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Original scientific paper

Levels and interactions of selected elements (Fe, Mn and Cu) in European hare tissue within different age classes from Serbian agricultural regions

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A B S T R A C T

The contents of the essential elements, iron, manganese and copper, were determined in the kidney and liver of the European brown hare (*Lepus europaeus).* The wild hares assayed were divided into five age classes ranging from 3 months to more than 36 months. The animals were collected during 2010/2011 from 21 different hunting terrains and originated mainly from arable and agricultural biotopes in Serbia. The mutual interactions of the metals obtained from kidneys and livers of 157 individual hares in age groups were calculated. The mean levels of Fe, Mn and Cu (mg/kg, wet weight) registered in kidney (K) and liver (L) were: Fe (K) 103.3±42.1; Fe (L) 138.5±52.7; Mn (K) 1.75±0.66; Mn (L) 2.36±0.85; Cu (K) 3.32±0.62; Cu (L) 4.16±1.40. No statistically significant differences ($p>0.05$) were found between the age groups with regard to the Fe, Mn and Cu contents in the kidneys and liver of brown hares (within the same organs). Statistically significant differences between levels in liver and kidney (between different organs) were registered in all age groups (in favour of higher levels in the liver over the kidney) of hares, except for Fe contents in both organs in the age groups of 3–6 and 12 months. Correlations between the content of elements within the age groups were determined using the Pearson test for normal distributions. The correlation patterns between the essential elements in the hare liver and kidney showed both positive and negative significant correlations among some single or different elements within the same organ and among the elements between the two organs. Within age groups, we registered seven different statistically significant mixed associations (FeK-FeL, CuL-MnL, MnL-FeL, CuK-MnK, MnK-FeL, CuK-FeL, and CuL-FeL).

1. Introduction

In much of the literature, the term "essential metals" has been used to signify those metals required by living organisms, and the absence of which produces specific deficiency symptoms (*Duffus,* 2002). Furthermore, "trace elements" are defined as essential, i.e., Mn, Fe, Cu, Zn, Se, Co, Mo and I, for plants or animals (*Zoroddu et al.,* 2019). The essential elements in herbivore tissues have attracted a great deal of attention in basic and applied biology. These elements are known to play important functions in the body, such as storage, regulation and supply of energy (*Rai et al.,*2021*; Tibbett et al.,*2021*; Nunes et al.,*2022).

Fe, Cu and Mn play important roles in most biological process, be it structural or enzymatic. These elements also play a role in oxygen transport and reduction, as they each take part in the composition or functioning of respiratory pigments. As essential metals in animals, however, they can also create a level of toxicity (*Pajarillo et al.,* 2021*; Jomova et al.,* 2022). The liver and kidney are the sites of metal metabolism in the body. The proteins that are involved in

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the fluxes of Fe, Mn and Cu in mammals have been observed mostly in these two organs in both the absorption and the excretion processes. Therefore, it is important to understand the role of these elements in these organs to comprehend the overall status of essential metals in the body (*Fu and Xi,* 2020*; Liu et al.,* 2020*; Cygan and Szczegielniak,* 2021).

Many previous field studies established that different heavy metals (considered as toxic or essential) have to be present in the body and different organs of the European hare (*Venäläinen et al.,* 1996*; Massanyi et al.,* 2003*; Pedersen et al.,* 2006*; Kolesarova et al.,* 2008*; Dlugaszek et al.,* 2013*; Le Fidalgo et al.,* 2015*; Demirbaş and Erduran,* 2017*; Wajdzik et al.* 2017*; Długaszek,* 2019). Some studies also investigated the distribution and levels of Fe, Mn and Cu in the organs of various animal species, including hares (*Lopez Alonso et al.,* 2004*; Kompiš and Ballova* 2021*; Squadrone et al.* 2022). However, from the literature reviewed for this study, there is limited information on the specific interactions between these elements within or between the target organs in the European hare, including narrower differentiation of age groups. Understanding these interactions is crucial, as Fe, Mn and Cu play essential roles in numerous biochemical and physiological processes. Furthermore, information on trace element levels in animal tissues is a valuable resource in animal toxicology. Fe is vital for oxygen transport and energy production, Mn participates in antioxidant defence and enzyme activation, while Cu is involved in enzyme function and cellular signalling (*Pilarczyk et al.,* 2020*; Kalisińska et al.,* 2023*; Stepanova et al.,* 2023).

By studying the background of Fe, Mn and Cu in animal physiology, we can try to establish a foundation for exploring their interactions within the hare's organs and optimal levels to ensure the normal growth and development of hares. The primary goals of this research were to examine: (1) the levels of Fe, Mn and Cu in kidney and liver of a hare population divided into five age groups that represent the natural life span of this wildlife species; (2) how Fe, Mn and Cu interact in the liver and kidney of European hares within the same and different organs and try to uncover the factors that affect these interactions derived from statistical data. Our focus was on analysing the amounts and patterns of Fe, Mn and Cu in these organs and determining whether there are any connections or interdependencies among the three metals. Furthermore, we aimed to comment on how factors such as diet composition, environmental conditions and especially age factors might impact these interactions.

2. Materials and methods

2.1 Studied wild hares and the collection locations

A total of 157 European brown hares (*Lepus europaeus*) were collected during the regular hunting season in the fall and winter of 2010/2011. The hares were collected mainly from habitats characteristic of the European brown hare, namely near agricultural land and other open areas such as meadows, clearings, plains, bushes and shrubs. The hares were from 21 Serbian regions at the locations shown on the map (Fig. 1). Of the study animals, 84 hares were collected from central Serbia, from the territories of 11 hunting associations (1–11: Užice, Bajina Bašta, Ub, Obrenovac, Mladenovac, Belgrade, Šabac, Ćićevac, Kuršumlija, Vranje and Prokuplje), while 73 hares were from the northern province of Vojvodina, from the territories of 10 hunting associations (12–21: Sonta, Aleksa Šantić, Sombor, Novi Sad, Pančevo, Putinci, Nikinci, Buđanovci, Mali Radinci, Voganj).

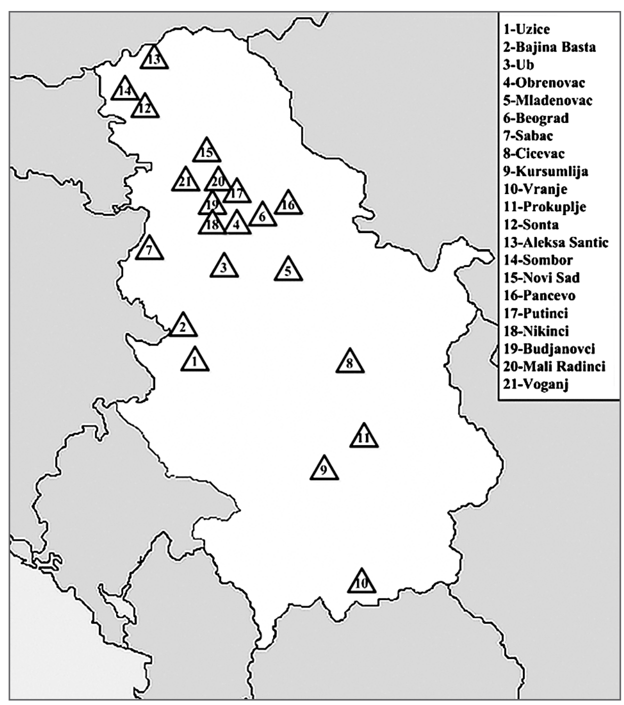

Figure 1. Locations in Serbia where hares were collected

2.2 Age determination of the studied hares

The detailed methodology for determining the age of the studied brown hare population was described in previous studies (*Suchentrunk and Hartl, 1991; Petrović et al., 2013, 2014*). The examined hares were divided into five age groups: 3–6 months, 12 months, 12–24 months, 24–36 months, older than 36 months.

2.3 Sample preparation

From the 157 hares, a total of 157 kidneys and 157 livers were taken, i.e., a total of 314 organs. The entire liver and both kidneys were removed from each animal. The livers and kidneys were stored at −20 °C until analysis. After each entire organ was subjected to a standard homogenization process (the two kidneys from each animal were processed together), 1 g of homogenate was digested with 8 ml HNO_3 (65 %v/v, analytical grade, JT Baker, Center Valley, USA) and 2 ml H_2O_2 (30 %, analytical grade, Kemika, Zagreb, Croatia) using the acidic microwave digestion method. Homogenates were digested in a microwave digestion device (Milestone TC, EVISA, EU) with temperature control. The digestion program started at a power of 1,000 W and was then ramped up to 200 °C for 10 minutes. The digests were then held at 1,000 W and a temperature of 180 °C for 20 minutes. Digests were then stored frozen at −18 °C and thawed in a refrigerator for 2 h before elemental analysis.

2.4 Elemental analysis (iron, manganese and copper)

The elements were measured with an atomic absorption spectrophotometer (VARIAN SpectrAA 220): Fe at 248.3 nm, LOD (1.0 mg/kg), Mn at 279.5 nm, LOD (0.5 mg/kg) and Cu at 324.8 nm, LOD (0.1 mg/kg). The accuracy of the method was tested with the standard reference material (BCR No. 186—Community Bureau of Reference) and by a recovery test with spiked samples. The recoveries of the reference material were ±10 % of the certified mean values. The recoveries in the different sample materials were 96 %–101 % in kidney and 98 %–102 % in liver for Fe; 88–96 % in kidney and 81 %–94 % in liver for Mn; 95 %–98 % in kidney and 92 %–95 % in liver for Cu; the coefficient of variation was between 4 % and 9 %. Calibrations were prepared from commercial solutions in HNO_3 (0.2 %) containing 1,000 mg/l of each element (JT Baker). All results are reported on a wet weight (w.w.) basis.

2.5 Statistical analysis

The analysis was performed using the MINITAB software package (MINITAB INC, USA), version 17.0. Levels in organs were expressed as means, standard deviations, minimum and maximum. Before selecting the appropriate statistical test, the best individual distribution of the data series was determined (16 different distributions were analysed) based on the lowest value of the Anderson-Darling coefficient and the highest p-value (above 0.05) for the final selection of the distribution that best fit the normal distribution. One-way ANOVA and post hoc HSD Tukey test were used to examine statistical differences of element contents in organs between age groups. The significance of the correlations of Fe, Mn and Cu levels between organs was calculated using Pearson's correlation (Ps). Differences were considered statistically significant if the p-value was $\leq$0.05. Boxplots were used to represent both measures of central tendency and the variability of the data.

3. Results

The measured contents of the three elements (mg/kg weight) in the kidney and liver of the hares examined are shown in Table 1.

3.1. Iron (Fe) levels in kidney and liver

Statistically significant differences (Table 1) between the Fe content in the kidneys and liver were not registered within the age groups of hares of 3–6 months and 12 months ($p > 0.05$). Later in the hare's life, looking at the mean values for the kidneys from this study, the Fe decreased slightly and then remained relatively constant. The mean Fe contents in the liver of the different age groups showed an increasing trend throughout the hares' life (Table 1). The Fe contents in the liver of hares of the other age groups (12–24 months, 24–36 months, and over 36 months.) were statistically significantly higher than in the kidneys ($p \leq 0.05$). No statistically significant differences were found between the Fe contents in the kidneys and liver between the different age groups ($p>0.05$). The mean Fe levels (Table 2) including the whole population studied for kidney (K) and liver (L) were 103.3 mg/kg and 138.5 mg/kg, respectively.

3.2 Manganese (Mn) levels in kidney and liver

The mean Mn levels in the examined kidneys (1.75 mg/kg) were lower than those in the liver (2.36 mg/kg), and the difference in the determined Mn levels between the two organs was statistically significant ($p=0.001$). Statistically significant differences between the Mn content in the kidneys compared to the liver were also found in all age groups (p=0.001–0.022). No statistically significant differences were found between the age groups with regard to the Mn content in the kidneys and livers of hares (Table 1). The mean Mn levels in the kidneys and liver (Table 1) of the studied individuals by age groups (3–6 months,12 months, 12–24 months, 24–36 months, older than 36 months) were, respectively: for kidney: 1.96 mg/kg, 1.88 mg/kg, 1.59 mg/kg, 1.65 mg/kg and 1.55 mg/kg; for the liver: 2.47 mg/kg, 2.58 mg/kg, 2.39 mg/kg, 2.15 mg/kg and 2.30 mg/kg. The lowest Mn level in the kidneys was 0.54 mg/kg (Table 3, sampling site 7) and was recorded in a hare aged 24–36 months from the area of Šabac (Table 1; MnK 24–36 m). The highest measured Mn level in the kidneys was 2.70 mg/kg in a hare collected from Buđanovci (Table 3, sampling

Table 1. Overview of the content (mg/kg) of the analysed elements (Fe, Mn and Cu) in the kidneys (K) and liver (L) of brown hares according to age groups

Age	Statistical measure	Fe/K	Fe/L	Mn/K	Mn/L	Cu/K	Cu/L
3–6 months (n=28)	mean	110.2	128.7	1.96	2.47	3.33	4.26
	SD	39.4	50.5	0.80	0.78	0.66	1.48
	min	29.5	44.8	1.0	0.93	2.45	0.73
	max	203.8	269.2	2.70	3.79	5.20	8.06
12 months (n=41)	mean	109.2	132.0	1.88	2.58	3.32	4.27
	SD	51.3	62.5	0.79	0.99	0.79	1.60
	min	37.6	43.01	0.72	0.42	1.34	0.92
	max	324.1	313.1	2.28		5.34	9.34
12–24 months (n=22)	mean	107.9	145.6	1.59	2.39	3.41	3.93
	SD	36.2	41.8	0.42	0.73	0.48	0.99
	min	62.4	81.5	0.79	0.55	2.28	1.97
	max	186.4	237.3	2.23	4.13	4.33	6.32
24–36 months (n=51)	mean	94.5	140.7	1.65	2.15	3.26	4.16
	SD	34.6	46.2	0.55	0.73	0.55	1.47
	min	42.5	65.2	0.54	0.90	1.76	2.07
	max	163.9	298.7	2.64	3.97	4.43	8.89
36+ months (n=15)	mean.	92.5	155.0	1.55	2.30	3.34	3.94
	SD	47.1	64.2	0.47	1.04	0.45	0.93
	min	39.6	75.9	0.62	0.50	2.73	2.20
	max	176.8	283.9	2.31	4.11	4.24	6.19

Legend: K – kidney, L – liver, SD – standard deviation, n – number of hares included. The results are expressed on the basis of wet weight (w.w).

Table 2. Statistically significant differences between the element contents in kidney (K) and liver (L) according to hare age groups

Age (months)	FeK/FeL	MnK/MnL	CuK/CuL
3–6 months	p=0.134**	p=0.001*	p=0.003*
12 months	p=0.054**	p=0.003*	p=0.001*
12–24 months	p=0.002*	p=0.001*	p=0.029*
24–36 months	p=0.001*	p=0.001*	p=0.001*
36+ months	p=0.004*	p=0.022*	p=0.038*

Legend: * statistically significant differences between the metal contents in kidney (K) and liver (L) according to age groups ($p \leq 0.05$); ** Differences were not registered.

Table 3. Overview of the content (mg/kg) of the analysed elements (Fe, Mn and Cu) in the kidneys (K) and liver (L) of brown hares according to sampling sites

Locality	Statistical measure	Fe/K	Fe/L	Mn/K	Mn/L	Cu/K	Cu/L
1. Užice (n=10)	mean ± SD	100.1±48.3	144.7±51.7	1.95±0.82	2.45±0.57	3.32±0.47	3.72±0.60
	min – max	29.5–186.4	89.9–267.7	0.99–2.12	1.83–3.24	2.75–4.40	2.74–4.86
2. Bajina Bašta (n=6)	mean ± SD	103.1±45.6	171.8±61.9	1.62±0.53	1.64±1.47	3.00±0.49	2.84±2.67
	min – max	39.6–155.1	89.5–273.8	0.92–2.10	0.42–3.46	2.35–3.71	0.91–7.54
3. Ub (n=10)	mean ± SD	104.2±45.8	153.9±50.6	1.24±0.63	3.26±0.79	3.90±0.40	3.93±0.49
	min – max	58.3–185.3	81.5–258.4	0.62–2.62	1.82–4.13	3.29–4.59	3.24–4.68
4. Obrenovac (n=6)	mean ± SD	95.5±44.1	146.8±67.5	1.61±0.30	2.07±0.55	2.82±0.37	3.87±0.45
	min – max	46.3–176.8	85.7–232.9	1.14–20.01	1.38–3.00	2.14–3.21	3.44–4.60
5. Mladenovac (n=10)	mean ± SD	120.1±36.9	169.3±58.4	1.46±0.31	2.39±0.88	3.14±0.41	4.10±0.76
	min – max	54.4–181.8	89.0–269.2	1.00–2.05	1.36–3.67	2.45–3.97	2.66–5.17
6. Belgrade (n=7)	mean ± SD	144.2±88.2	216.6±106.2	1.60±0.38	1.98±0.76	3.42±0.28	3.43±1.73
	min – max	54.5–324.1	62.1–313.1	0.95–1.97	0.98–3.30	2.96–3.86	0.92–6.13
7. Šabac (n=9)	mean ± SD	92.0±23.2	121.6±26.5	1.48±0.68	2.28±0.20	2.47±0.44	3.22±0.98
	min – max	63.5–125.4	81.6–161.4	0.54–2.49	1.94–2.50	1.76–3.09	2.07–4.76
8. Ćićevac (n=7)	mean ± SD	64.8±16.5	153.6±8.15	1.83±0.30	1.77±0.20	3.36±0.36	3.36±0.36
	min – max	53.3–91.5	153.1–164.7	1.40–2.12	1.50–2.06	3.16–4.23	2.90–4.02
9. Kuršumlija (n=6)	mean ± SD	93.3±23.6	122.2±57.7	1.61±0.5	2.63±0.36	3.08±0.59	3.82±0.52
	min – max	61.8–131.7	48.3–213.5	1.0–2.20	2.22–3.14	2.54–4.05	3.42–4.80
10. Vranje (n=6)	mean ± SD	88.4±38.5	118.4±40.9	1.85±0.66	2.89±1.0	2.98±0.37	4.15±0.85
	min – max	42.0–155.1	74.6–173.8	0.66–2.42	1.38–3.97	2.52–3.64	2.53–4.76
11. Prokuplje (n=7)	mean ± SD	74.9±24.9	95.0±45.3	2.16±0.95	2.81±1.3	2.81±1.10	4.42±1.67
	min – max	37.6–101.2	43.0–163.3	1.45–2.28	1.27–5.08	1.34–4.36	2.03–6.64
12. Sonta (n=7)	mean ± SD	88.2±14.9	138.8±18.5	1.46±0.16	1.73±0.85	3.27±0.36	4.02±1.37
	min – max	68.8–113.4	118.5–173.3	1.16–1.62	0.96–3.08	2.65–3.58	2.85–6.19
13. Aleksa Šantić (n=9)	mean ± SD	126.3±42.11	149.3±68.1	1.63±0.24	1.98±0.78	3.74±0.46	3.90±1.39
	min – max	57.7–203.4	44.8–253.3	1.39–2.06	0.90–3.26	3.20–4.82	1.36–5.81
14. Sombor (n=9)	mean ± SD	85.5±23.9	110.4±40.2	2.18±0.35	2.65±0.90	3.07±0.45	3.74±1.39
	min – max	50.6–123.3	64.4–186.3	1.55–2.54	0.93–4.11	2.60–3.74	0.73–5.28
15. Novi Sad (n=6)	mean ± SD	69.3±25.7	99.9±21.6	2.03±0.33	2.77±0.74	3.70±0.23	7.49±1.38
	min – max	42.5–106.2	65.2–122.7	1.72–2.65	1.70–3.77	3.31–3.93	5.98–9.34
16. Pančevo (n=9)	mean ± SD	94.3±22.3	126.7±29.2	1.43±0.17	2.26±0.69	3.06±0.50	4.43±1.28
	min – max	69.0–141.5	78.0–160.6	1.14–1.60	1.35–3.65	2.25–3.60	2.75–6.94
17. Putinci (n=6)	mean ± SD	119.3±32.0	158.3±28.5	1.87±0.32	2.34±0.65	3.84±0.36	5.95±1.80
	min – max	74.2–163.6	115.5–196.4	1.21–2.04	1.54–3.06	3.46–4.43	3.71–8.84
18. Nikinci (n=9)	mean ± SD	138.9±29.4	116.1±34.9	1.62±1.15	1.67±0.41	3.41±0.32	3.64±0.34
	min – max	100.1–178.4	76.0–177.9	0.65–2.50	1.06–2.43	2.99–4.07	3.18–4.07
19. Buđanovci (n=6)	mean ± SD	80.0±22.1	122.1±32.0	1.73±0.70	2.42±0.51	3.94±1.13	4.89±0.98
	min – max	54.9–116.9	87.5–166.9	1.01–2.70	1.69–2.95	2.51–5.34	3.87–6.74
20. Mali Radinci (n=6)	mean ± SD	140.9±33.8	143.1±37.5	2.04±0.81	3.08±0.72	3.73±0.18	5.25±.1.50
	min – max	91.1–196.2	105.7–165.0	1.94–2.38	2.51–4.38	3.45–3.90	4.01–8.06
21. Voganj (n=6)	mean ± SD	134.9±35.2	124.2±42.6	1.69±0.38	2.77±0.47	3.33±0.22	4.73±1.21
	min – max	80.9–176.9	86.4–183.9	1.16–2.12	3.00–5.12	3.11–3.72	3.00–6.32
N= 157	(mean ± SD)	103.3±42.1	138.5±52.7	1.75±0.66	2.36±0.85	3.32±0.62	4.16±1.40
	min – max	29.5–324.1	43.0–313.1	0.54–2.70	0.42–5.12	1.34–5.34	0.73–9.34

Legend: K – kidney, L – liver, SD – standard deviation, n – number of samples, N – samples analysed in total; the results are expressed on the basis of wet weight (w.w) site 19), an individual aged 3–6 months (Figure 3, MnK 3–6m). The lowest Mn level in the liver was 0.42 mg/kg and was recorded in a hare from Bajina Bašta (Table 3, sampling site 2), an individual 12 months old (Figure 3., MnL 12m). The highest recorded Mn level in the liver was 5.08 mg/kg in a one-12 months -old individual from Prokuplje (Table 1, sampling site 11) (Figure 3, MnL 12m).

3.3 Copper (Cu) levels in kidney and liver

The mean Cu levels (3.32 mg/kg) in the examined kidneys of the brown hare were lower than those in the liver (4.16 mg/kg), and the difference in the determined Cu levels between the two organs was statistically significant (p=0.001). Statistically significant differences between the Cu content in the kidneys compared to the liver were also found in all age groups (p=0.001−0.038). No statistically significant differences were found between the age groups with regard to the Cu content in the kidneys and liver of hares (Table 1). The mean Cu levels in the kidneys and liver (Table 1) of the studied individuals by age groups (3–6 months, 12 months, 12–24 months, 24–36 months and older than 36 months) were: for kidney: 3.31 mg/kg, 3.32 mg/kg, 3.41 mg/kg, 3.26 mg/kg and 3.34 mg/kg; for the liver: 4.26 mg/kg, 4.27 mg/kg, 3.93 mg/kg, 4.16 mg/kg and 3.94 mg/kg. The lowest measured Cu level in the kidneys was 1.34 mg/kg (Table 3, sampling site 11) and was recorded in a hare aged 12 months from the Prokuplje area (Table 1; CuK 12 m). The highest measured Cu level in the kidneys was 5.34 mg/kg in a hare collected from the Buđanovci area (Table 3, sampling site 19), an individual aged 12 months (Fig 4, Cu K12 m). The lowest Cu level in the liver

Table 4. Mean element levels (mg/kg) measured in hare organs by other authors

Element (mg/kg)	Kidney	Liver	Country	Authors
Fe	119[a] (total) 130[a] immature 108[a] adults	198[a](total) 236[a] immature 179[a] adults	Poland	Myslek and Kalisinska, 2006
	480 [f]	600 [f]	Poland	*Wajdzik et al., 2017**
	242,6 [e] /345,9 [b] (215.35–257.79)[e] (223.56–668.43)[b]	207,1 [e] /307,9 [b] (172.02–233.61)[e] (172.02–233.61)[b]	Croatia	Linšak et al., 2022
Mn	2.00 [a](total) 2.19[a] immature 1.98[a] adults	2.51[a](total) 2.62[a] immature 2.50[a] adults	Poland	Myslek and Kalisinska, 2006
	6.00[a]	4.80[a]	Turkey	Demirbaş and Erduran, 2017
Cu	2.6 [a] 6.6 [b]	3.1 [a] 5.8 [b]	Poland	Krelowska-Kulas et al., 1994
	(3.76 – 4.64) [a] (4.64 – 5.32) [b]	(4.64 – 5.32) [a] (4.61 – 5.15) [b]	Finland	Venäläinen et al.,1996
	3.85(total) 3.90[a] immature 3.78[a] adults	3.97(total) 3.95[a] immature 3.99[a] adults	Poland	Myslek and Kalisinska, 2006
	2.60 (juvenile)[c] 2.89 (adults)[c]	2.96 (juvenile)[c] 3.04 (adults)[c]	Spain	Le Fidalgo et al., 2015
	1.91 [a]	2.34 [a]	Turkey	Demirbaş and Erduran, 2017
	15[f]	14.7[f]	Poland	*Wajdzik et al., 2017**
	14[d]	13.8[d]	Croatia	*Lazarus et al., 2022**

Legend: The letters a-f indicate the environmental conditions of the sampling area. a –unpolluted areas, b – industrial areas; c – cultivated lands, d – natural gas treatment plant, e – coastal unpolluted area, f – mixed area (agricultural and industrial); *- results expressed as dry matter content, the data given in parentheses indicate the interval of findings.

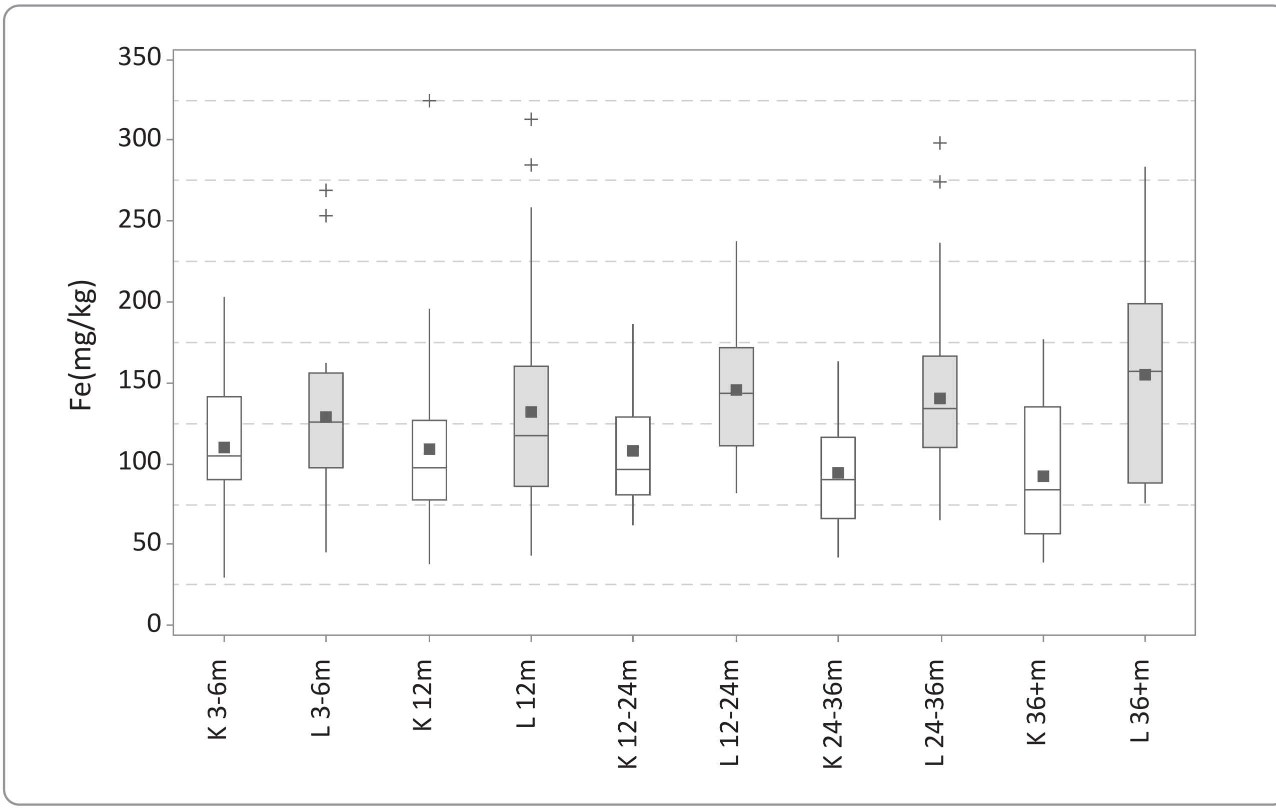

Figure 2. Iron (Fe) content (mg/kg, w.w.) in kidney (K) and liver (L) of hares grouped by age.

Legend: × indicates above-average values, ■ indicates mean values; a horizontal line within the box indicates median values

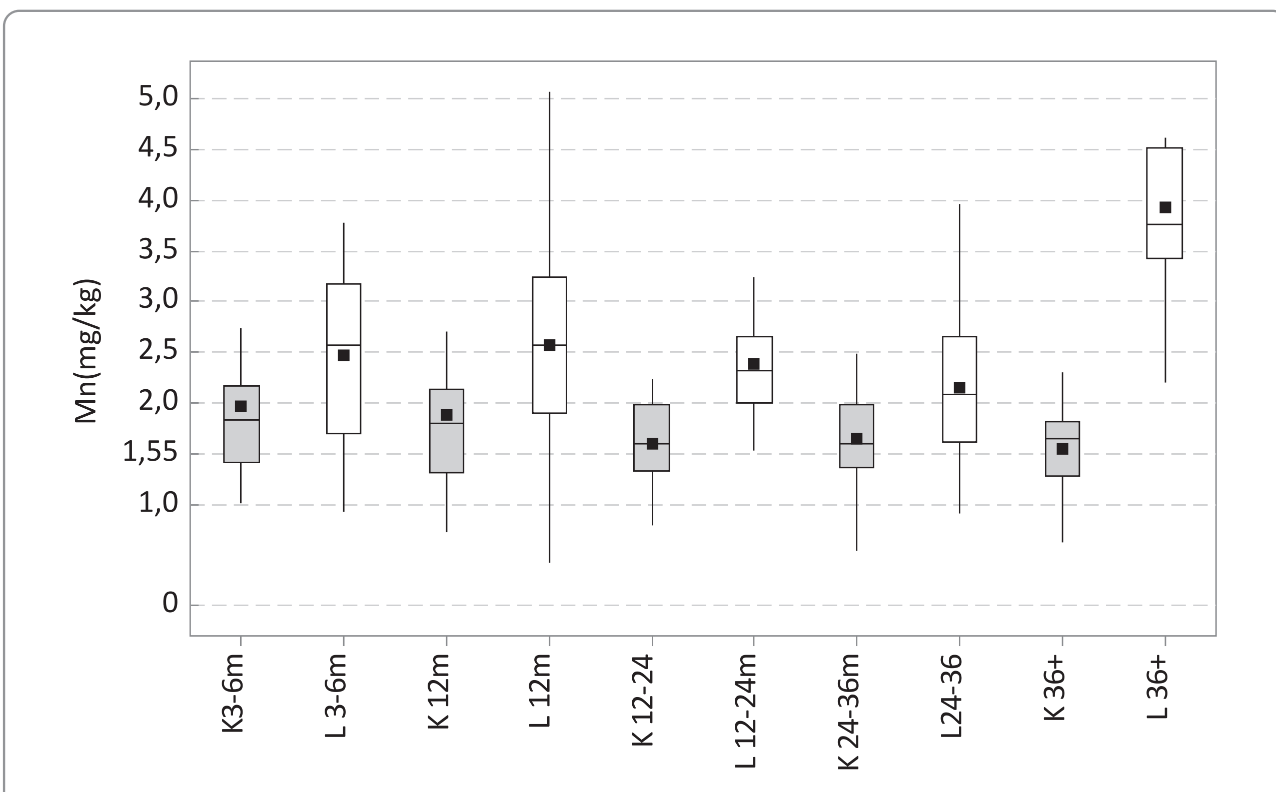

Figure 3. Manganese (Mn) content (mg/kg, w.w.) in kidney (K) and liver (L) of hares grouped by age.

Legend: × indicates above-average values, ■ indicates mean values; a horizontal line within a box indicates median values

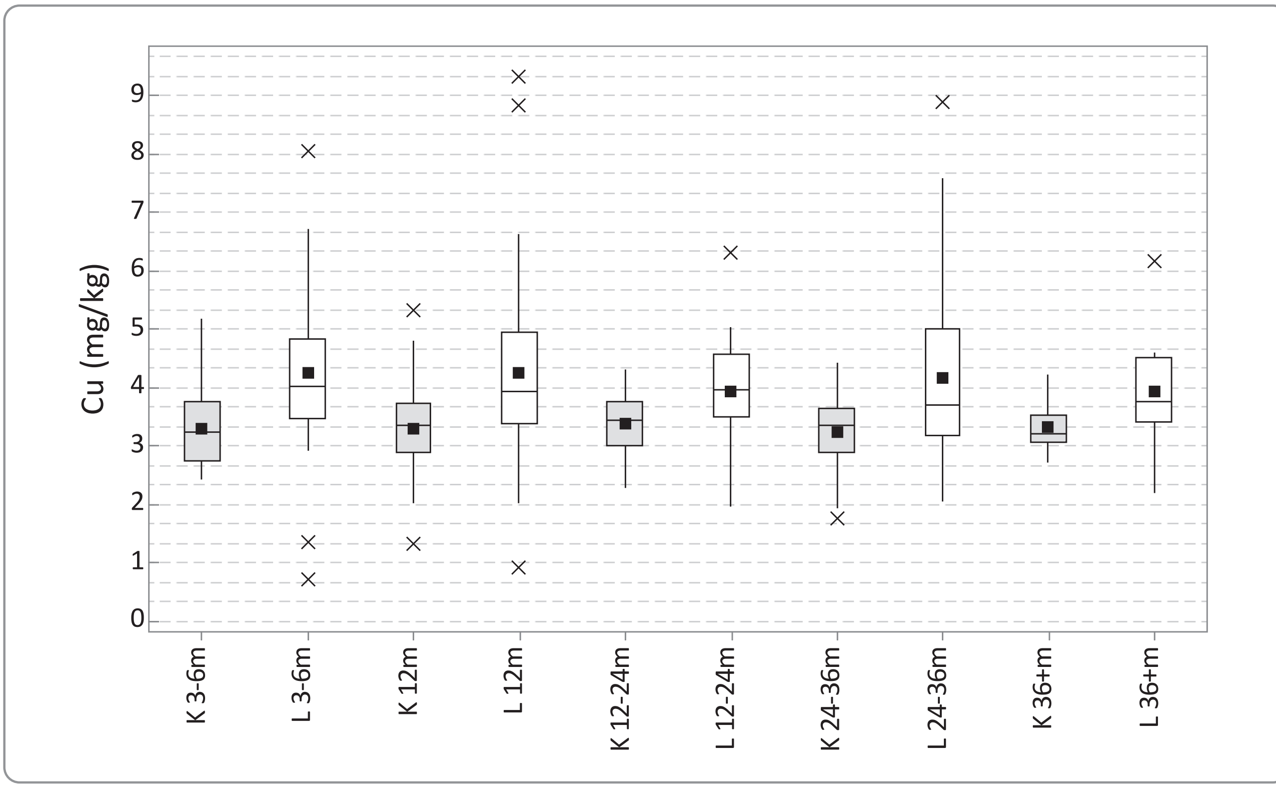

Figure 4. Copper (Cu) content (mg/kg, w.w.) in kidney (K) and liver (L) in hares grouped by age

Legend: × indicates above-average values, ■ indicates mean values; a horizontal line within a box indicates median values

was 0.73 mg/kg was recorded in a hare from Sombor (Table 3, sampling site 14), an individual 3–6 months old (Figure 4., CuL 3–6m). The highest recorded Cu level in the liver was 9.34 mg/kg in a one-year-old individual from the Novi Sad area (Table 1, sampling site 15) (Figure 4., CuL 12m).

The graphical representations of the Fe, Mn and Cu levels (mg/kg, w.w.) in the kidneys and liver of the examined hares according to age groups (3–6 months, 12 months, 12–24 months, 36 months and older than 36 months) can be found in Figs. 2–4.

3.4. Interactions between Fe, Mn and Cu in the kidney and liver of European hares

The correlation patterns between the essential elements in the liver and kidney of our hares were both positive and negative significant correlations among the single or different elements within the same organ and among the elements between the two organs (Table 5 and Table 6).

All registered correlations between liver and kidney were found when the Fe, Mn and Cu levels were correlated considering the whole population of hares studied (Table 5). All the mixed correlations between the three essential metals were found when the correlation matrix was formed by age groups (Table 6). The data analysis revealed a total of 12 different statistically significant associations (Table 5 and Table 6). Of these, seven different statistically significant correlations were observed for all samples analysed (Table 5) and all related to the association of different elements between kidney and liver.

Of these, only two common ones (FeK-FeL and MnK-FeL) occurred in correlations by age group (Table 6). The first common association, FeK-FeL, occurred in most age groups (3–6 m, Ps=0.56; 12 m, Ps=0.70; 24–36 m Ps=0.46), but was not found in groups of 12–24 m and over 36 m. Another common correlation, MnK-FeL, appeared once in the 12–24 m animals (Table 6). Within the age groups, the CuL-MnL correlation should be emphasised, which occurred four times (in the age groups: 3–6 m, Ps = 0.49; 12 m, Ps = 0.52; 24–36 m, Ps = 0.45 and 36 m+ , Ps = 0.68), but was not detected in the 12–24 m age group. The MnL-FeL correlation occurred twice (in the age groups 12 m, Ps= −0.31 and 12–24 m, Ps = −0.52). Other registered connections by age groups appeared once: CuK-MnK in 3–6 m, MnK-FeL in 12 m, CuK-FeL in 12–24 m and CuL-FeL in 24–36 m.

Of the seven correlation associations registered in the entire brown hare population studied, five out of a total of 12 were registered (Table 5), namely FeK-CuL, MnK-MnL, MnK-CuL, CuK-MnL and CuK-CuL, but did not appear in any correlation matrix formed by age groups. Most of these correlations were weak in relation to the whole population (Table 5), although they were weak to moderately strong within some age groups (± Ps correlation coefficient of −0.31 to 0.7). Weak to moderate negative correlations were also recorded in some age groups (Ps from −0.31 to −0.52). Note that all statistically significant correlations in

Table 5. Pearson (Ps) correlations between levels of different elements in kidney (K) and liver (L) of European hare within the whole examined population (n = 157)

Kidney – Liver		
Element/organ correlation	**Correlation coefficient**	**p value**
FeK-FeL	0.49	0.001[d]
FeK-CuL	−0.25	0.002[c]
MnK-FeL	−0.16	0.043[a]
MnK-MnL	0.21	0.010[b]
MnK-CuL	0.20	0.013[a]
CuK-MnL	0.19	0.018[a]
CuK-CuL	0.32	0.001[d]

Legend: [a]$p \leq 0.05$, [b]$p \leq 0.01$, [c]$p \leq 0.005$, [d]$p \leq 0.001$. Unless otherwise stated, the significance level is $p \leq 0.05$.

Table 6. Pearson (Ps) correlations between levels of elements in kidney (K) and liver (L) of European hare within particular age classes

Kidney-Liver, Kidney-Kidney, Liver-Liver (3–6 months. n=28)		
Element/organ correlation	**Correlation coefficient**	**p value**
FeK-FeL	0.56	0.002[d]
CuK-MnK	0.68	0.001[a]
CuL-MnL	0.49	0.009[b]
Kidney-Liver, Liver-Liver (12–24 months, n=22)		
Element/organ correlation	**Correlation coefficient**	**p value**
FeK-FeL	0.70	0.001[d]
CuL-MnL	0.52	0.001[a]
MnL-FeL	−0.31	0.049[a]
Kidney-Liver, Liver-Liver (12–24 months, n=22)		
Element/organ correlation	**Correlation coefficient**	**p value**
MnK-FeL	−0.47	0.026[a]
CuK-FeL	−0.47	0.029[a]
MnL-FeL	−0.52	0.014[a]
Kidney-Liver, Liver-Liver (24–36 months, n=51)		
Element/organ correlation	**Correlation coefficient**	**p value**
FeK-FeL	0.46	0.001[d]
CuL-FeL	−0.39	0.005[c]
CuL-MnL	0.45	0.001[d]
Liver-Liver (36+ months, n=15)		
Element/organ correlation	**Correlation coefficient**	**p value**
CuL-MnL	0.68	0.007[b]

Legend: [a]$p \leq 0.05$, [b]$p \leq 0.01$, [c]$p \leq 0.005$, [d]$p \leq 0.001$

the 12–24 month age group (MnK-FeL, Ps =−0.47; CuK-FeL, Ps= −0.46 and MnL-FeL, Ps= −0.52) were of medium strength and negatively correlated. The two recorded MnL-FeL correlations in the 12 m and 12–24 month age groups were negative. The largest number of mixed correlations between elements and between age groups appeared within the liver data (CuL-MnL, MnL-FeL and CuL-FeL) seven times, then between the liver and kidney data (CuK-MnL, MnK-FeL and CuK-FeL) five times, but only once within the kidney data (CuK-MnK).

4. Discussion

Our study documents Fe, Mn and Cu levels in kidney and liver within hare age groups and sampling sites as well as interactions within age and correlations between organs.

4.1. Iron (Fe) levels in kidney and liver

The content of Fe in the kidneys and liver did not differ in hares of 3–6 months and 12 months old. These results could be interpreted to mean that the content in these two organs is evenly built up until the animals are about 12 months old. Comparing the results of the present study with those of other studies (Table 4), it can be seen that the mean metal levels in our hares were lower than in those from Poland (*Myslek and Kalisinska,* 2006*; Wajdzik et al.,* 2017) and Croatia (*Linsak et al.,* 2017). In our study, the age difference between the studied groups was sharper and the mean Fe levels in the kidneys and liver (Table 1) of the studied individuals by age groups (3–6 months, 12 months, 12–24 months, 36 months and older than 36 months) were: for kidney (110.2 mg/kg; 109.2 mg/kg; 107.9 mg/kg; 94.5 mg/kg and 92.5 mg/kg) and for the liver (128.7 mg/kg; 132.0 mg/kg; 145.6 mg/kg; 140.7 mg/kg and 155.0 mg/kg), respectivly. The results for the Fe content in the kidneys of hares from the present study in relation to the age of 6 months to 24 months were similar to those from Poland (*Myslek and Kalisinska,* 2006). Other measured mean Fe levels in both organs were lower with respect to the studies selected for comparison (Table 4). It should be noted that comparisons of results should only be made between values expressed in the same way (i.e., wet weight or dry weight). In the study by *Falandysz et al.,* 1994), the Fe levels in the kidneys and liver of rabbits from farms in northern Poland were 27–83 mg/kg in kidney and 50–180 mg/kg in liver (similar to the present study, taking into account the interval results from different hunting areas).

In an older study (*Ferguson et al.,* 1962), the Fe levels in various organs (lung, kidney, liver, spleen) of laboratory-bred rabbits were investigated. The results for the kidney were 36–300 mg/kg and for the liver were 43–540 mg/kg. If we look at the Fe values determined in the kidneys and liver of the brown hares from the sampling sites in Serbia (Figure 2) and compare them with the study by *Ferguson* (1962), there are some similarities in the range. This comparison could lead us to the conclusion that the data from our study are close to the physiological Fe values in the examined organs of hares of different age groups from Serbia. The higher Fe levels (outliers) found in some hares (Figure 2) could be attributed to the state of the sample before homogenization, i.e., the high Fe could be due to excess blood on the surface of the organs.

4.2. Manganese (Mn) levels in kidney and liver

If we compare the Mn levels in the organs of brown hares from our study with the results of *Mislek and Kalisinska* (2006) from Poland, there was a very high degree of agreement, both in terms of the mean value (our study shows 1.75 mg/kg in the kidneys and 2.36 mg/kg in the liver, the Polish study 2.00 mg/kg and 2.51 respectively) and in terms of the levels in younger and older individuals. The Mn content in the kidneys and liver of young brown hares aged 3–6 months (Table 1) show, for example, that the levels (w.w.) in the kidney (1.96 mg/kg) and the liver (2.47 mg/kg) corresponded very well with those of younger individuals from Poland (kidney 2.19 mg/kg and liver 2.62 mg/kg). This similarity was also seen in older individuals. Thus, Mn levels of our study in hares aged 24–36 months (kidney 1.65 mg/kg, liver 2.15 mg/kg) were close to the Mn levels in older individuals from the Polish study of 2006 (Table 4), wherein the Mn levels in kidney and liver were 1.98 mg/kg and 2.50 mg/kg, respectively. Compared to the Turkish study (*Demirbaş and Erduran*, 2017), their registered mean Mn levels (kidney 6.00 mg/kg and liver 4.80 mg/kg) were somewhat lower in our study and also in the Polish study (*Mislek and Kalisinska,* 2006). In cattle and cervids, both ruminants, normal liver Mn levels were within the following ranges: 2.5–6.0 and 2.5–8.0 mg/kg (*Puls,* 1994).

Mn levels in the organs of brown hares can be influenced by the environment, as Europe consists of many land masses with different geomorphology and climate (*Vidus-Rosin et al.,* 2011*; Canova et al.,* 2020*; Kitowski et al.,* 2017*; Fattorini et al.,* 2021*; Buglione et al.,* 2022). Normal Mn levels in the kidneys of wild animals are lower than in the liver, but in some cases, the opposite is true (Table

4; *Demirbaş and Erduran,* 2017). The comparison of the results obtained in this study with regard to the Mn content in the kidney and liver of the European brown hare is consistent with other studies that have looked at the content of this element in various organs and animal species in the past. In the digestive system, the highest Mn levels were found in the stomach, followed by the liver and the kidneys, and the lowest levels were encountered in the intestines (*Ertl et al.,* 2016*; Kalisinska and Budis,* 2019). The Mn levels found in the organs of brown hares in this study were not so high as to have harmful effects (*Hackländer,* 2022*; Kompiš and Ballová,* 2021*; Selimovic and Arnold,* 2022).

Sporadically detected low Mn levels (0.42 mg/kg), e.g. in the liver of a 12-month-old hare (Figure 3) from the Bajina Basta region (Table 3, sampling point 2), could indicate a deficiency of this element in the diet or could be related to the health status of the individual. In fact, it is likely that brown hares suffer from malnutrition when they live in areas with monocultures that are poor in plant biodiversity (*Schai-Braun et al.,* 2015). It is well known that malnutrition has a negative impact on the survival rate of brown hares (*Edvards et al.,* 2000).

It appears from this study that the mean Mn levels in the organs of the brown hare by age classes are close to the physiological level and are not significant when the reported levels are considered in the context of the effect of this metal from the environment on the hare individuals studied, i.e. the toxicity of the registered amounts of Mn per individual. These results are in line with previous studies, where it was stated that the gastrointestinal and hepatobiliary systems play crucial roles in regulating and maintaining Mn organ levels within a relatively narrow physiologic range *(Aschner and Aschner* 2005*; Foster et al.,* 2015*; Zeman et al.* 2015).

From the literature reviewed, it appears there are not many field studies that have investigated Mn levels in different tissues and organs of the same animals and in different age groups of brown hares. Therefore, the data from our study are valuable data to show reference ranges for Mn levels in kidney and liver of European hares as they age.

4.3. Copper (Cu) levels in kidney and liver

A comparison of the mean Cu contents in the kidneys and liver of brown hares from our study with other reference studies (Table 4) shows a great similarity with the data from the Polish studies on hares from unpolluted areas (*Krelowska-Kulas et al.,* 1994*; Myslek and Kalisinska,* 2006) and slightly lower Cu levels in the kidneys and liver compared to in the organs of hares from unpolluted areas in Finland (*Venaelaeinen et al.,* 1996). In our study, Cu levels in kidney and liver were slightly higher on average, but still similar to levels in the Spanish study from hares collected from cultivated areas (*Le Fidalgo et al.,* 2015). Compared to the Turkish study that examined hares from unpolluted areas (*Demirbaş and Erduran*, 2017), the mean Cu levels in both examined organs in brown hares from Serbia were almost double.

In all hare age groups, some liver Cu levels (Fig. 4) were were outliers from the majority of the levels measured in the given population, i.e., 75% of the data from the third quartile and above the maximum levels (marked with the symbol x as outliers). This can be interpreted to mean that the excess Cu accumulates in the liver of these herbivores and in cases where Cu intake exceeds physiological limits (*Woolliams et al.,* 1983*; Grace et al.,* 1998). When comparing two groups of reared rabbits, one fed a basic diet (10 mg Cu/kg) and the other fed a pelleted diet with the addition of $CuSO_4 \times 5H_2O$ (140 mg/kg Cu — four times more than the maximum 35 mg/kg permitted by the EU regulation), *Skoivanova et al.* (2002) found 4.62 mg/kg in the liver of the control group, while the liver of rabbits fed a high dose of Cu contained 118.5±31.8 mg/kg of Cu. Moreover, the nutrient requirement for a rabbit in terms of Cu intake is 10 mg/kg per day (*Mateos and De Blas,* 1998). According to some studies, the Cu content in the diet had no significant effect on the Cu content in the liver (*Korish and Attia,* 2020*; López-Alonso and Miranda,* 2020*; Taylor et al.,* 2020). In conclusion, Cu supplementation increases liver Cu levels, while a non-supplemented diet prevents Cu accumulation in liver.

4.4. Interactions between Fe, Mn and Cu in the kidney and liver of European hares

There have been a number of previous studies on herbivorous species that revealed a large number of different correlations and interactive effects of essential element content in different tissues (*Goyer,* 1997*; Medvedev,* 1999*; Lopez et al.,* 2002a*,* 2004b*; Myslek and Kalisińska,* 2006). The explanation for the correlations found in this study seems to be related to a similar homeostatic mechanism of the so-called "cationic metals", a group of elements that are essential for body function (*Fairbrother et al.,* 2007). From these data alone, it is not possible to draw fundamental conclusions about the mutual kinetics of these elements (*Rahil-Khazen et al.,* 2002). A number of the elements studied produced interactive effects, evident in the brown hares from this study as well as in the herbivores from the earlier studies mentioned, with one element being able to influence the levels of another element in a predictable way.

5. Conclusions

The levels of Fe, Mn and Cu in kidneys and livers of brown hares collected in Serbian agricultural regions are within physiological limits and are comparable to other studies from other countries with similar biotopes and environmental conditions. The mean levels of all three investigated elements (Fe, Mn, Cu) between age groups within the same organ do not change significantly during the life span of the brown hare.

The content of Mn and Cu in hare liver is higher than in hare kidney in all age groups examined. For Fe, there are no statistically significant differences in the contents in these two organs in the first year of life, while after the first year of life, Fe contents in the liver and kidney are statistically significantly different, as is also the case for Mn and Cu.

The results of this study show that the correlations of the levels of the tested elements between different organs observed in the whole population of brown hares studied have a quantitatively lower strength than the strength of the relationship (Ps correlation coefficient) between different or within the same organs when the correlation matrix is formed separately for each age group. This finding suggests that a more precise age categorisation of brown hares gives a better picture of the registered associations and interactive effects. In this study, it was also observed that some correlations seen for the whole population did not occur between individual age groups in brown hares.

Određivanje nivoa i interakcije elemenata (Fe, Mn i Cu) u tkivu divljeg zeca u različitim starosnim grupama iz poljoprvrednih regiona Srbije

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INFORMACIJE O RADU

Ključne reči:
Mrki zec
Elementi
Bubreg
Jetra
Interakcije.

APSTRAKT

Određivan je sadržaj gvožđa, bakra i mangana u bubrezima i jetri evropskog divljeg zeca (Lepus europaeus). Ispitani zečevi su bili podeljeni u 5 starosnih grupa u rasponu od 3 meseca do starijih od 36 meseci. Prikupljeni su sa 21 različitog lovnog terena Vojvodine i Centralne Srbije, u blizini uglavnom obradivih i poljoprivrednih područja u Srbiji tokom 2010/2011. Studija je obuhvatila rezultate ispitanih koncentracija navedenih elemenata i međusobne interakcije u populaciji od ukupno 157 jedinki. Srednje vrednosti koncentracija Fe, Mn i Cu (mg/kg, vlažna masa) registrovane u bubrezima i jetri iznosile su: Fe (bubreg) 103,3±42,1, Fe (jetra) 138,5 ±52,7; Mn (bubreg) 1,75±0,66 Mn (jetra) 2,36±0,85; Cu (bubreg) 3,32±0,62, Cu (jetra) 4,16±1,40. Nisu bile registrovane statistički značajne razlike (p>0,05) između starosnih grupa u pogledu sadržaja Fe, Mn i Cu u bubrezima i jetri zečeva (unutar istog organa). Statistički značajne razlike između koncentracija elemenata u jetri i bubrezima (između različitih organa) registrovane u svim starosnim grupama u korist jetre u odnosu na bubreg zečeva osim koncentracije Fe u oba organa u starosnim grupama od 3–6 i 12 meseci. Obrasci korelacije između esencijalnih elemenata u jetri i bubrezima divljeg zeca u ovoj studiji pokazali su postojanje pozitivnih i negativnih statistički značajnih korelacionih povezanosti između pojedinačnih ili različitih elemenata unutar istog tkiva i pojedinih elemenata između različitih tkiva. Unutar starosnih grupa registrovano je 7 različitih statistički značajnih asocijacija (FeB-FeJ, CuJ-MnJ, MnJ-FeJ, CuB-MnB, MnB-FeJ, CuB-FeJ, CuJ-FeJ). Korelacione povezanosti između sadržaja elemenata u okviru organa unutar i između starosnih grupa određivane su primenom Pirsonovog testa za normalnu distribuciju.

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