levels in both of organs are likely due to the disparity in their distribution in tissues and organs along with other aspects (bioavailability, ingested form, seasonal variations in diet, age structure, local environmental presence of Cd and Hg). The age structure of the hare is an important indicator of growth, and as such is used in the planning of usage level of hares in hunting grounds. Although hares can live for 10–15 years, only 3 % hares reach the age of 4 years in the present conditions. Young hares (up to 1 year of age) consist 50–75 % of the autumn hare's population and the rest are mostly 1–2 years old. (Beuković et al. 2011). Topsoil Cd and Hg concentrations in sampling regions are probably affected by atmospheric deposition while anthropogenic sources could generally include agriculture in sampling areas (fertilizers, animal manures, pesticides). Cadmium from phosphate fertilizers seems to be a main source of Cd in soil because the phosphate rock is a substantial part of its processed phosphate (phosphoric acid and phosphate fertilizers) from third countries naturally consisting significant amount of Cd. Potential local sources of elemental and inorganic mercury released into the air would include smelting (the steelworks located in the region 4—close to sampling points), heating plants, gas and oil production plants (facilities located in the regions 3 and 5—close to sampling points), and combustion of fossil fuels (pathway of highway Belgrade-Zagreb passing through region 4 and pathway of highway E75 passing through regions 1 and 3—near to sampling points). Also, environmental levels of mercury in particular areas from which hares are collected are possibly influenced by different level of organic content and pH in local soils by application of fungicides in agriculture and periodical floods and also by seasonal burning of stubble and maize in fields.

## Conclusions

Models for predicting metal bioaccumulation by ingestion are primarily statistical in nature. We show through our analysis that the examined model result is consistent with the obtained data. It seems that polynomial regression is one method for modeling curvature in the relationship between Cd and Hg concentrations in the liver and kidney as a first approximation for bioaccumulation in hares.

Furthermore, age distribution suggests that the samples can be limited by age to include only those of animals with an exposure period of 2 or 3 years, given they are from the

same region, and then compare the obtained results to younger individuals.

During the first 6 months of life, there has been registered a distinctive rise of concentrations of Cd both in the liver and kidney (strong associations were registered). The strong correlations between hepatic levels of Cd and Hg in age groups 3–6 and 12–24 months would have been explained by joined actions both of toxic metals after the environmental exposure in these periods of their life span and also by their different mechanisms of excretion from hare body.

The age distribution suggests that the samples can be censored by age to include only those of animals with an exposure period of 2 or 3 years, given they are from the same region, and then compare the obtained results with young individuals (3–6 and 12 months, depending from particular tissue content). It may be important information if the hare organs are intended to be used for improving precision of environmental biomonitoring of Cd (using data for kidney and liver) and Hg (using data for liver).

Observed hares inhabiting the studied agricultural areas in Serbia show similar to lower Cd and Hg bioaccumulation compared to other biotopes in Europe.

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