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Jan Demeško

Zróżnicowanie ekotypów sarny (*Capreolus capreolus*): badanie porównawcze zawartości pierwiastków śladowych w tkankach twardych i plastyczności fenotypowej grubości szkliwa

Ecotypes variation in the European roe deer (*Capreolus capreolus*):
a comparative study in trace element content of hard tissues and phenotypic plasticity thickness of enamel

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Instytutu Ekologii i Ochrony Środowiska

Promotor:

- prof. dr hab. Janusz Markowski

Promotor pomocniczy:

- dr hab. Piotr Minias Prof. UŁ

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SPIS TREŚCI

1. STRESZCZENIE

1.1. Wstęp

Począwszy od XVIII w., tj. od czasu rewolucji przemysłowej, człowiek staje się coraz bardziej istotnym czynnikiem zmian zachodzących w środowisku Ziemi. Przejawem działalności człowieka były i są między innymi konsekwencje związane ze zmianą w użytkowaniu ziemi. W wyniku tej działalności kosztem środowisk naturalnych powstały rozległe i ciągle powiększające się obszary przemysłowe, zurbanizowane i rolnicze. Tak ukształtowane krajobrazy są źródłem skażeń substancjami toksycznymi przez przemysł: wydobywczy, metalurgiczny, chemiczny, spalanie na wielką skalę węgla, gazów i paliw płynnych, rolnictwo (nawozy mineralne, środki ochrony roślin) oraz ładunki zanieczyszczeń powstających przy przemysłowym chowie zwierząt, itp. (Pacyna i Pacyna 2001; Pacyna i in. 2007; Norgate i in. 2007; Garcia i in. 2011; Chauhan i in. 2012). Zanieczyszczenia przemysłowe o charakterze gazowym i pyłowym zwłaszcza te drugie, zawierające metale ciężkie, na skutek emisji do atmosfery stają się źródłem skażenia gleb, wody i żywych organizmów (Markert 1993; Kabata-Pendias i Pendias 2001; Palczewska-Komsa i in. 2016). Podwyższona zawartość tych pierwiastków w środowisku kumuluje się w tkankach roślin, a następnie są one włączane do łańcucha pokarmowego obejmującego zwierzęta oraz ludzi (Bowen 1979; Mankovska 1980; Holm i Wester 1988; Pokorny i in. 2004) i mogą stać przyczyną problemów zdrowotnych (np. Fraga 2005; Czeczot i Skrzycki 2015).

Ponieważ sarna europejska *Capreolus capreolus* spełnia szereg kryteriów organizmu wskaźnikowego, takich jak: szeroki zasięg geograficzny, stosunkowo małe terytoria (16–80 ha) (Jeppesen 1990; Pandini i Cesaris 1997), osiadłość - zwłaszcza samic (Ellenberg 1978 i Kurt 1991), ograniczoną strategię żerowania, dostępność podstawowych danych populacyjnych, oraz prostą procedurę pobierania próbek, gatunek ten jest powszechnie uznawany za dobry (np. Grodzinska i in. 1983, Tataruch 1991, Findo i in. 1993) lub nawet doskonały (Wren 1986) bioindykator skażeń środowiska metalami ciężkimi oraz innymi pierwiastkami śladowymi. W monitoringu tego rodzaju wykorzystuje się zarówno tkanki twarde ciała, takie jak: zęby (np. Zaccaroni i in. 2008; Sobota i in. 2011), poroże (np. (Sawicka-Kapusta 1979; Tataruch 1995; Kierdorf oraz Kierdorf 2003; Pokorny 2006; Pokorny i in. 2009; Jabłońska i in. 2016), włosy (Kucharczak i in. 2003, 2004, 2006), odchody (Babińska-Werka i Czarnowska 1988; Pokorny i in. 2004a, b), krew (Baroni i in. 2000; Žele i Vengušt 2012; Humann-Ziehank i in. 2008) oraz tkanki miękkie (np. Pokorny 2000; Pokorny i Ribarič-Lasnik 2002; Baloš i in. 2015; Durkalec i in. 2015; Lehel i in. 2016).

Dotychczasowe badania wskazują, że pierwiastki śladowe, w tym metale ciężkie, kumulowane są w tkankach organizmów z różną szybkością (Ericson i in. 1991; Komarnicki 2000; Garcia i in. 2011; Kubaszewski i in. 2014), a tempo ich akumulacji może zależeńć od płci i wieku zwierząt (Kierdorf i in. 1989; Gasparik i in. 2004; Rudy 2010; Garcia i in. 2011; Jarzyńska i Falandysz 2011; Lanocha i in. 2012).

Chociaż pierwotnymi siedliskami saren były różnorodne typy lasów, to gatunek ten przystosował się do życia w otwartym krajobrazie rolniczym na znacznych obszarach Europy (Pielowski 1984; Ellenberg 1978). Przeprowadzone badania populacji zamieszkujących tak odmienne siedliska pozwoliły na odnotowanie między nimi różnic w organizacji populacji oraz behawiorze, co pozwoliło na wyodrębnienie ekotypu „leśnego" i „polnego" (Pielowski 1984). Te dwa ekotypy wykazują różnice w rozmiarach i masie ciała, ekologii i fizjologii (Zejda i Homolka 1980; Fruziński i in. 1982; Majewska i in. 1982; Kałuziński 1982; Pielowski i Bresiński 1982; Zejda i Bauerova 1985; Hofmann i in. 1988; Pėtelis i Brazaitis 2003; Flis 2011). Ekotypy sarny leśnej i polnej różnią się także preferencjami żywieniowymi (Tixier i Duncan 1996). Dieta ekotypu polnego oparta jest przede wszystkim na roślinach uprawnych (pszenica, żyto, jęczmień, kukurydza, owies, gryka, warzywa), które stanowią 66% całkowitej masy ich paszy. Dieta sarny leśnej zawiera więcej twardszych fragmentów roślin, w tym drzew (grab zwyczajny, dąb szypułkowy, brzoza, klon zwyczajny, osika pospolita, świerk), krzewów i krzewinek (trzmielina, leszczyna pospolita, jeżyna, jagoda czernica), które stanowią około 11–13% całkowitego spożycia pokarmu i aż do 25% pokarmu spożywanego jesienią i zimą (Gębczyńska 1980). Wykazano również, że sarna leśna chętnie uzupełnia swoją dietę w sezonie jesienno-zimowym innymi twardymi pokarmami, takimi jak żołędzie (Krasnov i in. 2015). Sarny żyjące w otwartym krajobrazie, gdzie warunki klimatyczne są surowsze i bardziej zmienne, a znalezienia odpowiedniego schronienia jest ograniczona, są większe i cięższe w porównaniu do osobników ekotypu leśnego (Fruziński i in. 1982, Narauskaite i Petelis 2010). Z kolei badania kraniometryczne są mniej jednoznaczne, z jednej strony Petelis i Brazaitis (2003) oraz Kulak i Wajdzik (2009) wskazali na znaczne różnice w wymiarach czaszki między ekotypami, z drugiej strony inne badania takich różnic nie potwierdziły (Sabalinkiene i in. 2017).

Warto zauważyć, że badacze wskazujący na znaczne różnice w wymiarach czaszki między ekotypami wykazali je przede wszystkim w obrębie aparatu gnatostomatycznego (długość górnego i dolnego rzędu zębów oraz długość żuchwy). Uzębienie u ssaków jest morfologicznie i funkcjonalnie dostosowane do diety i zachowań żywieniowych. Zęby sarny składają się z trzech tkanek: szkliwa, zębiny i cementu. Szkliwo jest warstwą bezkomórkową

złożoną z mocno zmineralizowanej ciasno upakowanej masy kryształów hydroksyapatytu, która tworzy zewnętrzną krystaliczną powierzchnię zębów i jest najtwardszą tkanką zęba (Winkler i Kaiser 2015). Można zatem oczekiwać różnic między ekotypami sarny w cechach związanych z cechami morfologii zębów, takimi jak grubość szkliwa, która jest cechą pewolucyjnie lastyczną i zdolną do szybkiej adaptacji w odpowiedzi na zmianę diety (Hlusko 2004).

1.2. Podsumowanie publikacji

Niniejsza rozprawa doktorska składa się z cyklu trzech artykułów dotyczących zagadnień związanych z zawartością wybranych pierwiastków śladowych w różnych tkankach twardych sarny europejskiej, a także porównania zmienności pomiędzy różnymi ekotypami zamieszkującymi teren Polski środkowej i okolic Wilna na Litwie. Przy każdej pozycji podano wartość współczynnika Impact Factor oraz punktację Ministerstwa Nauki i Szkolnictwa Wyższego aktualne dla roku publikacji.

W skład rozprawy doktorskiej wchodzą następujące artykuły:

1. Jan Demesko, Janusz Markowski, Mirosława Słaba, Janusz Hejduk, Piotr Minias. 2017. Age Related Patterns in Trace Element Content Vary Between Bone and Teeth of the European Roe Deer (*Capreolus capreolus*). Archives of Environmental Contamination and Toxicology 74(2): 330-338. (IF 2017 = 2,497, punkty MNiSW = 25, lista A).

Zwierzęta łowne, takie jak sarna, są często wykorzystywane jako bioindykatory skażenia środowiska. Jak dotąd większość badań ekotoksykologicznych na zwierzętach kopytnych koncentrowała się na zawartości pierwiastków śladowych w tkankach miękkich i porożu, natomiast są jedynie fragmentaryczne informacje na temat tego, czy i w jaki sposób stężenia pierwiastków śladowych zmieniają się w zależności od wieku i jak kształtują się wzorce akumulacji dla różnych rodzajów tkanek. Celem tego badania było określenie stężenia siedmiu metali śladowych (baru, miedzi, żelaza, ołowiu, manganu, strontu, cynku) i fluoru w kościach i zębach sarny oraz ustalenie, czy wykazują one zmienność w zależności od wieku. Do badań posłużyły stałe zęby trzonowe i fragmenty kości żuchwy od 130 samic sarny pozyskanych w latach 2009-2015 w centralnej Polsce. Wiek osobnika był ustalony według starcia zębów. Przygotowane próbki tkanek twardych (zęby i fragmenty kości) przepłukiwano w wodzie dejonizowanej, a następnie suszono w piecu w temperaturze 70°C przez 48 godzin.,

po czym zmineralizowano je w grafitowym bloku mineralizującym. Następnie zawartość pierwiastków mierzono za pomocą spektrofotometru absorpcji atomowej. Średnie stężenia pięciu metali śladowych (miedź, ołów, mangan, stront i cynk) wykazały statystycznie istotne różnice między tkanką kostną z zębami. W szczególności stężenia miedzi i ołowiu były wyższe w tkance kostnej, podczas gdy stężenia manganu, strontu i cynku były wyższe w zębach. W pięciu z siedmiu pierwiastków śladowych (bar, żelazo, mangan, stront, cynk) wykazano istotną zależność od wieku badanych osobników. Różnice w bioakumulacji elementów śladowych związanych z wiekiem pomiędzy tkanką kostną a zębami można najprawdopodobniej wytłumaczyć znacznie szybszemu obrotowi materii w tkance kości w porównaniu ze strukturą zęba.

2. Jan Demesko, Janusz Markowski, Eva Demesko, Mirosława Słaba, Janusz Hejduk, Piotr Minias. 2018. Ecotype Variation in Trace Element Content of Hard Tissues in the European Roe Deer (*Capreolus capreolus*). Archives of Environmental Contamination and Toxicology 76(1): 76-86. (IF 2018 = 2,497, punkty MNiSW = 25, lista A).

Zwierzęta żyjące w siedliskach antropogenicznych ponoszą wiele kosztów, które są bezpośrednio lub pośrednio związane z działalnością człowieka. Należy do nich między innymi podwyższone narażenie na zanieczyszczenie środowiska, które może mieć negatywne konsekwencje dla kondycji i przeżywalności osobników. Celem tego badania było przetestowanie różnic w zawartości pierwiastków śladowych między ekotypem polnym a leśnym sarny europejskiej. Aby oszacować poziom ogólnego zanieczyszczenia środowiska w każdym siedlisku, zmierzyliśmy zawartość metali śladowych we czterech gatunkach roślin, będących ważnym składnikiem diety sarny. Stężenia trzech pierwiastków (miedzi, żelaza i ołowiu) były istotnie lub niemal istotnie powiązane z czynnikiem lesistości u wszystkich czterech badanych gatunków roślin, gdzie rośliny z obszarów o niższej lesistości (związanych z występowaniem ekotypu polnego sarny) wykazywały wyższe stężenia ww. pierwiastków. Podobny, negatywny związek między lesistością a stężeniem wykazywały również bar i cynk, ale jedynie u niektórych z analizowanych gatunków roślin. Analiza pierwiastków śladowych u saren przy uwzględnieniu czynników: ekotyp, wiek oraz typ próby (kość vs. zęby), wykazała, że stężenia pięciu pierwiastków (czterech metali: miedzi, żelaza, ołowiu, strontu oraz fluoru) pozostawały w istotnej statystycznie zależności z ekotypem i były wyższe u saren ekotypu polnego, zarówno w próbkach tkanki kostnej, jak i zębów. Te wyniki wskazują na ścisłe powiązanie zawartości elementów śladowych w tkankach saren z zawartością wybranych

metali w spożywanym przez nie pokarmie, a sarny ekotypu polnego cechują się wyższą koncentracją pierwiastków śladowych w stosunku do saren ekotypu leśnego.

3. Jan Demesko, Marta Kurek, Patrycja Podlaszczuk, Janusz Markowski. 2020. Enamel thickness differs between field and forest European roe deer *Capreolus capreolus*. Polish Journal of Ecology 68(1):100-107. doi.org/10.3161/15052249PJE2020.68.1.009. (IF 2018 = 0,590, punkty MNiSW = 40, lista A).

Niektóre cechy morfologiczne zębów, takie jak grubość szkliwa, uznawane są za cechy ewolucyjnie plastyczne, zdolną do szybkiej adaptacji w odpowiedzi na zmianę diety. Ponieważ uzębienie ssaków jest morfologicznie i funkcjonalnie dostosowane do diety oraz zachowań żywieniowych, w niniejszej pracy podjęto próbę przetestowania wpływu bytowania w siedliskach leśnych i polnych na grubość szkliwa u sarny. Szkliwo to warstwa złożona z mocno zmineralizowanej ciasno upakowanej masy kryształów hydroksyapatytu, która tworzy zewnętrzną krystaliczną powierzchnię zębów i jest najtwardszą tkanką zębową. Stąd uznaje się, że stanowi ono strukturalne wzmocnienie zębów, chroniące przed zużyciem i rozprzestrzenianiem się pęknięć. Dieta ekotypu polnego sarny oparta jest przede wszystkim na roślinach uprawnych, które stanowią 66% całkowitej masy ich pokarmu, natomiast dieta sarny leśnej zawiera więcej twardszych fragmentów roślin, w tym drzew. W obecnej pracy wykazaliśmy, że osobniki ekotypu polnego miały istotnie niższe średnie wartości grubości szkliwa (0,19 mm ± 0,063 [SE]) w porównaniu do osobników ekotypu leśnego (0,21 mm ± 0,052 [SE]). Analizę zróżnicowania wiekowego przeprowadzono dla dwóch kategorii – osobników młodych (od 2 do 4 roku życia) oraz starych (5 lat i powyżej 5 roku życia). Osobniki młode wykazywały tendencję do posiadania grubszego szkliwa niż osobniki stare. Cieńsza warstwa szkliwa korony trzeciego dolnego trzonowca u sarny ekotypu polnego może być wyjaśniana z jednej strony adaptacją do diety realizowanej w środowisku polnym, a z drugiej strony efektem stresu na jaki są narażone ze względu na mniejszą możliwość ukrycia się. Częsta ekspozycja na stres może znacznie zakłócić wydzielanie ameloblastów, a tym samym wpłynąć na grubość szkliwa

1.3. Introduction

Since the industrial revolution of the eighteenth century, man has exerted a growing influence on the changes occurring in the Earth's environment. Human activity is most clearly reflected in widespread changes in land use, characterized by the extensive and constant

growth of industrial, urbanized and agricultural areas at the expense of natural environments. Landscapes shaped in this way are a source of contamination by toxic substances produced by a range of industries, including those associated with mining, metallurgy and chemical production, as well as the large-scale combustion of coal, gases and liquid fuels; heavy pollution loads are also exerted by the agriculture sector in the form of mineral fertilizers and plant protection products, and by industrial animal husbandry (Pacyna & Pacyna 2001; Pacyna et al. 2007; Norgate et al. 2007; Garcia et al. 2011; Chauhan et al. 2012). Further contamination of soil, water and living organisms also occurs through the emission of industrial gas and dust pollutants, the latter containing heavy metals (Markert 1993; Kabata-Pendias & Pendias 2001; Palczewska-Komsa et al. 2016). These elements accumulate in plant tissues due to their increased content in the environment, and then enter the food chain including animals and humans (Bowen 1979; Mankovska 1980; Holm & Wester 1988; Pokorny et al. 2004) and may cause health problems (e.g. Fraga 2005; Czeczot and Skrzycki 2015).

As the European roe deer (*Capreolus capreolus*) fulfils a number of the criteria required for an indicator organism: their wide geographical range, relatively small territories of only 16-80 ha (Jeppesen 1990; Pandini and Cesaris 1997), the sedentary habits of females (Ellenberg 1978; Kurt 1991), limited feeding strategy, availability of basic population data and a simple sampling procedure. The species is widely recognized as a good (see: Grodzinska et al. 1983; Tataruch 1991; Findo et al. 1993) or even an excellent (Wren 1986) load indicator of heavy metals and other trace elements in the environment. Such pollutant monitoring is typically performed on hard body tissues such as teeth.

Research to date indicates that trace elements, such as heavy metals, accumulate in the tissues of organisms at different rates (Ericson et al. 1991; Komarnicki 2000; Garcia et al. 2011; Kubaszewski et al. 2014). This rate also depends on the sex and age of the animals (Kierdorf et al. 1989; Gasparik et al. 2004; Rudy 2010; Garcia et al. 2011; Jarzyńska & Falandysz 2011; Lanocha et al. 2012).

Although roe deer originally inhabited various types of forests, the species gradually adapted over time to life in the open agricultural landscape of large areas of Europe (Pielowski 1984; Ellenberg 1978). Differences have since been identified in the organization and behavior of the populations inhabiting such different habitats, and these have allowed the "forest" and "field" ecotypes to be distinguished (Pielowski 1984). These two ecotypes show differences in body size, weight, ecology and physiology (Zejda & Homolka 1980; Fruziński et al. 1982), as well as in their food preferences (Tixier & Duncan 1996). The field ecotype

diet is based on crops (wheat, rye, barley, corn, oats, buckwheat, vegetables), which constitute 66% of the total weight of their feed; in contrast, the forest roe deer consume harder plant fragments, including trees (European hornbeam, European oak, birch, Norway maple, aspen, spruce), shrubs and bushes (common spindle, hazel, blackberry, blueberry); these harder fragments constitute about 11– 13% of total food consumption, rising to 25% in autumn and winter (Gębczyńska 1980). It has also been shown that in the autumn and winter season, deer willingly supplement their diet with other hard foods such as acorns (Krasnov et al. 2015). The field-type roe deer living in an open landscape, where climatic conditions are more severe and variable, and lacking shelter, are larger and heavier than those of the forest ecotype (Fruziński et al. 1982, Narauskaite & Petelis, 2010).

Interestingly, the findings of craniometric studies are more contradictory: Petelis and Brazaitis (2003) and Kulak and Wajdzik (2009) note significant differences in skull size between ecotypes, while Sabalinkiene et al. (2017) report no such difference.

It is noteworthy that the studies indicating such significant differences in skull dimensions were based on measurements of the gnatostomatical apparatus, such as the lengths of the upper and lower rows of teeth, and the length of the jaw. Roe deer teeth consist of three tissues: enamel, dentin and cement. The hardest of these, enamel, is a cell-free layer composed of a highly mineralized, tightly packed mass of hydroxyapatite crystals that serves as the outer crystalline surface of the tooth (Winkler & Kaiser 2015). As the teeth of mammals are morphologically and functionally adapted to diet and feeding behavior, it would be reasonable for tooth-related features, such as enamel thickness, which is an evolutionary plastic feature and capable of rapid adaptation in response to a change in diet, to differ between the two roe deer ecotypes (Hlusko 2004).

1.4. Articles synopsis

This doctoral dissertation consists of a series of three articles on issues related to the content of selected trace elements in various hard tissues of European roe deer, as well as analyses of the variability between different ecotypes living in central Poland and the vicinity of Vilnius in Lithuania. For each item, the Impact Factor value and the Ministry of Science and Higher Education scores are current for the year of publication.

The doctoral dissertation consists of the following articles:

1. Jan Demesko, Janusz Markowski, Mirosława Słaba, Janusz Hejduk, Piotr Minias.

2017. Age Related Patterns in Trace Element Content Vary Between Bone and Teeth of the European Roe Deer (Capreolus capreolus). Archives of Environmental Contamination and Toxicology. 74 (2): 330-338. (IF 2017 = 2.497, MNiSW points = 25, list A)

Game animals such as roe deer (*Capreolus capreolus*) are used as bioindicators of environmental contamination. So far, most ecotoxicological studies on ungulates have focused on the content of trace elements in soft tissues and antlers, but little information exists about whether, and how, the concentrations of trace elements vary according to age and tissue type. The purpose of this study was to evaluate the concentrations of seven trace metals, *viz.* barium, copper, iron, lead, manganese, strontium, zinc and fluoride, in bone and tooth tissue samples from deer and to determine whether they show significant relation to age. The analysis was performed on the permanent molars and fragments of mandibular bones of 130 female roe deer obtained by regular hunting in 2009-2015 in central Poland. The age of the individuals was determined by tooth wear.

Briefly, the hard tissue samples, i.e. the tooth and bone fragments, were rinsed in deionized water and then dried in an oven at 70°C for 48 hours. They were then mineralized in a graphite mineralization block and their content was analyzed using an atomic absorption spectrophotometer. The mean values of five trace metal concentrations, *viz.* copper, lead, manganese, strontium and zinc, differed significantly between the sampled bone tissue and teeth: the copper and lead concentrations were higher in bone tissue, while manganese, strontium and zinc concentrations were higher in teeth. In addition, five of the seven studied trace elements (barium, iron, manganese, strontium, zinc) demonstrated a significant relationship with the age of the tested individuals. Such age-related differences in trace element levels between bone and teeth can most likely be explained by the much faster rotation of matter in bone tissue compared to teeth.

2. Jan Demesko, Janusz Markowski, Eva Demesko, Mirosława Słaba, Janusz Hejduk, Piotr Minias. 2018. Ecotype Variation in Trace Element Content of Hard Tissues in the European Roe Deer (Capreolus capreolus). Archives of Environmental Contamination and Toxicology. 76(1):76-86. (IF 2018 = 2,497, punkty MNiSW = 25, lista A).

Animals living in anthropogenic habitats bear a lot of the costs that are directly or indirectly related to human activities, including increased exposure to environmental pollution; such exposure can have negative consequences for free-living populations. The

purpose of this study was to examine the differences in trace element content between the field and forest ecotype of the European roe deer. To estimate the level of overall environmental pollution in each habitat, the study determined the content of trace metals in four plant species constituting an important component of the roe deer diet. The concentrations of three elements (copper, iron and lead) were significantly or almost significantly related to the forest cover factor in all four tested plant species: they were found at higher concentrations in plants from areas with lower forest cover, i.e. those associated with the occurrence of the roe deer ecotype. A similar, negative relationship between forest cover and concentration was also observed for barium and zinc, but only in some of the analyzed plant species.

A further analysis was performed of the concentration of trace elements with regard to the ecotype and age of deer, and the tissue type of the sample (bone vs. teeth). It was found that the levels of five elements, *viz.* the four metals copper, iron, lead and strontium, and the non-metal fluorine, were significantly higher in the bone and tooth samples from the field ecotype than those of the forest ecotype. These results indicate a close relationship between the content of trace elements in roe deer tissues and the content of selected metals in the food they eat. Roe deer living in the field are characterized by higher concentrations of trace elements those inhabiting forests.

3. Jan Demesko, Marta Kurek, Patrycja Podlaszczuk, Janusz Markowski. 2020. Enamel thickness differs between field and forest European roe deer *Capreolus capreolus*. (IF 2018/2019 = 0,590, punkty MNiSW = 40, lista A).

Some morphological features of the teeth, such as enamel thickness, are considered evolutionary plastic features capable of rapid adaptation in response to a change in the diet. As mammalian teeth are morphologically and functionally adapted to diet and feeding behavior, the present study examined the impact of living in forest and field habitats on the thickness of roe deer tooth enamel. The enamel is a layer consisting of a highly-mineralized, tightly-packed mass of hydroxyapatite crystals, which creates the outer crystalline surface of the teeth and is the hardest dental tissue. Therefore, it is considered to strengthen the structure of the teeth, protecting against wear and propagation of cracks. The diet of the field ecotype of roe deer is primarily based on crop plants, which constitute 66% of the total mass of their food; in contrast, roe deer tend to consume harder plant fragments, including trees.

Our findings indicate that field ecotype individuals showed significantly lower average enamel thickness values (0.19 mm ± 0.63 SE) compared to those in the forest ecotype (0.21 mm ± 0.52 SE). The analysis of age diversity was carried out based on two categories: young deer, i.e. those aged from two to four years, and old ones, i.e. those aged five years and over. Young individuals tended to have thicker enamel than the older ones. The presence of a thinner enamel crown on the third lower molar in a roe deer field ecotype can be explained on the one hand by adaptation to a diet implemented in a field environment, and on the other hand by the stress effect they are exposed to due to less concealment. More frequent exposure to stress can significantly disrupt the secretion of ameloblasts, and thus affect the thickness of the enamel.

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2. Wnioski/ Conclusions

1. Sarny ekotypu polnego miały wyższe średnie stężenia czterech pierwiastków śladowych: miedzi, żelaza, ołowiu, strontu i fluoru w porównaniu z sarną leśną. Różnice te były zgodne ze zróżnicowaniem stężeń metali ciężkich w środowisku (w obwodach łowieckich o różnym stopniu lesistości), co zostało ocenione na drodze analizy ich poziomu w organach czterech gatunków roślin będących ważnym elementem diety sarny.
2. Badania ujawniły różnice we wzorcach bioakumulacji pierwiastków śladowych między tkankami twardymi u sarny. Stężenia baru, magnezu, cynku i fluoru w zębach rosło wraz z wiekiem, podczas gdy nie odnotowano takich trendów w przypadku tkanki kostnej.
3. Wykazana istotnie statystycznie cieńsza warstwa szkliwa korony trzeciego dolnego trzonowca u sarny ekotypu polnego może być wyjaśniana z jednej strony adaptacją do diety realizowanej w środowisku polnym, a z drugiej strony efektem stresu na jaki są narażone ze względu na mniejszą możliwość ukrycia się. Częsta ekspozycja na stres może znacznie zakłócić wydzielanie ameloblastów, a tym samym wpłynąć na grubość szkliwa.
4. Badania wskazują, że tkanki twarde: kostna i zęby sarny można wykorzystać jako wiarygodny wskaźnik zanieczyszczenia środowiska.
5. Można oczekiwać, że dalszy wzrost zanieczyszczeń środowisk antropogenicznych może mieć negatywne konsekwencje dla populacji zwierząt łownych, a także dla konsumentów dziczyzny.
6. Nasze badanie potwierdza również potrzebę uwzględnienia czynnika jakim jest wiek w badaniach ekotoksykologicznych dziko żyjących zwierząt.

1. The field-ecotype roe deer display higher mean concentrations of four trace elements, *viz.* copper, iron, lead, strontium and fluorine, compared to those of a forest ecotype. These differences were consistent with the level of their concentration in the environment, in hunting districts, which was determined by analyzing their levels in the organs of four plant species.
2. Differences in bioaccumulation patterns of trace elements were observed between hard tissues in roe deer. The concentrations of barium, magnesium, zinc and fluorine increased with age in teeth; however, no such trends were found in bone tissue.
3. The layer of the enamel crown of the third lower molar was found to be significantly thinner in field ecotype deer; this can be explained, on the one hand, by adaptation to a diet implemented in a field environment, and on the other hand, by the stress effect they experience due the lack of hiding spaces. Frequent exposure to stress can significantly disrupt the secretion of ameloblasts, and thus affect the thickness of the enamel.
4. Our research indicates that hard tissues of roe deer, e.g. bone and tooth tissue, can be used as important indicators of environmental pollution.
5. It can be expected that a further increase in pollution by anthropogenic environments may have negative consequences for the wild game animal population, as well as for venison consumers.
6. Our study also confirms the need to take age into account as an important factor in ecotoxicological studies of free-living animals.

3. Publikacje wchodzące w skład rozprawy doktorskiej

3.1. Jan Demesko, Janusz Markowski, Mirosława Słaba, Janusz Hejduk, Piotr Minias. 2017. Age Related Patterns in Trace Element Content Vary Between Bone and Teeth of the European Roe Deer (*Capreolus capreolus*). Archives of Environmental Contamination and Toxicology 74(2): 330-338. DOI: 10.1007/s00244-017-0470-1

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Age-Related Patterns in Trace Element Content Vary Between Bone and Teeth of the European Roe Deer (*Capreolus capreolus*)

Jan Demesko[1] · Janusz Markowski[1] · Mirosława Słaba[2] · Janusz Hejduk[1] · Piotr Minias[1]

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Abstract Game animals, such as the roe deer (*Capreolus capreolus*), have long been used as bioindicators of environmental contamination. Most ecotoxicological research on ungulates has focused on trace element content in soft tissues and antlers. Also, only fragmentary information exists about whether and how trace element concentrations vary with the age of wild-living animals and whether these age-related patterns are similar for different types of tissues. The purpose of this study was to measure concentrations of seven trace metals (barium, copper, iron, lead, manganese, strontium, zinc) and fluoride in bone and teeth of roe deer and to determine whether significant variation is evident with individual age. For this purpose, we collected permanent molars and fragments of mandible bone from more than 130 female roe deer in Central Poland. We found that concentrations of four trace elements (barium, manganese, zinc, and fluoride) in teeth of deer showed positive linear relationships with individual age. No such trends were recorded for trace element content in bone. We suggest that these striking differences in age-related patterns of trace element bioaccumulation between bone and permanent teeth of roe deer might be explained by higher turnover rate and constant remodelling of bone tissue. The results suggest that analysis of permanent teeth may be useful for assessing throughout-life intoxication by environmental pollution in the roe deer and possibly in other mammal species. Our study reinforces the need to carefully account for age-related variation in ecotoxicological research on wild-living animals.

Technological developments and changes that occur in the modern world have a significant impact on natural environment. It is widely acknowledged that urbanization constitutes one of the most important threats to wildlife and its biodiversity in the present day (McKinney 2008). Expanding urban landscape, industrialization, and modern agriculture practices are all associated with growing emission of gaseous and particulate matter to the environment (Pacyna and Pacyna 2001; Pacyna et al. 2007; Norgate et al. 2007; Garcia et al. 2011). Contamination from burned fuel and other sources gets to the air, water, and plants (Markert 1993; Kabata-Pendias and Pendias 2001; Palczewska-Komsa et al. 2016). Increasing concentrations of heavy metals in the environment often leave a detectable trace in human and animal organisms (Bowen 1979; Maňkovská 1980; Holm and Wester 1988; Pokorny et al. 2004a, b).

Game animals, such as the European roe deer (*Capreolus capreolus*), have long been used as indicators of environmental contamination (Sawicka-Kapusta 1979; Maňkovská et al. 2012). Roe deer are found in high abundance across almost all of Europe and the Middle East. Roe deer show large behavioural plasticity and can occupy wide range of habitats, including those strongly affected by human activities (Burbaitė and Csanyi 2009). Despite broad geographical distribution, individual roe deer typically have small home ranges of 16–80 ha (Jeppesen 1990; Pandini and Cesaris 1997). Thus, it

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✉ Jan Demesko
jan.demesko@biol.uni.lodz.pl

[1] Department of Biodiversity Studies and Bioeducation, Faculty of Biology and Environmental Protection, University of Łódź, Banacha 1/3, 90-237 Łódź, Poland

[2] Department of Industrial Microbiology and Biotechnology, Faculty of Biology and Environmental Protection, University of Łódź, Banacha 12/16, 90-237 Łódź, Poland

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seems likely that concentrations of trace elements in roe deer tissues could reflect concentrations of pollutants in the local environment (Kucharczak et al. 2006). However, different trace elements might be deposited in different tissues at a varying rate (Ericson et al. 1991; Komarnicki 2000; Garcia et al. 2011; Kubaszewski et al. 2014). Also, there might be large age-related variation in the trace element content in different types of tissues (Kierdorf et al. 1989; Gasparik et al. 2004; Bilandžić et al. 2009; Rudy 2010; Garcia et al. 2011; Jarzyńska and Falandysz 2011; Lanocha et al. 2012).

Ecotoxicological studies of roe deer have mostly focused on heavy metal concentrations in blood (Baroni et al. 2000; Žele and Vengušt 2012; Humann-Ziehank et al. 2008) and soft tissues, such as liver, kidney, and muscle (Pokorny 2000; Pokorny and Ribarič-Lasnik 2002; Baloš et al. 2015; Durkalec et al. 2015; Lehel et al. 2016). This is understandable, because venison is allowed for consumption within the European Union (EU) and must comply with the EU standards (Ziembińska and Krasnowska 2007; Lehel et al. 2016). Trace element concentration was also studied in deer hair (Kucharczak et al. 2003, 2004, 2006) and feces (Babińska-Werka and Czarnowska 1988; Pokorny et al. 2004a, b). Finally, extensive ecotoxicological research has been performed on deer antlers (Sawicka-Kapusta 1979; Tataruch 1995; Kierdorf and Kierdorf 2000, 2001, 2002, 2003; Pokorny 2006; Pokorny et al. 2009; Jabłońska et al. 2016), possibly due to wide availability of hunting trophies. Other hard tissues, such as skeletal bones and teeth, have been much less researched (Maňkovská 1980; Zaccaroni et al. 2008; Sobota et al. 2011; Maňkovská et al. 2012). It remains unknown whether concentrations of trace elements in tissues change over the life cycle of roe deer and whether these age-related trends, if present, are similar for different types of tissues. We believe that such knowledge is crucial to draw reliable conclusions about the exposure of individuals or populations to environmental pollution while investigating concentrations of trace elements in animal tissues.

The purpose of this study was to measure concentrations of seven trace metals (barium, copper, iron, lead, manganese, strontium, zinc) and fluoride in skeletal bone and teeth of roe deer and to determine if they show significant variation with individual age. For this purpose, we collected permanent molars and fragments of mandible bone from more than 130 female roe deer in Central Poland. Although we had no quantitative data on environmental pollution in our sampling area, the trace elements chosen for this study are all toxic and potentially toxic, and they are among those in greatest commercial use or emission, likely exerting ecotoxicological effects on humans and wildlife (Wong et al. 2006).

Materials and Methods

Study Area

All samples were collected in Łódź voivodship, Central Poland. In terms of physiography, Łódź voivodship is situated on the border of two major units: the Central European Lowland and the Polish Highlands (Kondracki 2002). The entire study area has the lowest share of forests (21.1%) in the country. The rate of urbanization is 63.8%, and the share of agricultural area is 60.4%. Samples were collected in seven Game Breeding Centres (GBCs): Brzeziny (51°45′ N, 19°43′ E), Kolumna (51°34′ N, 19°13′ E), Kutno (52°14′ N, 19°08′ E), Poddębice (51°54′ N, 18°53′ E), Smardzewice (51°26′ N, 19°60′ E), Spała (51°31′ N, 20°11′ E), and Wieluń (51°11′ N, 18°44′ E), all managed by the Regional Directorate of State Forest in Łódź. All GBCs were located relatively close (25–100 km) to a large urban centre, Łódź (51°46′ N, 19°28′ E; 293 km^2, 708,500 inhabitants).

Sample Collection and Processing

Female roe deer were culled during regular hunting period from 30 September to 15 January in accordance with local hunting plans and regulations. A total sample consisted of 132 female skulls obtained in 2009–2014. Skull preparation followed standard procedures: boiling in water for 2–2.5 h, cleaning from soft tissues, rinsing in clean water, bleaching in oxidized water, and air drying for 24 h. Age of sampled specimens was evaluated based on dental wear (Przybylski 2008) by the members of the Regional Commission for Hunting Evaluation in Łódź. Tooth wear forms the mechanistic basis of senescence in ungulates (Gaillard et al. 1993) and have been frequently used for age determination in many cervid species (Brown and Chapman 1991; Ericsson and Wallin 2001; Høye 2006). Although tooth wear in roe deer has been reported to show some interpopulation variation due to differences in diet and habitat (Hewison et al. 1999), our samples were collected within small geographical area, which was characterized by relatively uniform environmental conditions. Age of roe deer in our sample varied between 2 and 12 years. For the purpose of analyses, animals were grouped into four age classes: (1) 2 years old ($n = 49$), (2) 3–4 years old ($n = 35$), (3) 5–6 years old ($n = 27$), and (4) > 6 years old ($n = 19$). The third permanent molar and a small fragment of mandible bone were collected from each skull and used in further analyses. The teeth were usually collected from the left side, but in a few cases teeth from the right side were extracted, because those on the left side were mechanically damaged, missing, or exhibited pathological alterations.

Measurements of Trace Metal and Fluoride Concentrations

All teeth and bone fragments were rinsed in deionized water to remove externally absorbed elements. The samples used for the measurements of metal concentrations were dried in an oven at 70 °C for 48 h and then weighed to the nearest 0.01 g. Average mass of dried samples was 0.71 ± 0.02 [SE] g and 0.48 ± 0.01 [SE] g for teeth and bone, respectively. Dried samples were dissolved in the proportion of 1:15 in 65% nitric acid, kept in 20 °C for 24 h, and then digested at 105 °C for another 24 h using a graphite digestion block (DigiPREP Mini, SCP Science, Quebec, Canada). After digestion, all samples were diluted with deionized water to the total volume of 30 mL and stored in polypropylene metal-free vials at 20 °C until analysis.

Concentrations of barium, copper, iron, lead, manganese, strontium, and zinc were measured in the samples using atomic absorption spectrophotometer SpectrAA 300A AAS, GTA-96 graphite tube atomizer, and programmable sample dispenser (Varian Techtron, Melbourne, Australia). The analyses were performed in the Laboratory of Computer and Analytic Techniques, Faculty of Biology and Environmental Protection, University of Łódź. For copper, iron, lead, manganese, and zinc, we used certified reference material (ERM-186 pig kidney) from the Institute for Reference Materials and Measurements (Geel, Belgium). For strontium, we used Strontium Standard for AAS (TraceCERT®, 1000 mg/L Sr in nitric acid), and for barium, we used Barium Standard for AAS (TraceCERT®, 1000 mg/L Ba in nitric acid) to verify the quality and accuracy of the analyses. Recovery rates for the certified reference materials were within an acceptable margin.

Measurements of fluoride concentration followed methodology recommended by Campus et al. (2007). First, we powdered 1.2 g of each tooth and bone sample in a ball mill Mixer Mill MM 400 (Retsch, Germany) with zirconium oxide beads (frequency 25 Hz, time 60 s). Powdered samples were transferred to a volumetric flask, dissolved in 8 mL of 37% HCl solution, and then diluted with deionized water to the total volume of 10 mL. A 5-mL aliquot of the above solution was transferred to another volumetric flask, diluted 1:1 with deionized water, neutralized with a 6 M NaOH solution to pH 4.5, and diluted with deionized water to the total volume of 25 mL. Sample solution was diluted with TISAB (1:1) and fluoride concentration was measured with ion-selective fluoride electrode (Hydromet S.C., Gliwice, Poland). All trace element concentrations were expressed in mg per kg dry mass.

Statistical Analyses

Before analyses, we identified outliers using criteria of > 4 SD. Outlier analyses were conducted separately for teeth and bone samples. Between one and three outliers were identified in 10 of 16 analysed measurements, whereas 6 measurements showed no outliers (Fig. S1 in the Electronic Supplementary Material). All outliers were removed from the dataset. After outlier removal, measurements with high (> 1) skewness (copper, iron, lead, manganese, and fluoride) were log-transformed to improve normality.

The effects of age and sample type (teeth vs. bone) on trace metal and fluoride concentrations were analysed with general linear mixed models (GLMMs). Because teeth and bone samples were collected from the same individuals, we included individual identity as a random factor to avoid pseudoreplication (Hurlbert 1984). The effect of year also was included as a random factor to control for interannual variation in the collected measurements. Age and sample type were included as fixed factors. To test whether age-related differences were similar for both sample types, we entered age-sample interaction in each model. GLMM models were fitted using the restricted maximum likelihood (REML) method. With this approach, denominator degrees of freedom are calculated by using a Satterthwaite approximation, which can result in fractional degrees of freedom (Satterthwaite 1946). The results of full models were reported. For measurements that showed significant age-related variation, we used contrast analysis (following recommendations by Ruxton and Beauchamp 2008) to test for an a priori hypothesis of linear increase or decrease with age. We also used Tukey post hoc comparisons to test for pairwise differences between age classes. All statistical analyses were conducted in JMP 12.1.0 (SAS Institute Inc., Cary, NC).

Results

We found that mean concentrations of five trace metals (copper, lead, manganese, strontium, and zinc) differed significantly between teeth and bone (Tables 1, 2). Specifically, concentrations of copper and lead were higher in bone, while concentrations of manganese, strontium, and zinc were higher in teeth (Tables 1, 2). No differences between teeth and bone were recorded in the concentration of barium, iron, and fluoride (Tables 1, 2).

Significant age variation was found in the concentrations of five of seven trace metals (barium, iron, manganese, strontium, and zinc), although in most cases the effect of age depended on the sample type (Table 2). Concentration of only one trace metal, iron, varied with age irrespectively of sample type. Concentration of iron in both teeth and

Table 1 Mean (±SE) concentrations of seven trace metals and fluoride in bone (mandible) and teeth (third permanent molar) in roe deer from Central Poland

Trace element	Bone		Teeth	
	Mean ± SE	*n*	Mean ± SE	*n*
Barium	209.25 ± 6.12	131	200.74 ± 6.39	132
Copper	5.74 ± 0.40	130	5.28 ± 0.27	130
Iron	21.71 ± 0.61	130	20.68 ± 0.49	132
Lead	0.62 ± 0.04	131	0.51 ± 0.04	130
Manganese	6.33 ± 0.18	132	82.78 ± 6.70	131
Strontium	89.09 ± 1.98	132	92.03 ± 2.29	132
Zinc	94.52 ± 1.19	130	107.49 ± 2.10	132
Fluoride	4.82 ± 0.45	129	3.81 ± 0.38	130

All concentrations are given in mg per kg dry mass

bone showed a decreasing linear trend with age (contrast analysis: $F_{1,122.5} = 8.99$, $p = 0.003$), but the only significant difference was between youngest individuals (2 years old) and older age classes (Tukey: all $p < 0.05$; Fig. 1c). The effect of age on all other trace metal concentrations depended on the type of sample, as indicated by significant age-sample interactions (Table 2). In all of these cases, we recorded no age-related variation in trace metal concentrations in bone (barium: $F_{3,126.3} = 0.99$, $p = 0.40$; manganese: $F_{3,124.9} = 1.56$, $p = 0.20$; strontium: $F_{3,126.4} = 1.16$, $p = 0.33$; zinc: $F_{3,122.3} = 0.70$, $p = 0.55$; Fig. 1). In contrast, teeth samples showed significant age-related variation in the concentrations of barium ($F_{3,125.3} = 4.75$, $p = 0.004$; Fig. 1a), manganese ($F_{3,125.4} = 4.85$, $p = 0.003$; Fig. 1e), and zinc ($F_{3,126.2} = 23.31$, $p < 0.001$; Fig. 1g), whereas no age variation was found for strontium concentration ($F_{3,126.9} = 1.03$, $p = 0.38$; Fig. 1f). Concentrations of barium, manganese, and zinc in teeth showed significant linear increase with age, as indicated by the contrast analysis (barium: $F_{1,126.2} = 8.78$, $p = 0.004$; manganese: $F_{1,125.4} = 7.70$, $p = 0.006$; zinc: $F_{1,126.2} = 57.35$, $p < 0.001$). An interaction between age and sample type for fluoride concentration approached significance (Table 2). Analysis of fluoride concentrations separately for the two types of samples revealed no age-related differences in bone ($F_{1,122} = 0.97$, $p = 0.33$) and a significant increase with age in teeth (contrast analysis: $F_{1,120.4} = 13.40$, $p < 0.001$; Fig. 1h). No age-related differences were recorded for copper and lead (Table 2; Fig. 1b, d).

Discussion

Our study provided strong evidence for age-related variation in the concentrations of several trace elements in permanent teeth of roe deer from Central Poland. Concentrations of three trace metals (barium, manganese, and zinc) in teeth of deer showed positive linear relationships with animal's age, indicating that some elements can accumulate in tooth structure throughout life. A similar trend was observed for fluoride concentration in teeth. In contrast, none of trace elements in bone showed an age-related increase in concentration, suggesting that bone and teeth show different patterns of trace element accumulation.

Differences in age-related patterns of bioaccumulation between bone and teeth can be most likely attributed to much higher turnover rate of bone when compared with tooth structure (Malara et al. 2016). Bone tissue, mainly formed from carbonated hydroxyapatite, is remodelled throughout entire animal life and its microelements can be transported to other tissues or excreted from organism. Specifically, bone can serve as a metal reservoir, because trace metals accumulated in this tissue are released into the bloodstream during its reconstruction. We are not aware of any quantitative data on bone turnover rate in the roe deer. However, it is estimated that more than 10% of total human bone tissue is remodelled each year, making an adult skeleton completely

Table 2 Effects of age and sample type (bone vs. tooth) on the concentrations of seven trace metals and fluoride

Factor	Barium		Copper		Iron		Lead	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Age	7.70	0.065	0.68	0.57	**6.01**	**< 0.001**	1.23	0.30
Sample type	0.44	0.51	**5.16**	**0.025**	0.89	0.35	**15.46**	**< 0.001**
Age*Sample type	**10.19**	**< 0.001**	0.37	0.77	0.73	0.53	0.69	0.56
	Manganese		Strontium		Zinc		Fluoride	
	F	*p*	*W*	*p*	*F*	*p*	*F*	*p*
Age	**2.99**	**0.033**	0.77	0.51	**13.63**	**< 0.001**	**4.31**	**0.006**
Sample type	**1105.3**	**< 0.001**	**7.35**	**0.008**	**97.41**	**< 0.001**	2.80	0.097
Age*Sample type	**4.74**	**0.004**	**2.77**	**0.045**	**20.87**	**< 0.001**	2.25	0.086

Significant terms are marked in bold

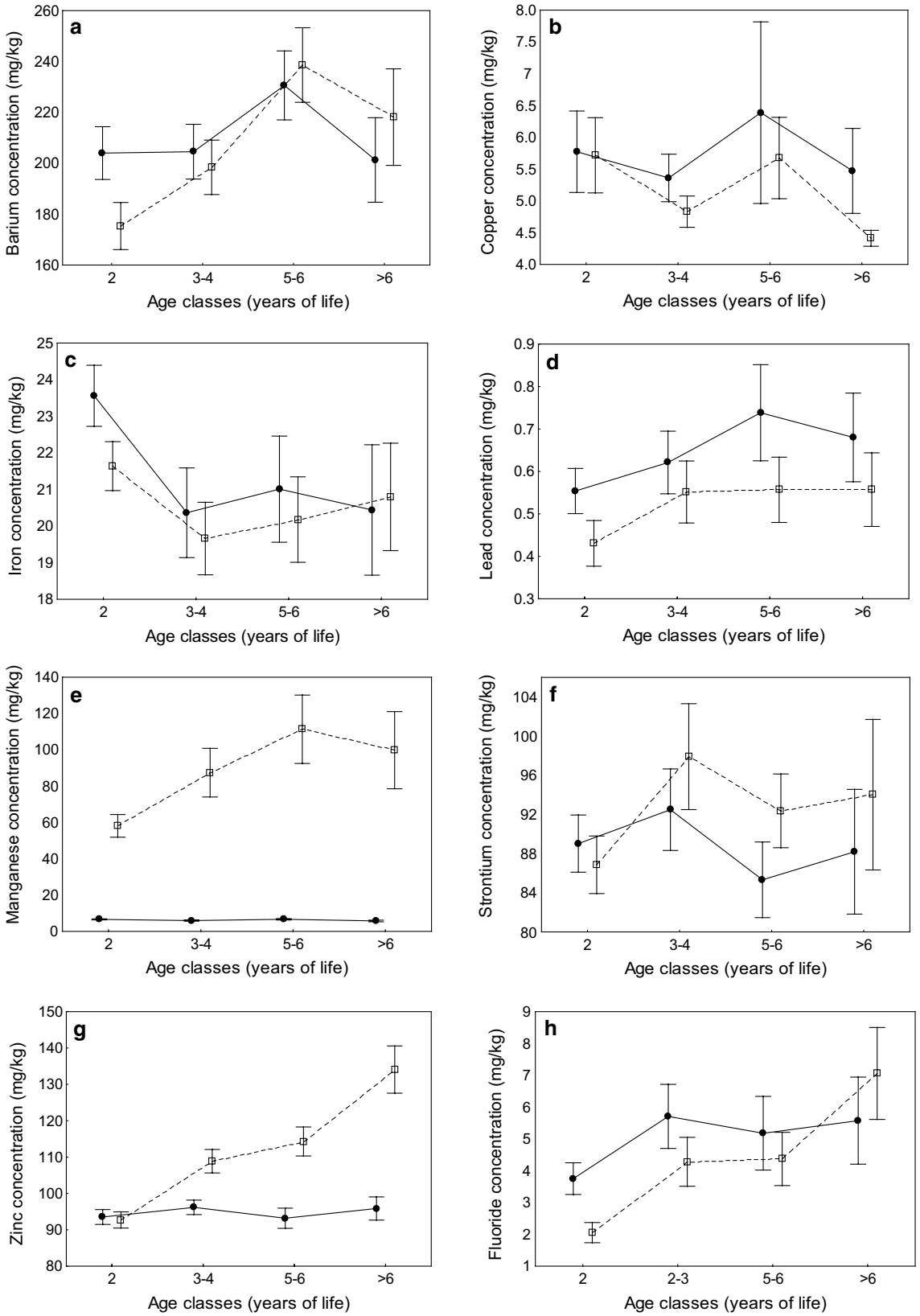

Fig. 1 Age-related variation in the concentrations of seven trace metals (**a** barium, **b** copper, **c** iron, **d** lead, **e** manganese, **f** strontium, **g** zinc) and fluoride (**h**) in bone (solid line, line, filled circles) and teeth (dotted line, open squares) of roe deer. Means ± SE are presented

rebuilt in less than 10 years (Arnett and Henderson 1998). In animals with shorter lifespan, such as the roe deer (maximum recorded lifespan of 17.5 years according to the AnAge database; De Magalhaes and Costa 2009), the period of the total skeletal turnover might be even shorter. Bone tissue is characterized by longer redevelopment period than most soft tissues, and thus, its trace element content is thought to reflect long-term exposure to environmental pollution (Glimcher 2006; Zaichick et al. 2011; Malara et al. 2016). Nevertheless, our study of roe deer suggests that, at least in this species, bone might not be a reliable indicator of throughout-life exposure to contaminants.

This is in sharp contrast to our findings for permanent teeth. Although ungulates have two generations of teeth, the deciduous set of teeth is replaced relatively early in the postnatal development and permanent teeth usually start to erupt before individuals finish their first year of life (Loe et al. 2004). When permanent teeth are developed their structure does not undergo remodelling and they are likely to bioaccumulate trace elements effectively from food and air. Consequently, long-term exposure of an organism to certain pollutants is likely to produce positive relationships between trace element concentrations in permanent teeth and individual age. Although we are aware that trace element content of permanent teeth cannot capture an exposure to pollutants in early postnatal period (first year of life), we suggest that permanent teeth are likely to more reliably, compared with bone tissue, indicate contamination that occurs throughout the entire adult life of roe deer. High reliability of teeth as the marker of environmental pollution by heavy metals also has been reported for small mammals, e.g., the bank vole (*Clethrionomys glareolus*) (Appleton et al. 2000).

Information on age-related variation in trace element concentrations in roe deer and other wild-living mammals is scarce and fragmentary. Many authors investigated trace elements in antlers, bone, and soft tissues of roe deer, but they either neglected age of animals in their studies (Sileo and Beyer 1985; Babińska-Werka and Czarnowska 1988; Zaccaroni et al. 2008; Baloš et al. 2015; Durkalec et al. 2015) or they distinguished two broad age categories of young and adults (Nowicka et al. 2006; Millán et al. 2008; Sobota et al. 2011). For example, Garcia et al. (2011) tested for differences in the contamination of liver, kidney, and muscle by cadmium, lead, and zinc between young (< 3 years old) and adult roe deer. Age-related differences were found for all tissues; cadmium and lead concentrations were higher in adults, whereas zinc concentration was higher in young individuals (Garcia et al. 2011). While separation of age into the categories of young and adults can provide some insight into age variation in trace element content, it is certainly insufficient to draw any detailed conclusions on how trace element concentrations change during an adult life. In contrast, our classification of age into four relatively narrow categories allowed us to effectively explore age-related trends in trace element bioaccumulation by roe deer.

In our study, age-related accumulation of trace elements in permanent teeth has been recorded for barium, manganese, and zinc. Consistently, mean concentrations of manganese and zinc were significantly higher in teeth than in bone tissue, and the same pattern was found for strontium. In fact, all these elements are thought to be important for hard tissues, especially for enamel (Lynch 2011) but possibly also for bone. For example, Marie et al. (2001) showed that application of large strontium dose had a positive effect on the hardness of the bone tissue in humans. However, two other trace elements, copper and lead, showed the opposite trends, as higher concentrations were recorded in bone that in teeth. Similar results were found in the forest reindeer (*Rangifer tarandus fennica*) from Karelia, Russia, where lead concentration was higher in bone than in teeth and antler (Medvedev 1995). In our study, neither copper nor lead showed any age-related variation in bone or teeth samples. Consistent with these results, an analysis of antlers and bone tissue by Sobota et al. (2011) provided no support for differences in lead concentration between young and adult roe deer. In contrast, Kierdorf and Kierforf (2002) found that lead concentration in antlers of young roe deer was significantly lower than in older individuals. Apparent inconsistency between these two studies suggests that age-related patterns in trace element content may vary between populations, possibly as a result of spatial and temporal variation in environmental pollution. In fact, trace element concentrations in roe deer have been reported to depend on the soil and water contamination and to show large geographical variation (Anderson et al. 2011). Accumulation rate of trace elements is also acknowledged to depend on the physiological state of animals (Zakrzewska et al. 2005), which may show some interpopulation variation. Finally, iron was the only element in our study of roe deer that had higher concentration in young than adult individuals, both in teeth and bone. While the mechanism responsible for this pattern merits further research, similar trends were found in other wild-living mammals, such as red fox *Vulpes vulpes* (Lanocha et al. 2012) and domestic mouse *Mus musculus* (Kennedy et al. 1986).

Conclusions

Our study revealed striking differences in age-related patterns of trace element bioaccumulation between bone and permanent teeth of roe deer. Most importantly, we found that concentrations of several trace elements in teeth increased with individual age, whereas no such trends were recorded for bone tissue. The results suggest that permanent teeth are likely to indicate reliably throughout-life intoxication

by environmental pollution in the roe deer and possibly in other mammal species. Our study also reinforces the need to account carefully for age-related variation in ecotoxicological research on wild-living animals.

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Ecotype Variation in Trace Element Content of Hard Tissues in the European Roe Deer (*Capreolus capreolus*)

Jan Demesko[1] · Janusz Markowski[1] · Eva Demesko[2] · Mirosława Słaba[3] · Janusz Hejduk[1] · Piotr Minias[1]

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Abstract
Animals living in anthropogenic habitats bear a multitude of costs, which are directly or indirectly associated with human activities. Among others, an elevated exposure to environmental pollution can have negative consequences for wildlife populations. We examined the differences in the concentrations of trace elements between the field and forest ecotype of the European roe deer (*Capreolus capreolus*). Naturally, roe deer inhabited various types of woodlands (forest ecotype), but within the last century, they adapted to life in a human-transformed agricultural areas (field ecotype), which could be associated with an increased exposure to pollution. In this study, we measured concentrations of seven trace metals (barium, copper, iron, lead, manganese, strontium, zinc) and fluoride in skull bones and permanent teeth of more than 230 roe deer from 8 study plots in East-Central Europe. We found that field roe deer had higher concentrations of four trace metals (copper, iron, lead, strontium) and fluoride compared with forest roe deer. These differences were consistent with variations in the general level of environmental contamination within the study plots, as assessed with trace element content in wild plants. Our study indicates that bone and teeth of the European roe deer can be used as a valid indicator of environmental pollution. Also, we expect that elevated exposure of field roe deer to environmental pollution can have negative consequences for wild populations of this species, as well as for the consumers of venison.

Rapidly increasing urbanization and industrialization in the second half of XX and the first decade of XXI century have led to large amounts of toxic contaminants being released into the environment worldwide. Many toxic elements occur naturally in the environment, but their concentrations can increase dramatically as a result of anthropogenic activities, such as mining, metal smelting, coal-based energy production, solid waste incineration, industrial manufacturing, as well as erosion of road surfaces by traffic and the abrasion of brakes and tires (Nriagu 1996; WHO 2013; Clemens and Ma 2016). Modern agricultural practices also contribute to increasing environmental pollution via application of agrochemicals and inorganic fertilizers (Chauhan et al. 2012). As a result, environmental concentrations of toxic elements can substantially exceed their normal background level, which disturbs biological balance of ecosystems and produces harmful effects on wildlife and human health (Tchounwou et al. 2012; Jaishankar et al. 2014), requiring implementation of pollution monitoring procedures (Wolkers et al. 1994; Srebočan et al. 2011). Wild animals, especially game species, are used relatively often as bioindicators of environmental pollution. In Europe, extensive ecotoxicological research has been conducted on cervids, which usually have wide geographical distribution and relatively long life-span (Sawicka-Kapusta 1979; Frank 1986; Tataruch and Kierdorf 2003). Also, their meat is a valuable and desired component of the human diet (Jarzyńska and Falandysz 2011), which has to comply with the World Health Organization standards for the content of heavy metal as pollutants (Lehel et al. 2016).

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✉ Jan Demesko
jan.demesko@biol.uni.lodz.pl

[1] Department of Biodiversity Studies and Bioeducation, Faculty of Biology and Environmental Protection, University of Łódź, Banacha 1/3, 90-237 Lodz, Poland

[2] Faculty of Medicine with Dentistry Division, Medical University of Lublin, Al. Racławickie 1, 20-059 Lublin, Poland

[3] Department of Industrial Microbiology and Biotechnology, Faculty of Biology and Environmental Protection, University of Łódź, Banacha 12/16, 90-237 Lodz, Poland

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The European roe deer (*Capreolus capreolus*) is recognized as one of the most ecologically plastic species among cervids, because it can tolerate strong anthropogenic pressure and can thrive in a human-impacted landscape (Augustine and McNaughton 1998; Tinoco Torres et al. 2011). Although the natural habitats of roe deer include a wide variety of forest types, the species has adapted to life in an intensively cultivated agricultural land across large areas of Europe (Pielowski 1984; Ellenberg 1978). Based on divergence in habitat selection, field and forest dwelling roe deer were identified as different ecotypes (Pielowski 1984). These two ecotypes have been reported to show remarkable phenotypic differences in morphology and anatomy (Fruziński et al. 1982; Hofmann et al. 1988; Pėtelis and Brazaitis 2003; Flis 2011), ecology (Zejda and Homolka 1980, Pielowski and Bresiński 1982; Kałuziński 1982; Zejda and Bauerova 1985), and physiology (Majewska et al. 1982). Jeppesen (1990) estimated that home range of forest ecotype varies from 15 to 85 ha, while it was at least twice bigger in the field ecotype. Forest and field ecotypes of roe deer also differ in feeding preferences (Tixier and Duncan 1996). The diet of the field roe deer is primarily based on cultivated plants, which may constitute up to 66% of the total feed mass (Kałuziński 1982). In contrast, forest deer ecotype typically feeds on the shoots of shrubs and trees, as well as wild herbaceous grasses and plants (Gębczynska 1980).

Heavy metal content in wildlife can be assessed across different types of tissues, which substantially vary in an average turnover time of their elements. For example, analysis of body fluids (e.g., serum, urine, or cerebrospinal fluid), which have the highest turnover rate, is only useful for evaluation of short-term exposure to pollutants, and thus, these tissues are rarely used in ecotoxicological research (Baroni et al. 2000; Humann-Ziehank et al. 2008; Žele and Vengušt 2012). In contrast, analysis of soft tissues and internal organs, especially liver and kidney, which accumulate toxic elements, can capture longer periods of exposure to contamination and these types of tissues have commonly been used in ecotoxicological monitoring of roe deer (Kryński et al. 1982; Frank 1986; Babińska-Werka and Czarnowska 1988; Pokorny and Ribarič-Lasnik 2002; Pompe-Gotal and Prevendar-Crnić 2002; de Mendoza et al. 2011; Srebočan et al. 2011; Długaszek and Kopczyński 2013; Wieczorek-Dabrowska et al. 2013; Durkalec et al. 2015; Lehel et al. 2016). Finally, hard tissues, such as bone or teeth, have the lowest turnover rates, and they are known to accumulate trace elements over years or decades (Glimcher 2006). For example, the biological half-life of trace elements in human bone tissue is up to 30 years, and the content of heavy metals in bones is known to comprise up to 90% of their total body content (Zaichick et al. 2011). In cervids, heavy metal content has been commonly assessed in antlers, because they are regularly collected as hunting trophies and can easily be used as research material (Kierdorf and Kierdorf 2002, 2004, 2006). However, cervids usually produce new antlers each year, and thus, they are not particularly suitable to investigate long-term exposure to pollutants. Taking all this into account, bone and teeth are expected to more reliably indicate long-term bioaccumulation of pollutants, and consistent with this prediction, we have recently shown that heavy metal content of permanent teeth reliably indicate throughout-life intoxication by environmental pollution in the European roe deer (Demesko et al. 2018).

The purpose of this study was to test for the differences in trace element content between the field and the forest ecotype of the Eurasian roe deer. For this purpose, we measured concentrations of seven trace metals (barium, copper, iron, lead, manganese, strontium, zinc) and fluoride in skull bones and permanent teeth of more than 230 roe deer from 8 study plots in East-Central Europe. We predicted higher trace element concentrations in the field ecotype of roe deer, which could be due to: (1) differences in general environmental pollution between areas inhabited by the two ecotypes of roe deer, or (2) difference in the ecology of the two roe deer ecotypes, i.e., an alteration in diet composition from wild forest plants (forest ecotype) to cultivated crops (field ecotype). To estimate the level of general environmental pollution within each study plot, we measured trace metal content in the common forest plants (2 species of trees and 2 genera of wild fruit plants) that are an important component of roe deer diet.

Materials and Methods

Study Area and Classification of Ecotypes

Samples were collected in seven game breeding centres from Łódź voivodship, Central Poland: Brzeziny (51°45′N, 19°43′E; $n = 25$), Kolumna (51°34′N, 19°13′E; $n = 11$), Kutno (52°14′N, 19°08′E; $n = 26$), Poddębice (51°54′N, 18°53′E; $n = 11$), Smardzewice (51°26′N, 19°60′E; $n = 32$), Spała (51°31′N, 20°11′E; $n = 13$), Wieluń (51°11′N, 18°44′E; $n = 21$), and in one game breeding centre from Vilnius area, Lithuania: Mickunai forest (54°41′N, 25°35′E; $n = 94$). Polish game breeding centres were located relatively close (25–100 km) to a large urban centre, Łódź (51°46′N, 19°28′E; 293 km^2, 708 500 inhabitants), while Mickunai forest was located ca. 20 km from the Vilnius city (54°41′N, 25°17′E; 401 km^2, 574,200 inhabitants). The share of urbanized areas within the study plots ranged from 5.2 to 26.8% (mean $9.0 \pm 2.6\%$). The distinction between the field and forest roe deer ecotype was based on the share of woodland area within the study plots. Forest ecotype was defined as inhabiting areas with $> 50\%$ share of woodland, while field ecotype was identified in the study plots with $< 35\%$ share

of woodlands (there were no study plots with 35–50% share of woodlands). Determination of roe deer ecotypes was consistent with legal classification of forest and field hunting units (Flis 2011) and with morphological variation of roe deer within our dataset, showing that forest individuals were significantly smaller than field individuals (as measured with height at the withers and chest circumference; $P < 0.05$). Mean share of woodland and agricultural areas was $58.2 \pm 4.6\%$ versus $36.1 \pm 4.7\%$ for the forest ecotype, and $22.7 \pm 4.8\%$ versus $66.2 \pm 5.3\%$ for the field ecotype. In total, samples for the field ecotype were collected in five study plots (Brzeziny, Kolumna, Kutno, Poddębice, and Wieluń), while forest ecotype was sampled in three study plots (Mickunai, Smardzewice, and Spała). There were no significant differences in the level of environmental contamination, as measured with concentrations of six heavy metals (barium, copper, iron, lead, strontium, and zinc) in wild forest plant species (see details below) between Polish and Lithuanian forest study plots (all $P > 0.05$), which provided support for our joint analysis of these data. The only difference was found for the manganese concentration in wild forest plants, which was higher in Polish than Lithuanian forest study plots ($P < 0.001$).

Sample Collection

Roe deer were culled during regular hunting period and in accordance with local hunting plans and regulations during 2009–2015. A total sample of 233 skulls was collected (139 and 94 samples for the forest and field ecotype, respectively). Age of sampled specimens varied between 2 and 12 years, as assessed based on dental wear by the members of the Regional Commissions for Hunting Evaluation (details in Demesko et al. 2018). For the purpose of analyses, four age classes were recognized: (i) 2 years old ($n = 77$), (ii) 3–4 years old ($n = 70$), (iii) 5–6 years old ($n = 49$), and (iv) > 6 years old ($n = 27$). A small part (ca. 0.7 g of dry mass) of mandible located between foramen mental and front edge of premolar, as well as the left third permanent molar were collected from each animal using a diamond saw. Material from the entire teeth was included in the analysis, because there may be differences in the mineral composition between crown and root, as well as between dentine and enamel (Vieira et al. 2004, 2005).

To assess the level of environmental pollution within the study plots, we also collected samples of four wild forest plant species. A total of 96 plant samples were collected in the corresponding 8 game breeding areas during June 2015. Plant specimens collected included silver birch (*Betula pendula*)—leaves, Scots pine (*Pinus sylvestris*)—needles, blackberry (*Rubus ssp.*)—entire plant, and European blueberry (*Vaccinium myrtilus*)—entire plant. Samples from each plant taxon were collected from three specimens located in different parts of each study plot; however, three samples of blueberry and one sample of pine were excluded from analyses for technical reasons. Ten leaves from birch and ten needles from pine were collected at the height of up to 1 m from the ground.

Measurements of Trace Metal and Fluoride Concentrations

All samples were washed in deionized water to remove any elements absorbed at the surface. Bone and tooth samples were powdered in a ball mill Mixer Mill MM 400 (Retsch, Germany) with zirconium oxide beads (frequency 25 Hz, time 60 s) and dried in an oven at 70 °C for 48 h. Plant samples were dried at 70 °C for 24 h. After drying, all samples were weighed to the nearest 0.01 g and 0.1 g of each sample was taken for the measurements of seven trace metal concentrations (barium, copper, iron, lead, manganese, strontium, and zinc). First, each sample was dissolved in solution of nitric acid (65%) and deionized water in the proportion of 1:15, kept in 20 °C for 24 h, and then digested at 105 °C for another 24 h using a graphite digestion block (DigiPREP Mini, SCP Science, Quebec, Canada). After digestion, all samples were diluted with deionized water to the total volume of 30 mL and stored in polypropylene metal-free vials at 20 °C until analysis.

Trace metal concentrations were measured with atomic absorption spectrophotometer SpectrAA 300A AAS, GTA-96 graphite tube atomizer, and programmable sample dispenser (Varian Techtron, Melbourne, Australia). The analyses were performed in the Laboratory of Computer and Analytic Techniques, Faculty of Biology and Environmental Protection, University of Łódź. Certified reference material from the Institute for Reference Materials and Measurements (Geel, Belgium) were used for each measurement as a quality control: ERM-186 pig kidney for copper, iron, lead, manganese, and zinc; Strontium Standard for AAS (TraceCERT®, 1000 mg/L Sr in nitric acid) for strontium; and Barium Standard for AAS (TraceCERT®, 1000 mg/L Ba in nitric acid) for barium. Recovery rates for the certified reference materials were within an acceptable margin.

Measurements of fluoride concentration followed the methodology recommended by Campus et al. (2007): 1.2 g of each bone and tooth sample was transferred to a volumetric flask, dissolved in 8 mL of 37% HCl solution, and then diluted with deionized water to the total volume of 10 mL. A 5 mL aliquot of the above solution was transferred to another volumetric flask, diluted 1:1 with deionized water, neutralized with a 6 M NaOH solution to pH 4.5, and diluted with deionized water to the total volume of 25 mL. Sample solution was diluted with TISAB (1:1), and fluoride concentration was measured with ion-selective fluoride electrode (Hydromet S.C., Gliwice, Poland). Fluoride concentrations

were not measured for plant samples. All trace element concentrations were expressed in mg per kg dry mass (Table 1).

Trace Element Distributions and Outlier Analysis

Since distributions of most trace element concentrations showed strong right-skewness (mean skewness 2.09 ± 0.58 [SE] and 2.47 ± 1.00 [SE] for bone/tooth and plant samples, respectively), we performed an outlier analysis on the dataset. We used conservative criteria (> 5 SD) for outlier detection. Outlier analyses were conducted separately for tooth and bone samples, while all plant species were analysed jointly. We identified between one and three outliers for the concentrations of barium, copper, and manganese in bone, while two outliers were identified for the copper concentration in tooth samples. Also, two outliers were identified for the concentration of barium in plants. No outliers were identified for any other measurement. All outliers were removed from the dataset. Measurements that retained strong (> 1) right-skewness after outlier removal were log-transformed to improve normality.

Interspecific Variation in Plant Trace Elements

There were significant differences in trace element content of different plant species. Concentrations of all trace elements, except for lead, showed significant differences between plant species, as assessed with the analysis of variance (ANOVA). In most cases (copper, iron, lead, manganese, and strontium), trace element concentrations were lowest in pine (Table 2). Post-hoc Tukey HSD comparisons showed that iron, manganese, and strontium concentrations in pine were significantly lower than in all other plant species (all $P < 0.05$), whereas copper concentrations in pine were significantly

Table 1 Mean (± SE) concentrations and sample sizes for seven trace metals and fluoride in bone (mandible) and teeth (third permanent molar) of field and forest ecotypes of the European roe deer

Trace element	Bone				Teeth			
	Forest ecotype		Field ecotype		Forest ecotype		Field ecotype	
	Mean ± SE	*n*	Mean ± SE	*n*	Mean ± SE	*n*	Mean ± SE	*n*
Barium	204.1 ± 7.0	128	198.4 ± 7.6	91	196.9 ± 6.9	128	197.9 ± 7.8	92
Copper	4.73 ± 0.12	124	5.45 ± 0.22	91	4.56 ± 0.08	127	4.96 ± 0.17	88
Iron	18.15 ± 0.52	128	22.98 ± 0.77	90	17.15 ± 0.38	128	21.87 ± 0.55	91
Lead	0.32 ± 0.02	127	0.69 ± 0.05	91	0.29 ± 0.02	127	0.58 ± 0.05	91
Manganese	7.22 ± 0.58	121	6.07 ± 0.19	92	64.9 ± 5.6	125	65.0 ± 5.9	92
Strontium	86.5 ± 3.3	128	95.2 ± 2.1	92	88.6 ± 3.3	128	98.9 ± 2.8	92
Zinc	98.1 ± 1.6	128	94.1 ± 1.4	90	105.9 ± 1.8	128	109.1 ± 2.4	91
Fluoride	2.76 ± 0.30	134	4.95 ± 0.56	90	2.14 ± 0.23	133	3.42 ± 0.39	89

All concentrations are given in mg per kg dry mass

Table 2 Mean (± SE) concentrations for seven trace metals in four plant species collected from areas with low and high woodland cover

Woodland cover	Trace element	Silver birch Mean ± SE	Scots pine Mean ± SE	Blackberry Mean ± SE	European blueberry Mean ± SE
Low	Barium	34.05 ± 4.37	123.5 ± 55.49	17.06 ± 3.51	54.65 ± 8.66
	Copper	6.68 ± 0.38	5.74 ± 0.30	8.71 ± 0.71	7.18 ± 0.71
	Iron	80.72 ± 6.43	48.31 ± 5.13	98.52 ± 8.81	75.67 ± 7.36
	Lead	0.54 ± 0.20	0.46 ± 0.29	0.48 ± 0.13	0.58 ± 0.18
	Manganese	1107.7 ± 186.1	210.8 ± 34.6	706.2 ± 143.5	1308.0 ± 295.9
	Strontium	14.60 ± 1.64	2.95 ± 0.45	9.31 ± 1.56	7.17 ± 1.75
	Zinc	200.9 ± 21.1	37.33 ± 3.18	44.84 ± 3.59	31.82 ± 3.38
High	Barium	35.46 ± 4.84	10.17 ± 3.37	93.21 ± 70.66	50.41 ± 6.82
	Copper	5.07 ± 0.42	4.55 ± 0.25	5.36 ± 1.00	5.40 ± 0.93
	Iron	75.96 ± 6.71	39.54 ± 4.09	95.03 ± 5.62	53.23 ± 3.22
	Lead	0.67 ± 0.41	0.16 ± 0.07	0.41 ± 0.24	0.37 ± 0.19
	Manganese	1437.1 ± 389.2	217.1 ± 58.1	1623.2 ± 692.2	2280.2 ± 554.2
	Strontium	12.09 ± 2.55	2.38 ± 0.29	14.27 ± 2.54	5.73 ± 1.34
	Zinc	216.3 ± 27.2	41.34 ± 2.44	58.11 ± 13.66	19.37 ± 1.93

All concentrations are given in mg per kg dry mass

lower compared with blackberry ($P=0.010$). Zinc concentrations were lowest in blueberry (Table 2; Tukey comparisons with other plant species: all $P<0.05$), whereas barium concentrations were lowest in birch (Table 2; nonsignificant differences in Tukey comparisons: all $P>0.05$). Maximum trace element concentrations were found in blackberry (copper and iron), blueberry (manganese), birch (lead, strontium, and zinc), and pine (barium) (Table 2). Because of these differences, we included plant species as a fixed factor in all further analyses of trace element content in plants.

Statistical Analyses

We used general linear mixed models (GLMMs) to test for the ecotype variation in trace element content in bone and teeth of the roe deer. Ecotype, sample type (bone vs. teeth), and age were entered as fixed factors. To test whether ecotype-related differences in trace element content were similar for both sample types, we also entered an ecotype-sample type interaction in each model. As age-related bioaccumulation rate of trace elements can vary between bone and teeth of the roe deer (our previous research on roe deer provided support for positive correlations between trace element concentrations and age in teeth, but not in bone (Demesko et al. 2018)), we also included age-sample interaction to account for these differences. Because teeth and bone samples were collected from the same individuals, we included individual identity as a random factor to avoid pseudoreplication (Hurlbert 1984). The effect of year was included as the second random factor to control for interannual variation in the collected measurements. All GLMM models were fitted using the restricted maximum likelihood (REML) method. With this approach, denominator degrees of freedom are calculated using a Satterthwaite approximation, which can result in fractional degrees of freedom (Satterthwaite 1946). Significance of independent variables was assessed with Wald χ^2 statistic.

Differences in trace element content of plants collected in the study plots with low (<35%) and high (>50%) woodland cover (consistent with field and forest deer ecotypes) were assessed with general linear models (GLMs). Plant species and the binary effect of woodland cover (low vs. high) were entered as fixed factors. To test whether the effect of woodland cover on trace element concentrations was similar for different plant species, we also included an interaction term between these two factors.

All GLMMs were run using lmer function as implemented in lme4 package (Bates et al. 2015) developed for R statistical environment (R Development Core Team 2013). We used *car* package (Fox and Weisberg 2011) to obtain Wald χ^2 statistics and p values for all independent variables. GLMs were conducted in Statistica 10.0 (StatSoft, Tulsa, OK, USA). The results of full models were reported. All values are shown as mean ± SE.

Results

After accounting for age-related variation in trace element content, we found that concentrations of four trace metals (copper, iron, lead, strontium) and fluoride in roe deer significantly varied with ecotype (Table 3). The effects of ecotype on the concentrations of these elements were similar for bone and tooth samples, as indicated by nonsignificant ecotype-sample type interactions (Table 1). In all these cases, trace element concentrations were significantly lower in the forest ecotype compared with the field ecotype, both in bone and teeth of roe deer (Table 1, Fig. 1). There was a significant ecotype-sample type interaction for zinc concentration (Table 3), but no significant effect of ecotype on zinc concentration was found in separate analyses of bone and tooth samples (all $P>0.05$). Also, we failed to find any differences in the concentrations of barium and manganese between the forest and field ecotypes of roe deer.

Concentrations of two trace elements, copper and iron, in plants varied with woodland cover irrespectively of sampled plant species (Table 4). Specifically, plants collected in areas with lower woodland cover had higher concentrations of copper (7.10 ± 0.30 mg/kg vs. 5.10 ± 0.35 mg/kg) and iron (76.3 ± 4.2 mg/kg vs. 65.9 ± 4.3 mg/kg) (Fig. 2). Lead concentration in plants showed nearly significant variation with woodland cover ($P=0.061$; Table 4), as plants from areas with lower woodland cover had a tendency for higher concentrations of lead (0.51 ± 0.10 mg/kg vs. 0.40 ± 0.13 µg/kg). Relationships between woodland cover and concentrations of two other trace elements, barium and zinc, varied between plant species, as indicated by significance of appropriate interaction terms (Table 4). Barium concentration in pine and zinc concentration in blueberry varied nearly significantly or significantly (respectively) with woodland cover (barium: $F_{1,21}=3.18$, $P=0.089$; zinc: $F_{1,19}=7.60$, $P=0.013$), and in both cases, element concentrations were higher in areas with lower woodland cover (barium: 123.5 ± 55.5 mg/kg vs. 10.2 ± 3.4 mg/kg; zinc: 31.8 ± 3.4 mg/kg vs. 19.4 ± 1.9 mg/kg). Barium and zinc concentration in other plant species did not vary with woodland cover (all $P>0.10$). Manganese and strontium concentrations in plants did not differ between areas of low and high woodland cover (Table 4).

Table 3 The results of general linear mixed models assessing the effect of ecotype and other factors (sample type and age) on the concentrations of seven trace metals and fluoride in bone and teeth of the European roe deer

Factor	Barium		Copper		Iron		Lead	
	W	*P*	*W*	*P*	*W*	*P*	*W*	*P*
Ecotype	0.08	0.77	**16.08**	**< 0.001**	**81.17**	**< 0.001**	**39.92**	**< 0.001**
Ecotype*sample type	1.37	0.24	1.91	0.17	0.01	0.93	3.35	0.067
Age	1.99	0.57	6.56	0.087	**44.58**	**< 0.001**	2.58	0.46
Age*sample type	**59.80**	**< 0.001**	1.15	0.77	4.46	0.22	7.45	0.059
Sample type	2.71	0.10	**16.41**	**< 0.001**	**4.10**	**0.043**	2.04	0.15
Factor	Manganese		Strontium		Zinc		Fluoride	
	W	*P*	*W*	*P*	*W*	*P*	*W*	*P*
Ecotype	0.49	0.48	**4.71**	**0.030**	0.37	0.54	**16.49**	**< 0.001**
Ecotype*sample type	1.52	0.22	0.30	0.58	**4.47**	**0.035**	1.26	0.26
Age	**9.97**	**0.019**	3.65	0.30	**58.66**	**< 0.001**	**21.18**	**< 0.001**
Age*sample type	**16.08**	**0.001**	**26.51**	**< 0.001**	**102.1**	**< 0.001**	**9.30**	**0.026**
Sample type	**729.1**	**< 0.001**	**6.47**	**0.011**	**67.23**	**< 0.001**	**14.28**	**< 0.001**

Individual identity was entered as a random factor in each model

Significant terms are marked in bold

Discussion

The results of our study clearly indicate that field and forest ecotypes of the European roe deer showed significant differences in trace element content. Field roe deer had higher concentrations of four trace metals (copper, iron, lead, strontium) and fluoride in both bone and teeth compared with forest roe deer. Animal data were consistent with respective plant data, which indicated higher environmental contamination of areas inhabited by the field ecotype of roe deer.

It is widely accepted that animals living in heavily transformed habitats bear many costs, which are directly or indirectly associated with human activities (Gaillard et al. 1993; Benhaiem et al. 2008; Demesko et al. 2018). These costs may include direct human threat (e.g., hunting or poisoning with human waste; Kie 1999; Burbaitė and Csanyi 2009), pollution with light and noise that may cause elevated levels of physiological stress (De Vires 1995; Lima 1998; Pierce et al. 2004; Benhaiem et al. 2008), and exposure to novel predators, such as feral dogs and cats (May and Norton 1996; Apfelbach et al. 2005). The results of our study provide support for the hypothesis that intoxication with harmful substances of anthropogenic origin can be an important cost for wild animal populations from human-altered landscapes. So far, urbanization and human-related land alteration (e.g., intensive agricultural activities) has been associated with increasing intoxication level in a relatively wide spectrum of wildlife. For example, red foxes (*Vulpes vulpes*) and stone martens (*Martes foina*) from suburban area had higher content of copper and lead in soft tissues (muscle, liver, and kidney) compared with individuals originating from rural populations (Bilandžić et al. 2010). Analysis of trace element content in hair of three bat species indicated highest concentrations of lead and zinc in those species that collected food in human-dominated landscapes, including cities (Flache et al. 2015). Similarly, rook (*Corvus frugilegus*) eggshells from colonies located in large cities had significantly higher concentrations of chromium, nickel, and lead than those from villages (Orłowski et al. 2014). These results provided support for a huge variation in the habitat-dependent bioaccumulation of heavy metal in avian eggs, which occurred as a result of the clear pollution gradient from rural to urbanized areas (Orłowski et al. 2014). Lead and cadmium concentrations also were higher in blue tit (*Cyanistes caeruleus*) and great tit (*Parus major*) nestlings raised in urban parkland site than in the suburban semi-natural woodland site (Markowski et al. 2014). The levels of lead found in the tissues of urban house sparrows (*Passer domesticus*) were significantly higher than in an agricultural control group in Vermont, USA (Chandler et al. 2004) and Finland, where concentrations of several heavy metals were generally higher in urban than rural groups (Kekkonen et al. 2012). We are also aware of two previous studies on the European roe deer, which tested for associations between trace element content in deer tissues and habitat-related environmental contamination. First, roe deer from the major industrial sites in Poland had much higher (up to an order of magnitude) concentrations of lead and cadmium in tissues and stomach content than deer from the natural lake-forest ecosystems (Durkalec et al. 2015). The same study revealed similar patterns in

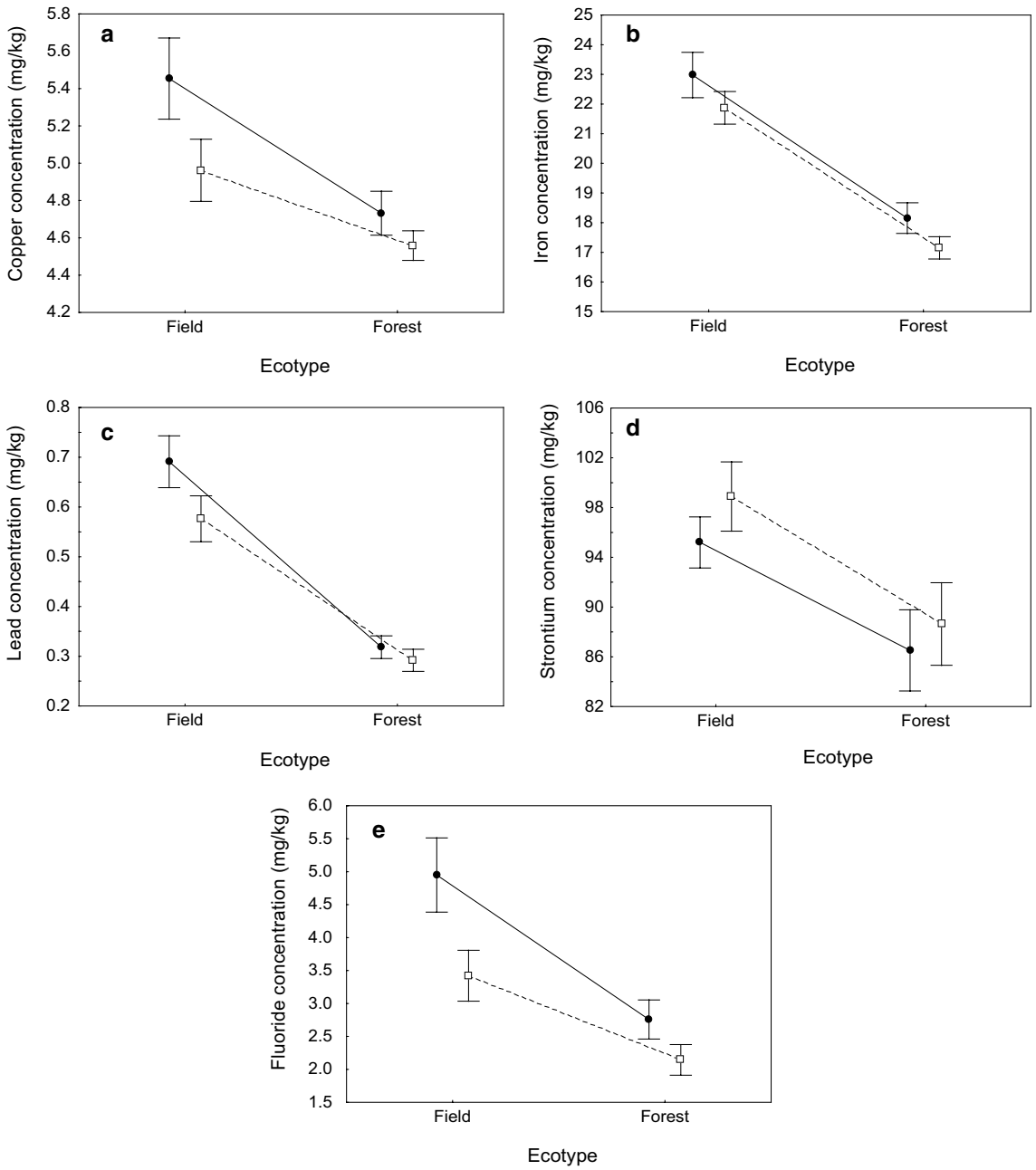

Fig. 1 Concentrations of trace elements (**a** copper, **b** iron, **c** lead, **d** strontium, and **e** fluoride) in bone (solid line, filled circles) and teeth (dotted line, open squares) of the two ecotypes (field and forest) of the European roe deer. Mean ± SE are presented

trace element content of the wild boar (*Sus scrofa*) tissues (Durkalec et al. 2015). The second study provided evidence for higher concentrations of arsenic and chromium in teeth of roe deer that lived in a closer proximity to agricultural and industrial areas (Zaccaroni et al. 2008). We provided convincing evidence for high concentrations of several trace elements in the field ecotype of roe deer, which primarily feeds on crop fields and pastures. In contrast, trace element concentrations were significantly lower in the forest ecotype of deer, which prefers more natural ecosystems, such as various types of woodlands.

Five of eight measured trace elements (copper, iron, lead, strontium, and fluoride) had concentrations significantly higher in the field than forest ecotype of roe deer. Although the patterns were consistent between tooth and bone samples, we suggest that our results for fluoride should be treated with caution. The mean content of fluoride in the samples ranged from 2.14 ± 0.23 to 4.95 ± 0.56 mg/kg

Table 4 The results of general linear models assessing the effect of woodland cover on the concentrations of seven trace metals in four wild plant species

Factor	Barium		Copper		Iron		Lead	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Forest cover	0.33	0.57	**17.74**	**< 0.001**	**4.47**	**0.037**	3.59	0.061
Species	**6.30**	**< 0.001**	0.94	0.43	**29.37**	**< 0.001**	1.62	0.19
Forest cover*species	**3.47**	**0.020**	0.76	0.52	1.23	0.30	0.55	0.65
Factor	Manganese		Strontium		Zinc			
	F	*P*	*F*	*P*	*F*	*P*		
Forest cover	0.93	0.34	0.01	0.94	0.24	0.62		
Species	**12.88**	**< 0.001**	**28.62**	**< 0.001**	**1114.7**	**< 0.001**		
Forest cover*species	0.55	0.65	1.65	0.18	**2.75**	**0.048**		

Significant terms are marked in bold

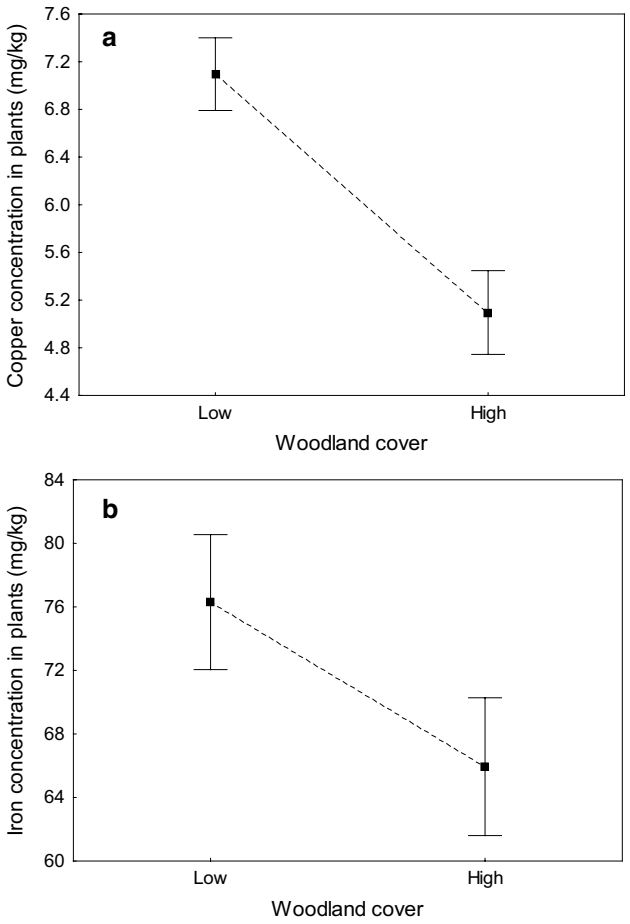

Fig. 2 Concentrations of copper (**a**) and iron (**b**) in four wild plant species sampled in study plots with low (< 35%) and high (> 50%) woodland cover. Mean ± SE for all taxa combined are presented

of dry matter, depending on the type of tissue and ecotype (Table 1). In contrast, fluoride concentrations previously reported for different wildlife ungulate and non-ungulate species coming from unpolluted European areas often achieved hundreds or thousands mg/kg of dry matter (Kierdorf et al. 2000, 2012; Jelenko and Pokorny 2010; Kalisinska and Palczewska-Komsa 2011; Vieira et al. 2005). On the other hand, our fluoride measurements yielded higher average values than reported by Sobota et al. (2011) for skull bone and antler samples from roe deer collected in West Pomerania, Poland (< 1 mg/kg d.m.). Also, many authors (Kierdorf and Kierdorf 2003, 2009; Dabkowska et al. 1995; Jelenko and Pokorny 2010) emphasized that environmental concentrations of fluoride have been significantly decreasing in the recent decades, which could possibly be responsible for low fluoride content in our samples.

In our study, large differences in trace element content between the two deer ecotype were consistent with differences in the general level of environmental contamination, suggesting that bone and teeth of the European roe deer can be used as a valid indicator of environmental pollution. We found that wild forest plant collected from areas with low woodland cover (characteristic for field ecotype of deer) showed significantly higher concentrations of copper and iron, and a nearly significantly higher concentration of lead. Also, concentrations of barium and zinc were higher in the areas with lower woodland cover but only in specific plant taxa (pine and blueberry, respectively). It is possible that differences in the environmental contamination between more and less wooded areas could be directly explained with various intensity of farming activities. It is widely known that chemicals (fertilizers and pesticides) commonly applied in agriculture can lead to the higher accumulation of elements, such as iron, manganese, copper, or zinc in the soil (Singh 1994; Kabata-Pendias 1995; Romic and Romic 2003; Micó et al. 2006; Martiniaková et al. 2011). Also, agriculture often is associated with bigger traffic, which can further increase air pollution (Bunce 1985; Sobota et al. 2011). Finally, trees act as biological filters, removing a lot of airborne particles

and, at the same time, improving the quality of air in polluted areas (Nowak et al. 2006). Thus, in most cases a negative correlation between woodland cover and environmental contamination should be well expected (Beckett et al. 1998), resulting both from lower exposure to contaminants and their more efficient removal in the wooded areas.

We did not study differences in foraging by the two ecotypes. However, large differences in trace element content of wild plants sampled from predominantly agricultural and predominantly wooded study plots suggests that high concentrations of trace minerals in hard tissues of field roe deer may not be a direct consequence of variation in diet between the two ecotypes. Foraging strategy and diet composition differs considerably between the two ecotypes (Kałuziński 1982), whereby field roe deer predominantly forage on agricultural plants and herbs with a small share of bushes and trees, while forest roe deer prefer grasses, as well as shoots and bark of woody plants (Kałuziński 1974, 1982; Szmidt 1975). The two ecotypes also differ in many other aspects of ecology, which under certain scenarios could possibly affect their exposure of contamination. For example, forest deer has much smaller home range, which was estimated at only 3–8 ha in dense coniferous/deciduous woods, although it may be a few times larger in more fragmented landscape (Tufto et al. 1996). The two ecotypes also differ in the level of sociality (field deer form large winter groups of up to 100 individuals, while forest deer are more solitary; Zejda 1978; Bresinski 1982) and behaviour, e.g., antipredator strategies (Fruziński et al. 1983; Pielowski and Bresiński 1982; Aulak and Babińska-Werka 1990; Hewison et al. 2001). While it might be difficult to offer specific predictions about how these differences could affect trace mineral content in deer, we cannot exclude that factors, such as the size of home range and the level of sociality, for example, could determine movement propensity of animals across different habitats, which could possibly alter their exposure to environmental contamination.

Conclusions

Our study provided correlational evidence for increased concentrations of trace elements in the field ecotype of the European roe deer, which primarily inhabits human-transformed agricultural landscape. Although the field ecotype of the European roe deer was first described in 1929 (Kałuziński 1974), its population size is thought to have considerably grown over the past decades, mainly due to the increasing fragmentation of forest habitats and greater availability of human-derived food. Future monitoring of European roe deer is warranted to explore ecotoxicological differences between field and forest roe deer.

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Compliance with Ethical Standards

Conflict of interest All the authors declare that they have no conflict of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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Enamel thickness differs between field and forest European roe deer *Capreolus capreolus*

Jan DEMESKO[1*], Marta KUREK[2], Patrycja PODLASZCZUK[1] and Janusz MARKOWSKI[1]

[1] Department of Biodiversity Studies and Bioeducation, Faculty of Biology and Environmental Protection, University of Łódź, Banacha 1/3, 93-237 Łódź

[2] Department of Anthropology, Faculty of Biology and Environmental Protection, University of Łódź, Banacha 12/16, 93-237 Łódź

*e-mail: jan.demesko@biol.uni.lodz.pl (*corresponding author*)

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ABSTRACT

Dental enamel is the hardest tissue of the mammalian body, consisting of 96–98% inorganic compound. As the dentition is functionally adapted to diet and feeding behaviour, relative differences in enamel thickness can reflect dietary adaptations. We hypothesize that differences in enamel thickness are related to adaptation for diet associated with habitat quality dwelling of European roe deer *Capreolus capreolus*. To test this hypothesis, 49 first permanent left lower molars were extracted from the mandible of roe deer (from Lithuania – 28 and Poland – 21 molars) inhabiting two type of habitats: field and forest. The linear thickness of total enamel (mean value of enamel thickness measured at three different points) was found to differ between the roe deer from the field and forest habitats, irrespective of age, with the animals of field ecotype tend to have thinner enamel ($F_{(1,26)} = 6.845$, $P = 0.025$). This suggests that there is an adaptation in enamel thickness to various types of diet in the field and forest habitat. On the other hand, roe deer from the field habitat can be also more exposed to stress, due to the lower possibility to hide or are more vulnerable to potential threats. More frequent exposure to stress can significantly disrupt ameloblasts secretion and thus affect the thickness of the enamel.

The dentition in mammals is morphologically and functionally adapted to diet and feeding behavior. Most mammalian teeth are composed of three dental tissues: enamel, dentine and cementum. Enamel is acellular layer made up of highly mineralized a tightly packed mass of hydroxyapatite crystals, which forms the outer crystalline surface of teeth and is the hardest dental tissue (Winkler and Kaiser 2015). Hence it is recognized to constitute a structural reinforcement of teeth, to protect against to wear and crack propagation (Koenigswald *et al.* 1987, Maas and Dumont 1999, Simmer *et al.* 2001). The enamel thickness, as rarely other dental trait, have elicited more interest in the research of origin and evolution in adaptation to different habitats of mammals over the past several decades. They mainly included extinct and extant taxa: as primates (e.g. Dumont 1995, Shimizu 2002, Grine *et al.* 2005, Ungar 2008, Constantino *et al.* 2009, McGraw *et al.* 2012, Galbany *et al.* 2014), ungulates (e.g. Archer and Sanson 2002, Heywood 2009, Kaiser *et al.* 2010, Famoso *et al.* 2013, Winkler *et al.* 2013, Gailer and Kaiser 2014), rodents (e.g. Flynn *et al.* 1987, Grayson *et al.* 1990, Martin 1993, Moinichen *et al.* 1996, Sander *et al.* 2008) as well as chiropterans (Dumont 1995), carnivores (Koenigswald 1996, Crossley 1995, Stefen and Rensberger 1999) and marsupialia (Stefen 1999).

The European roe deer, *Capreolus capreolus* L. is a medium sized ungulate and the most abundant species among cervids family in Europe (Burbaitė and Csányi 2009). It is considered as species preferring wooded landscapes (Hewison *et al.* 2001), but also recognized as one of the most ecologically adaptable species of deer (e.g. Pielowski 1977, Lehmann and Sägesser 1986, Kurt 1991). Its ability to adapt to a wide range of environmental gradients allows it to colonize on intensively cultivated agricultural land (cf. Pielowski 1977, Ellenberg 1978, Hewison *et al.* 2001) and even suburban zones. Field and forest dwelling roe deer have been identified as different ecotypes and showing distinct phenotypic variability and differences in breeding biology and social organization (e.g. Pielowski 1977, Fruziński *et al.* 1982, Fruziński and Łabudzki 1982, Pielowski and Bresiński 1982, Kurt 1991) and in some physiological parameters (Majewska *et al.* 1982). The two ecotypes also differ with regard to their feeding preferences (Kałuziński 1982, Tixier and Duncan 1996).

Generally, roe deer consume from 250 to 400 species of plants depending on the geographical area (Mihalusev *et al.* 1997, Krasnov *et al.* 2015). The diet of the field ecotype is based on cultivated plants (wheat, rye, barley, corn, oats, buckwheat, vegetables), which constitute 66% of the total mass of their feed. This proportion is the highest in the summer and winter, while in spring the roe deer of this ecotype prefer grasses and other plants (creeping buttercup, wood anemone, marsh-marigold, other grasses and sedges species (Kałuziński 1982). In contrast, the diet of the forest roe deer contains more harder plant fragments, including trees (European hornbeam, common oak, Birch, Norway maple, common aspen, European spruce) and shrubs (European spindle, common hazel, brambles, European blueberry), which constitute about 11–13% of the total food intake up to 25% in autumn and winter (Gębczyńska 1980). It has been also shown that forest roe deer eagerly supplements their diet with other hard food items, such as acorns in the autumn and winter seasons (Krasnov *et al.* 2015).

Roe deer dwelling in the field have adapted to live in an open landscape where climate conditions are harsher due to lack of shelter, thus individuals are larger and heavier than individuals of the forest ecotype (Fruziński *et al.* 1982, Narauskaite and Petelis 2010).

In turn, craniometric studies are more ambiguous, on the one hand Petelis and Brazaitis (2003) and Kulak and Wajdzik (2009), who pointed out significant differences in the dimensions of the skull between ecotypes, while on the other hand Sabalinkiene *et al.* (2017) emphasized the lack of such differences. Also Aragon *et al.* (1998), who studied the variability of roe deer skulls throughout Europe showed that individuals with comparatively short and wide skulls feeds selectively on trees and shrubs throughout the year in opposition to individuals with longer and narrow skulls feeding both on woody and herbaceous plants. It is worth noting that researchers indicating significant differences in cranial dimensions between ecotypes showed them within the gnatostomatic apparatus, such as the length of upper and lower teeth row, mandible length. Thus it can be expected also the differences in dental related features as enamel thickness, which is an evolutionary plastic trait and capable to rapid adaptation in response to diet change (Hlusko 2004). Therefore an attempt this study was made here to elucidate the influence of dwelling in forest and field habitats on enamel thickness in roe deer. We expected that roe deer from field ecotype may have a thinner enamel as and less differences between age groups as an adaptation to consume more herbaceous plants. We verified this assumption on a sample of females that are less mobile than males and are therefore strongly associated with the habitat occupied (Ellenberg 1978 and Kurt 1991).

The study was conducted on the first lower permanent molar (M_1) of 49 females of roe deer obtained during regular hunt and management culls in the six Game Management Centres (Central Poland): Brzeziny (51°45′N, 19°43′E, Kolumna (51°34′N, 19°13′E), Kutno (52°14′N, 19°08′E), Poddębice (51°54′N, 18°53′E), Smardzewice (51°26′N, 19°60′E), Spała (51°31′N, 20°11′E), managed by the Regional Directorate of State Forest in Lodz (N = 21 molars) and one from Lithuania – Vilnius district (54°41′N, 25°35′E) managed by the local Vilnius Hunting and Fishing Department (N = 28 molars) during the period 2009 to 2014.

Central Poland is situated in transitory belt between Central Polish Lowland and Polish Upland in the south and the less diverse fragment of Lakeland in the north (Kondracki 2002). Afforestation of Game Management Centers varied from 26.7 to 61.2%; except Kutno where only 0.1% area is forested. In Central Poland dominate the coniferous stands (65%) represents mainly by fresh coniferous forest (30%) and fresh mixed coniferous forest (26.5%). The habitat with deciduous forest stands cover 35% of forest area and the most common is fresh forest – 20.6% (WIOS 2004). Vilnius district is located in southeast Lithuania within the East Baltic-Belarussian Lowlands (Kondracki 2002), and afforested in 80% with predomination of fresh coniferous stands (http://vilmu.lt/about-us/about-us).

The age hunted animals was estimated by dental wear (Pielowski 1999) and corrected by the experts of the Regional Commission for Hunting trophies in Lodz and Vilnius.

In total, 49 female mandibles of roe deer aged between 2 and 8 years were at our disposal. The first molars (M_1) were extracted from the alveolus – mainly left (n = 42), but in a cases when the tooth was mechanically damage or exhibited pathological changes we used the tooth from the right side (n = 7).

Following this, the histological samples were prepared. Each tooth was degreased in a bath containing 70% alcohol for 24 hours and dried with compressed oil-free air. Sections of the collected teeth were cut off using a 0.5 mm diamond-wafering blade (BuehlerIsoMet 1000) after prior embedding in epoxy resin (Biodur®). The molars were sectioned along the long axis in the labiolingual plane. The sections passed through the highest point of the posterior (distal) cusp. The cutting surface of one tooth half was polished using a series of abrasive papers (grades 600, 1200, 2400, 5000) and polishing paste, and were mounted on a glass slide. The block was then removed from the slide using a rotary saw, leaving behind an approximately 300 μm thick section. This section was ground and polished to a final thickness of approximately 100 μm and cover-slipped.

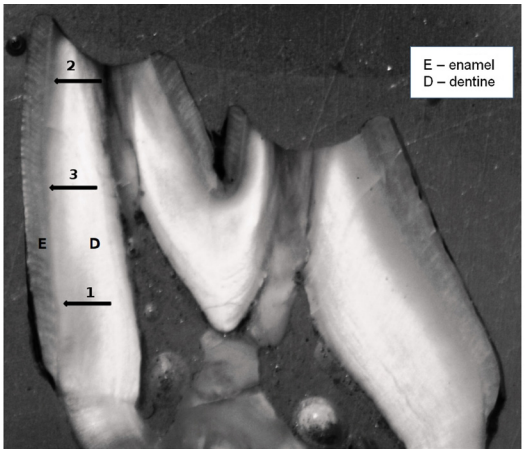

Fig. 1. A distal cusp of the lower molar of European roe deer *Capreolus capreolus* showing measurements of enamel thickness at buccal surface at: 1) the cervical level; 2) the lateral region near to the dentin horn; 3) the midpoint between these two levels.

The sections were examined under an optical microscope (Delta Optical Evolution 300) under 10× and 40× magnification. For each section, photographs were taken with a Delta Optical HDCE – 50B camera attached to the light microscope with an apochromatic objective lens 40×/0.65 ∞/0.17.

The thickness of the enamel was initially measured in the lingual and buccal surface at three levels from the cement-enamel junction: at the cervical level, at the lateral region near to the dentin horn, and at the midpoint between these two levels (Fig. 1). The analysis of the measurements in the subsample (n = 10) revealed a significant correlation between the buccal and lingual sides of the distal cusp (r = 0.64, P = 0.045, n = 10); therefore, further measurements were performed on the lingual surface.

All measurements were taken at the distal cups, along enamel prisms. For each tooth, the mean enamel thickness (MET) was calculated from the measurements performed at the described locations on the crown. All enamel measurements were performed by one researcher (PP) using Coolview 2.0 software with an accuracy of up to 0.1 mm. All sections were re-measured twice, with negligible discrepancies (Fig. 1)

Because the measurements of enamel thickness: a) concerned the same in terms of type and location tooth (M_1); b) in our material there was no significant correlation between the enamel thickness and tooth size as height of lingual cusp (r = -0.421, P = 0.825, n = 30), we used directly calculated its mean

value to compare this parameter between ecotypes value.

The values of all the analysed measurements of enamel linear thickness were characterised by a homogeneity of variance ($P > 0.05$ in the Levene test) and a normal distribution ($P > 0.05$ in the Shapiro-Wilk test). For the assessment of differences in enamel parameters between roe deer sample from Poland and Lithuania, the Student's t-test was used. As no significant differences were observed between the two samples ($t = 1.07$, $df = 32$, $P = 0.29$), all specimens from the two countries were gathered together for further analysis.

The relationship between the thickness of the enamel and ecotype was analysed with general linear mixed models (GLMMs). Ecotype (field when < 35% of habitat are covered by forests (n = 14; only in Poland); forest when ≥ 35% of habitat are covered by forests (in Poland n = 7; in Lithuania n = 28) and age class (Class 1 – one to three years; Class 2 – over three years) were included as fixed factors. We also included interactions between ecotype and age. Since data from each location are not non-independent, we added sampling location as a random factor in the analysis. The effect of location was nested within ecotype, as all specimen collected from each location were associated with a single ecotype (either field or forest).

All statistical analyses were performed using STATISTICA 12.0 software.

The individuals living in the field ecotype displayed lower values for mean enamel thickness than those of the forest ecotype. The mean thickness of enamel was 0.19 mm ± 0.63 SE in the field ecotype deer (minimum 0.13 mm and maximum 0.23 mm) and 0.21 mm ± 0.52 SE in the forest ecotype deer (minimum 0.15 mm, maximum 0.29 mm). The mean thickness was significantly thinner in field ecotype deer ($F_{(1,26)} = 6.845$, $P = 0.025$; Fig. 2). Regarding age, the mean thickness was found to be 0.22 mm ± 0.70 SE in younger deer and 0.20 mm ± 0.52 SE in older deer. Age was not found to have any general effect on enamel thickness ($F_{(1,26)}=1.412$, $P = 0.242$) and no significant relationship was found between age-ecotype interaction and enamel thickness ($F_{(1,26)} = 1.904$, $P = 0.176$). The mean thickness did not vary significantly with sampling location ($F_{(1,26)} = 0.82$, $P = 0.543$).

Our results are consistent with the hypothesis that thickness of dental enamel indicating that the habitat in which an animal lives can affect facilitates a diet resistant to fracture. Our findings indicate that dental enamel tended to be thinner in the field roe deer, suggesting that the habitat in which an animal lives can affect the thickness of tooth enamel. In addition, no significant relationship was found between thickness of enamel and age, indicating that the difference between ecotypes does not result in abrasion of the enamel; however, it is possible that microevolutionary adaptations can occur in response to differences in diet.

The development of the first permanent molars begins in the prenatal period (about one month *in utero*) and the crown of the tooth is completed four months after birth. Females usually give birth in June, typically to two fawns, after a 10-month gestation period. Both sexes reach sexual maturity in the second year, which coincides with the growth of antlers. As the first molar is formed early, it is unlikely that pregnancy or antler formation are responsible for disturbances in its enamel layer (Pielowski 1999).

Tooth wear is caused by a cumulative loss of enamel and dentine caused by the action of opposing teeth and the friction of hard and abrasive food objects (Lucas *et al*. 2008). The course of tooth wear is connected with the consumption of hard food objects; the energy needed in the process of effective chewing and the direction of energy both influence the surface of the tooth (Maas and Dumont 1999). In the case of herbivores, the degree of tooth attrition significantly influences the biological condition. A high level of tooth attrition will influence the effectiveness of mastication, and hence the quantity of nutrition absorbed by the animal. Research conducted on koala bears by Logan and Sanson (2002) has shown that scavenging time may extend in response to changes in enamel thickness, and Veiberg *et al*. (2007) report that roe deer animals demonstrating a lower level of tooth attrition have a longer expected lifetime. The survival and reproduction of animals is dependent on condition of the teeth. Kaiser *et al*. (2008) in his article argues that captive individuals have harder-to-kill teeth than free animals that have a more varied diet. He

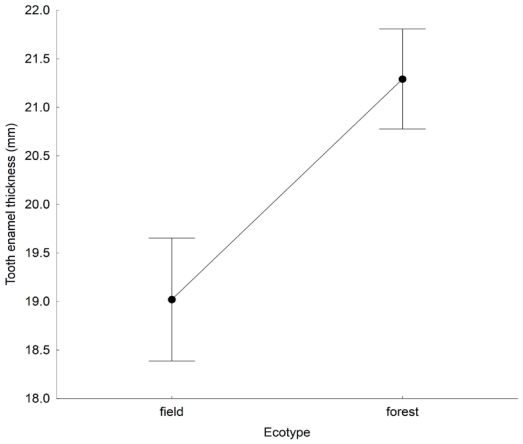

Fig. 2. Differences in tooth enamel thickness between two ecotypes of European roe deer (*Capreolus capreolus*). Means ± SE are presented. $F_{(1,26)} = 6.845$, $P = 0.025$.

argues that the non-natural tooth wear pattern can be important for animals released in re-stocking programs. Burak *et al.* (1999) reports that food intake is important in the abrasion process.

The first molars of roe deer wear down earlier than other molars. In addition, the thickness of enamel has been found to vary between associated forms of primates and this variation can be related to the hardness of food intake (Dumont 1995). This was confirmed by Vogel *et al.* (2008) in a study based on the analysis of food intake by chimpanzees and orangutans. A comparison of the diet and enamel thickness revealed that chimpanzees consuming a greater proportion of soft parts of plants are characterized by thinner enamel than gorillas, who tend to consume the harder parts of plants.

Although differences in enamel thickness between field and forest roe deer are likely to be primarily explained with variation in diet, we cannot exclude the role other mechanisms. For example, roe deer from the field habitat can be also more exposed to stress, due to the lower possibility to hide or escape from danger (own observations). Numerous studies of wild living animals have shown that the noises caused by motor vehicles and some human activities can result in elevated levels of the stress hormone cortisol (Creel *et al.* 2002, Barja *et al.* 2007, Freeman 2008, Zwijacz-Kozica *et al.* 2013) and higher levels of such metabolites as glycogen and lipid products (Majewska *et al.* 1982). Cortisol is a glucocorticoid, an example of a class of compounds which allow the organism to adapt to stress and maintain homeostasis. Moreover, it has an anti-inflammatory and immunosuppressive activity, inhibits bone formation and delays healing (Fabue *et al.* 2010). An increased level of cortisol reduces the absorption of calcium from the gastrointestinal tract and reduces its concentration in the blood. Such a reduction has been found to result in calcium being expelled in the urine (Górski 2010). Research has shown greater bone mineral density loss when higher cortisol levels are found in the blood (Dennison *et al.* 1999). Also, Markowski (1993) reports that field ecotypes tend to demonstrate a higher level of variable asymmetry, which is widely used as an indicator of developmental stability, one that defines the potential of the body to accurately express genetically-determined developmental pathways, despite environmental disturbances . This was taken into account in a discussion of environmental stress.

This situation could also lead to the disruption of tooth development, because calcium receptors (CaSR) are located in ameloblasts, allowing them to react to disorders in the level of calcium. Kukita *et al.* (1992) report that primary cultured rat ameloblasts responded to high concentrations of Ca^{2+} by changing their morphology, suggesting that the process of enamel maturation could be controlled by the rate of transport through the ameloblasts into the matrix. Mathias *et al.* (2001) note that CaR was expressed in ameloblasts in the early secretory stage; hence, extracellular Ca^{2+} may help modulate the function of early secretory ameloblasts. Therefore, the administration of corticosteroids to rats resulted in accentuated surface perikymata and the increased spacing of incremental lines (Hallett and Hall 1995). More frequent exposure to stress can significantly disrupt ameloblasts and thus affect the width of the enamel.

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4. Dorobek naukowy

4.1. Artykuły lista A MNiSW

- Jan Demesko, Janusz Markowski, Mirosława Słaba, Janusz Hejduk, Piotr Minias. 2017. Age Related Patterns in Trace Element Content Vary Between Bone and Teeth of the European Roe Deer (*Capreolus capreolus*). Archives of Environmental Contamination and Toxicology 74(2): 330-338. DOI: 10.1007/s00244-017-0470-1
- Jan Demesko, Janusz Markowski, Eva Demesko, Mirosława Słaba, Janusz Hejduk, Piotr Minias. 2018. Ecotype Variation in Trace Element Content of Hard Tissues in the European Roe Deer (*Capreolus capreolus*). Archives of Environmental Contamination and Toxicology 76(1): 76-86. DOI: 10.1007/s00244-018-0580-4
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4.2. Postery

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5. Oświadczenia współautorów

Łódź, dn. 14.05.2020r.

Jan Demeško
Katedra Badania Różnorodności Biologicznej
Dydaktyki i Bioedukacji
Wydział Biologii i Ochrony Środowiska
Uniwersytet Łódzki
ul. Banacha 1/3
90-237 Łódź

Oświadczenie o udziale w publikacji

Oświadczam, że jestem współautorem artykułu:

Jan Demesko, Janusz Markowski, Mirosława Słaba, Janusz Hejduk, Piotr Minias. 2017. Age Related Patterns in Trace Element Content Vary Between Bone and Teeth of the European Roe Deer (*Capreolus capreolus*). Archives of Environmental Contamination and Toxicology 74(2): 330-338. (IF 2017 = 2,497, punkty MNiSW = 25, lista A),

a mój udział w pracy oceniam na 45%. Polegał na: zbieraniu materiału, przeprowadzeniu badań, pisaniu artykułu.

Demeško Jan

Łódź, dn. 14.05.2020r.

Jan Demeško
Katedra Badania Różnorodności Biologicznej
Dydaktyki i Bioedukacji
Wydział Biologii i Ochrony Środowiska
Uniwersytet Łódzki
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Demeško Jan

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Jan Demeško
Katedra Badania Różnorodności Biologicznej
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a mój udział w pracy oceniam na 30%. Polegał na: zbieraniu materiału, przeprowadzeniu badań, pisaniu artykułu.

Demeško Jan

Łódź, dn. 14.05.2020r.

Janusz Markowski
Katedra Badania Różnorodności Biologicznej
Dydaktyki i Bioedukacji
Wydział Biologii i Ochrony Środowiska
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Łódź, dn. 14.05.2020r.

Janusz Markowski
Katedra Badania Różnorodności Biologicznej
Dydaktyki i Bioedukacji
Wydział Biologii i Ochrony Środowiska
Uniwersytet Łódzki
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Janusz Markowski
Katedra Badania Różnorodności Biologicznej
Dydaktyki i Bioedukacji
Wydział Biologii i Ochrony Środowiska
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Łódź, dn. 14.05.2020r.

Dr hab. Piotr Minias, prof. UŁ
Katedra Badania Różnorodności Biologicznej
Dydaktyki i Bioedukacji
Wydział Biologii i Ochrony Środowiska
Uniwersytet Łódzki
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a mój udział w pracy oceniam na 20%. Polegał na współudziale w analizie wyników, a także na współtworzeniu artykułu i jego korekcie.

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Dr hab. Piotr Minias, prof. UŁ
Katedra Badania Różnorodności Biologicznej
Dydaktyki i Bioedukacji
Wydział Biologii i Ochrony Środowiska
Uniwersytet Łódzki
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Łódź, dn. 14.05.2020r.

Eva Demesko
Wydział Lekarsko - Dentystyczny
Uniwersytet Medyczny w Lublinie
ul. Chodźki 19
20-093 Lublin

Oświadczenie o udziale w publikacji

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Jan Demesko, Janusz Markowski, Eva Demesko, Mirosława Słaba, Janusz Hejduk, Piotr Minias. 2018. Ecotype Variation in Trace Element Content of Hard Tissues in the European Roe Deer (*Capreolus capreolus*). Archives of Environmental Contamination and Toxicology 76(1): 76-86. (IF 2018 = 2,497, punkty MNiSW = 25, lista A),

a mój udział w pracy oceniam na 5%. Polegał na: zbieraniu danych w terenie, przygotowaniu bazy danych.

Eva Demesko

Łódź, dn. 14.05.2020r.

Janusz Hejduk
Katedra Badania Różnorodności Biologicznej
Dydaktyki i Bioedukacji
Wydział Biologii i Ochrony Środowiska
Uniwersytet Łódzki
ul. Banacha 1/3
90-237 Łódź

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a mój udział w pracy oceniam na 5%. Polegał na:
zbieraniu i preparowaniu czaszek sarny użytych do analiz oraz stworzeniu bazy danych.

Łódź, dn. 14.05.2020r.

Janusz Hejduk
Katedra Badania Różnorodności Biologicznej
Dydaktyki i Bioedukacji
Wydział Biologii i Ochrony Środowiska
Uniwersytet Łódzki
ul. Banacha 1/3
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a mój udział w pracy oceniam na 5%. Polegał na:
zbieraniu i preparowaniu czaszek sarny użytych do analiz oraz tworzeniu bazy danych.

Łódź, dn. 14.05.2020r.

Marta Kurek
Katedra Antropologii
Wydział Biologii i Ochrony Środowiska
Uniwersytet Łódzki
ul. Banacha 1/3
90-237 Łódź

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a mój udział w pracy oceniam na 30%. Polegał na: przygotowaniu preparatów odontologicznych, pomiarach parametrów szkliwa na zdjęciach wykonanych pod mikroskopem optycznym, przygotowaniu bazy danych oraz części wstępu i dyskusji.

Kurek Marta

Łódź, dn. 14.05.2020r.

Mirosława Słaba
Katedra Mikrobiologii Przemysłowej i Biotechnologii
Wydział Biologii i Ochrony Środowiska
Uniwersytet Łódzki
ul. Banacha 12/16
90-237 Łódź

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a mój udział w pracy oceniam na 10%. Polegał na:

- konsultacjach odnośnie zakupu elementówniezbędnych do homogenizacji prób,
- homogenizacji prób w homogenizatorze kulowym,
- korekcie i uściśleniu opisu metody homogenizcji.

Łódź, dn. 14.05.2020 r.

Mirosława Słaba
Katedra Mikrobiologii Przemysłowej i Biotechnologii
Wydział Biologii i Ochrony Środowiska
Uniwersytet Łódzki
ul. Banacha 12/16
90-237 Łódź

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a mój udział w pracy oceniam na 5%. Polegał na:

- homogenizacji materiału,
- wprowadzeniu niewielkich korekt końcowej wersji manuskryptu

M. Słaba

Łódź, dn. 14.05.2020r.

Patrycja Podlaszczuk
Katedra Badania Różnorodności Biologicznej
Dydaktyki i Bioedukacji
Wydział Biologii i Ochrony Środowiska
Uniwersytet Łódzki
ul. Banacha 1/3
90-237 Łódź

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a mój udział w pracy oceniam na 20%. Polegał na współudziale w pomiarach szkliwa, analizie statystycznej, pisaniu i redagowaniu manuskryptu.