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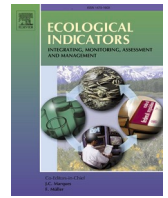
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## Minor and trace element concentrations in roe deer hair: A non-invasive method to define reference values in wildlife

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### ABSTRACT

Most chemical elements are crucial for life maintenance, but the intake of non-essential elements or inadequate concentrations of essential ones can have major consequences on wildlife health. However, concentrations of minor and trace elements remain largely unknown in free-ranging animals. This study aimed to establish the first reference values for 22 essential (Ca, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, Se, Zn) and non-essential (Al, As, Cd, Hg, Pb, Sb, Sr, Ti, Tl) elements in roe deer hair (*Capreolus capreolus*), to assess the degree of covariation between elements, and to investigate how individual characteristics and environmental context influence minor and trace element profiles. To do so, 758 hair samples from 542 individuals were collected between 2016 and 2019 in five roe deer populations (i.e. Ain, Chizé, Châteauvillain-Arc-en-Barrois, Jura and Trois-Fontaines) inhabiting contrasted habitats in France. Compared to the scarce literature on minor and trace elements in roe deer hair, concentrations were generally lower, and correlations between elements were mostly positive. Element concentrations were also heterogeneously distributed among populations, with overall greater heterogeneity in two (i.e. Jura and Châteauvillain-Arc-en-Barrois) of the five populations, which could be explained by their specific environmental characteristics (e.g. vegetation, climate, altitude). Overall, minor and trace element concentrations did not differ according to age and sex. Further studies on element bioavailability are needed to clarify whether the observed heterogeneity of element concentrations in roe deer hair is primarily driven by individual or by geographical characteristics.

### 1. Introduction

With increasing anthropogenic pressures, there is growing concern about biodiversity loss and emerging diseases in wildlife, and a need to improve our understanding of the factors that can influence the health and demography of wild populations (Deem et al., 2001; Tegler et al.,

2001; Stephen, 2014; Decker et al., 2016). In this context of global change and with the emergence of the “One health” concept (i.e. health at the interface between animals, humans and their environment; Hinchliffe, 2015), it has become critical to develop practical tools for assessing wildlife health that can be implemented on a large scale as part of wildlife population monitoring (Ryser-Degiorgis, 2013). Yet, such an

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initiative requires the identification of reliable and non-invasive health indicators, which can prove challenging when dealing with free-ranging animals. So far, the most commonly used methods are based on characterisation of blood parameters, assessment of body condition, physical examination, or faecal material analyses (Kophamel et al., 2022). Assessing minor and trace element status can also be relevant to inform on the health of individuals in animal population as done in humans (Fraga, 2005). Indeed, minor and trace elements encompass both inorganic nutrients (i.e. minerals) that are classified as essential or beneficial elements because of their tight link to health, survival and reproduction, and non-essential elements that can be toxic even at low dose (Underwood, 2012). Although non-essential elements occur naturally in the environment, most contamination stems from anthropogenic activities (e.g. fossil fuel combustion, smelters, mining, agriculture, waste dumps, urban development; He et al., 2005; Engwa et al., 2019; Shahid et al., 2019).

Essential elements are required for normal physiological functions. They are involved in several biochemical pathways (e.g. catalysis or inhibition of enzymatic reactions, hormonal control, regulation of gene transcription; McClure, 2008) and are thus crucial for life maintenance (Underwood, 2012). Therefore, inadequate intake of essential elements can have a major and lasting impact on the physiology of individuals and, consequently, on their health. Deficiencies in essential elements (such as copper (Cu), selenium (Se), zinc (Zn)) have been shown to impair the immune system, leading to diseases, poor reproductive performance and even death in mice, chicken or several ungulate species, for instance (Prohaska and Lukasewycz, 1981; Flueck, 1994; O'Hara et al., 2001; Grace and Wilson, 2002; Frank, 2004; Johnson et al., 2007; Zhang et al., 2012). On the other hand, these essential elements can also become toxic above a critical concentration in tissues, as is the case for chromium (Cr), cobalt (Co), Cu, iron (Fe), manganese (Mn), molybdenum (Mo), nickel (Ni), Se, or Zn (mainly showed in humans; Sandstead, 1995; Valko et al., 2005). At the cellular level, such toxicity can result in DNA damage, lipid peroxidation or depletion of proteins (Valko et al., 2005; Feng et al., 2020). However, toxic effects from non-essential elements are rather observed when non-essential elements (e.g. lead (Pb), cadmium (Cd), arsenic (As), mercury (Hg)) accumulate in tissues, i.e. when their rate of absorption and storage exceeds their rate of metabolism or excretion (Govind, 2014). These elements have no established biological functions and are toxic to all living forms, even at low concentrations (Raikwar et al., 2008). They are responsible for important physiological damages through interactions with cell components and several enzymes involved in metabolic functions and detoxification and damage repair mechanisms (Tchounwou et al., 2012).

In human health, the use of chemical elements as biomarkers to assess physiological status is common. Although it remains less employed in animals, the desire to develop tools for animal health monitoring has increased in recent years, as human health is often linked to animal health (i.e. One health approach; Frazzoli et al., 2015). Assessment of essential and non-essential element concentrations in farmed and wild animals can provide information on their exposure to environmental stressors such as diet deficiencies or pollution, for instance. Although the concentrations of essential and non-essential elements in livestock are well described (e.g. Gupta and Gupta, 1998; Hostetler et al., 2003; Bolan et al., 2004; Raikwar et al., 2008; Gupta, 2021; Suttle, 2022), there is less data available on wild species. Few studies have reported minor and trace element values in blood, organs or epidermal appendages (e.g. feathers, hair) in birds (Ohlendorf, 1993; Kim and Oh, 2016; Samaraweera et al., 2022), in fish (Thorrold and Shuttleworth, 2000; Agusa et al., 2005) or in wild ungulates (O'Hara et al., 2001; Roug et al., 2015; Durkalec et al., 2018; Pacyna et al., 2018; Cygan-Szczegieliński et al., 2018; Jutha et al., 2022), but reference values for minor and trace element concentrations in wildlife are rarely known. However, these values are necessary to assess wildlife health for diagnostic purposes in the context of wild population monitoring. They could be used to detect deficiencies or detrimental accumulation of

elements in wildlife, and therefore to better understand potential individual or population issues (e.g. disease occurrence, demography patterns). In this context, the use of hair and feather have become increasingly popular in wildlife research, to gain knowledge on wildlife's exposure to environmental contaminants (in feathers: Burger, 2007; Poesel et al., 2008; in hair: Dietz et al., 2006; Kempson and Lombi, 2011; Draghi et al., 2023). Unlike blood or organ sampling, hair and feathers collection are non-invasive procedures, and their storage (at ambient temperature) does not require a complex logistic chain. Hair accumulates elements throughout its growth period since the last molt, which allows the assessment of an animal's status in chemical elements over a wide temporal window (Combs et al., 1982; Combs, 1987; Cygan-Szczegieliński et al., 2014; Tête et al., 2014). Element concentrations in hair are therefore expected to reflect element contents of the habitat (Demesko et al., 2019).

In this study, we focused on the roe deer (*Capreolus capreolus*), a common and widely distributed mammal in Europe. Roe deer are strongly territorial (Linnell and Andersen, 1998; Saïd et al., 2009; Gaudry et al., 2018) and highly tolerant to human activities (Linnell and Andersen, 1995; Bonnot et al., 2013). Therefore, roe deer might be exposed to a variety of anthropogenic contaminants, and can be considered as a sentinel species for monitoring chemical elements in the environment (Draghi et al., 2023). Moreover, roe deer are harvested for human consumption and their meat represents an important food source in some European countries (Tomasevic et al., 2018). Thus, finding a way to better characterize the health of these animals is not only of ecological relevance, but also of significant socio-economic interest (Tataruch and Kierdorf, 2003). Literature on roe deer mainly focused on non-essential element accumulation in organs of hunted individuals (in kidney and liver: Pokorny, 2000; Wieczorek-Dąbrowska et al., 2013; in kidney, liver & muscle: Durkalec et al., 2015; in game meat: Gerofoke et al., 2018; in antlers: Cappelli et al., 2020; Ludolph et al., 2021). However, reference values for minor and trace elements are lacking in this species, especially in hair, since only a couple of studies provided reference concentrations for certain essential and non-essential elements (Cygan-Szczegieliński and Stasiak, 2022; Draghi et al., 2023).

The aim of this study was to establish reference values for a large set of essential and non-essential elements measured in roe deer hair. To do so, we measured the concentrations of 22 essential (calcium (Ca), Co, Cr, Cu, Fe, potassium (K), magnesium (Mg), Mo, Mn, sodium (Na), Ni, Se, Zn) and non-essential (aluminium (Al), As, Cd, Hg, Pb, antimony (Sb), strontium (Sr), titanium (Ti), thallium (Tl)) elements in roe deer hair collected from five French populations, with contrasting environmental conditions. We then evaluated the degree of covariation between the different elements in order to reveal their possible correlations (Cygan-Szczegieliński et al., 2014, 2018; Cygan-Szczegieliński, 2021). Finally, we investigated whether element concentrations were dependent on individual characteristics and environmental contexts by comparing the data obtained from the five study sites.

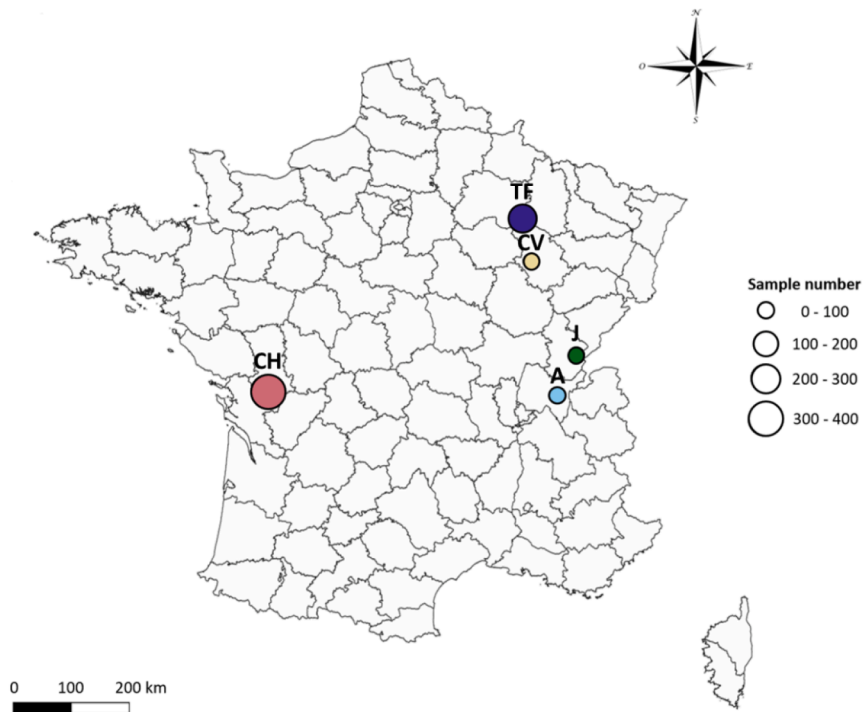
## 2. Materials & methods

### 2.1. Study populations

Roe deer hair samples were collected on individuals from five populations in France: "Réserve Biologique Intégrale of Chizé" (CH), "Territoire d'Étude et d'Expérimentation of Trois-Fontaines" (TF), Jura (J), Châteaullain-Arc-en-Barrois (CV) and Ain (A) (Fig. 1). These study sites share the same geological substrate, i.e. sedimentary rock (BRGM, 2023). However, they differ in a number of environmental characteristics such as altitude, type of habitat, vegetation characteristics, soil composition and climate (Table 1).

### 2.2. Hair sample collection

Hair samples from the populations of Trois-Fontaines, Chizé, Jura



**Fig. 1.** Study site locations in France: Ain (A), Chizé (CH), Châteauvillain-Arc-en-Barrois (CV), Jura (J), Trois-Fontaines (TF). The dots size is proportional to the number of samples collected per site.

**Table 1**  
Geographical and environmental characteristics of the five study sites.

Name of population	Coordinates	Altitude (m)	Size (ha)	Type of habitat (% of forest cover)	Main vegetation	Soil	Climate
Ain	45°99'N, 5°55'E	400–950	11,000	Open mountain forest (69 %)	Oak ( <i>Quercus</i> spp.), beech ( <i>Fagus sylvatica</i> )	Marno-calcaerous	Semi-continental (mountain influence)
Chizé	46°12'N, 0°42'W	50–100	2640	Enclosed lowland forest (98 %)	Oak, hornbeam ( <i>Carpinus betulus</i> ), maples ( <i>Acer monspessulanum</i> , <i>Acer campestre</i> ) & beech	Calcaerous, shallow	Oceanic
Châteauvillain-Arc-en-Barrois	48°02'N, 5°00'E	200–400	30,700	Open lowland forest (98 %)	Oak, beech	Calcaerous, shallow	Continental-Oceanic
Jura	46°66'N, 6°02'E	500–1100	21,000	Open mountain forest (62 %)	Conifers (e.g. <i>Abies alba</i> , <i>Picea abies</i> ), oak, beech	Calcaerous, shallow	Semi-continental (mountain influence)
Trois-Fontaines	48°72'N, 4°92'E	150–250	1360	Enclosed lowland forest (98 %)	Oak, beech, hornbeam	Calcaerous, deep, fertile	Continental

and Ain were collected as part of capture-mark-recapture programs that take place each year between October and March, i.e. during the non-reproductive season. In these populations, one-year-olds are sexed and marked with ear tags at first capture, so that age can be determined on subsequent captures. Samples were also collected during hunting seasons between October and February in Châteauvillain-Arc-en-Barrois and in Jura from individuals after shooting. In those cases, individuals were sexed and their age class was assessed based on tooth eruption patterns and presence or absence of tooth wear characters (Maublanc et al., 1991; Delorme et al., 2007). Roe deer captures were carried out in accordance with the French Environmental Code (Art. R421-15 to 421-31 and R422-92 to 422-94-1) and are approved by the Prefecture of Paris (agreement n°2009-14, n°2013-118, n°2019-02-19-003). Handling time was minimized to reduce animal stress in accordance with the guidelines for the ethical use of animals in research (Sikes and the Animal Care and Use Committee of the American Society of Mammalogists, 2016).

All hair samples were taken from the white patch around the rump of the animals. A tuft of hair was pulled out, being careful to harvest the

bulb. Roe deer undergo molt twice a year, in late spring and then in early autumn (Johnson and Hornby, 1975). Animals are expected to have completed most of their autumn moult by October, meaning that all individuals of our study already displayed their winter fur when sampled (Johnson and Hornby, 1975). Thus, in each sample, the accumulation of chemical elements during hair growth was complete, and we do not anticipate any bias in accumulation due to the time of hair collection. All these sampling conditions allow to avoid the effect of hair colour, collection site and seasonal changes in hair growth on element concentrations (Combs et al., 1982; Combs, 1987; Wells et al., 1990).

### 2.3. Minor and trace element analysis

Minor and trace element analyses were performed at the PEA<sup>2</sup>t platform from Chrono-environnement laboratory (UMR CNRS 6249, Université de Franche-Comté).

The samples were first washed in six successive baths of alternating acetone and ultrapure water using an ultrasonic cleaning device (140/

280 W, 37 kHz, Fisher Scientific, S-LINE 4.25 L) to remove potential external contamination so that only non-essential elements accumulated in (and not deposited on) hair were measured (Nageeb Rashed and Soltan, 2005, Amaral et al., 2008, Carneiro et al. 2011). Washed samples were then dried at 60 °C in an oven before being mineralized in closed tubes placed in a block digestion system (DigiPREP, SCP Sciences, Courtaboeuf, France). A gradual heating mode is used to achieve a final temperature of 100 °C (total run of 265 min). Hair samples weighed on average  $118 \pm 37$  mg. Samples with a mass higher than 125 mg were digested with an acid-oxidant mixture (Optima™ for ultra-traces, Fisher Scientific) composed of 3.5 ml of nitric acid (65 %) and 1 ml of hydrogen peroxide (30–32 %), those with a lower mass were digested with volumes divided by two. They were then diluted by adding 25 or 12.5 ml of ultra-pure water (Elga, 18.2 MV/cm<sup>2</sup>), respectively. Finally, a filtration to 1 µm was performed.

In total, the concentrations of 22 elements were assessed. We measured the concentrations of the following 18 minor and trace elements using inductively coupled plasma-mass spectrometry (ICP-MS, X Series II model, ThermoFisher Scientific, Courtaboeuf, France) after dilution by a factor 10: Al, As, Cd, Co, Cr, Cu, Fe, Hg, Mn, Mo, Ni, Pb, Sb, Se, Sr, Ti, Tl, and Zn. The following 4 element concentrations were measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES, iCAP 6500 radial model, ThermoFisher Scientific, Courtaboeuf, France) without dilution: Ca, K, Mg, and Na. Repeatability (within-run precision) and reproducibility of the measurements were surveyed (checking of relative standard deviation values and use of internal standards), and quality control (control solutions and blanks to check for the absence of drift) was conducted during measurements. Analysis accuracy was checked using two certified reference materials (CRM), i.e. oriental tobacco leaves (INCT-OBTL-5) and fish proteins (NRCC DORM-3). The percentage of recovery between certified values and average of measured values was considered satisfactory between 75 and 125 % (USEPA, 2000). Therefore, caution should be paid when considering the results for sodium and mercury, as they exhibit a low (46 %) and a high (173 %) average recovery, respectively, in OBTL-5 CRM (Table S1). For Hg, this is probably due to very low concentration in this CRM (0.0209 µg/g), and recovery rate is much better (92 %) in DORM-3 CRM where Hg concentration is higher (0.409 µg/g, Table S1). Concentrations of Na and Hg can nevertheless be included in inter-population comparisons but they cannot be regarded as established accurate reference values in roe deer hair. Blanks (acid + ultra-pure water) and CRM were prepared and analysed using the same methods as the samples. The concentrations of minor and trace elements in hair are expressed as µg/g or ng/g dry mass. For 3.9 % of measures, concentrations were below the quantification limits (i.e. As, Cd, Cr, Hg, Mg, Mo, Ni, Sb, Se, Sr, Tl). For those values, half of the quantification limit value was assigned for the statistical analyses (Table S1; USEPA, 2000).

Subsequently, to carry out the analyses, we established two categories of elements: essential ones (i.e. Ca, Co, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, Se, Zn) and non-essential ones (i.e. Al, As, Cd, Hg, Pb, Sb, Si, Sr, Ti, Tl) (following Underwood, 2012 for the classification of elements in human and animals).

#### 2.4. Statistical analyses

All statistical analyses were performed using R (version 4.2.1; R core Team, 2021). To maximize sample size, descriptive statistics (i.e. mean, standard deviation (sd), median, range), reference intervals and correlations were computed from all 758 samples (i.e. including repeated measurements). Data from Chizé and Trois-Fontaines populations effectively included samples from the same individuals that had been collected in different years, but results remained overall similar when analyses were performed with and without these repeated measurements. Reference intervals for the 22 elements were assessed using the *referenceIntervals* package (Finnegan, 2022) with a 95 % coverage for the

calculated reference interval. Outliers were detected using Horn's method (Horn et al., 2001) and Tukey's interquartile fences (Tukey, 1949) on a Box-Cox transformation of the data (Box and Cox, 1964). If a measurement lied outside 1.5 times the interquartile range from the first or third quartile point, it was considered an outlier. Outliers remained nonetheless in the dataset as we wanted to explore the full variability of the data to calculate reference intervals as representative as possible. Finally, correlations between elements were assessed using Spearman's rank correlation coefficient (Spearman, 1906).

When testing for age (i.e. juveniles under 1 year old *versus* adults), sex and population differences in element concentrations, we chose to consider only one randomly selected measurement per individual to prevent pseudo replication issues (leaving us with a final sample size of  $n = 542$ ; except when testing for sex differences where  $n = 540$ , since the sex of two individuals was not reported). Since most element concentrations did not meet the assumptions of normal distribution nor those of homoscedasticity (statistically verified with the Levene test using the *LeveneTest* package), we used the non-parametrical Kruskal-Wallis test by ranks to examine potential differences among populations (Kruskal and Wallis, 1952). Afterwards, a test for multiple comparisons, i.e. post-hoc Dunn test (Dunn, 1964), was applied to decipher the differences between the five populations using the *FSA* package (Ogle et al., 2022). Finally, we used the non-parametric Mann-Whitney test (Mann and Whitney, 1947) to test for potential differences in element concentrations between age and sex categories. *P-values* were adjusted using the Holm adjustment method (Holm, 1979) to counteract the increase in type I error after multiple testing.

### 3. Results

#### 3.1. Minor and trace element status of roe deer

In total, 758 samples were collected ( $n_A$ : 28,  $n_{CH}$ : 363,  $n_{CV}$ : 37,  $n_J$ : 42,  $n_{TF}$ : 288,  $n_{juveniles}$ : 261,  $n_{adults}$ : 497,  $n_{females}$ : 413,  $n_{males}$ : 343). These samples corresponded to 542 different individuals, of which 88 had 2 measurements in two different years, 46 had 3 and 12 had 4. Descriptive statistics for essential and non-essential elements are presented in Tables 2 and 3, respectively. Results include statistics for all sites combined, but also for each population, age class (juveniles *versus* adults) and sex.

#### 3.2. Determination of reference intervals

The reference intervals determined for the 22 minor and trace elements and their specific outliers are presented in Table 4. A total of 177 outliers were detected among values for all essential elements, with 80 measurements below the lower limit and 97 above the upper one, representing 1.8 % of measurements (i.e. 177 outliers out of 758 samples times 13 essential elements). Chromium was the element with the highest number of outliers below the reference interval ( $n = 15$ ), while Ni was the element with the highest number of outliers detected above ( $n = 25$ ). Calcium was the only essential element that showed no outliers.

For non-essential elements, a total of 179 outliers were identified, with 94 measurements below the lower limit and 85 above the upper limit, representing 2.6 % of measurements (i.e. 179 outliers out of 758 samples times 9 non-essential elements). Thallium was found to be the non-essential element with the highest number of concentrations above the reference interval ( $n = 27$ ), and As the one with the most outliers ( $n = 65$ ) (all elements combined). Cadmium was the only non-essential element that showed no outliers.

#### 3.3. Correlations between elements

Most elements were positively correlated with each other (Fig. 2). Iron and Ti were highly correlated, as were Fe and Al, Ti and Al, and Co



**Table 2**  
Essential element concentrations determined by ICP-MS or ICP-AES (dry mass) in roe deer hair samples ( $n = 758$ ) collected between 2016 and 2021 in France. Data were collected on juveniles (Juv) and adults (Ad) of both sexes (females: F, males: M) and from 5 different populations (A = Ain, CH = Chizé, CV = Châteauvillain-Arc-en-Barrois, J = Jura, TF = Trois-Fontaines).

	Unit		All	Population					Age		Sex	
				A	CH	CV	J	TF	Juv	Ad	F	M
Ca	(µg/g)	Mean ± sd	625 ± 582	833 ± 1138	596 ± 421	755 ± 604	970 ± 818	573 ± 616	679 ± 641	569 ± 548	646 ± 556	599 ± 614
		Median	479	540	494	531	686	447	509	461	503	443
		Range	239–7285	249–6209	244–4890	296–3066	293–5114	239–7285	249–7285	239–6209	249–6209	239–7285
Co	(ng/g)	Mean ± sd	47.6 ± 59.5	42.2 ± 53.4	32.1 ± 19.7	61.6 ± 48.1	29.1 ± 32.6	68.5 ± 85.5	49.4 ± 54.4	46.6 ± 62.0	47.9 ± 67.8	47.1 ± 47.8
		Median	32.1	24.8	26.8	40.7	18.7	44.6	34.3	30.9	30.7	33.3
		Range	7.31–1046	12.2–292	8.89–156	8.63–221	7.31–177	16.2–1046	7.45–533	7.31–1046	7.86–1046	7.31–533
Cr	(ng/g)	Mean ± sd	220 ± 297	230 ± 343	204 ± 230	214 ± 265	178 ± 237	248 ± 368	217 ± 202	222 ± 336	225 ± 339	216 ± 237
		Median	145	137	148	133	111	151	155	139	141	149
		Range	3.12*–4857	3.12*–1883	6.49*–2639	4.72*–1325	3.12*–1412	3.74*–4857	3.12*–4857	3.12*–4857	3.12*–4857	3.12*–2358
Cu	(µg/g)	Mean ± sd	6.34 ± 2.31	6.21 ± 0.630	6.54 ± 3.06	6.06 ± 1.16	6.85 ± 2.85	6.05 ± 0.834	6.58 ± 1.54	6.21 ± 2.62	6.26 ± 2.83	6.43 ± 1.47
		Median	6.12	6.10	6.22	5.97	6.15	5.96	6.34	6.00	6.03	6.27
		Range	3.36–60.4	4.11–78.4	4.11–60.4	3.36–8.45	5.30–24.0	4.15–12.0	4.68–24.0	3.36–60.4	3.36–60.4	3.36–24.0
Fe	(µg/g)	Mean ± sd	84.1 ± 109	113 ± 133	71.4 ± 51.6	218 ± 279	82.0 ± 110	80.5 ± 111	81.9 ± 98.3	85.3 ± 115	86.0 ± 116	81.3 ± 100
		Median	55.0	73.4	55.6	115	43.4	49.9	56.0	53.0	52.6	55.7
		Range	12.8–1367	31.1–730	14.1–354	14.6–1367	15.2–576	12.8–1246	15.8–1246	12.8–1367	12.8–1367	13.9–1246
K	(µg/g)	Mean ± sd	4409 ± 1369	4199 ± 1087	4653 ± 1442	4128 ± 1010	3134 ± 1131	4343 ± 1251	4504 ± 1504	4358 ± 1290	4568 ± 1463	4223 ± 1223
		Median	4183	4299	4480	3962	3182	4165	4332	4147	4393	4055
		Range	1080–13438	2543–6954	2186–13438	2090–6155	1080–5821	1434–9926	1080–13438	1434–10390	1434–13438	1080–9926
Mg	(µg/g)	Mean ± sd	276 ± 173	347 ± 163	238 ± 177	234 ± 175	228 ± 118	329 ± 160	284 ± 196	272 ± 159	295 ± 187	253 ± 152
		Median	238	349	190	152	225	307	248	231	258	226
		Range	28.4*–1961	75.1–738	46.3–1961	28.4*–742	59.6–589	70.2–1076	59.6–1961	28.4*–882	55.7–1961	28.4*–1077
Mn	(µg/g)	Mean ± sd	5.57 ± 7.45	5.34 ± 5.70	3.62 ± 4.68	6.07 ± 4.68	3.09 ± 3.76	8.33 ± 9.89	6.14 ± 9.81	5.26 ± 5.84	6.11 ± 8.29	4.90 ± 6.27
		Median	3.56	2.89	2.49	5.58	1.60	5.42	3.69	3.48	3.80	3.38
		Range	0.361–84.3	1.07–27.7	0.395–71.7	0.361–21.1	0.648–17.7	1.00–84.35	0.684–84.3	0.361–60.2	0.361–71.7	0.648–84.3
Mo	(ng/g)	Mean ± sd	112 ± 619	253 ± 622	49.0 ± 219	640 ± 1936	145 ± 120	104 ± 632	131 ± 716	102 ± 563	119 ± 638	102 ± 599
		Median	39.5	69.5	29.3	133	110	44.7	41.4	38.6	40.2	38.0
		Range	3.93*–11,367	32.1–2924	3.93*–3876	52.8–11367	33.4–580	14.8–10526	9.29–10526	3.93*–11,367	3.93*–11,367	3.93*–10,526
Na <sup>§</sup>	(µg/g)	Mean ± sd	267 ± 286	238 ± 205	281 ± 133	828 ± 957	381 ± 297	164 ± 92.6	239 ± 152	282 ± 335	268 ± 243	264 ± 330
		Median	199	163	247	401	251	141	192	206	206	190
		Range	72.3–3984	94.6–1081	92.1–992	116–3984	133–1514	72.3–987	72.3–992	75.0–3984	86.5–2802	72–3984
Ni	(ng/g)	Mean ± sd	248 ± 313	260 ± 236	245 ± 302	237 ± 207	214 ± 149	256 ± 359	264 ± 358	239 ± 286	247 ± 300	250 ± 329
		Median	166	177	166	197	182	161	168	164	172	161
		Range	4.68*–3760	87.9–1130	7.27*–2812	4.68*–1135	43.4–7456	15.8*–3760	16.3*–3760	4.68*–2881	7.27*–2881	4.68*–3760
Se	(µg/g)	Mean ± sd	0.263 ± 0.153	0.190 ± 0.0493	0.273 ± 0.178	0.178 ± 0.0907	0.120 ± 0.0445	0.293 ± 0.121	0.255 ± 0.163	0.270 ± 0.147	0.270 ± 0.156	0.258 ± 0.149
		Median	0.229	0.174	0.223	0.168	0.115	0.261	0.216	0.241	0.239	0.224
		Range	0.0192*–1.34	0.129–0.320	0.0678–1.34	0.0254*–0.374	0.0230*–0.275	0.0192*–0.794	0.0192*–1.34	0.0192*–1.13	0.0230*–1.34	0.0192*–1.13
Zn	(µg/g)	Mean ± sd	77.6 ± 17.1	76.6 ± 8.62	76.8 ± 11.9	76.4 ± 12.6	87.2 ± 13.9	77.5 ± 22.8	76.9 ± 15.3	78.0 ± 17.9	79.1 ± 19.0	75.8 ± 14.1
		Median	75.2	77.2	75.6	73.4	86.1	72.7	74.8	75.3	76.6	73.4
		Range	44.6–296	57.4–91.0	46.2–123	60.7–103	63.3–137	44.6–296	44.6–138	45.2–296	44.6–296	44.8–141

\* Values under the quantification limit (UQL).

§ Values must be considered for their order of magnitude but not as accurate reference values because of low (Na) and high (Hg) analytical recovery (see Table S1).

**Table 3**

Non-essential element concentrations determined by ICP-MS or ICP-AES (dry mass) in roe deer hair samples ( $n = 758$ ) collected between 2016 and 2021 in France. Data were collected on juveniles (Juv) and adults (Ad) of both sexes (females: F, males: M) and from 5 different populations (A = Ain, CH = Chizé, CV = Châteauvillain-Arc-en-Barrois, J = Jura, TF = Trois-Fontaines).

	Unit		All	Population					Age		Sex	
				A	CH	CV	J	TF	Juv	Ad	F	M
Al	(µg/g)	Mean ± sd	62.3 ± 71.8	69.0 ± 67.6	65.6 ± 71.2	77.4 ± 59.7	45.4 ± 62.9	57.9 ± 75.2	60.9 ± 59.0	63.0 ± 77.7	62.2 ± 69.0	62.3 ± 75.3
		Median	41.4	42.1	47.3	62.2	21.0	36.8	39.9	42.4	40.2	42.5
		Range	7.12–1020	16.6–328	8.58–1020	8.86–238	7.12–337	7.98–870	8.37–386	7.12–1020	7.12–870	8.12–1020
As	(ng/g)	Mean ± sd	63.9 ± 106	78.6 ± 93.7	58.0 ± 113	135 ± 157	63.9 ± 110	60.7 ± 85.1	60.6 ± 78.5	65.6 ± 118	68.9 ± 125	57.7 ± 77.9
		Median	38.2	47.3	34.6	76.2	25.6	40.2	42.5	35.5	37.4	39.0
		Range	2.87*–1475	17.4–500	3.38*–1475	6.53*–723	2.87*–644	3.59*–748	2.87*–741	2.87*–1475	2.87*–1475	2.87*–741
Cd	(ng/g)	Mean ± sd	55.3 ± 119	24.2 ± 29.4	36.0 ± 67.4	14.2 ± 11.9	188 ± 268	68.6 ± 133	58.0 ± 116	53.9 ± 121	53.6 ± 118	57.8 ± 120
		Median	19.9	9.71	15.8	13.9	41.8	31.1	28.3	16.0	19.6	23.7
		Range	1.11*–1181	2.81–124	1.11*–962	2.68*–47.6	3.64*–1149	1.11*–1181	1.11*–1149	1.11*–1181	1.11*–1181	1.11*–1122
Hg <sup>§</sup>	(ng/g)	Mean ± sd	112 ± 984	67.0 ± 74.7	62.7 ± 247	10.7 ± 10.9	93.3 ± 51.4	195 ± 1569	80.7 ± 313	129 ± 1193	127 ± 1296	95.1 ± 342
		Median	44.7	25.9	42.5	6.57	85.8	52.1	43.8	45.0	43.8	45.9
		Range	1.83*–26,066	2.59*–220	4.71*–4116	2.59*–49.9	2.59*–266	1.83*–26,066	1.83*–4998	2.59*–26,066	2.59*–26,066	1.83*–4998
Pb	(µg/g)	Mean ± sd	0.394 ± 2.11	0.345 ± 0.843	0.312 ± 0.278	2.44 ± 9.29	0.323 ± 0.498	0.238 ± 0.379	0.284 ± 0.294	0.445 ± 2.60	0.343 ± 0.825	0.448 ± 3.01
		Median	0.197	0.167	0.230	0.242	0.146	0.151	0.203	0.191	0.191	0.203
		Range	0.0407–55.7	0.0661–4.62	0.0502–2.49	0.0434–55.7	0.0407–2.87	0.0509–5.45	0.0520–2.87	0.0407–55.7	0.0407–13.8	0.0526–55.7
Sb	(ng/g)	Mean ± sd	29.1 ± 93.3	13.9 ± 10.4	35.3 ± 89.3	57.8 ± 217	20.0 ± 22.7	20.5 ± 81.4	23.2 ± 37.5	32.2 ± 112	32.7 ± 104	24.9 ± 78.3
		Median	12.8	11.4	17.8	10.4	12.7	8.84	13.9	12.2	13.2	12.3
		Range	0.803*–1321	2.69*–47.8	0.803*–915	1.66*–1321	1.27*–121	0.959*–1305	0.959*–359	0.803*–1321	0.803*–1305	0.959*–1321
Sr	(µg/g)	Mean ± sd	0.509 ± 0.608	0.749 ± 1.34	0.406 ± 0.368	0.532 ± 0.596	0.674 ± 0.888	0.574 ± 0.647	0.570 ± 0.617	0.468 ± 0.585	0.529 ± 0.621	0.473 ± 0.569
		Median	0.389	0.421	0.344	0.318	0.441	0.436	0.443	0.359	0.405	0.374
		Range	0.0294*–7.38	0.185–7.38	0.0681*–4.51	0.0366*–2.62	0.0294*–4.92	0.0681*–6.17	0.0681*–6.17	0.0294*–7.38	0.0294*–7.38	0.0366*–6.17
Ti	(µg/g)	Mean ± sd	1.02 ± 0.847	1.17 ± 0.902	0.896 ± 0.564	1.71 ± 1.23	0.783 ± 0.844	1.10 ± 1.00	1.03 ± 0.824	1.01 ± 0.854	1.03 ± 0.894	1.00 ± 0.779
		Median	0.769	0.796	0.736	1.33	0.495	0.795	0.772	0.766	0.737	0.782
		Range	0.166–9.27	0.380–4.12	0.199–4.87	0.301–6.01	0.166–4.25	0.241–9.27	0.166–6.28	0.186–9.27	0.166–9.27	0.199–6.28
Tl	(ng/g)	Mean ± sd	1.61 ± 2.72	2.05 ± 1.50	1.51 ± 3.10	1.65 ± 1.10	4.70 ± 3.81	1.23 ± 1.83	1.81 ± 2.94	1.50 ± 2.59	1.62 ± 3.08	1.59 ± 2.21
		Median	0.954	1.38	1.01	1.49	3.27	0.730	1.00	0.938	0.969	0.932
		Range	0.0467*–34.7	0.595–6.26	0.112*–34.7	0.223*–4.46	0.505–15.4	0.0467*–14.2	0.0467*–32.1	0.0467*–34.7	0.0467*–34.7	0.0467*–15.3

\* Values under the quantification limit (UQL).

§ Values must be considered for their order of magnitude but not as accurate reference values because of low (Na) and high (Hg) analytical recovery (see Table S1).

**Table 4**Reference Intervals for essential and non-essential elements ( $\mu\text{g/g}$  of dry mass) in roe deer hair samples collected between 2016 and 2021 in France ( $n = 758$ ).

		Unit	Lower reference limit (2.5 %)	Upper reference limit (97.5 %)	Outliers under lower RI	Outliers above upper RI
Essential elements	Ca	( $\mu\text{g/g}$ )	274	1718	0	0
	Co	( $\text{ng/g}$ )	11.0	176	3	2
	Cr	( $\text{ng/g}$ )	30.3	809	15	16
	Cu	( $\mu\text{g/g}$ )	4.78	8.64	12	9
	Fe	( $\mu\text{g/g}$ )	19.5	344	4	2
	K	( $\mu\text{g/g}$ )	2219	7352	7	9
	Mg	( $\mu\text{g/g}$ )	68.7	647	1	1
	Mn	( $\mu\text{g/g}$ )	0.881	22.2	2	1
	Mo	( $\text{ng/g}$ )	14.8	398	8	20
	Na <sup>§</sup>	( $\mu\text{g/g}$ )	95.3	840	1	2
	Ni	( $\text{ng/g}$ )	50.6	1045	10	25
	Se	( $\mu\text{g/g}$ )	0.0808	0.619	8	3
	Zn	( $\mu\text{g/g}$ )	56.4	110	9	7
	Non-essential elements	Al	( $\mu\text{g/g}$ )	10.8	240	1
As		( $\text{ng/g}$ )	3.59	385	45	20
Cd		( $\text{ng/g}$ )	1.11	351	0	0
Hg <sup>§</sup>		( $\text{ng/g}$ )	3.41	235	18	8
Pb		( $\mu\text{g/g}$ )	0.0656	1.32	3	2
Sb		( $\text{ng/g}$ )	2.19	119	5	13
Sr		( $\mu\text{g/g}$ )	0.0760	1.62	5	14
Ti		( $\mu\text{g/g}$ )	0.288	3.23	2	1
Tl		( $\text{ng/g}$ )	0.150	7.91	15	25

<sup>§</sup> Values must be considered for their order of magnitude but not as accurate reference values because of low (Na) and high (Hg) analytical recovery (see Table S1).

and Mn. The strongest correlations involving two non-essential elements were Al and As, Ti and As, Al and Pb, Ti and Pb, Cd and Hg, Pb and Sb, and finally As and Cd. Overall, Mg, Zn and Cu were less correlated to the other elements. Mg was weakly, but significantly negatively correlated with Na and Cu primarily, and to a lesser extent to Pb and Sb.

### 3.4. Comparison of element concentrations according to population, age and sex

When comparing average element concentrations among populations (Table S2), significant differences were found for 20 out of 22 elements. Only the concentrations of Cr and Ni were not significantly different between the five populations. Results remained similar when considering repeated measurements as well ( $n = 758$ ; see Table S4 for statistical outputs). The Dunn test allowed us to identify which populations differed from the others for each element (Figs. 3 & 4; see Table S3 for detailed statistical results).

Regarding essential elements, Co, Mo, Na and Se concentrations were the most different among populations. Some individuals, from Chizé and Jura especially, also showed very high concentrations of Cu. Zinc concentrations were significantly higher in the Jura population than in the other populations, with the exception of the Ain population. Likewise, Fe concentrations in individuals from Châteauvillain-Arc-en-Barrois were significantly higher, except when compared to the Ain population. Manganese concentrations in individuals from Trois-Fontaines were significantly higher, except when comparing to those from Châteauvillain-Arc-en-Barrois. On the other hand, individuals from Chizé and Jura populations exhibited respectively significantly lower Mo and Se concentrations. Individuals from Jura had significantly less K, and individuals from Ain and Trois-Fontaines significantly less Na, than those from other populations. Jura and Trois-Fontaines stood out with the highest number of elements exhibiting significantly different concentrations with the other populations.

In the case of non-essential elements, Hg and Tl concentrations differed the most among populations, and the highest values for those elements were found in the Jura population. The highest As and Ti concentrations were found in the Ain and Châteauvillain-Arc-en-Barrois populations. Aluminium concentrations were significantly higher in individuals from Chizé and Châteauvillain-Arc-en-Barrois when compared to Jura and Trois-Fontaines, and Sb concentrations were

significantly higher in individuals from Chizé when compared to those from Châteauvillain-Arc-en-Barrois and Trois-Fontaines. Overall, the Jura population differed the most from the others. Indeed, significant differences in concentrations were found with at least one population for all non-essential elements but Sb and Sr.

When comparing average element concentrations between age and sex categories (Table S2), significant differences were found between juveniles and adults, and between females and males, for Cu only. More specifically, juveniles ( $W = 25027$ ;  $p < 0.001$ ) and males ( $W = 29318$ ;  $p = 0.003$ ) had significantly higher Cu concentrations than adults and females, respectively.

### 3.5. Distribution of median element concentrations

An overview of the distribution of minor and trace elements in the five roe deer populations is given in Fig. 5. Element concentrations in roe deer hair were not distributed the same depending on the study site. Indeed, individuals from Châteauvillain-Arc-en-Barrois and Jura seemed to have overall a greater heterogeneity in their element distribution than the other populations. The Jura population had higher median element concentrations for Ca, Cd, Hg, Tl and Zn, but on the other hand, the medians were the lowest of all populations for Al, As, Co, Cr, Fe, K, Mn, Se and Ti. Similarly, the Châteauvillain-Arc-en-Barrois population showed the highest median concentrations for Al, As, Fe, Mo, Na, Ni, Pb and Ti but also the lowest for Hg, Mg and Sr.

## 4. Discussion

This study defines for the first time the reference values for 22 essential and non-essential elements in a wild ungulate, the roe deer, using hair as a non-invasive and easily reproducible sampling method. In addition, we also explored the influence of environmental context on element concentrations by comparing values of five populations in France.

### 4.1. Minor and trace element profiles

Overall, the literature on minor and trace element concentrations in hair is limited in ungulates, particularly in roe deer. Concerning essential elements, the concentration of Cu ( $6.34 \pm 2.31 \mu\text{g/g}$ ) and Zn ( $77.6$



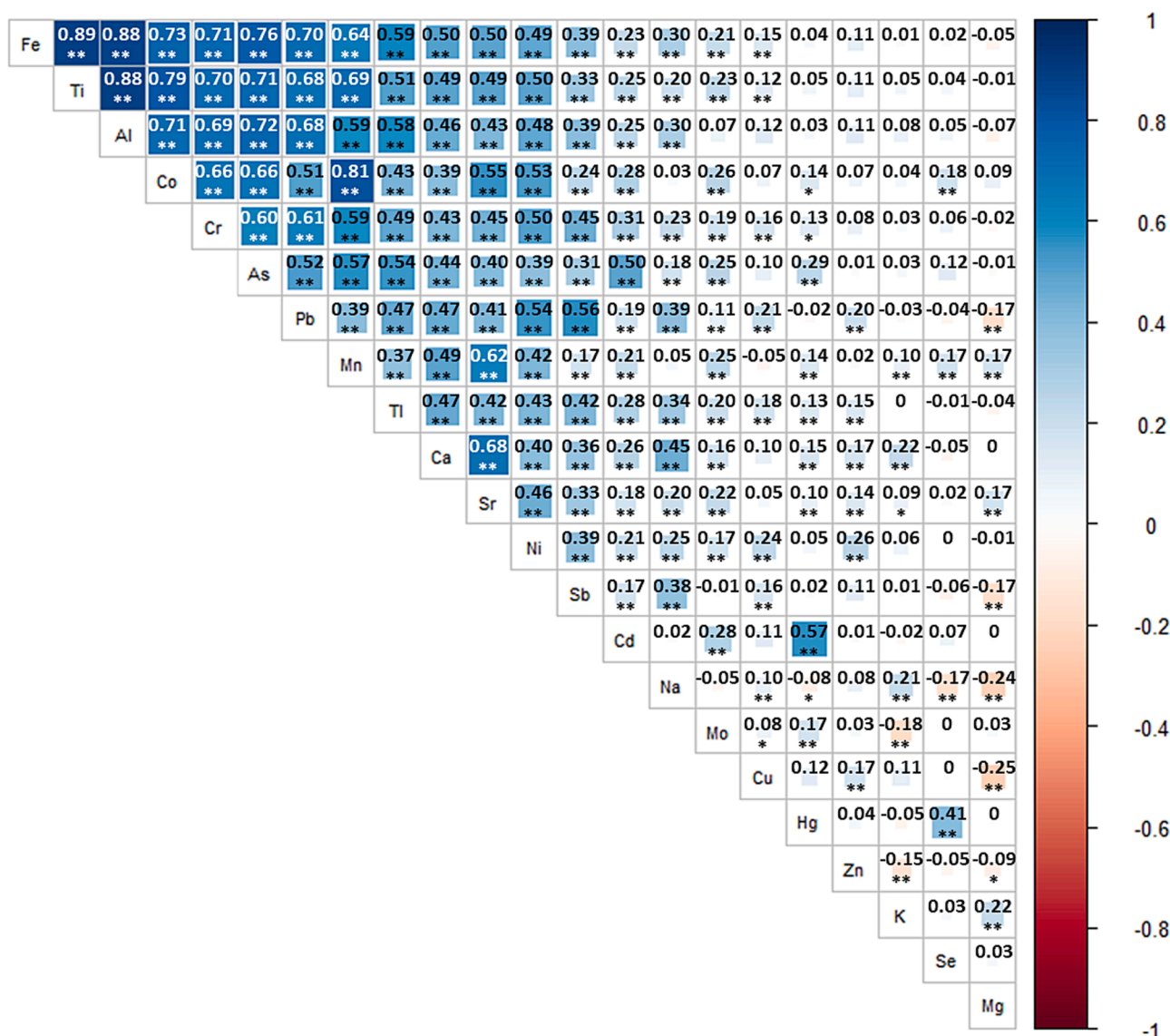
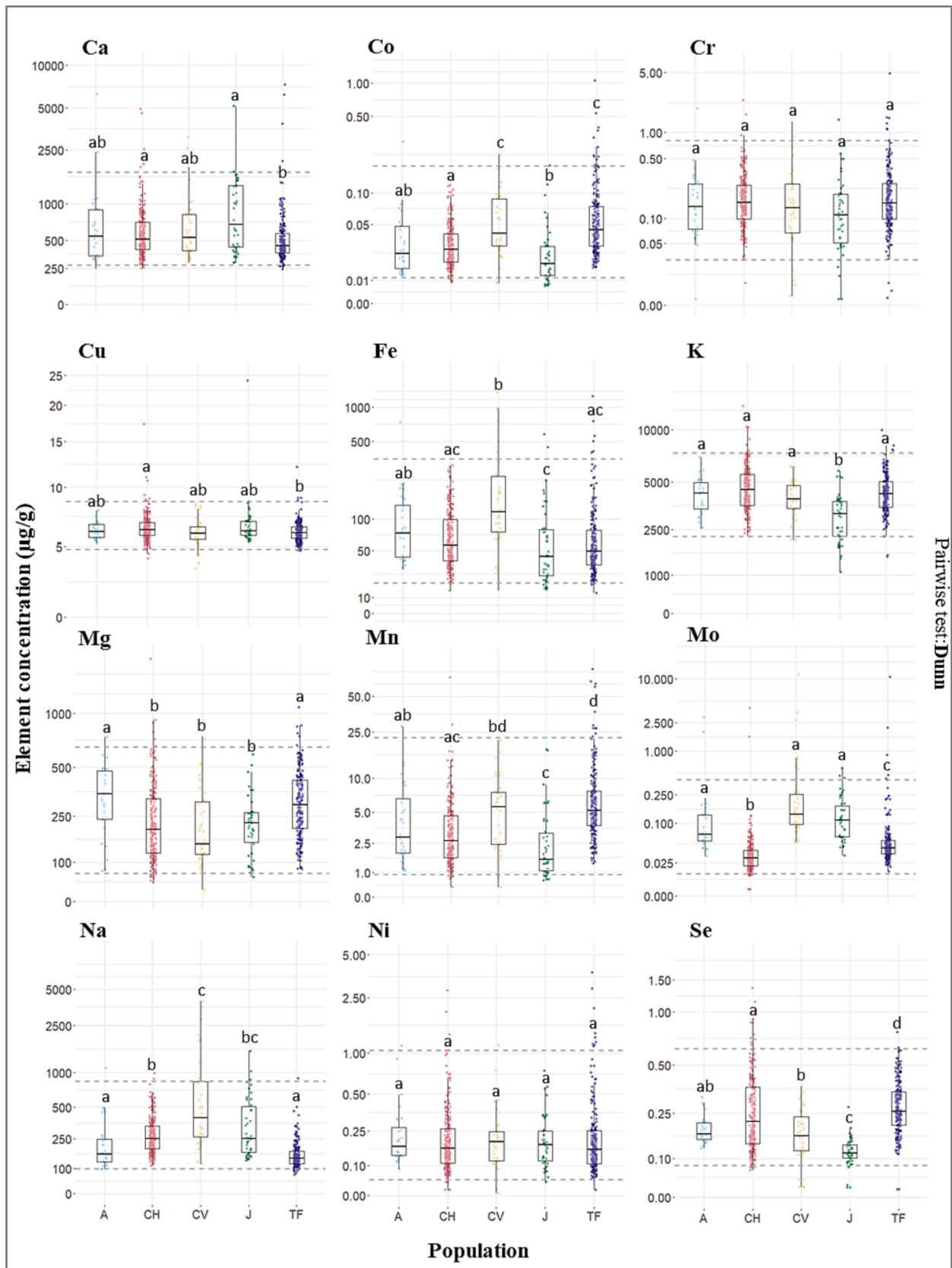


Fig. 2. Correlation plot between concentrations of minor and trace elements measured in roe deer hair. The colour of the squares indicates whether the correlation is positive (i.e. blue) or negative (i.e. red), and its size is proportional to the strength of the correlation (i.e. Spearman's rho coefficient). Correlation coefficients are specified in each square. Significant differences between elements are shown with stars (\*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

$\pm 17.1 \mu\text{g/g}$ ) measured in our study are on average two and three times lower than those found in roe deer in Poland (Cu and Zn of  $13.7 \mu\text{g/g}$  and  $240 \mu\text{g/g}$  dry mass, respectively; (Cygan-Szczegieliński and Stasiak, 2022), but were very close to those reported in roe deer in Italy (Cu:  $7.81 \pm 2.04 \mu\text{g/g}$  in the urbanized area and  $7.18 \pm 1.48 \mu\text{g/g}$  in the rural area; Zn:  $70 \pm 10 \mu\text{g/g}$  both urbanized and rural areas; Draghi et al., 2023). Mean Cr ( $0.220 \pm 0.297 \mu\text{g/g}$ ), Ni ( $0.248 \pm 0.313 \mu\text{g/g}$ ) and Se ( $0.263 \pm 0.153 \mu\text{g/g}$ ) concentrations in our study were also lower than those described in Italy (Cr:  $2.01 \pm 1.33 \mu\text{g/g}$  and  $1.23 \pm 0.43 \mu\text{g/g}$ ; Ni:  $0.68 \pm 0.58 \mu\text{g/g}$  and  $0.96 \pm 1.49 \mu\text{g/g}$ ; Se:  $1.70 \pm 1.42 \mu\text{g/g}$  and  $1.52 \pm 1.06$  for the urbanized and rural areas, respectively; Draghi et al., 2023). Mean Fe concentrations in our study ( $84.1 \pm 109 \mu\text{g/g}$ ) were found close to those from the rural area ( $90 \pm 60 \mu\text{g/g}$ ) but lower than those of the urbanized area ( $220 \pm 170 \mu\text{g/g}$ ; Draghi et al., 2023). Finally, mean Mg ( $276 \pm 173 \mu\text{g/g}$ ) and Mn ( $5.57 \pm 7.45 \mu\text{g/g}$ ) concentrations in our study were comparable to those in the urbanized area in Italy (Mg:  $260 \pm 140 \mu\text{g/g}$  and  $200 \pm 100 \mu\text{g/g}$ ; Mn:  $4.35 \pm 2.79 \mu\text{g/g}$  and  $1.91 \pm 1.05 \mu\text{g/g}$  for the urbanized and rural areas, respectively; Draghi et al., 2023). Overall, there are contrasting concentrations of minor and trace

elements between studies which may reflect local environmental differences leading to varying dietary compositions and/or bioavailability of elements.

Compared with published data, our concentration values for non-essential elements rarely meet or exceed those found in roe deer from polluted or unpolluted environments. In our study, mean concentrations of Cd and Pb ( $0.0553 \pm 0.119 \mu\text{g/g}$  and  $0.394 \pm 2.11 \mu\text{g/g}$ , respectively) measured in hair collected between October and March on roe deer living in forest environments were in the same range as those measured in hair from roe deer ( $0.06 \mu\text{g/g}$  and  $1.04 \mu\text{g/g}$  of dry mass, respectively) hunted between May and January in a moderately industrialized region in Poland (Cygan-Szczegieliński and Stasiak, 2022). However, higher Pb concentrations were found in hair of roe deer hunted between June and December in both urbanized ( $1.99 \pm 2.09 \mu\text{g/g}$ ) and rural areas ( $0.83 \pm 0.70 \mu\text{g/g}$ ) in northern Italy, while mean Cd concentrations were close to our values ( $0.10 \pm 0.22 \mu\text{g/g}$  in the urbanized area and  $0.06 \pm 0.05 \mu\text{g/g}$  in the rural area; Draghi et al., 2023). In this same study, mean Al and As concentrations in hair (Al:  $220 \pm 390 \mu\text{g/g}$  in the urbanized area and  $70 \pm 60$  in the rural area; As:  $2.26 \pm 2.05 \mu\text{g/g}$  in the urbanized area and



**Fig. 3.** Comparison of essential element concentrations measured in roe deer hair samples ( $n = 542$ ) collected between 2016 and 2021 in 5 populations in France: A = Ain ( $n = 28$ ); CH = Chizé ( $n = 240$ ); CV = Châteauvillain-Arc-en-Barrois ( $n = 37$ ); J = Jura ( $n = 42$ ); TF = Trois-Fontaines ( $n = 195$ ). Y axis was log transformed for readability purposes. Box plots show median concentrations (black solid line) within interquartile ranges. The horizontal grey dotted lines represent upper and lower reference limits, respectively. Populations sharing the same lowercase letter do not exhibit significant differences in their average concentrations ( $p > 0.05$ ).

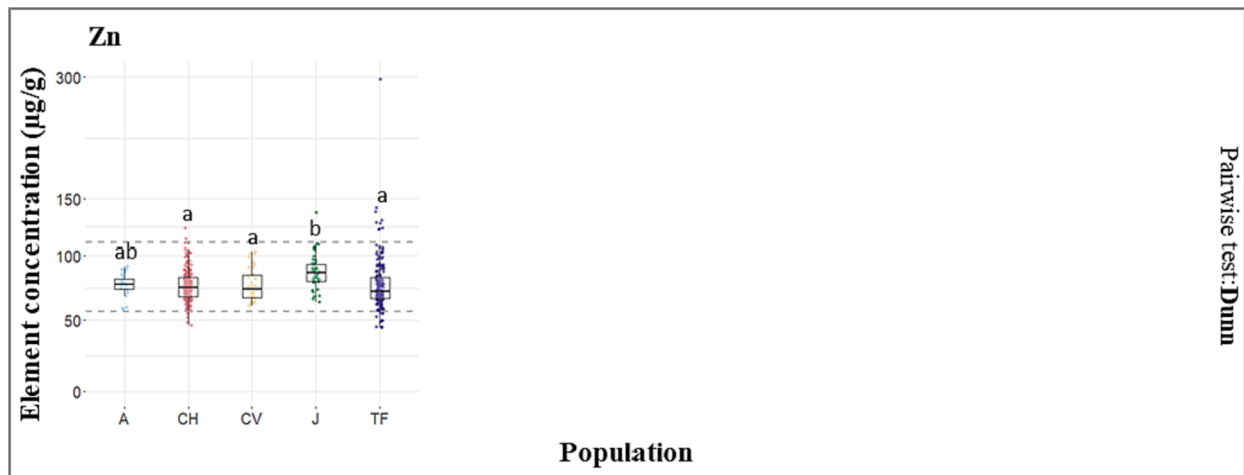


Fig. 3. (continued).

$1.69 \pm 1.24$  in the rural area; Draghi et al., 2023) were also higher than in our populations (Al:  $62.3 \pm 71.8$  µg/g; As:  $0.0639 \pm 0.106$  µg/g). Concentrations seem to be influenced by the degree of anthropization of roe deer habitats, with higher burdens in urbanized areas. However, the time of hair sampling could also partly explain these differences between studies (Pokorny and Ribarič-Lasnik, 2002). Finally, we did not find any study reporting Hg concentrations measured in roe deer hair.

When comparing to other wild ungulate species, our values were in the reported ranges for Cu, Fe, Mg, Se and Zn measured in the tail hair of free-ranging mule deer (*Odocoileus hemionus*) in California (Roug et al., 2015). Median concentrations for Cd, Co, Cu, Mo and Se of our roe deer populations were also close to those measured in the shoulder's hair of free-ranging woodland caribou (*Rangifer tarandus caribou*; Jutha et al., 2022). Fe, Mn and Pb concentrations were notably higher in our study than in caribou's hair, but median Zn concentration was higher in caribou. Compared to our data, the concentrations of Ca, Mg, Na and K measured in the hair of red deer (*Cervus elaphus*) from a moderately industrialized region in Poland were much higher. Conversely, K concentration was three times lower. Nevertheless, the concentrations of all the elements measured in red deer, with the exception of Ca, are included in our value range (Cygan-Szczegieliński et al., 2018).

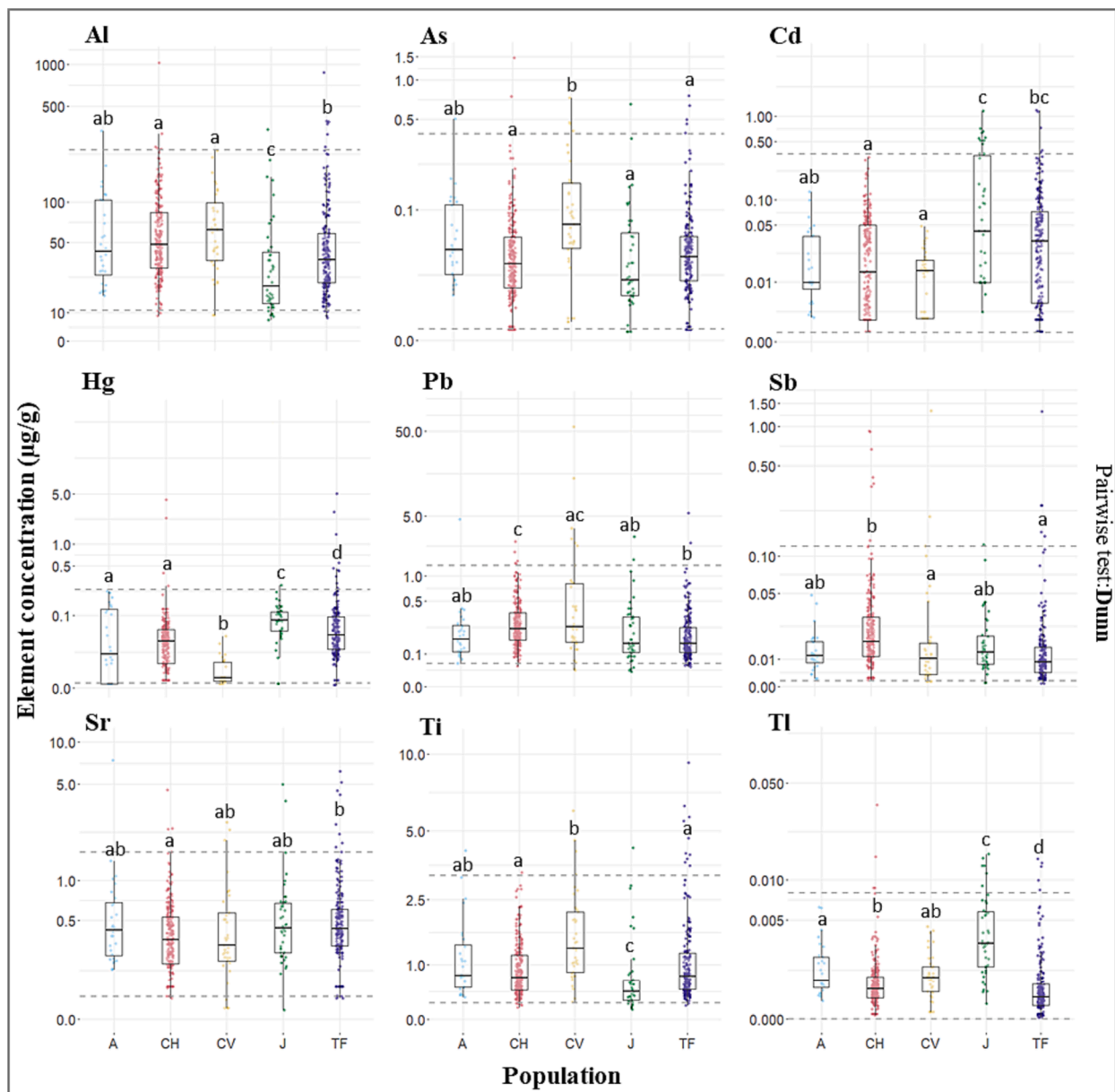
Comparing with domestic ungulate species, mean concentrations of Al, As, Hg and Ti in our roe deer populations were close to those found in sheep wool (Kołacz et al., 1999; Patkowska-Sokoła et al., 2009). However, concentrations of Ni and Sr were lower, while that of Cr were higher (Kołacz et al., 1999; Patkowska-Sokoła et al., 2009). Measurements carried out in Germany on wool from sheep grazing on contaminated soils show a mean As concentration 8 times higher than that measured in our study, and a mean Hg concentration close to that measured in our study (As:  $0.57$  µg/g of dry mass; Hg:  $0.107$  µg/g of dry mass; Gebel et al., 1996). Concentrations of Pb, Cd, Fe, Mn, Co and Ni measured in sheep wool in Egypt were also consequently higher in three out of four studies locations, and similar in one (Nageeb Rashed and Soltan, 2005). Finally, we did not find any values for Sb and Tl measured in hair of a wild or domestic ungulate species in the literature to compare our data with.

Although these studies give us some insight into roe deer's element status compared to closely related species, there is still a general lack of reference values for the concentrations of a wide range of minor and trace elements in hair, whether it concerns domestic or wild mammals. Yet, roe deer is considered as a bioindicator (Cygan-Szczegieliński and Stasiak, 2022; Draghi et al., 2023; García et al., 2011) and such data are crucial for the long-term monitoring of essential element deficiencies or non-essential element contaminations. In wild populations, most reference intervals of chemical elements are given for blood (e.g. Ratliff and Hartup, 2019; Villa et al., 2017). However, to our knowledge, there is no

study describing reference intervals in hair, although this matrix is indicative of the accumulation of elements and recognised as a suitable bioindicator for determining element status in vertebrates (Tête et al., 2014). The only reference intervals of chemical elements that have been assessed in hair are for humans and some livestock animals for specific agronomic issues (e.g. Mikulewicz et al., 2013; Miroshnikov et al., 2020). Thus, evaluating health and potential ecotoxicological issues in wild populations through element status using a non-invasive method requires a preliminary study that lays the groundwork to fill in the existing gap on reference intervals. By comparing with the literature, we found that our populations had mostly lower element concentrations, which may be attributed to the fact that they live in forested areas, far from industrial sites, qualifying them as poorly contaminated and with a low risk of deficiencies. From this observation, we were entitled to determine reference values from our data.

#### 4.2. Reference intervals for minor and trace element concentrations

Livestock studies have shown that deficiencies in essential elements often lead to the impairment of fertility, embryo viability, immune responses and an increased risk in disease development which ultimately may result in death (Hidiroglou, 1979; McClure, 2008). In our case, Cr is the element with the most suspected outliers under our lower reference limit (i.e. 15 values ranging between  $0.0303$  and  $0.809$  µg/g). The median concentration was several times lower than the one reported in Draghi et al. (2023), and our reference interval was also slightly lower than the one reported in cow hair in Miroshnikov et al., (2020;  $0.053$ – $1.121$  µg/g). Finally, when comparing to the values of essential elements determined in cows, our reference intervals remained in the same range as theirs for Co, Fe, Ni, Se and Zn, with the exception of Mn for which our reference interval was much lower than theirs ( $7.5$ – $100.1$  µg/g in Miroshnikov et al., 2020). Nonetheless, these differences may be attributed to species-related physiological characteristics, rather than to deficiencies in given elements such as Cr or Mn, for instance. Essential elements can also become toxic when their concentration exceeds a given threshold. For example, Se is a constitutive element of selenocysteines and plays a key role in redox homeostasis through the synthesis of the glutathione peroxidase, a major antioxidant enzyme (Valko et al., 2005; Kośła et al., 2019). However, taken in excess, Se causes toxic effects referred to as selenosis, which results in several clinical symptoms and lesions that can lead to death (Sun et al., 2014). Toxic concentrations of Se in livestock have been set at  $\geq 5$  µg/g in cattle hair and  $4.0$ – $6.0$  µg/g in sheep wool and pig hair (Reis et al., 2010). Although there is no threshold value for roe deer, it is worth noting that our maximum value ( $1.343$  µg/g) was below the toxic concentrations for livestock. Moreover, 9 individuals had very high Cu concentrations (the



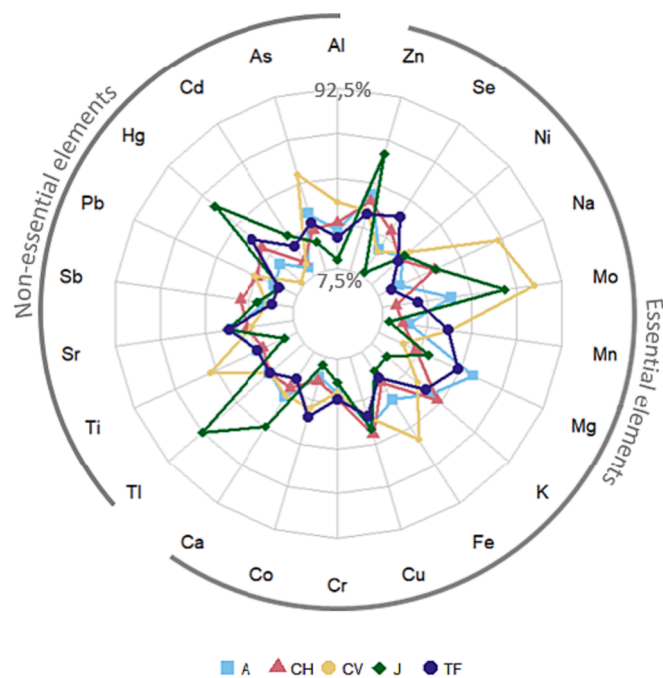
**Fig. 4.** Comparison of non-essential element concentrations measured in roe deer hair samples ( $n = 542$ ) collected between 2016 and 2021 in 5 populations in France: A = Ain ( $n = 28$ ); CH = Chizé ( $n = 240$ ); CV = Châteauvillain-Arc-en-Barrois ( $n = 37$ ); J = Jura ( $n = 42$ ); TF = Trois-Fontaines ( $n = 195$ ). Y axis was log transformed for readability purposes. Box plots show median concentrations (black solid line) within interquartile ranges. The horizontal grey dotted lines represent upper and lower reference limits, respectively. Populations sharing the same lowercase letter do not exhibit significant differences in their average concentrations ( $p > 0.05$ ).

highest one being  $60.443 \mu\text{g/g}$ ), even though our upper reference limit is  $8.642 \mu\text{g/g}$ . Copper is also needed in various metabolic pathways but liver and kidney damages may occur at high doses (Govind, 2014). However, an individual exceeding our upper limit will not necessarily indicate a toxicity, since toxicological data remains missing for roe deer, and wildlife in general.

Non-essential elements can have detrimental effects on all living forms, even at low doses of exposure. Toxic doses for acute and chronic exposure of non-essential elements are well described in livestock (Reis et al., 2010) and some commonly reported toxic effects of these elements include brain damage, kidney damage and reproductive impairments (Tchounwou et al., 2012; Govind, 2014). Our upper reference limit for Pb (i.e.  $1.32 \mu\text{g/g}$ ) was below the median concentrations found in roe deer hair in other studies (Cygan-Szczegiłniak and Stasiak, 2022; Draghi et al., 2023). However, the median concentration from the

Italian urbanized area and the maximal concentrations in both urbanized and rural areas did exceed our reference value (Median Pb:  $1.81 \mu\text{g/g}$ , Maximum Pb:  $9.42 \mu\text{g/g}$  in the urbanized area and  $2.25 \mu\text{g/g}$  in the rural area; Draghi et al., 2023). Out of a total of 542 individuals in our populations, two individuals from Châteauvillain-Arc-en-Barrois exhibited Pb concentrations above the upper limit (i.e.  $55.70$  and  $13.82 \mu\text{g/g}$ , respectively), which indicates that some roe deer may be subjected to local contamination in this area, potentially linked to the site's industrial history. This is not surprising as roe deer have a high tolerance to human activities (Linnell and Andersen, 1995; Bonnot et al., 2013) and are therefore likely to be exposed to various anthropogenic contaminants. When considering other non-essential elements, some individuals were found above our upper reference limits (e.g. maximum values for Al:  $1020$ ; As:  $1.48$ ; Hg:  $26.1$ ; Sb:  $1.32$ ; Sr:  $7.38$ ; Ti:  $9.27$ ; Tl:  $0.0347 \mu\text{g/g}$ ). When comparing with other studies, median





**Fig. 5.** Comparison of median element concentrations ( $\mu\text{g/g}$ , dry weight) in roe deer hair ( $n = 758$ ) from 5 populations in France: A = Ain ( $n = 28$ ); CH = Chizé ( $n = 363$ ); CV = Châteauvillain-Arc-en-Barrois ( $n = 37$ ); J = Jura ( $n = 42$ ); TF = Trois-Fontaines ( $n = 288$ ). For readability purposes, axes of the radar chart represent upper and lower reference values at 85 % coverage instead of 95 %.

concentrations for Al and Cd reported in the study of Italian roe deer were under our upper reference limit, whereas median concentration of As was far above, which was suggested as being due to a specific soil composition that can release higher burdens of this element (Draghi et al., 2023). Finally, to our knowledge, the single study that determined reference values in the hair of an ungulate (apart from humans) focused on cows (Miroshnikov et al., 2020). The reference limits are similar to ours for As (0.040–0.304  $\mu\text{g/g}$ ), lower for Cd (0.013–0.098  $\mu\text{g/g}$ ), Hg (0.002–0.042  $\mu\text{g/g}$ ) and Pb (0.096–0.681  $\mu\text{g/g}$ ) and much higher for Sr (3.4–29.1  $\mu\text{g/g}$ ), i.e. these elements being common to both our studies.

#### 4.3. Correlations between elements

Overall, the observed correlations were mostly positive and the few negative ones almost exclusively included Mg. The positive correlations between several non-essential elements strongly suggest their joint presence in the environment.

The literature is scarce and the few studies that investigated correlations between elements in hair of wild species often found correlations opposite to our results. Significant negative correlations between Cu-Pb ( $r_{xy} = -0.369$ ), Cd-Zn ( $r_{xy} = -0.531$ ) and Cu-Zn ( $r_{xy} = -0.409$ ) were found in the hair of roe deer in Poland (Cygan-Szczegielniak and Stasiak, 2022). A negative correlation was also found between Cu-Zn in the hair of red deer (Cygan-Szczegielniak et al., 2018; Cygan-Szczegielniak, 2021). This antagonism could be explained by the inhibition of Cu's intestinal absorption by Zn (Sandstead, 1995). However, a significant positive correlation between Cu and Zn, as in our study, has been found in the muscles and kidneys of wild boars (Gasparik et al., 2012), suggesting that relative bioavailability of Cu and Zn may influence the degree of their interaction.

Such contrasted results can stem from environmental differences in terms of element concentrations. Indeed, even if different locations exhibit the same mixture of elements, the local bioavailability patterns will depend on the soil geochemistry and the bioaccumulation rate in plants (Sach et al., 2020; Squadrone et al., 2020).

#### 4.4. Minor and trace element concentration differences according to environmental context and individual characteristics

Analysis of the concentrations of chemical elements in hair also provides information on the quality of the environment animals inhabit (Tataruch and Kierdorf, 2003). Minor and trace elements present in roe deer hair can be assimilated through various ways, for instance with the deposition of atmospheric particles that can cross the dermal barrier or be directly inhaled. These particles can also deposit in water, soil, mushrooms and plants, and be absorbed through feeding (French et al., 2017; Engwa et al., 2019). The circulation of elements through environment and food webs is, however, complex (Bañuelos and Ajwa, 1999). Their concentration in water, soil, rocks, fungus and plants can be modified by natural processes including the activity of microorganisms, temperature, pH-related adsorption-desorption (Chen et al., 2022), or by human activities such as industrial and agricultural practices (Bañuelos and Ajwa, 1999; Kabata-Pendias, 2000). Thus, in our study case, comparing element status of different populations evolving in the same geological background (i.e. sedimentary rock) but different environmental conditions in terms of climate, forage quantity/quality, soil composition and nearby anthropic activities and their accessibility (i.e. open versus closed study sites; see Table 1) could highlight the effect of the ecological context (McDowell, 1996). Although the five study populations occur in forested habitats, they effectively differ markedly in their element profiles, with only two elements (i.e. Cr and Ni) for which there was no significant differences.

The Jura population had the most different concentrations compared to the other populations, especially with the lowest concentrations for 9 elements (Al, As, Co, Cr, Fe, K, Mn, Se, Ti) and the highest ones for 5 elements (Ca, Cd, Hg, Tl, Zn) (Figs. 3, 4 & 5). This population differs from the others through various habitat characteristics, including altitude, snow frequency and forest stand. Located at higher altitude (500–1100 m) than the other populations except the Ain one, the presence of snow is notably more important and lasts longer during winter than in any other study sites. Moreover, this is the only territory where the forest is partly composed of conifers, while the other ones are mainly composed of deciduous trees. Heat loss in conifers can also be significantly lower than in bare hardwoods, which could modify roe deer activity rate and habitat use (and by extension their diet composition), as suggested in red squirrels (Wauters et al., 1992). Hardwood stands are also known to have on average higher aboveground biomass and higher leaf litter production than conifer stands (Park et al., 2008), which could result in contrasted soil composition and element contents. Thus, the particular environmental conditions of the Jura forest could influence roe deer element intake through different processes, and explain the marked contrast in the minor and trace element profiles of this population compared to the other ones.

The populations from Jura and Châteauvillain-Arc-en-Barrois seemed to have overall larger amplitudes in their minor and trace element concentrations than the other populations (Fig. 5), which may be due to greater environmental heterogeneity and/or small sample sizes in these study sites. Alongside with Jura, Châteauvillain-Arc-en-Barrois showed many elements for which the concentrations were on average higher or lower than in the other populations. It had the highest concentration of some non-essential elements (Al, As, Pb, Ti), an effect that may be attributed to nearby agricultural lands where roe deer can venture (unlike Trois-Fontaines and Chizé which are enclosed forests). Indeed, roe deer of Châteauvillain-Arc-en-Barrois live in an open lowland forest where agricultural activity is very present (Table 1). Arsenic and Pb are known to be associated with agricultural practices, with high concentrations found in pesticides, sewage, sludge and manure (Kabata-Pendias and Szeke, 2015). Roe deer that frequent open environments such as meadows or fields are more likely to be exposed to non-essential element pollution, whether from agriculture, road traffic or nearby industry. In future studies, it would therefore be interesting to link our data with local data related to the study sites (e.g. resource quality, soil



composition) and more specifically anthropogenic activities associated with agricultural and industrial practices.

On the other hand, Châteauvillain-Arc-en-Barrois and Ain populations were the most similar in terms of minor and trace element concentrations (i.e. only four elements with significantly different concentrations; see Table S3). This was somehow counter-intuitive, as Châteauvillain-Arc-en-Barrois is a lowland forest environment, whereas Ain is a mountainous one, with different soil types. The most obvious similarity between these two study sites is that the forests are mainly composed of oak and beech. This suggests that the forest stand may be an important factor driving the element status in roe deer. Another explanation could also be that the small number of samples in these two populations ( $n_A$ : 28,  $n_{CV}$ : 37) prevented us from detecting potential differences.

Finally, Chizé and Trois-Fontaines populations were the most dissimilar (i.e. 15 elements with significantly different concentrations), which may be explained by a great influence of the environmental context. While Trois-Fontaines is characterized by a continental climate with mild winters and hot but not dry summers, Chizé is exposed to an oceanic climate with mild winters, hot dry summers and frequent summer droughts (Gaudry et al., 2018). Meteorological conditions can modify forest structure and dynamics, and can in turn modulate habitat use by roe deer (Widmer et al., 2004; Gaudry et al., 2018). Climate can also influence concentrations of chemical elements in soil through its effect on the deposition of atmospheric particles (Jenny, 1994). The dominant trees present in these two forests are substantially the same (i.e. oak, beech, hornbeam), with only more maples in Chizé. Nevertheless, differences in wood productivity and soil structure can be observed between sites. Trois-Fontaines forest is highly productive and overall homogeneous, while forest productivity is lower in Chizé (Pettorelli et al., 2006). The difference in soil type is also marked between Trois-Fontaines (i.e. deep, fertile, calcareous) and Chizé (i.e. shallow, stony and calcareous). A soil too rich in limestone, for example, induces Fe deficiencies by retaining it, which could explain lower concentrations of Fe in Trois-Fontaines (Chen and Barak, 1982).

Thus, the observed differences between populations seem to be attributable to environmental differences likely leading to contrasted bioavailability of minor and trace elements. Studies that consider simultaneously element concentrations, environmental variables, habitat use and/or diet of wild animals are scarce, partly due to logistical constraints. Yet, understanding the links between individual element status and these factors is essential to assess the overall health of natural populations.

Chemical element status in hair may also be influenced by individual characteristics such as age and sex. Indeed, a study assessing several element concentrations in hair of roe deer showed significantly higher concentrations of Cr and Mg in older animals ( $\geq 36$  months old) compared to younger ones, and significantly higher concentrations of Cd, Cr, Cu, Fe and Mg in females than in males (Draghi et al., 2023). A review on cows also reported higher concentrations of Ca, Cu, K, Na and Zn in calves, and higher Mn and Fe concentrations in adults. In our study however, we did not find evidence for age nor for sex-specific differences in element concentrations, except for Cu. Our results are congruent with those of another study, which also revealed no age-related differences in Mg and Zn concentrations in cattle hair (Combs et al., 1982). Nevertheless, potential age- and sex-related differences in element concentrations should not be discarded at this stage, since proper identification of these effects might require specific investigation (i.e. using data from known-age individuals rather than from individuals assigned to reductive age categories).

## 5. Conclusion

Hair sampling represents an excellent non-invasive way of monitoring minor and trace element concentrations in wildlife populations where ethical and logistical contexts are constraining (Zemanova,

2020). This matrix is relevant for comparing cumulative levels, since hair remains metabolically inactive and chemically stable, unlike blood or urine, which reflect circulating levels. Using this matrix, we established the first reference intervals for essential and non-essential elements in roe deer. Although we studied populations living in forests only, we highlighted the importance of the environmental context on the element status of individuals. In future studies, it would be interesting to compare these results with data from roe deer populations inhabiting agricultural and urbanized areas, while ensuring the same sampling parameters (i.e. hair sampling location and season), or to add reference values for other contaminants such as pesticides, which have been shown to affect wildlife as well (Fritsch et al., 2022).

This work highlights the importance of wildlife studies in assessing ecosystem health, as wild animals may be considered sentinels for the environment, but also for domestic animals and even humans. Indeed, they may provide essential information on potential negative impacts of chemical and biological contaminants (Patz et al., 2004; Rabinowitz et al., 2010; Bossart, 2011). An elegant perspective to our study would be to investigate the extent to which concentrations of essential and non-essential elements outside established reference limits affect blood biochemical parameters and health indicators. On a population scale, the question also arises as to the effect of these element concentrations on population dynamics, for conservation purposes and risk assessment (Morrissey et al., 2023).

## CRedit authorship contribution statement

**Amandine Herrada:** Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing. **Léa Bariod:** Conceptualization, Writing – original draft, Writing – review & editing. **Sonia Saïd:** Conceptualization, Data curation, Funding acquisition, Project administration, Supervision, Writing – review & editing. **Benjamin Rey:** Conceptualization, Data curation, Supervision, Writing – review & editing. **Hervé Bidault:** Project administration. **Yoann Bollet:** Methodology. **Stéphane Chabot:** Project administration. **François Débias:** Methodology. **Jeanne Duhayer:** Methodology. **Sylvia Pardonnet:** Methodology. **Maryline Pellerin:** Data curation, Project administration. **Jean-Baptiste Fanjul:** Project administration. **Cyril Rousset:** Project administration. **Clémentine Fritsch:** Methodology, Writing – review & editing. **Nadia Crini:** Methodology, Writing – review & editing. **Renaud Scheifler:** Methodology, Writing – review & editing. **Gilles Bourgoïn:** Conceptualization, Data curation, Funding acquisition, Supervision, Writing – review & editing. **Pauline Vuarin:** Conceptualization, Data curation, Funding acquisition, Supervision, Writing – review & editing.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The authors affiliated with VetAgro Sup, LBBE, and OFB played key roles in study design, data collection and analysis, and manuscript writing.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ecolind.2024.111720>.

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