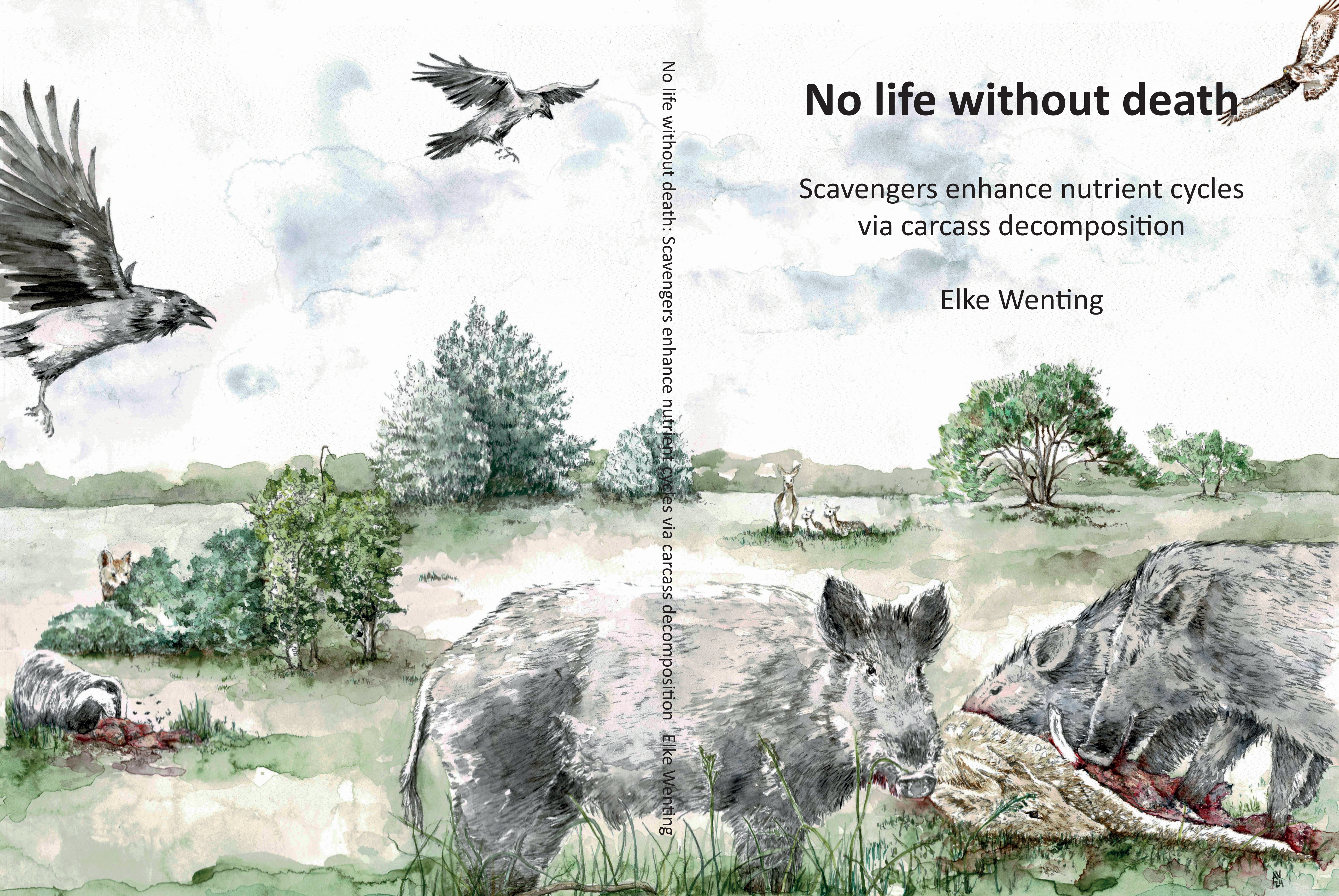

No life without death

Scavengers enhance nutrient cycles via carcass decomposition

Elke Wenting

No life without death: Scavengers enhance nutrient cycles via carcass decomposition Elke Wenting

Propositions

1. The circle of life of large animals is the major cause of heterogeneity in the landscape.
   (this thesis)

2. Biomonitoring based on a single element in a single tissue provides more uncertainty than useful insights.
   (this thesis)

3. Descriptive research deserves more appreciation.

4. Obtaining a PhD degree is a test of resilience and creativity rather than intelligence.

5. Productivity is independent of the number of working hours.

6. Mutual respect is the most important ingredient for productive cooperation.

Propositions belonging to the thesis, entitled:

No life without death: Scavengers enhance nutrient cycles via carcass decomposition

Elke Wenting
Wageningen, 7 June 2024

No life without death

Scavengers enhance nutrient cycles via carcass decomposition

Elke Wenting

Thesis committee

Promotors:
Prof. Dr F. van Langevelde
Professor of Wildlife Ecology and Conservation
Wageningen University & Research

Prof. Dr H. Siepel
Professor of Animal Ecology
Radboud University, Nijmegen

Co-promotor:
Dr P. A. Jansen
Associate professor, Wildlife Ecology and Conservation Group
Wageningen University & Research

Other members:
Prof. Dr L. Mommer, Wageningen University & Research
Prof. Dr H. Olde Venterink, Free University of Brussels, Belgium
Dr P. Mateo-Tomás, Oviedo University, Spain
Dr J. Sitters, Research centre B-WARE, Nijmegen

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No life without death

Scavengers enhance nutrient cycles via carcass decomposition

Elke Wenting

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Summary / Samenvatting

Summary

Macro elements such as nitrogen (N) and phosphorous (P) are no longer the major limiting factors of primary production in many regions, including the Netherlands. Increased soil acidification, partially due to excessive N deposition, has resulted in imbalanced availability of chemical elements. These elements can be essential for all life forms but scarce in nature, ecotoxic when taken up in excessive amounts, or both. The consequences of decades of acidification are becoming increasingly evident, and management efforts are (experimentally) applied to restore elemental cycles in protected areas. However, although animals play a crucial role in elemental cycles, their actual importance is still poorly understood.

I hypothesised that (vertebrate) animals play a crucial role in terrestrial elemental cycles by accumulating a wide range of elements in their bodies over their entire lifespan. These accumulated elements are released as carrion when animals die. The interplay between animals consuming carrion ("scavengers") and other decomposers ("detritivores") determines the fate of the carrion-stored elements. When consumed by scavengers, the elements are redistributed over larger areas before entering the soil via e.g. faeces. When scavengers are absent or too slow in colonising the carrion, detritivores would dominate the decomposition process. When dominated by detritivores, most carrion fluids leak locally into the soil. Plants take up elements from the soil, but there is a chance the elements leach into deeper layers when concentrations become too high. Then, the elements might become unavailable for plants and will not be invested in above-ground biomass. Thus, the presence of scavengers would strongly influence the fate of carrion-stored elements. However, their importance in determining the fate of the carrion-stored elements has never been investigated.

In this thesis, I address the three most important knowledge gaps concerning the importance of scavengers in the cycle of carrion-stored elements. First, I examine the elemental composition of (larger-sized) mammals - the "ionome" -, determining whether carcasses contain - as assumed - considerable concentrations of scarce elements that are valuable for other animals (including scavengers). Second, I examine whether the structure of the local scavenger community is important for carcass exploitation by vertebrate facultative scavengers, which would strongly influence via which pathway - dominated by scavengers or detritivores - carrion-stored elements cycle through ecosystems. Third, I examine whether a wide range of elements leak into the soil due to carrion decomposition, and whether that is influenced by the presence of scavengers. Particularly, I focus on Dutch

protected areas that are characterised by imbalanced nutrient availability, the absence of obligate scavengers and the absence of large carnivores.

In Chapter 2-4, I explore the first knowledge gap by examining the ionome of larger-sized mammals to assess the elements that eventually enter the carrion pool. Therefore, we dissected carcasses from various mammal species to collect samples of different organs and tissues, that we analysed for their elemental concentrations.
In Chapter 2, we assessed the ionome of fallow deer (*Dama dama*) and Eurasian otter (*Lutra lutra*), two species that differ considerably in their diet and prevalence, and found considerable variation between and within these species. This disproves the widely-held assumption that the elemental composition of animals is under relative tight homeostatic control and hardly variable.
We further explored the ionomic variation between species by assessing the ionome of red deer (*Cervus elaphus*) and wild boar (*Sus scrofa*), two co-occurring ungulates, in Chapter 3. Again, we found considerable ionomic variation between and within these species, with no single tissue accurately representing the elemental concentration in the entire animal. This questions commonly used methods of biomonitoring potential toxicities and deficiencies in (protected) areas. Moreover, a serious lack of reference values hindered us to put our findings in the context of their biological meaning.
In Chapter 4, we further explored the ionomic variation within species, focussing on the ionome of fallow deer - obtained from a single protected area - of different sex and age classes. We found that some, but not most, of the ionomic variation was related to sex and age.
Thus, based on the insights into the mammalian ionome, I concluded that the ionome of vertebrate animals - thus carcasses after death - can significantly vary. Elemental leakage due to carrion decomposition is expected to be highly variable as well.

In Chapter 5-6, I explore the second knowledge gap by examining the importance of the local scavenger community on the carrion decomposition speed, which is important in determining the fate of carrion-stored elements.
In Chapter 5, we evaluated the scavenger community in Dutch protected areas. We monitored fresh carcasses until depletion with camera traps, and monitored the vertebrate scavengers that visited the carcasses. The camera trap videos were annotated for the scavenger species, their behaviour and preferred tissues, and the stage of decay at the moment of visiting. We used these characteristics to identify groups of species with similar roles during the carcass decomposition process (“functional scavenger groups”). Linking the presence of these groups to the carcass decomposition speed, wild boar appeared to be the only scavenger species of which their presence can significantly speed up the carrion decomposition process.

Chapter 6 built on the results of Chapter 5, by examining the influence of tree cover - a habitat characteristic that might be important for scavengers - on carcass exploitation by different scavenger groups. The results showed that enhanced carcass exploitation, especially by wild boar, speed up the decomposition speed, but that this was not related to the amount of tree cover.
Thus, based on these results, I conclude that wild boar is an important scavenger species in Dutch protected areas and that carcass exploitation is not necessarily driven by habitat characteristics like the amount of tree cover.

In Chapter 7-8, I explore the third knowledge gap by empirically examining the elements that leak into the soil during carrion decomposition, and whether that is influenced by the presence of scavengers.
In Chapter 7, we tested whether leakage of a wide range of elements is measurable. We were able to measure higher elemental concentrations beneath decomposing dead mice. Maize plants grew up to nine times higher on the soils enriched with carrion fluids, indicating that elemental leakage from decomposing carrion can locally result in increased biomass production.
Chapter 8 reports a field experiment in which we investigated whether the elemental leakage of carrion fluids is influenced by the presence of different scavenger groups. We systematically excluded different subsets of vertebrate scavengers from decomposing carcasses: we excluded (1) no scavengers; (2) wild boar; (3) all mammals; and (4) all mammals and birds. When all vertebrates were excluded, we found that leakage of several (trace) elements showed distinct peaks. This implies that carrion-derived elements are dispersed over larger areas via vertebrate scavengers when they dominate the process, rather than locally leaking into the soil beneath the carrion. The scarcest elements, e.g. Cu and Zn, are particularly influenced by local carrion decomposition.
Thus, based on the insights into the carrion-related elemental leakage, I conclude that a wide range of (scarce) elements can indeed leak into the soil as a result of carrion decomposition, which especially occurs when invertebrate scavengers (insects) dominate the decomposition process.

I concluded that the overarching theme of my thesis is substantial variation: variation in the mammalian ionome (Chapter 2-4), variation in the carrion decomposition speed and scavenger behaviour (Chapter 5-6), and variation in the elemental leakage into the soil (Chapter 7-8). Larger-sized animals create and maintain the variation - "heterogeneity” - of the landscape through their natural circle of life and death.

Samenvatting

Macro-elementen zoals stikstof (N) en fosfor (P) zijn niet langer de voedingstoffen die het meest limiterend zijn voor primaire productie (biomassagroei) in veel natuurgebieden in de wereld, waaronder in Nederland. Overmatige stikstofdepositie heeft geleid tot een verstoring van de balans in de beschikbaarheid van voedingsstoffen in de natuur. Dit geldt voor alle essentiële voedingsstoffen die van nature schaars zijn maar die alle vormen van leven nodig hebben, chemische elementen die giftig zijn als ze in hoge concentraties worden opgenomen, of beide. De nadelige gevolgen van decennialange verzuring worden steeds duidelijker en om die tegen te gaan, worden steeds meer beheermaatregelen (experimenteel) toegepast om de kringloop van schaarse voedingsstoffen in natuurgebieden te herstellen. Dieren lijken een cruciale rol te spelen in deze kringlopen, maar hun daadwerkelijke rol wordt tot op heden nauwelijks begrepen.

In dit proefschrift onderzoek ik de hypothese dat grote dieren een cruciale rol spelen in de kringloop van (schaarse) voedingsstoffen doordat ze een breed scala aan voedingsstoffen in hun lichaam opslaan gedurende hun hele leven. Deze voedingsstoffen komen beschikbaar in de vorm van aas ("kadavers") op het moment dat dieren doodgaan. De wisselwerking tussen dieren die aas consumeren ("aaseters") en andere afbrekers ("detritivoren") bepaalt wat er gebeurt met de voedingsstoffen die in kadavers liggen opgeslagen. Als aaseters de kadavers opeten, worden de voedingsstoffen opgeslagen in de lichamen van deze aaseters. Op die manier worden de voedingsstoffen over een groter gebied verspreid, bijvoorbeeld via de uitwerpselen van deze aaseters, of via hun kadavers wanneer de aaseters doodgaan. Maar als het ontbindingsproces wordt gedomineerd door detritivoren, lekt een groot deel van de voedingsstoffen de bodem in onder het kadaver. Planten nemen de voedingsstoffen op uit de bodem. De kans bestaat dat de concentraties zó hoog worden, dat de plantenwortels de voedingsstoffen niet meer kunnen opnemen. Daardoor kunnen ze uitspoelen naar diepere lagen van de bodem. Plantenwortels kunnen er dan niet meer bij. Als dat gebeurt, kunnen planten ze niet meer opnemen en op die manier investeren in (bovengrondse) biomassa en is er sprake van een nettoverlies aan voedingsstoffen in het ecosysteem. Dat betekent dat de aanwezigheid van aaseters een grote invloed kan hebben op het lot van de (schaarse) voedingsstoffen die liggen opgeslagen in kadavers. Het is echter nog nooit onderzocht hoe groot dit veronderstelde effect is.

In dit proefschrift behandel ik de drie belangrijkste kennislacunes met betrekking tot het belang van aaseters in de kringloop van voedingsstoffen. Ten eerste onderzoek ik de elementaire samenstelling van (grotere) zoogdieren - het "ionoom" -, waarbij ik bestudeer of kadavers inderdaad aanzienlijke concentraties aan schaarse voedingsstoffen bevatten en daardoor een waardevolle voedselbron zijn voor

aaseters. Ten tweede onderzoek ik of de samenstelling van de lokale aasetergemeenschap belangrijk is voor de manier waarop gewervelde aasetersgebruik maken van kadavers. Dit heeft waarschijnlijk een groot effect op via welke route de kadavers, inclusief alle opgeslagen voedingsstoffen, worden gerecycled: via aaseters of via detritivoren. Ten derde onderzoek ik of een breed scala aan (schaarse) voedingsstoffen in de bodem lekt ten gevolge van de ontbinding van kadavers en hoe de aanwezigheid van aaseters dat beïnvloedt. Ik heb me hoofdzakelijk gericht op Nederlandse natuurgebieden, die worden gekenmerkt door een onbalans in de beschikbaarheid van voedingsstoffen, de afwezigheid van obligate aaseters (aaseters die afhankelijk zijn van aas, zoals gieren) en de afwezigheid van grote carnivoren.

In Hoofdstuk 2-4 wordt de eerste kennislacune onderzocht: de elementaire samenstelling van grote zoogdieren. De voedingsstoffen die in deze dieren liggen opgeslagen, zijn ook de voedingsstoffen die uiteindelijk beschikbaar komen in de vorm van kadavers. Hiertoe hebben we kadavers van verschillende zoogdieren ontleed en monsters genomen van hun organen en weefsels, die we (dat wil zeggen, ikzelf samen met de coauteurs) hebben geanalyseerd op hun elementaire samenstelling.
In Hoofdstuk 2 richten we ons op de elementaire samenstelling van damherten (*Dama dama*) en Europese otters (*Lutra lutra*). Dit zijn twee diersoorten die enorm van elkaar verschillen wat betreft dieet en leefomgeving. We vonden aanzienlijke variatie, zowel tussen de diersoorten als tussen individuen per soort. Dit weerlegt de wijdverbreide aanname dat de elementaire samenstelling van dieren nauwelijks variabel is - in tegenstelling tot planten - ten gevolge van homeostatische controlemechanismen.
We hebben de variatie in elementaire samenstelling verder onderzocht in Hoofdstuk 3, waar we ons richtten op het ionoom van edelherten (*Cervus elaphus*) en wilde zwijnen (*Sus scrofa*). Dit zijn twee diersoorten - beide hoefdieren - die voorkomen in hetzelfde leefgebied, maar een verschillend dieet en spijsverteringskanaal hebben. Opnieuw vonden we aanzienlijke variatie in de elementaire samenstelling van deze diersoorten, zowel tussen de soorten als tussen de individuen. Geen enkel weefsel gaf de elementaire concentratie in het hele dier nauwkeurig weer en was een goede proxy voor de totale concentratie in een dier. Dit zet vraagtekens bij veelgebruikte methoden voor biomonitoring, waarbij bepaalde weefsels en organen (zoals lever en nieren) van dieren worden gebruikt als maatstaf voor potentieel giftige stoffen en mogelijke tekorten in dieren en in natuurgebieden. Bovendien belemmerde een ernstig gebrek aan referentiewaarden ons om de gemeten concentraties in de context van hun biologische betekenis te plaatsen.
In Hoofdstuk 4 hebben we de elementaire variatie binnen één diersoort verder onderzocht. We gebruikten damherten, afkomstig uit hetzelfde natuurgebied, van verschillende geslachten en verschillende leeftijden. We ontdekten dat een deel van de variatie binnen een diersoort waarschijnlijk verband houdt met geslacht en/of leeftijd.
Op basis van de inzichten uit Hoofdstuk 2-4 concludeer ik dat de elementaire samenstelling van zoogdieren aanzienlijk kan variëren. Aangezien dit de voedingsstoffen zijn die

uiteindelijk via de ontbinding van kadavers beschikbaar komen, verwachtte ik dat de voedingsstoffen die de bodem in lekken tijdens de ontbinding van kadavers ook zeer variabel zullen zijn.

In Hoofdstuk 5-6 wordt de tweede kennislacune onderzocht: het belang van de lokale aasetergemeenschap op de ontbindingstijd van kadavers. De ontbindingstijd is een belangrijke indicatie voor het lot van de voedingsstoffen die liggen opgeslagen in kadavers: worden deze gerecycled via aaseters, of via detritivoren?
In Hoofdstuk 5 onderzoeken we de aasetergemeenschap in Nederlandse natuurgebieden. Hiertoe hebben we verse kadavers gemonitord totdat ze volledig waren vergaan. Met behulp van cameravallen volgden we het ontbindingsproces. De video's die werden opgenomen door deze cameravallen werden geanalyseerd op: (1) de diersoort - gewervelde aaseters - die het kadaver bezocht; (2) het gedrag dat deze aaseter vertoonde richting het kadaver; (3) indien van toepassing, de weefsels die werden opgegeten; en (4) het ontbindingsstadium op het moment van opname. Deze gegevens gebruikten we om de aanwezige aaseters in functionele groepen in te delen ("functionele aasetergroepen"). Dit zijn groepen met diersoorten die een vergelijkbaar gedrag vertoonden ten opzichte van de kadavers en daardoor een vergelijkbaar effect hebben op het ontbindingsproces. Er konden vijf aasetergroepen worden onderscheiden: (1) wilde zwijnen; (2) andere zoogdieren; (3) vogels; (4) kadavergasten; en (5) grote herbivoren. Daarna hebben we getest of de aan- of afwezigheid van de verschillende aasetergroepen gecorreleerd was aan de ontbindingstijd. We vonden dat wild zwijn de enige aaseter was die het ontbindingsproces significant kon versnellen. Maar het gedrag van aaseters is onvoorspelbaar: de aanwezigheid van wilde zwijnen betekende niet dat zij kadavers volledig opaten.
In Hoofdstuk 6 hebben we, voortbordurend op de resultaten van Hoofdstuk 5, onderzocht of de kroonbedekking belangrijk is voor de activiteit van aaseters. Dit was niet het geval. De activiteit - detectietijd en eetgedrag - van aaseters was wél belangrijk. Vooral als wilde zwijnen erg actief waren en veel van het kadaver aten, leidde tot een versneld afbraakproces.
Op basis van de inzichten uit Hoofdstuk 5-6 concludeer ik dat wilde zwijnen een belangrijke aaseter zijn in Nederlandse natuurgebieden. De mate waarin gewervelde aaseters actief zijn wordt niet bepaald door omgevingsfactoren zoals kroonbedekking, maar wel door de interacties tussen aaseters en hun eetgedrag.

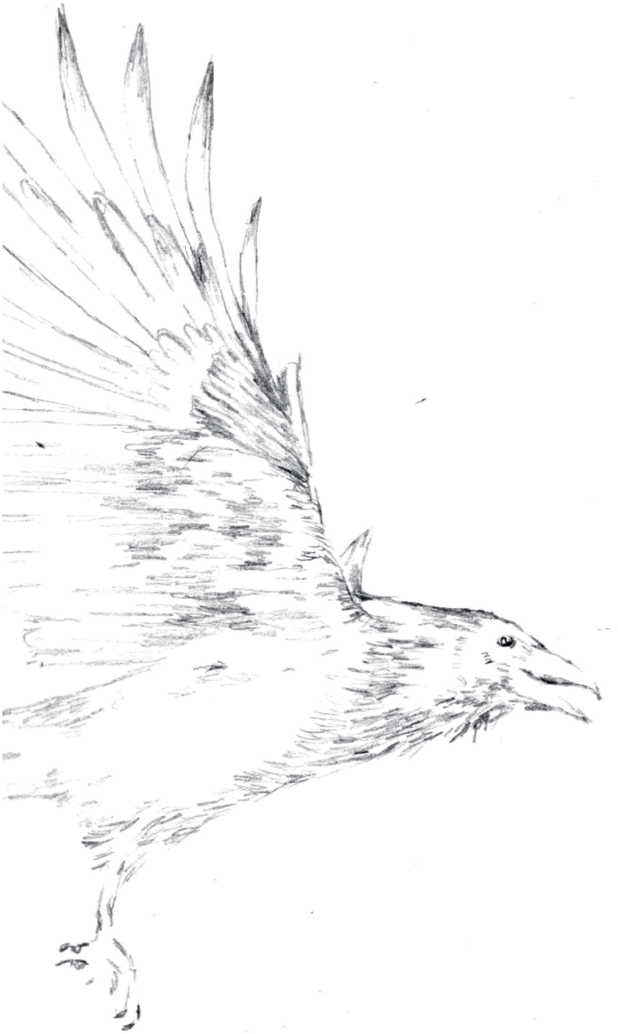
In Hoofdstuk 7-8 wordt de derde kennislacune onderzocht: in welke mate lekken voedingsstoffen de bodem in ten gevolge van ontbindende kadavers en wordt beïnvloed door de aanwezigheid van gewervelde aaseters?
In Hoofdstuk 7 hebben we getest of voedingsstoffen die uit een kadaver lekken meetbaar zijn in de bodem. Dit hebben we gedaan door middel van een experiment in een semi-

gecontroleerde omgeving. Als aas gebruikten we dode muizen. Het was inderdaad mogelijk om hogere concentraties van voedingsstoffen te meten onder de ontbindende muizen. Ook vonden we dat maïsplanten tot negen keer harder groeiden op bodems die waren verrijkt met kadavervocht. Dit betekent dat de ontbinding van kadavers kan leiden tot een verhoogde biomassaproductie op de plek waar een kadaver heeft gelegen.

Hoofdstuk 8 betreft een veldexperiment waarin we onderzochten of het uitlekken van voedingsstoffen in de bodem tijdens de ontbinding van kadavers wordt beïnvloed door de aanwezigheid van gewervelde aaseters. Daartoe hebben we systematisch verschillende groepen aaseters de toegang tot kadavers ontnomen: we hebben (1) geen aaseters uitgesloten; (2) alleen wild zwijn uitgesloten; (3) alle zoogdieren uitgesloten; en (4) zowel alle zoogdieren als alle vogels uitgesloten. We vonden enorme pieken in de concentraties van koper (Cu) en zink (Zn) - de meest schaarse voedingsstoffen - wanneer alleen ongewervelde aaseters (insecten; behandeling 4) toegang hadden tot kadavers. Dit zou betekenen dat, als gewervelde aaseters het grootste deel van een kadaver opeten, deze voedingsstoffen over een groter gebied worden verspreid via de aaseters. Met andere woorden, er lekken meer voedingsstoffen de bodem in als een kadaver alleen wordt gebruikt door insecten.

Op basis van de inzichten uit Hoofdstuk 7-8 concludeer ik dat het inderdaad mogelijk is om een breed scala aan voedingsstoffen in de bodem te meten onder ontbindende kadavers. Deze voedingsstoffen lekken voornamelijk de bodem in als ongewervelde aaseters (insecten) het ontbindingsproces domineren.

Het overkoepelende thema van mijn proefschrift is substantiële variatie: variatie in de elementaire samenstelling van zoogdieren (Hoofdstuk 2-4); variatie in de afbraaksnelheid van kadavers en het gedrag van aaseters (Hoofdstuk 5-6); en variatie in de concentraties van voedingsstoffen die de bodem in lekken ten gevolge van de ontbinding van kadavers (Hoofdstuk 7-8). Ik concludeer dat grote dieren de variatie - “heterogeniteit” - van het landschap creëren én versterken via hun natuurlijke cyclus van leven en dood.



Contents

Chapter 1

General introduction

Wenting, E.

1.1 Problem statement and theory

The nutrient cycle is one of the main drivers of ecosystem functioning (e.g. Ngai & Srivastava 2006; Marschner & Rengel 2007; Fagúndez 2013; Delgado-Baquerizo et al. 2020; Dantas de Paula et al. 2021). It consists of nutrient uptake by plants through the soil and carbon sequestered from the atmosphere by photosynthesis, and the return of these nutrients to the soil as dead plant or animal matter (Dighton 1995). Nutrients can exist in many forms, including carbohydrates, proteins, fats, vitamins, and elements (e.g. Hill et al. 2021). Especially elements are needed for all lifeforms to maintain their life functions (e.g. McDowell 1992; Baxter 2009; Huang & Salt 2016; Kaspari 2021), and occur as chemical elements. These chemical elements can be essential, ecotoxic, or both depending on the concentration (e.g. Robinson et al. 2009; Crowe & Bradshaw 2014). The term '(chemical) elements' is used in a general more neutral way, while the term 'nutrients' is used when elements serve as food particles and the term 'minerals' when elements are chemical compounds in the soil.

In general, carbon (C), nitrogen (N) and phosphorus (P) are the main building blocks of species biomass (Vogels et al. 2023). These elements, particularly N, are traditionally considered the major limiting elements for biomass production (e.g. Vitousek & Howarth 1991). However, these elements are no longer the main limiting factors of primary production in many regions in the world (e.g. Throop 2005). Increased soil acidification, partially caused by excessive N deposition, has resulted in imbalanced availability of essential elements (Gilliam et al. 2020). The consequences of decades of acidification are becoming more and more clear (e.g. Baines et al. 2016; Nijssen et al. 2017). N deposition, for instance, reduces both the relative and absolute quantities of other essential elements for vegetation and animals (Vogels et al. 2023). Management efforts are more and more (experimentally) applied to restore imbalanced nutrient cycles in protected areas (e.g. Weijters et al. 2018; Desie et al. 2020; Chakraborty et al. 2023). Moreover, the importance of (vertebrate) animals in (restoring) nutrient cycles is increasingly recognized (Beard et al. 2002; Vanni 2002; Abraham 2021), but still underexposed.

Animals play a crucial role in nutrient cycles by accumulating - both essential and ecotoxic - elements in their bodies over large areas (Figure 1.1, arrow i). These include elements that are essential for all organisms but are hard to gather due to their scarcity in nature. When animals die, the elements that they accumulated over their entire lifespan are released at a single point in time and space, causing local nutrient pulses (Yang et al. 2008). The elements stored in animal bodies thus enter the carrion pool, in the form of carcasses, as soon as animals die (arrow ii). The course of the carrion decomposition process will determine the fate of these carrion-stored elements, roughly via two possible pathways. The first pathway is that elements are taken up by other animals, so-called scavengers (arrow iii). Scavengers accumulate the carrion-derived elements in their bodies, or redistribute them over larger areas via their excreta - e.g. faeces - before the elements enter the soil nutrient pool (arrow iv). In this pathway, elements enter the soil nutrient pool in relatively low concentrations and more scattered throughout the landscape. As such, they can be taken up by plants, and ultimately the elements will be invested in above-ground biomass. Animals can, in turn, take up the elements when they consume plants (arrow i), accumulating these elements in their bodies until their death. The second pathway is that the carrion decomposition process proceeds on the spot where the animal died. In this pathway, scavengers are absent or too slow in colonizing the carrion for themselves, which allows detritivores - e.g. microbes, fungi, or nematodes - to dominate the carrion decomposition process (arrow v; Janzen 1977; Ruxton et al. 2014). As opposed to scavengers, these detritivores operate locally, meaning that the carrion-stored elements will directly leak into the soil, and so be reflected in the soil nutrient pool directly beneath the carrion (arrow vi). Carrion fluids can also directly leak into the soil (arrow vii). Consequently, due to the local release compared to the scavenger-dominated pathway, elements will leak into the soil in higher concentrations. This increases the chance that the elements leach to deeper soil layers (arrow viii), where they become unavailable for plants (e.g. Biederman & Harpole 2013; Bender & Van der Heijden 2015; Maharajan et al. 2021).

Which pathway dominates the process of carrion decomposition can thus strongly influence the terrestrial nutrient cycle. However, it remains unknown how the elements that are stored in animals, thus carcasses after their death, are recycled in terrestrial ecosystems (e.g. Barton et al. 2013; 2019; Wilson & Wolkovich 2011). The process of how elemental cycling depends on the presence of scavengers has never been investigated. Thus, the importance of carcasses and scavengers in the terrestrial nutrient cycle remains unknown.

1.2 Knowledge gaps

There are three major knowledge gaps that need to be addressed to gain a better understanding of the importance of carcasses and scavengers in the terrestrial nutrient cycle.

First, carcasses are considered as high-quality food sources (e.g. DeVault et al. 2003; Wilson & Wolkovich 2011; Turner et al. 2017), implying that they contain high-valuable nutrients. However, the mineral nutrient and trace elemental composition of organisms - the ionome (Lahner et al. 2003; Salt et al. 2008) - is poorly known. This is especially problematic in the case of free-ranging large mammals, which contribute substantially to the total carrion biomass in terrestrial ecosystems (Moleón et al. 2019).

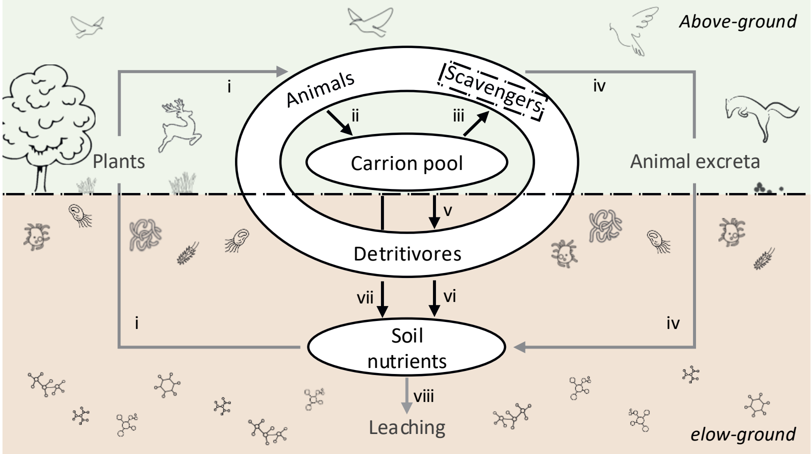


Figure 1.1 Conceptual model of the carrion-centred nutrient cycle. Arrows represent elemental fluxes. The outer circle represents an ecosystem, with animals of which some are called "scavengers". These animals predominantly operate above-ground ("green food web"). At some point, the animals die and the elements stored in their bodies enter the carrion pool, as represented in the inner circle. From there, these elements can be taken up by the scavengers or so-called "detritivores", i.e. all kind of other decomposers that operate below-ground ("brown food web"). When the carrion-stored elements leak into the soil locally, either directly or via detritivores, they enter the soil nutrient pool, as represented by the lower circle. From there, elements can be taken up by plants - contributing to above-ground biomass - or leach to deeper layers where they become unavailable for plants.

Second, the structure of the scavenger community that inhabits an ecosystem is supposed to be important in determining the carrion decomposition speed (e.g. Melis et al. 2007; Parmenter & MacMahon 2009). The decomposition speed is an important indicator of whether carrion-derived elements cycle via scavengers or detritivores. However, the importance of the scavenger species that form the scavenger community, and how these are affected by habitat characteristics, remains poorly understood.

Third, if scavengers indeed play a key role in the carrion decomposition process (e.g. DeVault et al. 2003; Farwig et al. 2014), their presence would strongly influence carrion-derived elemental fluxes into the soil. It has never been examined whether a wide range of elements that are stored in animal bodies actually leak into the soil due to the decomposition process, and whether this depends on the presence of different groups of scavengers.

In the next sections, I discuss these three knowledge gaps in more detail.

1.2.1 The mammalian ionome

The ionome is an important expression of the physiological state of organisms (Lahner et al. 2003). Like ecological stoichiometry - i.e. the study of the balance of energy and multiple chemical elements in ecological interactions (Elser 2006; Raubenheimer et al. 2009) -, ionomics relates to various biological and ecological processes, including life history plasticity (Jeyasingh & Weider 2005), population growth (Ågren 2004; Vrede et al. 2004), and foraging ecology (Ayotte et al. 2006). Where ecological stoichiometry focusses on C:N:P ratios, ionomics includes a much wider range of chemical elements. So far, ionomics has been predominantly applied to plants (e.g. Hänsch & Mendel 2009; Sha et al. 2012; Watanabe et al. 2016; Asis et al. 2017), invertebrates (e.g. Filipiak et al. 2017; Filipiak & Filipiak 2022), and fish (e.g. Yoshida et al. 2014; Du et al. 2019). Insights into the mammalian ionome, specifically larger-sized mammals, e.g. ungulates, are extremely limited and species-specific insights are, for most species, absent.

A widely held assumption is that the ionome of plants is much more influenced by environmental conditions than the ionome of vertebrate animals (e.g. Berman-Frank & Dubinsky 1999; Persson et al 2010). The mammalian ionome, in particular, is believed to be under relatively tight homeostatic control (Sterner & Elser 2002). This assumption is based on ecological stoichiometric insights and thus limited to C:N:P ratios (e.g. Jeyasingh et al. 2017). However, a growing number of studies have indicated that the assumption of ionomic homeostasis might not hold when including a wider range of elements (e.g. Ma et al. 2015). It also remains unclear to which extent ionomic variation would be inter- and intraspecific, and how chemical elements are distributed across the body.

1.2.2 Scavenger community interactions

Scavengers are believed to play a key role in the redistribution of carrion-derived nutrients over the landscape (e.g. Selva et al. 2003; 2005; Turner et al. 2017; Patterson et al. 2022). Vertebrates would be able to redistribute carcass parts over longer distances than invertebrates since vertebrates have larger bite sizes and home ranges than invertebrates (e.g. Peery 2000; Sessions & Kelly 2001). For this reason, the main focus of this thesis will be on vertebrate scavenger communities.

When scavengers are absent or too slow in detecting and consuming a carcass, detritivores can colonize the carcass for themselves before scavengers can do so. Microbes, in particular, are under strong selection to render a carcass as reprehensible or unusable for the scavengers as fast as possible (Janzen 1977). The carcass decomposition speed would therefore be an important factor, indicating whether carcass decomposition is dominated by scavengers or detritivores. Whether different scavenger species can dominate the decomposition process, and so redistribute carcass-derived elements over longer distances, depends on their traits and abilities to exploit carcasses (e.g. Vucetich et al. 2004; Selva & Fortuna 2007; Kane et al. 2014; Smith et al. 2014). Consequently, different scavenger species, thus different scavenger guilds, would differently influence the carcass decomposition process.

Several studies described that a more diverse scavenger community would result in more complete carcass decomposition (e.g. Selva & Fortuna 2007; Olson et al. 2012; 2016; Gutiérrez-Cánovas et al. 2020). These studies were carried out in areas where obligate scavengers - i.e. vultures - and/or large carnivores - e.g. wolves (*Canis lupus*) - are present. Habitat characteristics like the amount of tree cover, for instance, are mentioned as important factors influencing the ability of vultures and other avian scavengers to detect and consume carcasses (e.g. Pardo-Barquín et al. 2019; Oliva-Vidal et al. 2022). However, it remains unknown whether insights obtained from areas with vultures and large carnivores are applicable in areas with only facultative scavengers and in the absence of large carnivores.

1.2.3 Carcass-related elemental leakage

Decomposing carcasses can result in highly concentrated islands of fertility, that are associated with increased microbial activity and nematode abundance (Carter et al. 2007). Also, so far, it has been well demonstrated that the carcass decomposition process would influence the C and N cycle (e.g. Benninger et al. 2008; Parmenter & MacMahon 2009; Barton et al. 2013; Macdonald et al. 2014). Only some studies included other macro nutrients in their analysis (e.g. Melis et al. 2007; Bump et al. 2009). However, it has never been extensively studied how carcass decomposition influences the cycle of other elements, especially essential trace elements.

Existing studies mainly focused on the overall effect of carrion decomposition on elemental concentrations in the soil, i.e. biochemical changes after full decomposition (e.g. Danell et al. 2002; Melis et al. 2007). Only few studies examined the elemental fluxes over time during the decomposition process (e.g. Barton et al. 2019; Quaggiotto et al. 2019). For example, Macdonald et al. (2014) and Quaggiotto et al. (2019) found increased electrical conductivity (EC) in the soil beneath decomposing carrion. Although EC does not give any information about individual elements, an increased EC might be an expression of elevated elemental concentrations (e.g. Heiniger et al. 2003). Therefore, it remains unclear how the process of carrion decomposition influences the concentrations of a wide range of elements during the decomposition process (Carter et al. 2007). Moreover, it has never been studied how any potential changes in elemental concentrations depend on the presence of different groups of scavenger species.

1.3 Thesis outline

The aim of this thesis is to determine how the role of carrion decomposition in the terrestrial nutrient cycle is influenced by (vertebrate) scavengers. I focus on areas with disturbed nutrient balance, no obligate scavengers, and no (re-established) large carnivores. In such areas, I expect the impact of carrion-derived nutrients - especially trace elements - even more striking, hence detectable. In particular, I focus on Dutch protected areas, that I thus consider as a suitable model system for this thesis. In accordance with the identified knowledge gaps, this thesis is thematically divided in three parts.

In the first part, I focus on the ionome of larger-sized mammals to assess the elements that eventually enter the carrion pool. Chapter 2 examines the potential magnitude of the variability of the mammalian ionome by comparing two very unrelated species that differ considerably in their diet and prevalence: fallow deer (*Dama dama*) and Eurasian otter (*Lutra lutra*). Chapter 3 compares the ionome of two ungulate species - red deer (*Cervus elaphus*) and wild boar (*Sus scrofa*) - that co-occur in a single protected area but differ in their diet and digestive system. This chapter describes how a wide range of elements is distributed across the body. Chapter 4 examines whether and how intra-specific ionomic variation could be related to age and sex, by focussing on one species - fallow deer - from a single protected area.

In the second part, I focus on the structure and functioning of the (vertebrate) scavenger community. More specifically, Chapter 5 examines the vertebrate scavenger community in the Netherlands by determining functional scavenger groups, i.e. groups of scavenger species that share specific traits and preferences. The chapter relates the presence of these groups to the carcass decomposition speed. Chapter 6 investigates how carcass exploitation by different functional scavenger groups, as defined in Chapter 5, are influenced by the amount of tree cover.

In the third part, I focus on carrion-related elemental leakage into the soil. Chapter 7 examines whether a wide range of elements can indeed leak into the soil as a result of carrion decomposition. Chapter 8 reports a field experiment, focussing on the elemental fluxes during the decomposition process of carcasses, and whether these elemental fluxes are influenced by the presence of different scavenger groups.

Finally, in Chapter 9, I reflect on the results from Chapter 2-8. I discuss how the individual chapters contribute to the identified knowledge gaps. Also, I discuss how the insights from the individual chapters converge, linking the insights of the mammalian ionome and interactions among vertebrate scavengers to the process of carrion decomposition and elemental leakage. I end my thesis by synthesising the obtained insights, putting them in the context of nature conservation and management, and by providing some concluding remarks.

Chapter 2

Stoichiometric variation within and between a terrestrial herbivorous and a semi-aquatic carnivorous mammal

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Abstract

Background: The elemental composition of the mammalian body is widely believed to be more or less constant within and among species, yet reliable comparisons of elemental content are lacking. Here, we examine the elemental composition of two mammal species with different diet and provenance: terrestrial herbivorous Fallow deer (*Dama dama*) - collected from a single area - and semi-aquatic carnivorous Eurasian otter (*Lutra lutra*) - collected from different areas.

Methods: We compared twelve elemental contents for twelve different body tissues and organs, for four tissue samples per species. Homogeneous samples were tested for twelve elemental contents using ICP-OES.

Results: We found evidence for differences in elemental composition between species, between tissues, and between individuals. Herbivorous Fallow deer seemed more variable in its elemental composition compared to carnivorous Eurasian otter. The absolute concentration of some elements, e.g. Mn and Cu, showed differences between the species as well.

Conclusion: Since we found stoichiometric variation among the species, these findings question the widely held assumption that mammals are under relative tight stoichiometrically homeostatic control.

Keywords: Ecological stoichiometry, Ionomics, Minerals, Trace elements, Macro nutrients

2.1 Introduction

The nutrient composition of plants is widely believed to be more influenced by environmental nutrient supply - and therefore more variable - than the nutrient composition of vertebrate animals, particularly mammals (e.g. Reiners 1986; Berman-Frank & Dubinsky 1999; Person et al. 2010). The nutrient composition of mammals would be under relative tight homeostatic control, and the limited variation that does exist would be interspecific rather than intraspecific (e.g. Sterner & Elser 2002). The underlying reasoning is that mammals, unlike plants, can control their nutrient uptake by foraging selectively over large areas and long timespans (Daufresne 2013; Doughty et al. 2016). However, ecological stoichiometry - i.e. the study of the balance of energy and multiple chemical elements in ecological interactions (Elser 2006; Raubenheimer et al. 2009) - is generally based on C:N:P ratios rather than any other elements. Even if C:N:P ratios are rather constant in mammals (Sterner & Elser 2002), stoichiometric variation might still occur for other elemental contents.

Stoichiometric studies that consider trace elements are extremely scarce. We compared four studies that measured at least four trace elements for one or more different body tissues and organs (hereafter ‘tissues’) with a more or less balanced and complete dataset. Two of these studies analysed the nutrient composition for more than two tissues (Ammerman et al. 1974; Fick et al. 1976), and two incorporated only one or two tissues (Giuffrida-Mendoza et al. 2007; Tajik et al. 2010). Although the studies are not fully comparable in their methods, their results indicate that mammal species might differ in their elemental composition (Figure 2.1; Ammerman et al. 1974; Fick et al. 1976; Giuffrida-Mendoza et al. 2007; Tajik et al. 2010). It therefore seems worthwhile to investigate the mammalian elemental composition more extensively using a single method.

Sterner and Elser (2002) argued that two conditions must be met for animals to differ in their nutrient composition: (1) the elemental composition should differ between tissues, and (2) the contribution of different tissues to the total body mass should differ between species. The first condition might be met for N:P ratios (see Figure 4.12a in Sterner and Elser (2002), based on Elser et al. (1996) with original data from Bowen (1979)), yet Sterner and Elser (2002) reasoned that this variation would similarly apply to all vertebrates. However, variation in other elements has not been considered. It is thus possible that stoichiometric variation exists in elements other than C, N, and P.

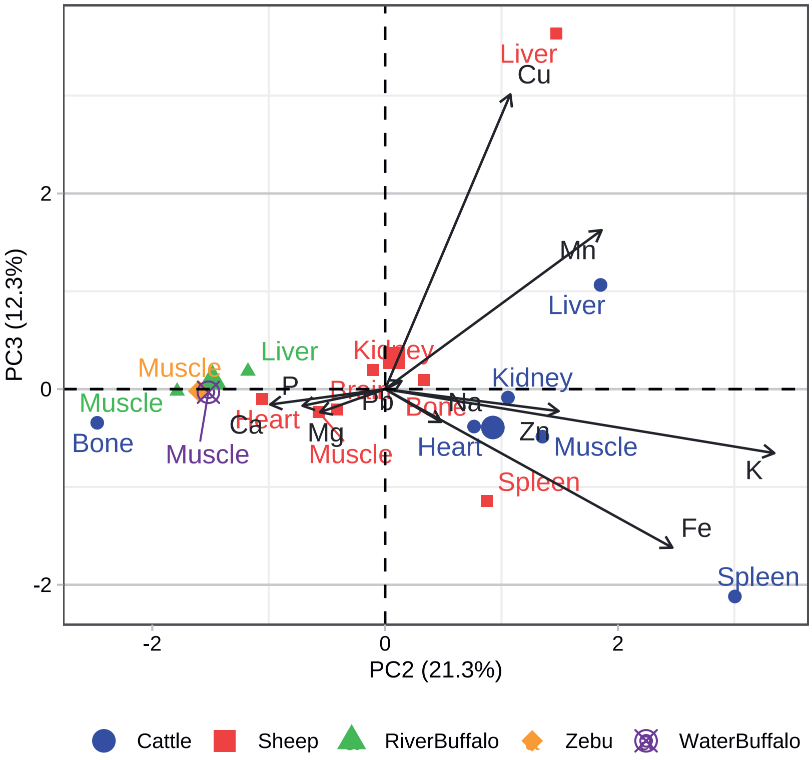

Figure 2.1 Principal component analysis (PCA) of published estimates of nutrient composition of body tissues of five herbivorous mammals: cattle (Ammerman et al. 1974), sheep (Fick et al. 1976), river buffalo (Tajik et al. 2010), Zebu-influenced cattle, and water buffalo Giuffrida-Mendoza et al. 2007). Only the nutrients measured in all four studies are included. Note that the second and third PCA axes are shown, as the first axis explained only differences between bones and other tissues.

The second condition might be met due to allometric variation in tissue contribution to body mass (see Figure 4.12b in Sterner and Elser (2002), based on Calder (1984) with original data from Pitts and Bullard (1968)). For example, digestive organs take up a greater proportion of the body in ruminants than in non-ruminants, and the mass proportion of bones generally increases with body mass (Prange et al. 1979). The relationship of tissue weight with body mass has been evaluated for 42 American mammal species from eight different orders, ranging in mass by three orders of magnitude, from 7 g to 17 kg (Schoenemann 2003). However, this sample still covers only a fraction of the entire body mass range in mammals, and is irrespective of diet (Schoenemann 2003). Overall, the prevailing knowledge seems inappropriate to test Sterner and Elser's (2002) two conditions.

Ionomics has been mostly based on plants (e.g. Salt et al. 2008), although some focussed on microbes (Jeyasingh et al. 2017; Rudman et al. 2019), fish (e.g. Rudman et al. 2019), amphibians (e.g. Prater et al. 2018), or amphipods (e.g. Goos et al. 2017). Ma et al. (2015) examined the mammalian ionome based on tissues samples of 26 mammalian species, 18 elements and four tissues. Although they found e.g. some lineage-specific patterns, and correlations between elements, tissues, and body mass, they did not extensively analyse intra- and inter-individual differences and variation. To our knowledge, there are no other studies on ionomics that reported such variation patterns based on two or more species and at least ten elements.

Here, we compare elemental contents between two mammal species with completely different diets and habitats to explore whether Sterner and Elser's (2002) two conditions might apply for more different species and elements. If stoichiometric variation between species occurs, we should be able to detect this when comparing these two species. Specifically, we compared Fallow deer (*Dama dama*), henceforth 'deer', a terrestrial herbivore with an adult body weight of 40 to 80 kg and a non-nomadic lifestyle (e.g. Focardi et al. 2006), and Eurasian otter (*Lutra lutra*), henceforth 'otter', a semi-aquatic apex predator with an adult body weight of 7 to 17 kg and a nomadic lifestyle (e.g. Beja 1997).

2.2 Material and Methods

2.2.1 Carcass collection

Four deer carcasses were obtained from culling at Veluwezoom National Park, the Netherlands. The deer carcasses - two juvenile males and two adult females - were frozen in the very fresh post-mortal stage of post-mortem rigidity (Levy et al. 2010). Five otter carcasses were obtained from Wageningen Environmental Research. These carcasses - two adult and a juvenile male, and an adult and a juvenile female - originated from different locations in the Dutch provinces of Drenthe and Overijssel. These carcasses were road kills - except for the juvenile female, which had drowned -, and were frozen in the stage of post-mortem rigidity - except for the juvenile male, which was in an early autolytic stage (e.g. Levy et al. 2010). The juvenile individuals of both species were long weaned at the moment of death. All the carcasses and later described tissues and tissue samples were frozen at minus 18 degrees Celsius.

2.2.2. Measurements

We sampled four sets of twelve tissues of each species, as to study intraspecific as well as interspecific variation (Harris 2007). We first weighed each carcass to determine its total body weight. Second, we dissected each carcass by tissue and weighed each tissue. All the dissections were performed in the dissection room of Wageningen Environmental Research using standard scalpels and other dissection tools, e.g. tweezers and scissors. Before grinding, the tissues were stored in plastic bags in the freezer. Third, we grinded each tissue with a blender to create homogeneous tissue samples. We took three table spoons - approximately 15 to 25 grams, depending on the tissue - of grinded tissue material from the blender that we stored in plastic bags. These tissue samples were frozen before they were freeze-dried. The blender was thoroughly cleaned with a detergent - not containing any of the elements we measured - and water, and dried after a tissue was grinded before we grinded the next one. All tissues were processed in the same way. We used bare sand - that was processed corresponding to the tissues - as blending control. Part of this sand was put on the dissection table and then grinded by the blender before we measured the same elemental concentrations - eight replicates each - as we did for the tissue samples. In case an elemental concentration was measured in this control material, this value was subtracted from the elemental concentrations of the tissue samples.

We considered twelve tissues belonging to different organ systems, representing different body functions (Reece et al. 2014): bone, skin and hair, muscle, brain, lungs, heart, spleen, kidney, liver, pancreas, stomach (including rumen for deer), and intestines. Some tissues of the otters were unusable due to the cause of death: liver, muscle, and intestines of the juvenile male; pancreas of an adult male; brain of the adult female; and bone, skin and hair, lungs, heart, spleen, kidney, and stomach of the juvenile female. However, overall, we were able to use four tissue samples per tissue per species, i.e. 96 tissue samples in total.

Before freeze-drying, we transferred the stored tissue samples to plastic tubes that we weighed with a precision of four decimal places to determine the fresh tissue weight. Then, the tissue samples were freeze-dried and weighed again to determine the dry weight. So far, we conducted the procedure, from dissecting the carcasses till freeze-drying the tissue samples, at Wageningen University & Research (with the dissection room of Wageningen Environmental Research located in the same building).

For further sample preparation, we transported the freeze-dried tissue samples - that were wrapped in ice blocks to prevent defrosting - to Radboud University. Here, we used a microwave destruction (aka digestion) method with 5 ml 65% nitric acid (HNO_3) and 2 ml 30% hydrogen peroxide (H_2O_2). Elemental contents were then measured using Inductively coupled plasma optical emission spectroscopy (ICP-OES) (Van de Wiel 2003). The accuracy of the ICP-OES was guaranteed by using the following quality controls (QC): Multi element standard IV, Merck 1.11355; Phosphate standard, Merck 1.19898; Sulphate standard, Merck 1.19813; and Silicium standard, Merck 1.70236. The QC matrices were considered to correspond to the sample matrices since for both, any contamination of HNO_3 and H_2O_2 was eliminated by using blanks. Moreover, we used additional spike and recovery experiments to measure the analytes in the real matrices (Appendix 2.1), which resulted in an error margin of 3.24% at maximum for all elemental contents measured, except for calcium (Ca) and phosphorous (P), which showed higher and lower recovery percentages, respectively. We do not consider this as a point of concern since this does not affect the core message of our study (see results section).

We measured twelve elemental contents; five macro elements: P, potassium (K), Ca, magnesium (Mg), and sodium (Na); and seven trace elements: cobalt (Co), copper (Cu), iron (Fe), manganese (Mn), selenium (Se), zinc (Zn), and lead (Pb). The choice of these elements is based on scarcity in the environment on the one hand (K, Co, Ca, Mg, Na and Mn, due to long-term acidification and leaching of these elements and more storing in organic matter, including P, due to N deposition and increased plant growth (Nijssen et al. 2017; Vogels et al. 2017; Siepel et al. 2018), and potential toxicity due to aerial pollution during past decades on the other hand (Cu, Zn and Pb, e.g. De Vries & Groenenberg 2009). Since the C:N:P stoichiometry should be rather constant between mammals (Sterner & Elser 2002), we did not measure the carbon (C) and nitrogen (N) content in this study.

Some elemental contents could not be measured for all tissues due to the detection limits of ICP-OES (Van de Wiel 2003). For instance, Co content could only be measured in bones, Cu content for all tissues except most bone samples, and Mn, Se and Pb content were not detectable for all tissue samples (Table 2.1). In cases that an elemental content was only detectable in one tissue of a species, we could not report the standard deviation. In some tissues, an elemental concentration was not detectable at all, e.g. Se in deer's bone, brain, lungs, and heart. In such cases, we reported no elemental content value for that specific tissue.

2.2.3 Statistical analyses

All statistical analyses were done in R Version 3.3.3 (R Core Team 2017). We tested for differences in elemental composition between tissues - Sterner and Elser's (2002) first condition - in three steps. First, we tested for differences in elemental concentration within each species using Kruskal-Wallis tests. Second, we used t-tests for each tissue-element combination to test for differences in elemental concentration between the species, which we visualised in a table and a PCA biplot using the factoextra package (Kassambara & Mundt 2017). Missing values were calculated with the Principal Components Analysis model, using the imputePCA function of the missMDA package (Husson & Josse 2017). We used the step-up Benjamini and Hochberg (Benjamini & Hochberg 1995) procedure to correct the alpha for multiple tests using the p.discrete.adjust function of the discreteMTP package (Heller et al. 2012). Last, we compared the percentage of explained variation - as visualised in the PCA biplot - between the two species. We calculated the percentage of total variation explained by each tissue per species by multiplying the percentage of variance of the PCA axes by the contribution to those axes per tissue sample. All the twelve PCA axes were used in this calculation to include all the variation in the data. We visualised these percentages in a ladder plot.

We tested for differences between species in tissue contribution to body mass - Sterner and Elser's (2002) second condition - in three steps. First, we tested for differences in tissue contribution - both fresh and dry weight ratios - within the sampled individuals of each species using Kruskal-Wallis tests. The tissue contributions were expressed in ratios of the total standardized body weight. Second, we used ANOVAs with Tukey post-hoc tests to analyse the dry weight ratios between the tissues per species. Last, we compared the tissue contribution in fresh and dry weight of both species using t-tests, which we visualised in scatter plots. We used the step-up Benjamini and Hochberg (Benjamini & Hochberg 1995) procedure to correct the alpha for multiple tests using the p.discrete.adjust function of the discreteMTP package (Heller et al. 2012).

2.3 Results

2.3.1 Elemental concentrations

We found differences in elemental concentration between tissues among individuals in neither deer (Kruskal-Wallis test, X^2=0.673, *df*=3, *p*=0.880) nor otter (X^2=0.297, *df*=4, *p*=0.990). However, we did find significant differences between the species in elemental contents per tissue (Table 2.1). For twelve of the tissue-element combinations, standard deviations were higher than the average elemental content (Table 2.1); five times for deer - bone's Co and Fe content, skin and hair's Mn content, stomach's Se content, and brain's Pb content -, and seven times for otter - lungs', stomach's and intestines' Ca content, intestines' Fe content, stomach's and intestines' Mn content, and pancreas' Se content. The Se content for deer was not detectable in any liver sample, while the mean and standard deviation were available for the Se content of otter's liver (Table 2.1). Furthermore, we found that all tissues of both species, except for skin and hair, differed in Mn content (Table 2.1).

There were no tissues for which we found differences in exactly the same elemental contents (Table 2.1). The most variable tissues were kidney, which differed in ten elemental contents (P, K, Ca, Mg, Na, Cu, Fe, Mn, Se, and Zn) and muscle, which differed in

Table 2.1 Concentrations of twelve elements (ppb) in twelve body tissues collected from a herbivore - Fallow deer (FD) - and a carnivore - Eurasian otter (EO). Significant differences between the* species *(p<0.05), ± standard deviation, are underlined.*

Element	P		K	
Tissue	FD	EO	FD	EO
Bone	190,683 ± 112,626	256,450 ± 23,891	684 ± 454	2,162 ± 655
Skin & Hair	5,524 ± 1,237	4,158 ± 1,518	12,379 ± 2,114	6,013 ± 2,434
Muscle	18,590 ± 1,325	16,223 ± 1,952	28,865 ± 2,706	25,035 ± 2,572
Brain	32,368 ± 2,443	26,603 ± 6,349	30,640 ± 5,827	25,563 ± 9,011
Lungs	17,388 ± 4,176	15,633 ± 4,658	19,828 ± 4,125	15,245 ± 4,110
Heart	18,978 ± 1,425	16,678 ± 1,509	22,308 ± 1,805	18,785 ± 3,453
Spleen	18,420 ± 1,721	25,475 ± 5,311	21,975 ± 1,305	25,093 ± 4,658
Kidney	27,018 ± 2,640	21,145 ± 1,152	29,560 ± 4,309	21,630 ± 621
Liver	23,455 ± 2,735	19,383 ± 2,362	17,488 ± 2,362	18,798 ± 3,632
Pancreas	19,043 ± 12,562	28,420 ± 2,076	21,673 ± 4,478	20,925 ± 2,378
Stomach	13,765 ± 2,461	21,190 ± 10,159	15,990 ± 3,868	21,293 ± 4,190
Intestines	13,865 ± 2,731	27,970 ± 11,423	33,265 ± 6,276	26,163 ± 8,988
Total	399,095	479,325	254,653	226,703

Table 2.1 Continued.

Element	Ca		Mg	
Tissue	FD	EO	FD	EO
Bone	415,278 ± 243,576	556,350 ± 47,412	6,814 ± 4,112	9,403 ± 1,308
Skin & Hair	1,032 ± 223	639 ± 159	1,436 ± 290	348 ± 164
Muscle	502 ± 108	796 ± 558	2,323 ± 128	1,855 ± 228
Brain	1,506 ± 806	1,129 ± 489	1,444 ± 231	1,273 ± 404
Lungs	1,139 ± 119	3,233 ± 4,304	965 ± 193	1,007 ± 269
Heart	955 ± 394	854 ± 117	1,750 ± 224	1,634 ± 170
Spleen	1,131 ± 75	772 ± 175	1,372 ± 54	1,577 ± 288
Kidney	1,319 ± 160	760 ± 78	1,951 ± 167	1,432 ± 54
Liver	701 ± 203	652 ± 124	1,087 ± 141	1,402 ± 265
Pancreas	3,692 ± 1,009	4,783 ± 3,749	1,986 ± 630	2,199 ± 250
Stomach	3,763 ± 1,486	11,210 ± 16,277	1,530 ± 330	1,877 ± 857
Intestines	8,680 ± 2,253	41,817 ± 51,763	3,840 ± 1,329	2,538 ± 1,445
Total	439,697	622,994	26,500	26,546

Table 2.1 Continued.

Element	Na		Co	
Tissue	FD	EO	FD	EO
Bone	9,775 ± 5,473	12,748 ± 792	1.66 ± 2.26	0.49 ± 0.31
Skin & Hair	5,104 ± 1,512	3,955 ± 1,874		
Muscle	4,911 ± 723	8,129 ± 2,106		
Brain	17,333 ± 2,912	13,910 ± 3,394		
Lungs	13,585 ± 3,267	16,020 ± 2,533		
Heart	9,357 ± 830	12,270 ± 911		
Spleen	12,018 ± 1,319	10,280 ± 2,431		
Kidney	18,010 ± 2,668	12,378 ± 1,184		
Liver	6,780 ± 414	9,366 ± 1,041		
Pancreas	13,538 ± 3,727	10,061 ± 1,234		
Stomach	18,350 ± 3,226	10,733 ± 2,369		
Intestines	25,108 ± 4,062	13,912 ± 4,813		
Total	153,866	133,760	1.66	0.49

Table 2.1 Continued.

Element	Cu		Fe	
Tissue	FD	EO	FD	EO
Bone	3.87		152 ± 80	44 ± 17
Skin & Hair	7.56 ± 2.11	6.41 ± 2.49	100 ± 89	114 ± 86
Muscle	16.01 ± 3.74	15.75 ± 6.26	198 ± 15	483 ± 38
Brain	18.45 ± 1.84	25.52 ± 5.64	227 ± 17	620 ± 400
Lungs	16.03 ± 4.99	10.28 ± 1.38	1,859 ± 370	2,538 ± 933
Heart	38.38 ± 6.47	32.68 ± 1.98	602 ± 153	890 ± 34
Spleen	15.61 ± 4.68	7.30 ± 2.35	3,151 ± 830	1,292 ± 414
Kidney	67.75 ± 17.16	27.13 ± 2.95	386 ± 83	1,082 ± 272
Liver	430.73 ± 190.43	77.86 ± 30.16	459 ± 107	1,267 ± 879
Pancreas	16.64 ± 10.83	12.82 ± 2.81	227 ± 51	438 ± 142
Stomach	11.21 ± 4.27	15.79 ± 6.17	159 ± 106	451 ± 253
Intestines	20.42 ± 4.84	19.57 ± 11.69	510 ± 153	818 ± 858
Total	662.64	251.11	8,031	10,037

Table 2.1 Continued.

Element	Mn		Se	
Tissue	FD	EO	FD	EO
Bone	5.16 ± 2.68		1.53	2.03
Skin & Hair	17.18 ± 21.42	0.03	5.28 ± 0.47	4.41 ± 3.81
Muscle	1.83 ± 0.57	1.65	0.46 ± 0.16	4.43 ± 3.15
Brain	4.79 ± 1.47	0.80 ± 0.52	1.91	4.31 ± 2.10
Lungs	39.21 ± 20.67		2.72	5.77 ± 2.25
Heart	16.23 ± 13.66	0.45 ± 0.28	1.51	3.11 ± 3.62
Spleen	236.81 ± 74.79	0.74 ± 0.94	3.01 ± 0.91	4.26 ± 3.10
Kidney	88 ± 36	4.14 ± 0.90	7.46 ± 1.70	11.64 ± 2.56
Liver	60.11 ± 17.30	16.22 ± 5.26		6.60 ± 4.93
Pancreas	448.46 ± 45.85	12.55 ± 3.87	0.66 ± 0.33	1.64 ± 2.22
Stomach	1,291 ± 552	17.89 ± 22.78	2.67 ± 4.07	3.68 ± 2.99
Intestines	1,821 ± 825	100.55 ± 145.54	2.86 ± 2.11	7.85 ± 4.00
Total	4,030	155.02	30.06	59.73

Table 2.1 Continued.

Element	Zn		Pb	
Tissue	FD	EO	FD	EO
Bone	170 ± 91	270 ± 32	16.00 ± 6.26	44.92 ± 13.58
Skin & Hair	149 ± 28	139 ± 31	0.70	1.78 ± 1.36
Muscle	180 ± 19	304 ± 11	1.88 ± 0.97	3.41 ± 1.44
Brain	131 ± 32	114 ± 34	2.79 ± 3.48	4.00 ± 3.59
Lungs	106 ± 16	112 ± 33	3.02	3.21 ± 0.92
Heart	137 ± 13	171 ± 5	2.98 ± 1.51	3.04 ± 2.81
Spleen	207 ± 13	209 ± 55	2.10 ± 0.72	2.49 ± 2.63
Kidney	255 ± 41	147 ± 9	5.62 ± 1.91	4.54 ± 2.82
Liver	207 ± 13	219 ± 61	2.05 ± 0.69	5.65 ± 3.67
Pancreas	166 ± 26	251 ± 45	4.23 ± 0.43	2.18 ± 0.92
Stomach	172 ± 12	180 ± 25	10.19 ± 5.79	0.84 ± 0.61
Intestines	205 ± 49	230 ± 79	12.65 ± 5.73	1.09 ± 1.02
Total	2,084	2,346	64.21	77.14

seven elemental contents (P, K, Mg, Na, Fe, Mn, and Zn; Table 2.1). The least variable tissues, differing in just three elements, were skin and hair (K, Ca, and Mg), brain (Cu, Fe, and Mn), and lungs (K, Cu, and Mn; Table 2.1).

Overall, intraspecific variation in elemental composition was greater in deer than in otter (Figure 2.2). The composition of bones was strongly correlated with the first PCA axis for both species, explaining more of the variation than the other tissues (Figure 2.2). The deer tissues were more correlated to the second PCA axis than otter tissues (Figure 2.2). Deer tissues explained 54% of the total variation in the PCA, compared to 46% for otter's tissues (Figure 2.3). We found that skin and hair contributed noticeably more to the explained variation for otter than for deer, while stomach contributed noticeably more to the explained variation for deer than for otter (Figure 2.3).

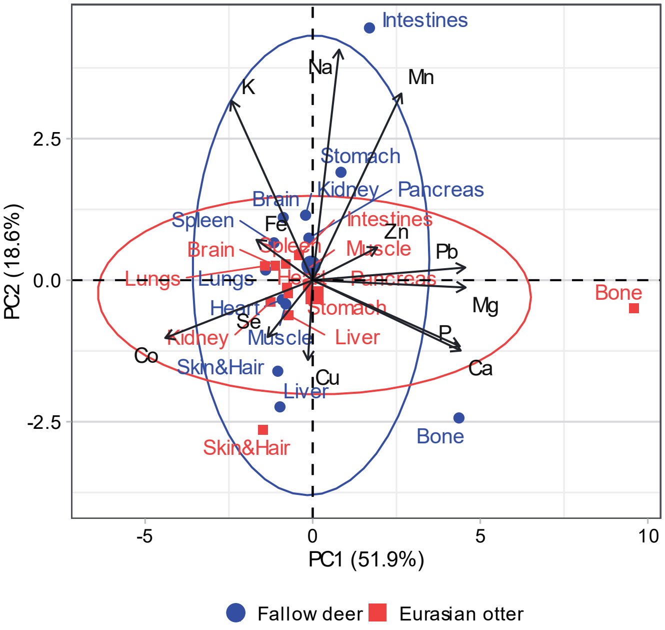

Figure 2.2 Principle component analysis (PCA) of the nutrient composition of body tissues collected from Fallow deer and Eurasian otter.

2.3.2 Relative weight of tissues

We found differences among individuals in proportional fresh weights of tissues neither for deer (Kruskal-Wallis test, X^2=0.303, *df*=3, *p*=0.960) nor for otter (X^2=0.118, *df*=4, *p*=0.998). This was also true for dry weight in both deer (X^2=0.427, *df*=3, *p*=0.935) and otter (X^2=0.667, *df*=3, *p*=0.879). However, in terms of dry weight, deer bone and skin and hair differed from each other and from the other ten tissues, while intestines differed from liver, muscle, and pancreas (ANOVA, *F*=99.92, *df*=11, *p*<0.000). For otter, we found that bone and skin and hair differed from each other and from the other ten tissues, but we found no differences between any of the other tissues (*F*=135, *df*=11, *p*<0.000). Between deer and otter, we found differences in fresh weight tissue contribution, except for brain, intestines, and spleen (Figure 2.4a). Most tissues, except for stomach, contributed more to the total body mass of otter compared to deer (Figure 2.4a). We found no differences in dry weight tissue contribution between the species (Figure 2.4b).

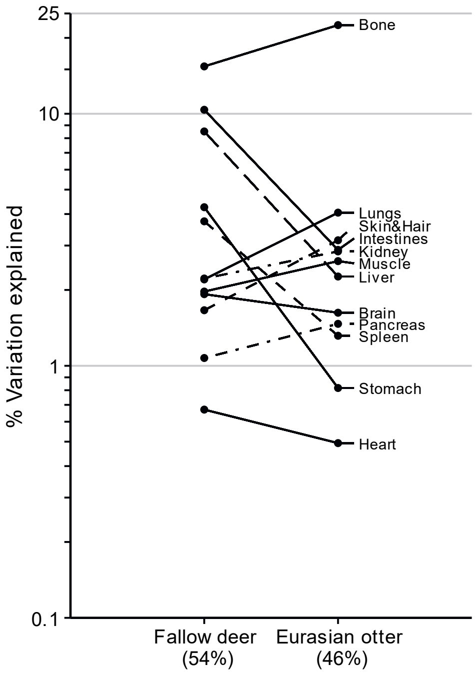

Figure 2.3 Percentage of total variation in tissue composition explained for Fallow deer and Eurasian otter. This percentage was calculated by multiplying the percentage of variance of the PCA axes by the contribution per tissue sample. All the twelve PCA axes were used in this calculation to include all the variation in the data.

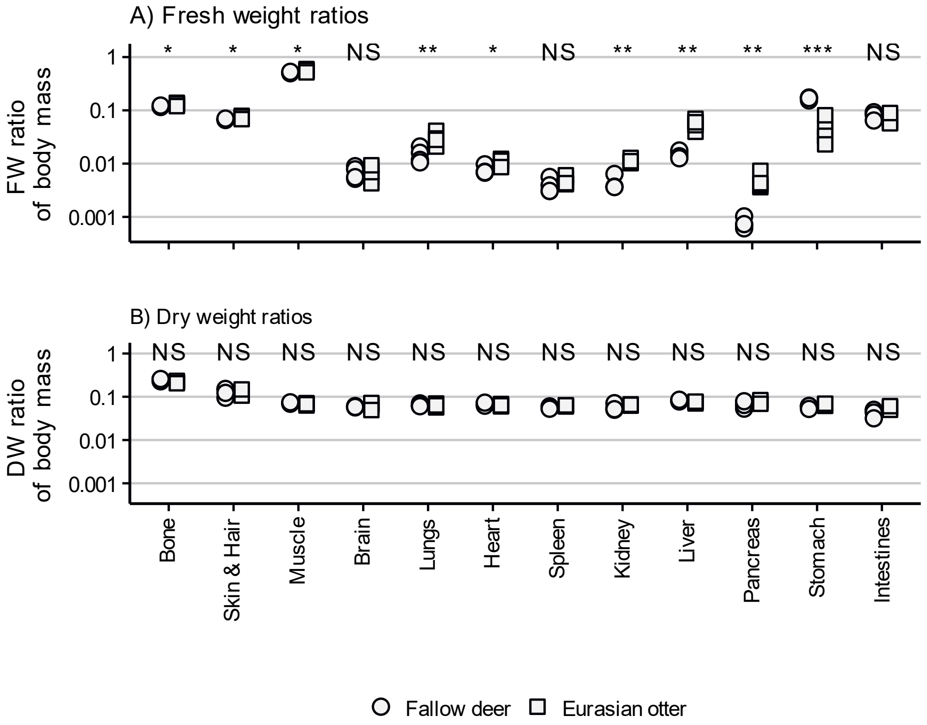

*Figure 2.4 Tissue contribution to total body mass of Fallow deer and Eurasian otter, in fresh weight (A) and dry weight ( ) ratios. *p<0.05, **p<0.01, and ***p<0.001.*

2.4 Discussion

The nutrient composition of the mammalian body is widely believed to be more or less constant within and among species (e.g. Sterner & Elser 2002), yet reliable comparisons of elemental content between tissues and species are lacking. We compared the elemental content of twelve tissues and tissue contribution to body weight between two mammal species with different diet and provenance. We found evidence for differences in elemental composition between tissues within and between the species, and for differences in tissue contribution to body mass between the species. Since we found stoichiometric variation among the species, these findings question, and encourage further investigation of, the widely held assumption that mammals are under relative tight stoichiometrically homeostatic control.

2.4.1 Elemental composition of tissues

We found that the elemental composition of tissues was less variable in otter than in deer (Figure 2.2+2.3). This was despite the fact that the otter carcasses came from the more different areas while the deer carcasses came from a single area. A plausible explanation is that species at higher trophic levels feed more homeostatically than species at lower trophic levels (Raubenheimer et al. 2009). Yet, deer and otter did overlap considerably in elemental composition (Figure 2.2). This is likely due to similarity of mammal species in their elemental requirements (e.g. Crowe & Bradshaw 2014; Reece et al. 2014). Nutrient intake and uptake rates might be subordinate to accomplished nutrient storage, although variation between species and individuals can still exist. More extensive surveys that include more species - within and among trophic levels - are needed to understand the effect of trophic level on the elemental composition of mammals. Overall, our results indicate that mammal species differ in the stoichiometric variation, and for some elements (e.g. Mn and Cu) also in total absolute elemental contents (Table 2.1).

We found substantial variation in elemental composition between tissues. This may be related to fundamental physiological and chemical processes within the body. For instance, bones did not only contain the highest Ca content (Table 2.1), as expected (e.g. Sterner & Elser 2002; Crowe & Bradshaw 2014), but were also the largest storage pools for Co and Pb (Table 2.1). These Co and Pb have relatively high electronegativity, and can bond to Ca ions (e.g. Crowe & Bradshaw 2014). The bone Pb concentration in otter even was at the edge of chronic poisoning (Flora et al. 2012), which can be explained by its position as apex predator in a contaminated environment. Co is one of the scarcest elements in nature (e.g. Pourret & Faucon 2016) and the essential element with the lowest concentration in the body (Crowe & Bradshaw 2014), which may explain why we were only able to detect the Co content in bones (Table 2.1). Thus, when elements, especially with high electronegativity, have a relatively strong tendency to form cations, they more easily bond to Ca ions, and thus form storage pools in bones (Crowe & Bradshaw 2014).

The substantial between-individual and between-tissue variation that we found indicates that examining the mammalian ionome should requires a rather complete dataset and adequate sample size. Although Ma et al. (2015) reported ionomic adjustments related to phylogeny, longevity, and body mass, their dataset was rather incomplete and unbalanced. This raises the question to which extent these patterns might be individual-specific rather than species-specific, and it would be worth to further examining this.

We also found substantial intraspecific variation in the elemental composition of tissues (Table 2.1). In deer, we found high variation in the P, Ca, Mg, Na, and Fe content of bones, and the Se content of stomach. In otter, we found relatively high variation in the P, Ca, and Mn content of stomach, and the P, Ca, Fe, and Mn content of intestines (Table 2.1). An open question is to which extent this variation is a consequence of differences in diet, for example because individuals forage in habitats with contrasting quality, or because a species has a wide nutritional niche (e.g. Senior et al. 2016). To our knowledge, no past studies compared the elemental composition of mammals directly to the elemental composition of their food sources.

The deer in our study came from an area with poor sandy soils in which many elements were scarce. Local game managers occasionally provide artificial salt licks (KNZ^{TM} Wild, Nouryon, Amsterdam, Netherlands) as bait for culling purposes. These salt licks contain salt (NaCl), Mg, Zn, Cu, iodine (I), Se, and Fe, but not Co, although Co is one of the scarcest elements (Pourret & Faucon 2016). Particularly Cu, Se, and Zn are essential trace elements that an animal body requires in small amounts (NRC 2001; Crowe & Bradshaw 2014; El-Ramady et al. 2016). As the salt licks contain relatively high amounts of these trace elements, e.g. 2,500 mg/kg Cu, 25 mg/kg Se, and 800 mg/kg Zn, they might have influenced the elemental composition of the deer that we sampled.

2.4.2 Relative weight of tissues

The tissue contribution to the body mass of individuals did not entirely match the prevailing knowledge (Figure 2.4a). While the contribution of bone is generally believed to increase with body mass, we found that bone contributed less to body mass in deer than in otter (Figure 2.4a). Although we did not include blood as a single tissue - and therefore we cannot judge whether blood, heart, lungs, and spleen together contribute equally to body mass for both species -, we found that heart and lungs contributed more to otter's body mass than to deer's (Figure 2.4a). Other factors than body mass - such as diet, digestive system, metabolism, and aquatic vs terrestrial habitat - may need to be included to better understand these patterns.

2.4.3 Limitations

One limitation of our study is that we lack reference data to check the validity of our measurements. Ammerman et al. (1974) and Fick et al. (1976) measured most of the tissues and elemental contents for cattle and sheep, respectively, as we did for deer and otter, but they used colorimetric determination method (Boltz & Mellon 1948) for P content and atomic absorption spectrophotometry method (Helfer & Rodgerson 1966) for K, Na, Ca, Mg, Fe, Cu, Zn, Mn, and Pb content, while we used ICP-OES for all elemental contents (Van de Wiel 2003). Furthermore, whereas we created homogeneous tissue samples from the whole organs, flank muscles, and tibia, Ammerman et al. (1974) used e.g. the middle lobe of liver, left ventricle of heart, and gracilis or cleido-occiptlalis muscle, and Fick et al. (1976) used the cerebrum, metacarpus, and portions of the anterior and posterior muscle from different body parts. Tajik et al. (2010) evaluated some elemental contents of the *longissimus dorsi thoracis* muscle and liver in River buffalos using the same spectrophotometry method as Ammerman et al. (1974) and Fick et al. (1976). Giuffrida-Mendoza et al. (2007) used *longissimus dorsi thoracis* muscle of Water buffalo and Zebu-influenced cattle, and measured P content by UV-vis spectrophotometry (AOAC 1990) and K, Na, Ca, Mg, Fe, Cu, Zn, and Mn content using the same spectrophotometry method as the previous mentioned studies. Due to these differences in methods and reported variation - i.e. standard deviation, standard error, or none -, it is hardly possible to use the reported elemental contents of these four studies as reference values for our measurements.

Another limitation is found in the detection limits of ICP-OES (Van de Wiel 2003). We were not able to detect all elemental contents for all tissues of deer and otter (Table 2.1). In retrospect, the use of Inductively coupled plasma mass spectrometry (ICP-MS) might have increased the detectability of some elements, due to the lower detection limits (Van de Wiel 2003). Therefore, we highly recommend the use of ICP-MS in further studies regarding the animal ionome, since our results demonstrate that extremely low elemental contents might be expected for some trace elements.

2.4.4 Conclusions

Overall, our results raise several new questions. Why did we find differences in elemental content between species, between tissues, and between individuals? Are differences detectable when comparing species within a trophic level, e.g. comparing different herbivores, or different carnivores? What is the influence of dietary intake and uptake? And how does this relates to elemental concentrations in animal excretions and internal reallocation or internal recycling? How affects this elemental dietary requirements? Can animals select their food sources based on their nutritional requirements? How does stoichiometric variation relate to stoichiometric homeostasis? Can we physiologically explain differences in tissue contribution to body mass between species? Do differences in tissue contribution to body mass only exist between species of different trophic levels, or also when comparing species within the same trophic level? And how does this all relate to ecological processes like the nutrient cycle and soil chemistry?

In general, we hypothesise that individual variation would be higher for scarce trace elements, as some individuals would be better in acquiring these elements than others. We predict that herbivores have higher variation among individuals than carnivores, as concentrations in their food sources are even lower than for species at higher trophic levels. Carnivores, and apex predators in particular, may be less prone to deficiencies as their food sources would contain on average higher quantities of these scarce trace elements. Especially species that forage on food sources containing high proportions of calcareous compounds - e.g. calcareous skeletons as for otter (Beja 1997) - are expected to contain relatively high amounts of scarce elements. Moreover, we predict that smaller-sized animals contain higher concentrations of scarce trace elements in their bodies, as they require relatively more of these elements since their food intake is lower due to their body size.

2.5 End sections

2.5.1 Author contributions

Elke Wenting: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Funding acquisition, Project administration. Henk Siepel: Conceptualization, Methodology, Resources, Writing - review & editing, Supervision. Patrick A. Jansen: Conceptualization, Writing - review & editing, Supervision.

2.5.2 Acknowledgements

We thank ARK Rewilding for the financial support for the chemical analyses, and everyone involved in the carcass provisioning. Special thanks to Dennis Lammertsma and Ruth van den Herik for their help with the dissections.

2.5.3 Conflicts of interest

No actual or potential conflicts of interest are declared by the authors.

Chapter 3

Variability of the ionome of Wild boar (*Sus scrofa*) and Red deer (*Cervus elaphus*) in a Dutch national park, with implications for biomonitoring

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Abstract

(1) The ionome - an important expression of the physiological state of organisms - is poorly known for mammals. The focus on particular tissues - such as liver, kidney, and bones - in biomonitoring of environmental pollution and potential deficiencies is based on widely held assumptions rather than solid knowledge of full mammalian ionomes.

(2) We examined the full ionome of Red deer (*Cervus elaphus*) and Wild boar (*Sus scrofa*), two commonly used mammals for biomonitoring, in a Dutch protected nature reserve (Veluwezoom). We used four individuals per species.

(3) We dissected 13 tissues and organs from each individuals (eight in total) of each species and measured 22 elemental concentrations in each. We assessed, for each element, how concentrations varied across tissues within and between individuals. Based on existing literature, we put our findings in the context of their function in the mammalian body.

(4) We found that the ionome was highly variable between as well as within the two species. For most elements, tissues containing the highest and lowest concentration differed between individuals. No single tissue accurately represented the accumulation of toxic elements or potential deficiencies in the bodies. Our assessment of the element’s biological roles revealed a serious lack of reference values.

(5) Our findings imply that analyses of commonly used tissues in biomonitoring do not necessarily capture bioaccumulation of toxins or potential deficiencies. We recommend establishing a centralised database of mammalian ionomes to derive reference values in future. To our knowledge, our study is one of the most complete assessments of mammalian ionomes to date.

Keywords: Ecological stoichiometry, ionomics, minerals, trace elements, macro nutrients, heavy metals

3.1 Introduction

The elemental composition of organisms - the ionome - is an important expression of their physiological state (Lahner et al. 2003; Salt et al. 2008). Like ecological stoichiometry - the study of the balance of energy and chemical elements in ecological interactions (Sterner & Elser 2002; Elser 2006; Raubenheimer et al. 2009) -, ionomics has been applied to population ecology to understand allocation and life history plasticity (Jeyasingh & Weider 2005; Jeyasingh et al. 2017) and population growth (Ågren 2004; Vrede et al. 2004). The ionome relates to a wide variety of ecological processes, including foraging, scavenging and carrion decomposition (Ayotte et al. 2006; Wenting et al. 2020). So far, ionomics and ecological stoichiometry have been widely applied to plants (e.g. Hänsch & Mendel 2009; Sha et al. 2012; Watanabe et al. 2016; Asis et al. 2017), invertebrates (e.g. Filipiak et al. 2017; Filipiak & Filipiak 2022), and fish (e.g. Yoshida et al. 2014; Du et al. 2019). Insights into the mammalian ionome, particularly large mammals, are limited.

The ionome reflects both the nutrient status and ecotoxic load of animals. While foraging across landscapes, large mammals accumulate a wide range of elemental nutrients in their bodies over their lifespan (Doughty et al. 2016). These include essential trace elements - e.g. cobalt (Co) and selenium (Se) - that are hard to gather for animals in sufficient amounts, especially in nutrient-poor areas. Large mammals may experience difficulties in acquiring sufficient amounts of essential elements, increasing the risk of deficiencies. This particularly applies to areas with unbalanced nutrient availability, for example due to decades of high nitrogen (N) deposition, which leads to acidified soils and leaching of cations to deeper horizons, hence increasing the risks of deficiencies for wildlife (Bobbink et al. 2010; Siepel et al. 2018; 2019).

While foraging, large mammals may also take up potential ecotoxic elements, such as arsenic (As), cadmium (Cd) and lead (Pb). Large mammals, especially ungulates that are the main consumers of vegetation (e.g. Reimoser & Putman 2011), are therefore commonly used for biomonitoring of such ecotoxic elements (Kuiters 1996). Biomonitoring of environmental pollution - i.e. ecotoxicity - is mainly based on the assumption that ecotoxic elements would accumulate in liver, kidney, bones or hairs of wildlife, in order to be excreted from the body as fast as possible (e.g. Catellino & Aloj 1964; Fassett 1975; Vahter & Concha 2001). These tissues are also most frequently used for assessing risks of elemental deficiencies of scarce elements (e.g. Gasparik et al. 2004; Malmsten et al. 2021). However, it is uncertain that this assumption holds given the high variation of the mammalian ionome (e.g. Ma et al. 2015; Wenting et al. 2020).

In Europe, ecotoxicity and potential deficiencies are commonly monitored by screening of particular tissues - mainly liver and kidney - of culled Red deer (*Cervus elaphus*) and Wild boar (*Sus scrofa*) (e.g. Ellen et al. 1989; Sobańska 2005; Yarsan et al. 2014; Cygan-Szczegielniak et al. 2018; Oropesa et al. 2022; Squadrone et al. 2022). For example, Nowakowska et al. (2016) used liver and kidney samples of Wild boar to examine environmental levels of Se in Poland. Vikøren et al. (Vikøren et al. 2005) used the liver as an indication for the cupper (Cu), Co and Se concentration in wild Red deer in Norway. However, due to the lack of knowledge of the full mammalian ionome, it is uncertain whether liver and kidney are indeed the proper focal tissues for ecotoxic and scarce elements. The lack of extensive overviews of the full mammalian ionome also limit the interpretation of current ionomic studies (Wenting et al. 2023c).

For both ecotoxic and scarce elements, the distribution over the mammalian body thus remains poorly known and extensive overviews are missing (e.g. Amici et al. 2012). Most ionomic studies thus use an incomplete approach, which could unintendedly cause severe bias in the assessment of ecotoxicology and potential deficiencies in ionomic studies dealing with large mammals. Also, a systematic overview of the biological relevance of chemical elements for the mammalian body, as well as signs of deficiency or toxicity, is missing. This is needed to put existing and new ionomic insights in the context of their biological and physiological role.

Here, we aimed to gain more insight in the ionome of large mammals, particularly in how elements are distributed across the body. We measured the concentrations of 22 elements in 13 different tissues from four individuals of Red deer and four individuals of Wild boar (henceforth ‘deer’ and ‘boar’). These individuals were culled in a national park in The Netherlands, a mineral- and nutrient-poor environment that experienced decades of high N deposition, where deficiencies are likely to occur. We also tried to put our findings in the context of their function in the mammalian body, based on the literature.

3.2 Methods

3.2.1 Study site and species

We focused on deer and boar in Veluwezoom National Park (henceforth ‘Veluwezoom’), the Netherlands (52°02’N, 6°01’E), a protected area of 5,000 ha situated on partly glacier deposits and partly on cover sands over these deposits, making the natural mineral availability limited to very scarce (mineral-poor cover sands). Veluwezoom is a former agro-silvopastoral landscape that became a national park in 1930 (Kuiters 2005). It contains a mosaic of dry grass-heathlands, pastures, abandoned crop fields, and woodland, grazed by free-ranging Scottish highland cattle (*o s taurus*) and Icelandic horses (*Equus ferus caballus*) - introduced in the 1980s - as well as by Roe deer (*Capreolus capreolus*), Fallow deer (*Dama dama*), Red deer and Wild boar (Bruinderink & Lammertsma 2001). Due to surrounding highways former pollution (especially before 1980's) with Pb might be possible. Samecka-Cymerman et al. (2005) found elevated levels of Pb in bryophytes collected from Veluwezoom.

The Red deer is a herbivorous ruminant ungulate that is associated with woodland habitats (e.g. Kamler et al. 2008; Bywater et al. 2010). Only the males have antlers, that cast in spring and regrow in late summer (e.g. Mitchell & Parish 1970). Single-born calves are born in spring. The mating season ("rut"), which costs lots of energy, is from September to November with a peak in October (Clutton-Brock et al. 1983; Alves et al. 2013).

The Wild boar is a monogastric, opportunistic omnivorous ungulate that is known for its highly plastic diet and their ability to adapt to diverse food and habitats (e.g. Ballari & Barrios-García 2014). Reproduction is less seasonal and litter sizes range from one to twelve piglets, depending on many factors including the maternal body weight and summer temperature (e.g. Frauendorf et al. 2016).

3.2.2 Carcass dissection

We used freshly culled carcasses that were obtained in the culling season 2019-2020 (culled between October 2019 and March 2020), and used four individuals of each species. Local hunters used Pb free RWS HIT ammunition. Culling at Veluwezoom is only used as an intervention against wildlife damage, i.e. culled individuals are not harvested but left to decompose in nature. For deer, we used two young females ('RD1' and 'RD2'), one young male ('RD3'), and one male calf ('RD4'). For boar, we used two male piglets ('WB1' and 'WB2') and two female piglets ('WB3' and 'WB4'). For each carcass, we dissected 13 tissues belonging to different organ systems (Reece et al. 2014): bone; skin and hair; muscle; brain; eyes; lungs; heart; spleen; kidney; liver; pancreas; stomach (including rumen for deer); and intestines. We were able to dissect all these tissues from all the carcasses, except the pancreas for the male calf (RD4) and a male piglet (WB1).

No animals were killed for the purpose of our study. According to the Animal Welfare Officer of Wageningen University & Research, our study is not considered as experimentation on animals (Appendix 3.1), and therefore permitted under Dutch law.

The dissection procedure was performed in two steps. First, we dissected seven of the tissues - skin and hair, muscle, lungs, heart, spleen, kidney, and liver - in a self-made dissection room at Veluwezoom. We also collected the entire guts, head and the right hind leg that we needed to create tissue samples for the other six tissues. The carcass remains after dissection were returned to nature. Second, we further dissected the guts - pancreas, stomach, and intestines -, the head - brain and eyes -, and the hind leg - bone - in the dissection room of Wageningen Environmental Research. For the bone, we sawed a piece of bone from the lower leg, that we cleaned by boiling it a few minutes. Once dissected, we stored all the collected tissues in the freezer at minus 18 °C until we further processed them into homogeneous tissue samples.

For culling purposes only, the local game wardens occasionally provide mineral licks for the deer and corn for the boar. We analysed these mineral licks - two different types - and the corn using the same procedure as described below since mineral licks and additional feeding might help ungulates to compensate for any deficiencies in their diet (e.g. Klaus & Schmidg 1998). However, since we can only speculate about the consequences for the ionome of deer and boar that we analysed, we do not discuss the elemental composition of these salt licks and corn (Appendix 3.2), and potential effects on the ionome of deer and boar, in detail in this study.

3.2.3 Measurements

Each collected tissue was homogenized in the dissection room of Wageningen Environmental Research by grinding it with a blender. We stored about 15-25 grams of the grinded tissue - three table spoons - in plastic bags. The tissue samples were frozen at minus 18 °C before we transferred them into plastic tubes for freeze-drying. The freeze-dried samples were transported to Radboud University, where we further prepared them for the chemical analysis.

We used a microwave digestion method with 5 mL 65% nitric acid (HNO_3) and 2 mL 30% hydrogen peroxide (H_2O_2) to prepare the tissue samples for measuring the elemental concentrations with Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) and Inductively Coupled Plasma Mass Spectroscopy (ICP-MS). We measured 22 elemental concentrations for all the tissue samples. We used ICP-OES to measure 7 elements: calcium (Ca), potassium (K), magnesium (Mg), sodium (Na), phosphorus (P), sulfur (S), and silicon (Si). The other 15 elements were measured using ICP-MS: aluminum (Al), As, boron (B), Cd, Co, chromium (Cr), Cu, iron (Fe), manganese (Mn), molybdenum (Mo), nickel (Ni), Pb, Se, strontium (Sr), and zinc (Zn). We used the same devices as in Wenting et al. (2020), meaning that the reported spike-and-recovery experiments also apply to this study. Correspondingly, the accuracy of these devices was guaranteed - besides using certified reference material for every microwave run - by using the following quality controls (QC): Multi element standard IV, Merck 1.11355; Phosphate standard, Merck 1.19898; Sulphate standard, Merck 1.19813; and Silicium standard, Merck 1.70236. The QC matrices were considered to correspond to the sample matrices since for both, any contamination of HNO_3 and H_2O_2 was eliminated by using blanks (see for more details, including spike-and-recovery experiments, Wenting et al. 2020).

3.3 Results

We present our results in a descriptive way due to the low sample sizes that we used, with four individuals of each species. First, we summarized the total concentrations per element per individual in a table (Table 3.1). This table revealed variation in the total concentrations that we measured, which may indicate variation amongst tissues as well. Second, we listed the highest and lowest concentration measured per element for deer (Table 3.2) and for boar (Table 3.3), including the tissues in which these were found. For most elements, the tissues containing the highest and lowest concentrations varied within and between the species. Third, we used wind rose diagrams to visualize how the total elemental concentration - as noticed in Table 3.1 - is distribution over the 13 tissues (Figure 3.1-3.22).

As we aimed to put our findings in the context of their biological function, we report them per element separately, including a description of the biological relevance of each element for the mammalian body, as well as signs of toxicity and deficiency.

Table 3.1 Total concentrations (µg Kg^{-1}) of elements in individuals of red deer (RD1-RD4) and wild boar (W 1 -W 4) collected from Veluwezoom National Park, the Netherlands. Elements are in alphabetical order.

	RD1	RD2	RD3	RD4	WB1	WB2	WB3	WB4
Al	287	1,172	433	1165	895	393	526	1,141
As	0.22	0.29	0.01	0.43	24.07	0.8	12.06	26.66
B	32.51	88.37	32.87	16.02	75.24	43.46	65.2	55.65
Ca	351,232	171,542	155,282	302,348	435,317	348,739	469,061	256,532
Cd	27.25	39.98	15.08	5.12	19.57	15.19	27.3	20.54
Co	1.02	1.9	1.17	1.92	0.24	0.12	0.68	0.99
Cr	117.39	39.77	16.5	15.73	10.49	16.29	17.84	23.03
Cu	329.94	345.54	374	237.93	263.71	252.6	292.33	251.46
Fe	6,420	7,928	7,250	8,665	8,494	8,643	9,127	6,403
K	305,535	259,502	274,613	278,729	268,186	240,455	299,686	264,669
Mg	26,420	22,522	21,626	19,317	26,182	21,398	23,628	21,948
Mn	3,331	5,462	2,785	5,027	1,535	698	1,902	1,130
Mo	59.7	58.77	54.1	47.3	59.43	45.76	35.63	57.89
Na	152,728	145,511	149,330	156,760	139,680	117,545	146,317	158,860
Ni	105	85.02	72.92	77.5	146	80.14	92.23	111
P	376,183	273,821	286,736	356,541	399,327	344,298	223,948	320,518
Pb	4.67	14	619.94	13.61	24.89	4.82	9.01	21.41
S	193,954	174,096	182,296	187,548	169,640	144,988	170,016	168,743
Se	11.44	14.55	11.93	10.35	14.55	14.76	27.53	22.29
Si	1,768	3,054	1,362	2,391	1,911	1,407	1,797	3,153
Sr	163	106	78.71	107	182	234	27.18	129
Zn	2,207	1,822	2,418	1,972	1,667	1,939	1,938	1,671

Table 3.2 Highest and lowest concentrations (µg Kg^{-1}), with corresponding tissue, in individuals of red deer collected from Veluwezoom National Park, the Netherlands.

	RD1				RD2			
	Highest		Lowest		Highest		Lowest	
Al	50.92	Skin	8.75	Liver	450	Intestines	15.69	Liver
As	0.156	Pancreas	0.06	Skin	0.137	Pancreas	0.04	Heart
B	5.5	Stomach	0.507	Eyes	24.34	Pancreas	0.995	Liver
Ca	329800	Bone	249	Liver	148300	Bone	302	Liver
Cd	19.8	Kidney	0.065	Eyes	34.78	Kidney	0.079	Stomach
Co	0.352	Liver	0.015	Lungs	0.643	Liver	0.027	Muscle
Cr	20.01	Spleen	0.823	Bone	26.1	Intestines	0.353	Lungs
Cu	121	Liver	2.68	Intestines	156	Liver	2.67	Bone
Fe	1944	Lungs	49.59	Bone	2394	Lungs	57.65	Bone
K	43000	Spleen	638	Bone	32980	Spleen	417	Bone
Mg	5371	Bone	439	Skin	5735	Intestines	395	Skin
Mn	1887	Stomach	1.89	Bone	3133	Intestines	0.571	Eyes
Mo	34.09	Pancreas	0.053	Intestines	38.96	Heart	1.21	Intestines
Na	22640	Brain	3694	Muscle	17440	Eyes	3805	Muscle
Ni	31.37	Pancreas	1.81	Bone	17.35	Intestines	0.97	Eyes
P	154900	Bone	3949	Skin	69890	Bone	3532	Eyes
Pb	1.65	Bone	0.029	Brain	4.28	Intestines	0.038	Eyes
S	21890	Skin	2002	Intestines	30890	Skin	2533	Bone
Se	3.73	Kidney	0.227	Skin	6.44	Kidney	0.011	Bone
Si	401	Pancreas	33	Heart	1135	Pancreas	14.6	Brain
Sr	125	Bone	0.116	Liver	50.27	Bone	0.267	Liver
Zn	408	Pancreas	28.3	Intestines	291	Liver	24.86	Eyes

Table 3.2 Continued.

	RD3				RD4			
	Highest		Lowest		Highest		Lowest	
Al	99.32	Intestines	10.02	Heart	366	Skin	13.72	Muscle
As	0.013	Liver	0.013	Liver	0.231	Stomach	0.07	Skin
B	5.66	Lungs	1.02	Kidney	5.21	Stomach	0.003	Kidney
Ca	138100	Bone	295	Muscle	279400	Bone	264	Muscle
Cd	13.13	Kidney	0.02	Spleen	4.77	Kidney	0.006	Intestines
Co	0.37	Liver	0.018	Heart	0.408	Liver	0.046	Muscle
Cr	3.39	Skin	0.572	Brain	3.2	Lungs	0.599	Kidney
Cu	137	Liver	3.69	Bone	51.39	Lungs	1.28	Bone
Fe	2362	Lungs	80.51	Bone	2678	Lungs	50.31	Bone
K	35220	Brain	844	Bone	34800	Spleen	29.4	Pancreas
Mg	2581	Intestines	685	Eyes	4852	Bone	2.4	Pancreas
Mn	1245	Intestines	1.334	Bone	3895	Intestines	1.71	Bone
Mo	42.16	Liver	0.057	Stomach	28.44	Stomach	0.919	Muscle
Na	23590	Brain	3386	Muscle	33820	Eyes	3937	Muscle
Ni	18.64	Lungs	1.49	Brain	20.82	Lungs	1.3	Eyes
P	65530	Bone	5561	Eyes	135000	Bone	5021	Eyes
Pb	535	Lungs	0.016	Brain	2.62	Bone	0.147	Muscle
S	27680	Skin	3463	Bone	30520	Skin	19.7	Pancreas
Se	5.23	Kidney	0.17	Skin	5.76	Kidney	0.066	Muscle
Si	507	Intestines	20.1	Bone	881	Intestines	18.7	Heart
Sr	50.87	Bone	0.194	Muscle	78.56	Bone	0.225	Muscle
Zn	464	Kidney	43.28	Bone	245	Liver	53.74	Eyes

Table 3.3 Highest and lowest concentrations (µg Kg^{-1}), with corresponding tissue, in individuals of wild boar collected from Veluwezoom National Park, the Netherlands.

	WB1				WB2			
	Highest		Lowest		Highest		Lowest	
Al	367	Intestines	9.64	Brain	45.31	Intestines	18.32	Bone
As	11.55	Intestines	0.07	Brain	0.4	Bone	0.06	Bone
B	16.65	Intestines	3.31	Liver	6.28	Skin&Hair	1.27	Heart
Ca	421300	Bone	523	Muscle	336500	Bone	298	Skin&Hair
Cd	6.97	Kidney	0.4	Intestines	12.76	Kidney	0.06	Bone
Co	0.11	Intestines	0.01	Kidney	0.08	Liver	0.04	Spleen
Cr	1.97	Liver	0.3	Bone	2.41	Brain	0.4	Bone
Cu	42.82	Kidney	2.03	Bone	56.76	Kidney	1.95	Bone
Fe	1996	Liver	83.82	Bone	4115	Lungs	43.1	Skin&Hair
K	35310	Spleen	3916	Bone	31350	Brain	498	Skin&Hair
Mg	6506	Bone	362	Skin&Hair	5118	Bone	65.1	Skin&Hair
Mn	953	Intestines	1.43	Bone	286	Stomach	1	Skin&Hair
Mo	40.75	Bone	0.12	Lungs	17.96	Liver	0.27	Spleen
Na	25690	Eyes	3701	Muscle	18280	Eyes	376	Skin&Hair
Ni	58.27	Intestines	2.81	Eyes	31.39	Lungs	1.67	Brain
P	200000	Bone	3787	Skin&Hair	155200	Bone	494	Skin&Hair
Pb	7.74	Bone	0.8	Heart	1.76	Bone	0.03	Brain
S	18780	Heart	6153	Bone	18720	Muscle	705	Skin&Hair
Se	6.28	Kidney	0.4	Intestines	9.83	Kidney	0.18	Eyes
Si	686	Intestines	47.1	Brain	161	Intestines	54.4	Skin&Hair
Sr	150	Bone	1.24	Liver	220	Bone	0.8	Kidney
Zn	210	Liver	46.49	Skin&Hair	330	Liver	13.44	Skin&Hair

Table 3.3 Continued.

	WB3				WB4			
	Highest		Lowest		Highest		Lowest	
Al	160	Bone	14.64	Brain	661	Stomach	12.61	Spleen
As	2.71	Stomach	0.03	Brain	19.69	Intestines	0.01	Heart
B	29.62	Bone	1.31	Pancreas	22.64	Stomach	0.83	Heart
Ca	454500	Bone	591	Heart	236700	Bone	478	Liver
Cd	19	Kidney	0.02	Heart	16.03	Kidney	0.05	Heart
Co	0.3	Intestines	0.07	Kidney	0.75	Stomach	0.01	Heart
Cr	6.95	Intestines	0.34	Brain	3.27	Stomach	0.46	Bone
Cu	43.97	Bone	5.68	Eyes	47.39	Heart	1.45	Bone
Fe	2929	Lungs	51.43	Skin&Hair	1241	Liver	40.24	Bone
K	34200	Bone	4406	Skin&Hair	36270	Muscle	1068	Bone
Mg	5805	Bone	242	Skin&Hair	3485	Bone	295	Skin&Hair
Mn	1293	Bone	2.19	Brain	561	Stomach	1.2	Bone
Mo	24.48	Eyes	0.71	Bone	32.17	Spleen	0.19	Intestines
Na	18540	Eyes	3560	Skin&Hair	22810	Eyes	4245	Muscle
Ni	22.22	Lungs	0.93	Skin&Hair	46.79	Spleen	1.1	Skin&Hair
P	25780	Kidney	2791	Skin&Hair	110500	Bone	3711	Skin&Hair
Pb	2.41	Bone	0.19	Heart	9.48	Stomach	0.03	Brain
S	19950	Kidney	6064	Skin&Hair	22640	Heart	3861	Bone
Se	13.48	Kidney	0.04	Skin&Hair	10.12	Kidney	0.06	Liver
Si	485	Bone	49.7	Liver	1335	Stomach	47.1	Bone
Sr	12.04	Bone	0.6	Heart	104	Bone	0.15	Liver
Zn	251	Liver	41.5	Skin&Hair	224	Liver	49.56	Eyes

3.3.1 Aluminum (Al)

Although Al is the third most common element in the earth's crust, it is poorly absorbed in the animal body and there is no clear role described for this element (NRC 2001; Pérez-Grenados & Vaquero 2002). Due to long-lasting acidification of the environment due to excess N deposition mainly from agriculture, industry and traffic, pH values dropped significantly below 3.5 (Siepel et al. 2018), which is much lower than the given pH value of 4.2 below which Al becomes soluble from soil aluminum oxides and hydroxides (De Graaf et al. 1997) and is thus potentially taken up by the vegetation, and so by animals. Although Al toxicity is associated with malabsorption of e.g. P (Allen 1984, cited in NRC 2001), it remains unclear which and how other elements interplay with it to cause toxicity (NRC 2002). Most described symptoms of Al toxicity include inappropriately decreased feelings of thirst, refusal to swallow, and movement disorders such as hypokinesia (e.g. Hewitt et al. 1990; Yellamma et al. 2010).

Thurston et al. (1972) and Pérez-Granados & Vaquero (2002) described that Al will be mainly accumulated in bone when renal function is compromised. However, although the digestive organs tended to be important target tissues for some individuals (Figure 3.1a-h; Table 3.2+3.3), we found no particular tissue that contained most of the Al for the individuals we measured.

3.3.2 Arsenic (As)

Contrary to Al, As is well absorbed but has no specific role in the animal body (NRC 2002), although Frost et al. (1955) described that As may function as antibiotic and anti-coccidial in swine and poultry. By our knowledge, it has not been used for that purpose in other animals (NRC 2002). Since it is well absorbed, As toxicity is likely to occur when food is contaminated with As (e.g. Ventura-Lima et al. 2011), for instance from Cu and Pb smelters (Lillie 1970). When As exposure builds up slowly, animals may get used to it and ruminants may even develop a taste for it (Clarke & Clarke 1975). As appears to be most toxic in inorganic form (Shi et al. 2004; Valko et al. 2005), and affects most organs, although kidney is mentioned as the most sensitive organ for As toxicity (Cohen et al. 2006). Commonly described symptoms of As toxicity include straining, abdominal pain, bone marrow depression with anemia, skin pigmentation changes, and diarrhea containing blood and mucus (e.g. Selby et al. 1977; Hutton & Christians 1983; Hall 2002).

Although the As concentration in WB2 was noticeable lower compared to the other boar, we found noticeably higher As concentrations in boar compared to deer (Table 3.1). It has been traditionally thought that As accumulates in the hairs (e.g. Hindmarsh 2002), however skin and hair did not contain the highest concentration for any individual (Figure 3.2a-h; Table 3.2-3.3). As was rather randomly scattered throughout the whole body for WB3 (Figure

3.2g), and the guts appeared to be the main target tissues for the other individuals (Figure 1.2a-h). We were not able to detect As in any other tissue than liver for RD3 (Figure 3.2c; Table 3.2).

3.3.3 Boron (B)

B is an essential trace element that is important for numerous life functions, including bone density, wound healing, embryonic development and metabolism of sex steroids and vitamin D (e.g. Goldbach & Wimmer 2007; Pizzorno 2015; Abdelnour et al. 2018). Although symptoms of B toxicity are poorly understood in animals (e.g. Nielson 1997), acute B toxicity has been described for humans, including symptoms as nausea, vomiting, diarrhoea, and lethargy (Linden et al. 1986). Chronic B toxicity is associated with symptoms including weight loss, reduced reproduction, and decreased appetite (Hunt 1993, cited in Nielsen 1997). However, for both humans and animals, no critical intake values have been described, which also applies to potentially B deficiency (e.g. Goldbach & Wimmer 2007). Signs of B deficiency, however, are correlated with low immune function and increased mortality risk due to high incidence of osteoporosis (Abdelnour et al. 2018).

Although it has been suggested that B is mostly accumulated in bone, nails and hair (e.g. Ku et al. 1991; Uluisik et al. 2018), we found it scattered throughout the whole body (Figure 3.3a-h). The B concentration was the lowest for RD4 (Table 3.1), which tended to accumulate more in bone, eyes, intestines and stomach (Figure 3.3d). B was not detectable in the bone and heart of RD3 but was present in any other tissue (Figure 3.3c). Moreover, WB3 was the only individual with the highest B concentration in bone (Table 3.3; Figure 3.3g), while the B concentration was notably the highest in the stomach and intestines for WB4 (Table 3.3; Figure 3.3h).

3.3.4 Calcium (Ca)

Ca has many functions in the animal body, including the formation of skeletal tissues, transmission of nervous tissue impulses, excitation of skeletal and cardiac muscle contraction, and blood clotting (e.g. NRC 2002; Simon et al. 2014). Excessive dietary intake is not associated with any specific signs of (NRC 2002). Ca toxicity - aka hypercalcemia - can manifest in many different forms, including renal stones, bone pain, gastrointestinal abdominal moans, neuromuscular psychic groans, and cardiovascular issues (e.g. Carroll & Schade 2003). Ca deficiency - aka hypocalcemia - is most dangerous for young animals, which leads to reduced mineralization of new bones and therefore reduced growth (NRC 2002; Schenck et al. 2006). Like a deficiency of vitamin D or P, a deficiency of Ca can also contribute to rickets (NRC 2002; Schenck et al. 2006).

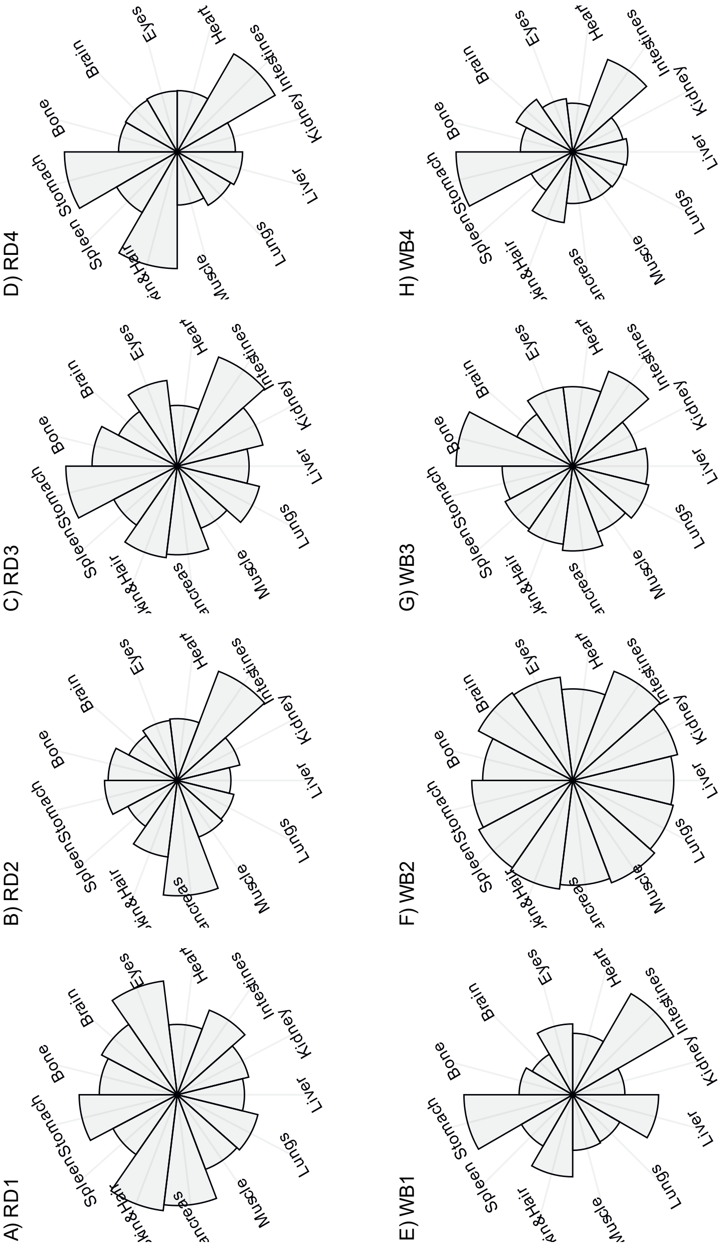

Figure 3.1 Distribution of aluminium (Al) per tissue per individual.

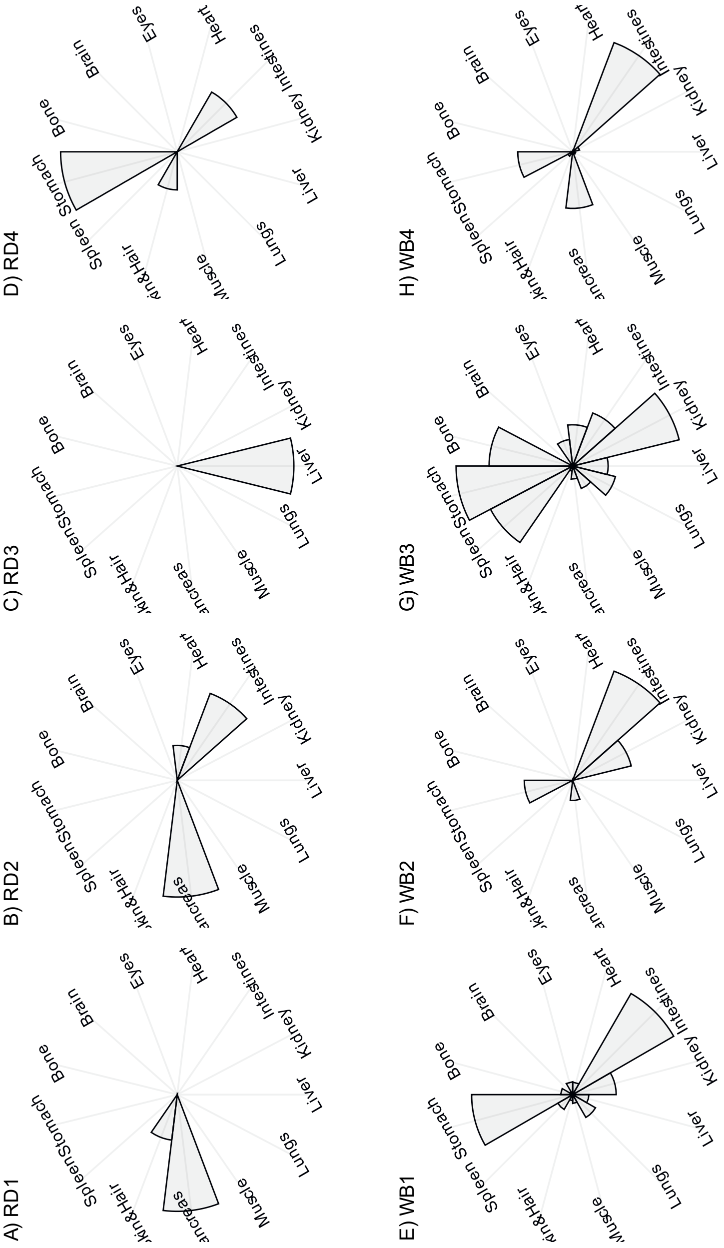

Figure 3.2 Distribution of arsenic (As) per tissue per individual.

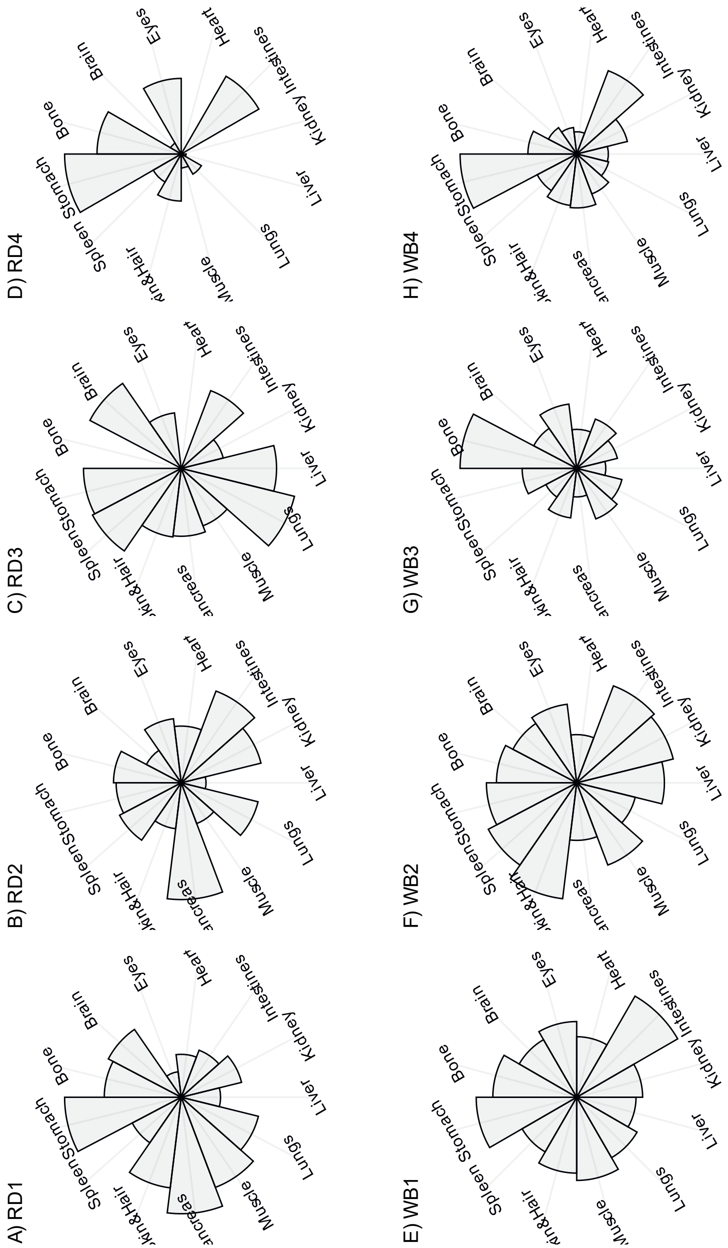

Figure 3.3 Distribution of boron ( ) per tissue per individual.

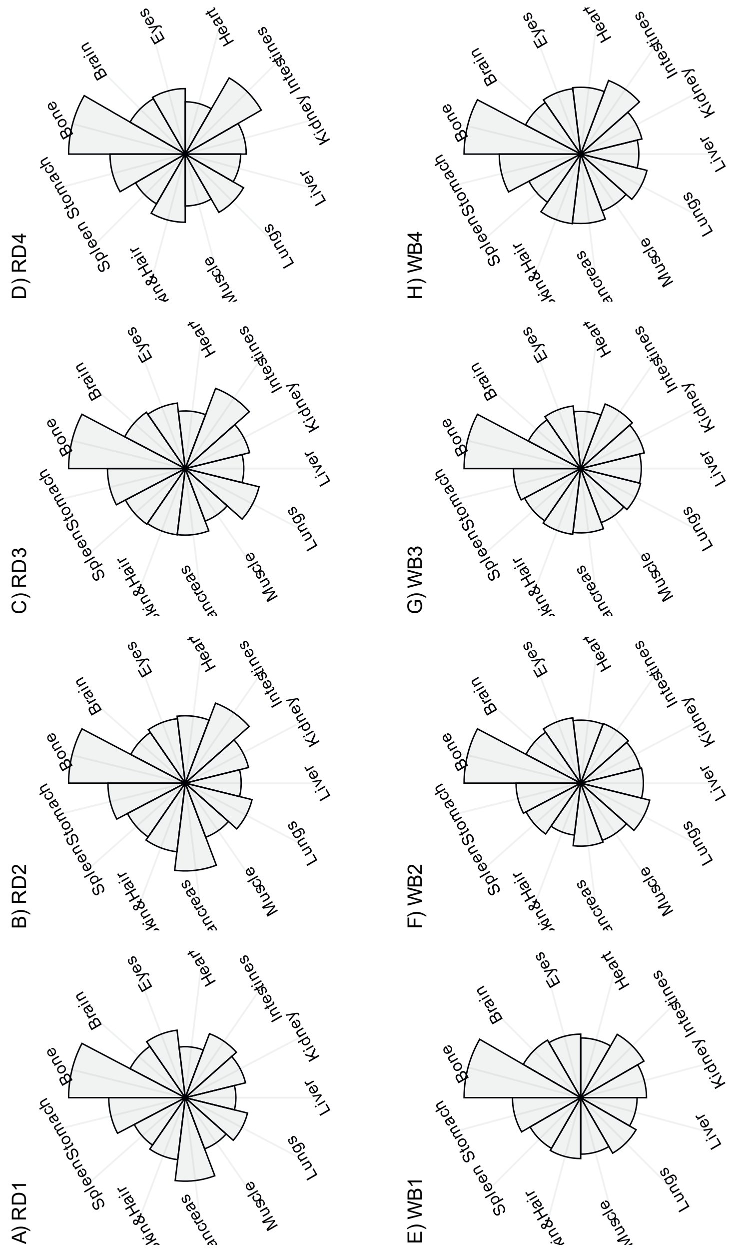

Figure 3.4 Distribution of calcium (Ca) per tissue per individual.

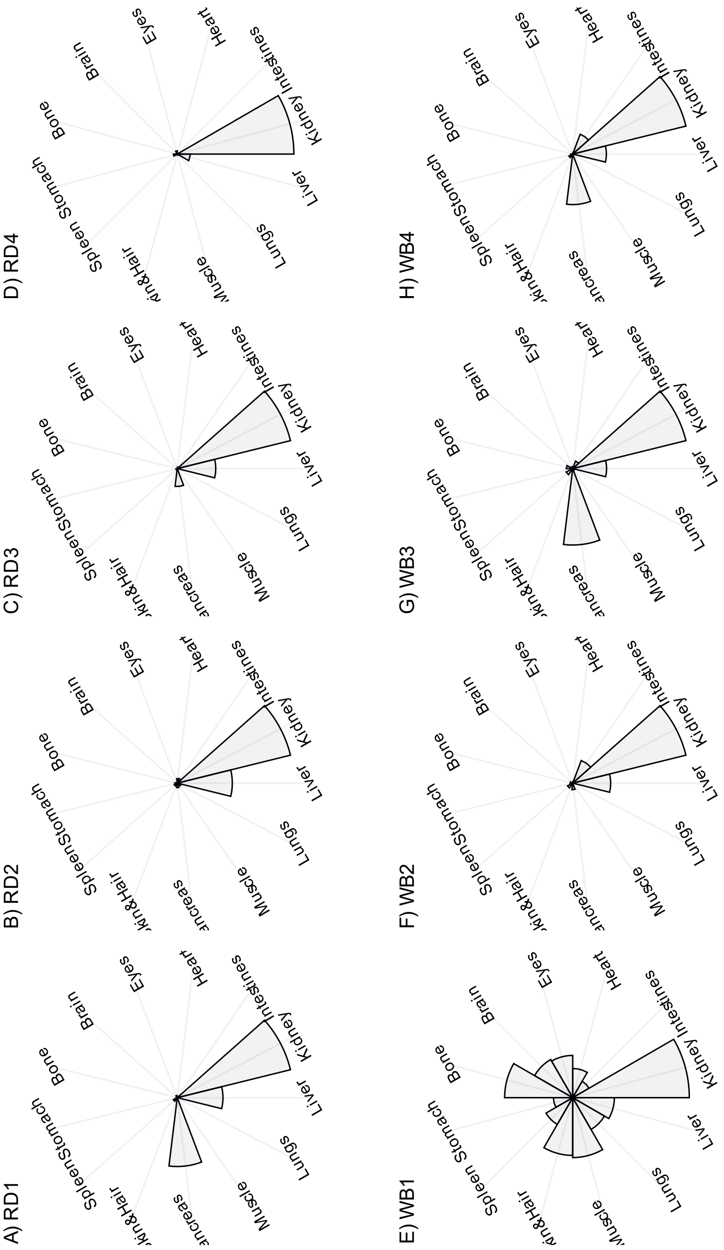

Figure 3.5 Distribution of cadmium (Cd) per tissue per individual.

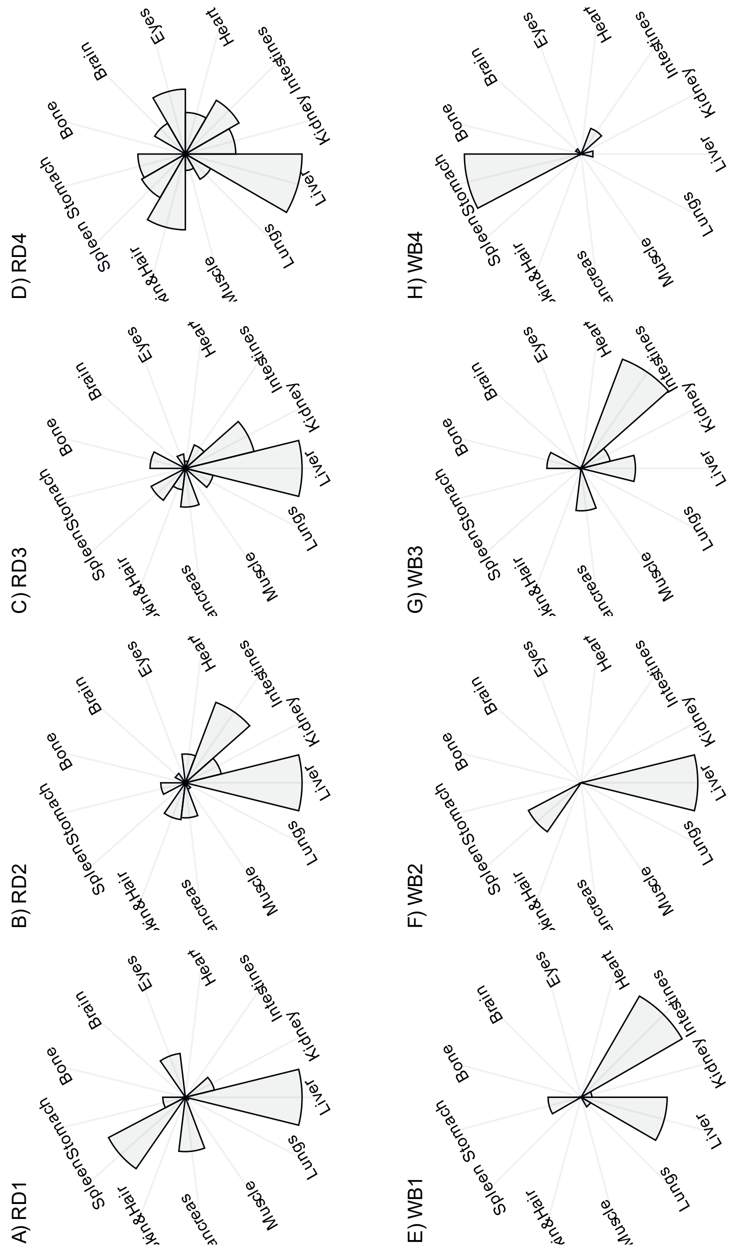

Figure 3.6 Distribution of cobalt (Co) per tissue per individual.

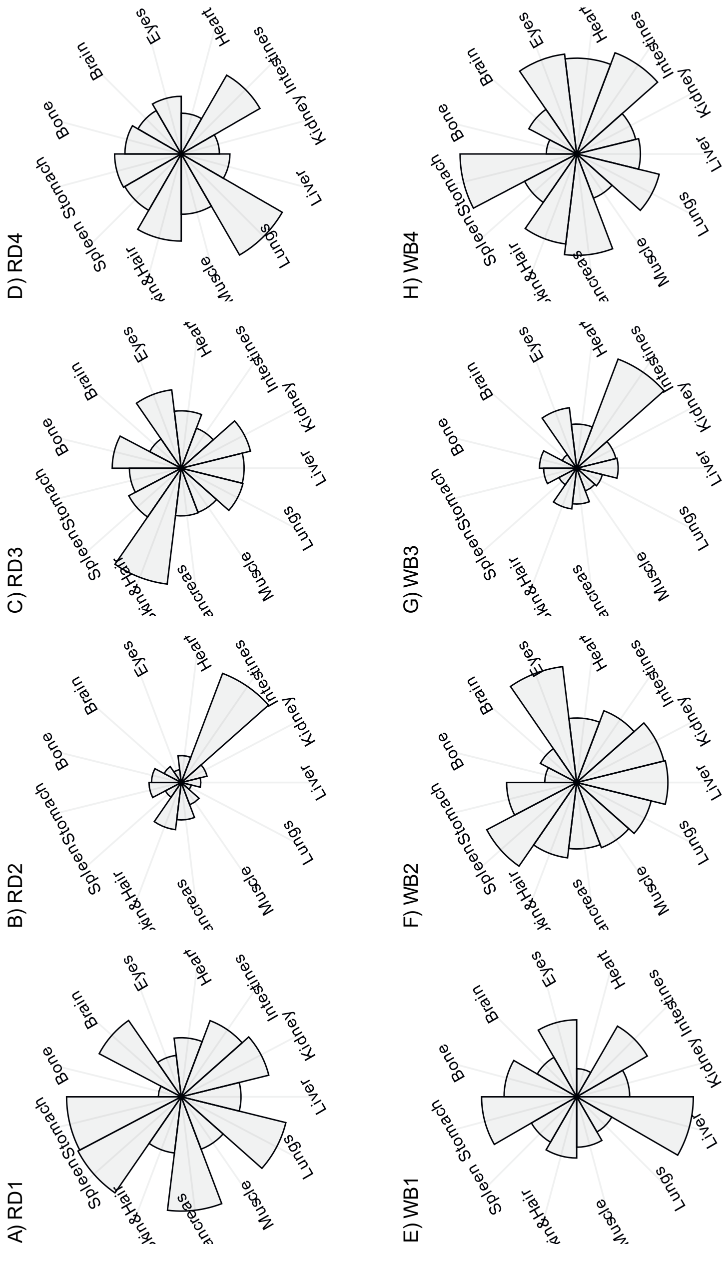

Figure 3.7 Distribution of chromium (Cr) per tissue per individual.

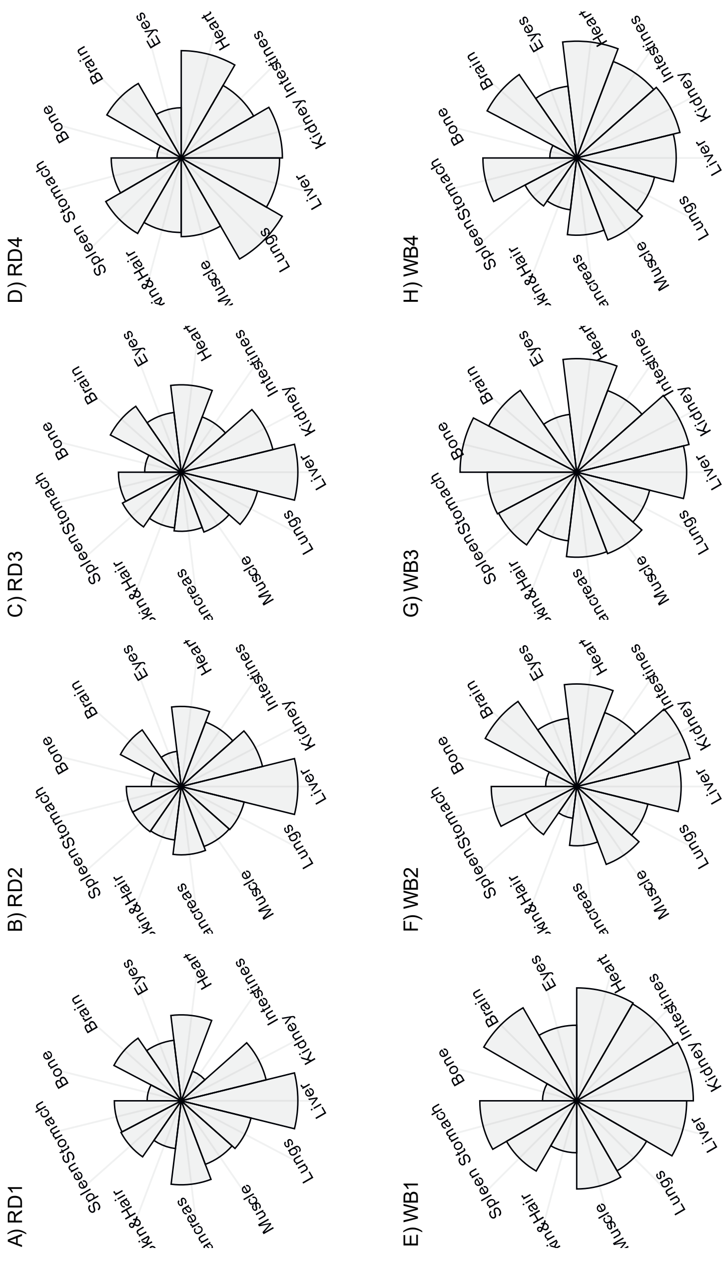

Figure 3.8 Distribution of copper (Cu) per tissue per individual.

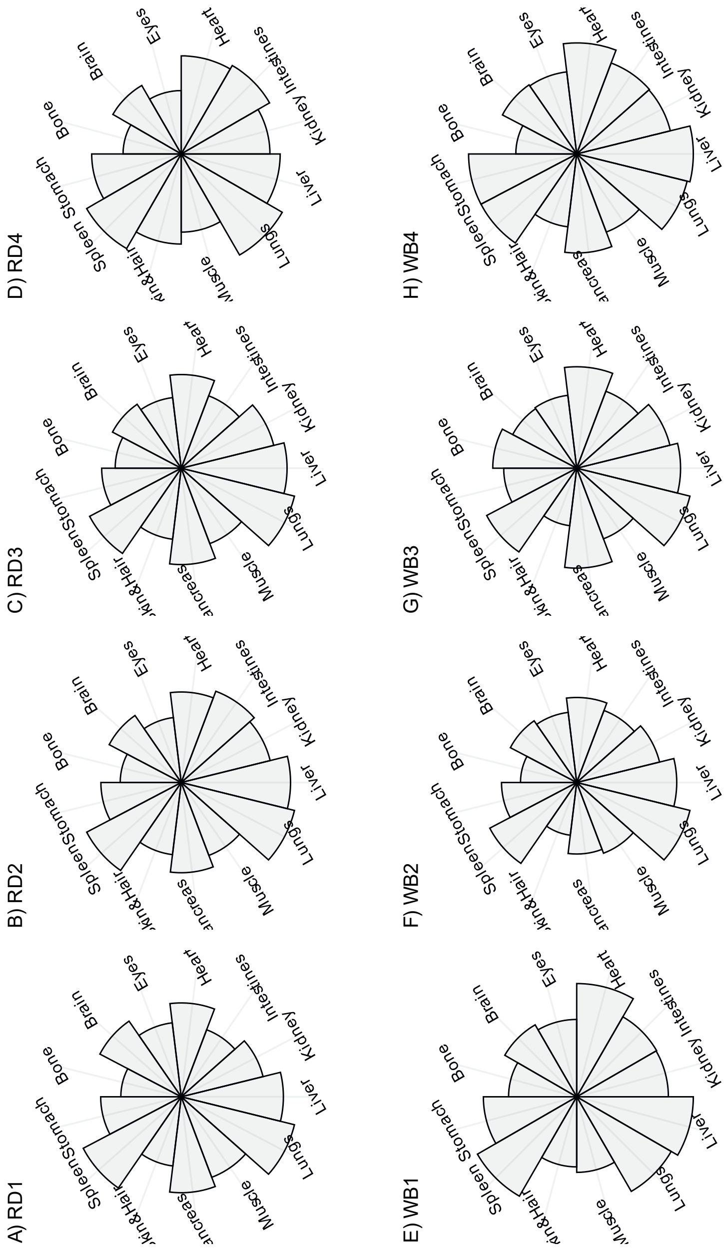

Figure 3.9 Distribution of iron (Fe) per tissue per individual.

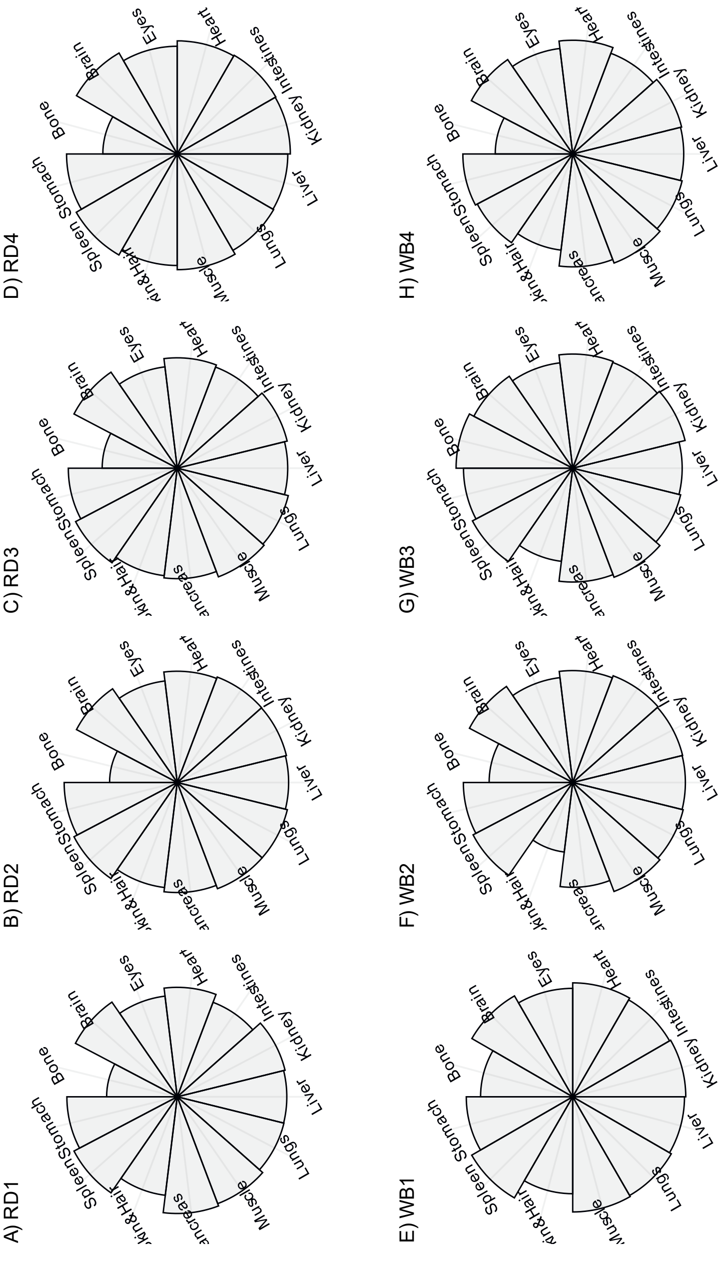

Figure 3.10 Distribution of potassium (K) per tissue per individual.

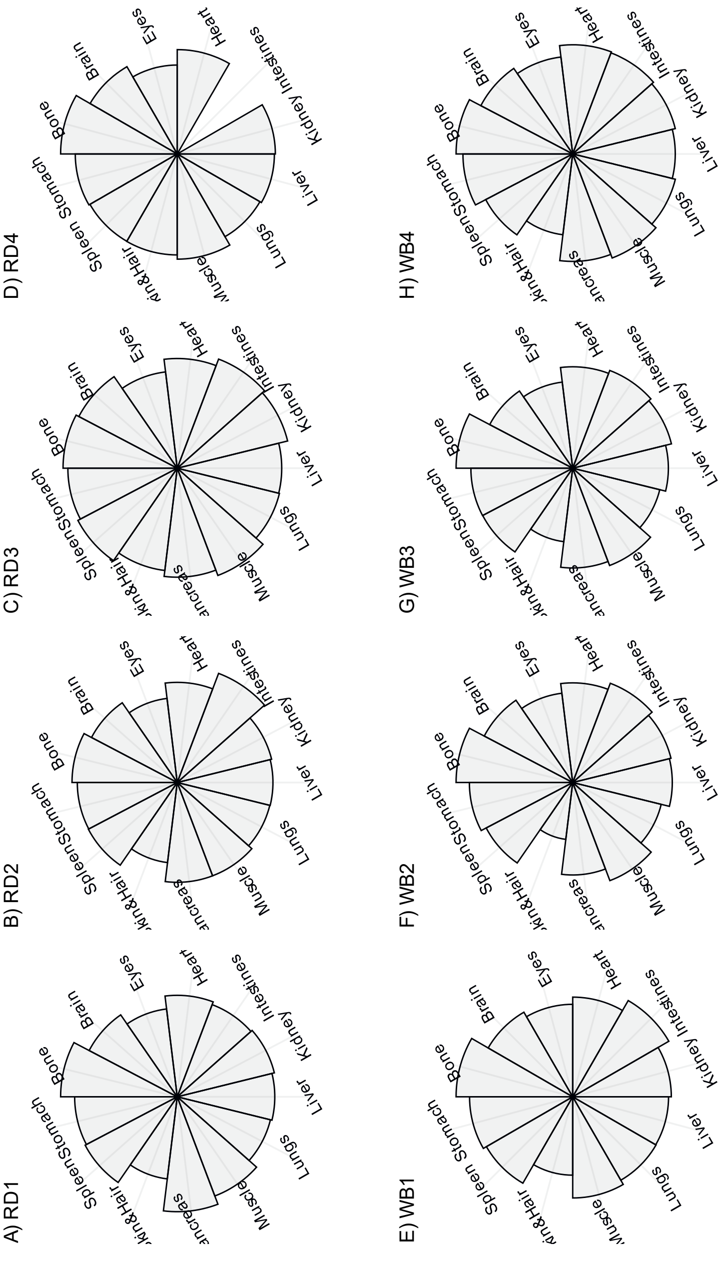

Figure 3.11 Distribution of magnesium (Mg) per tissue per individual.

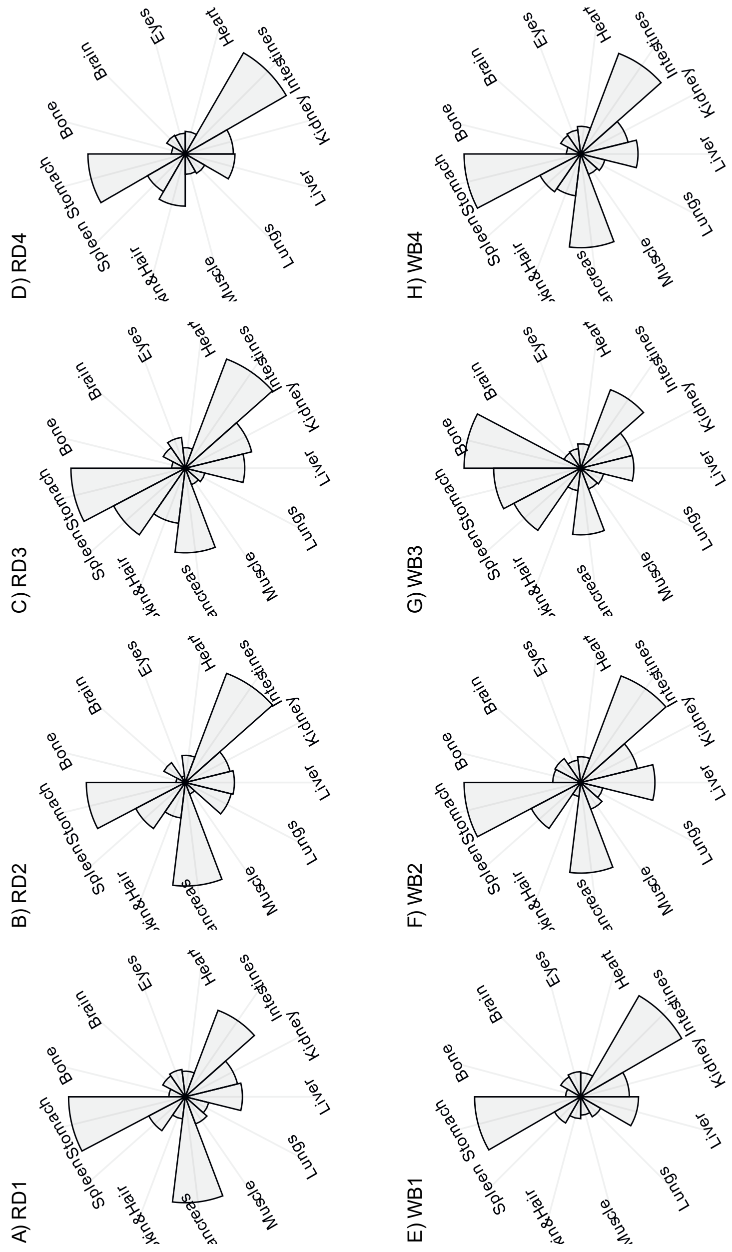

Figure 3.12 Distribution of manganese (Mn) per tissue per individual.

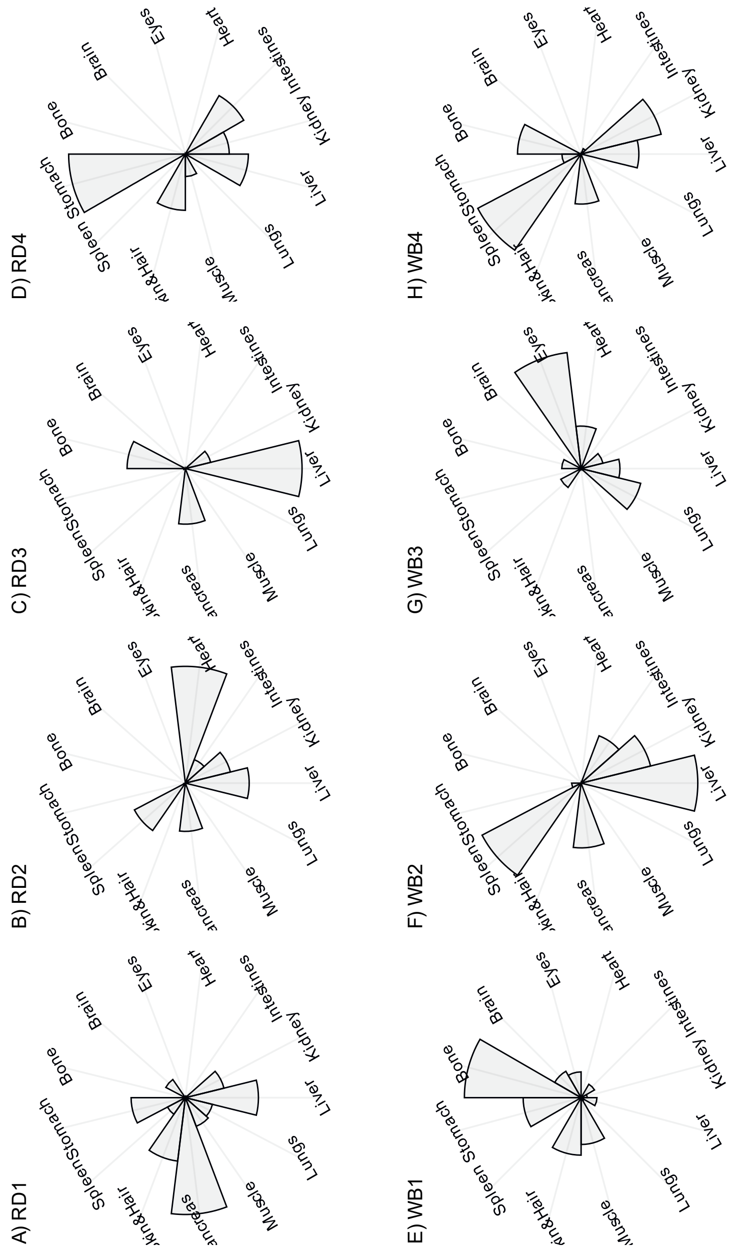

Figure 3.13 Distribution of molybdenum (Mo) per tissue per individual.

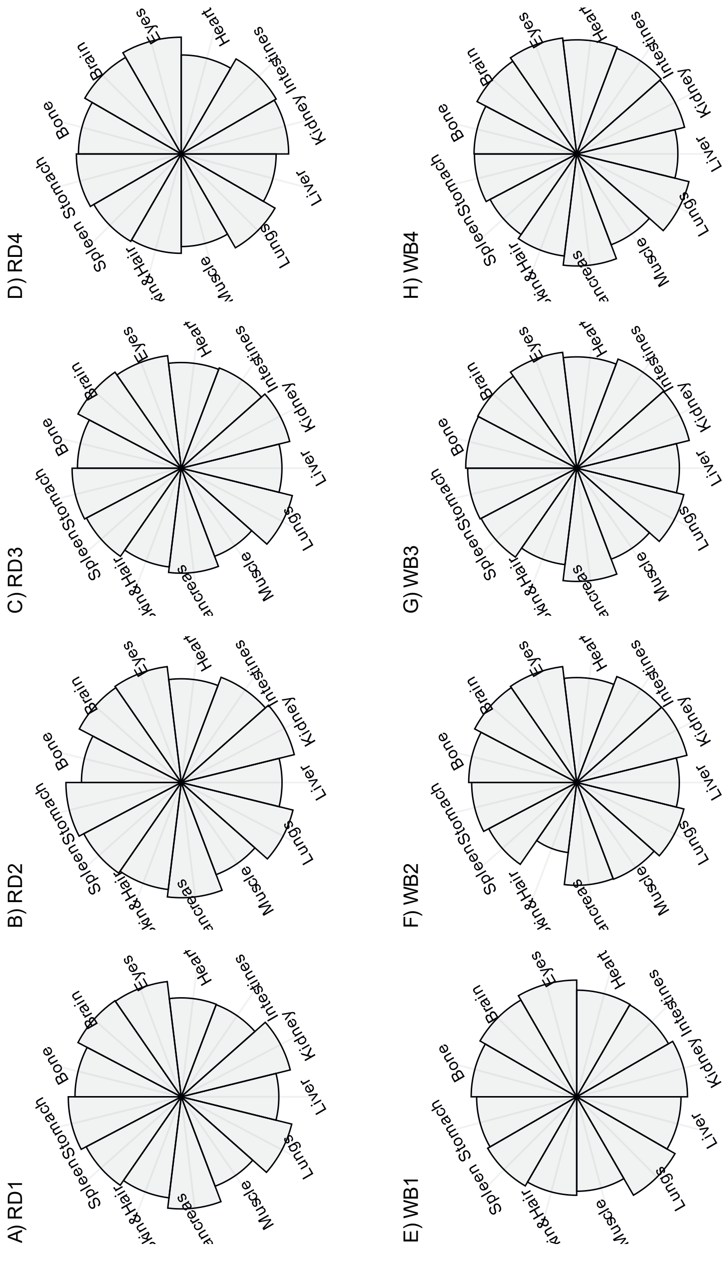

Figure 3.14 Distribution of sodium (Na) per tissue per individual.

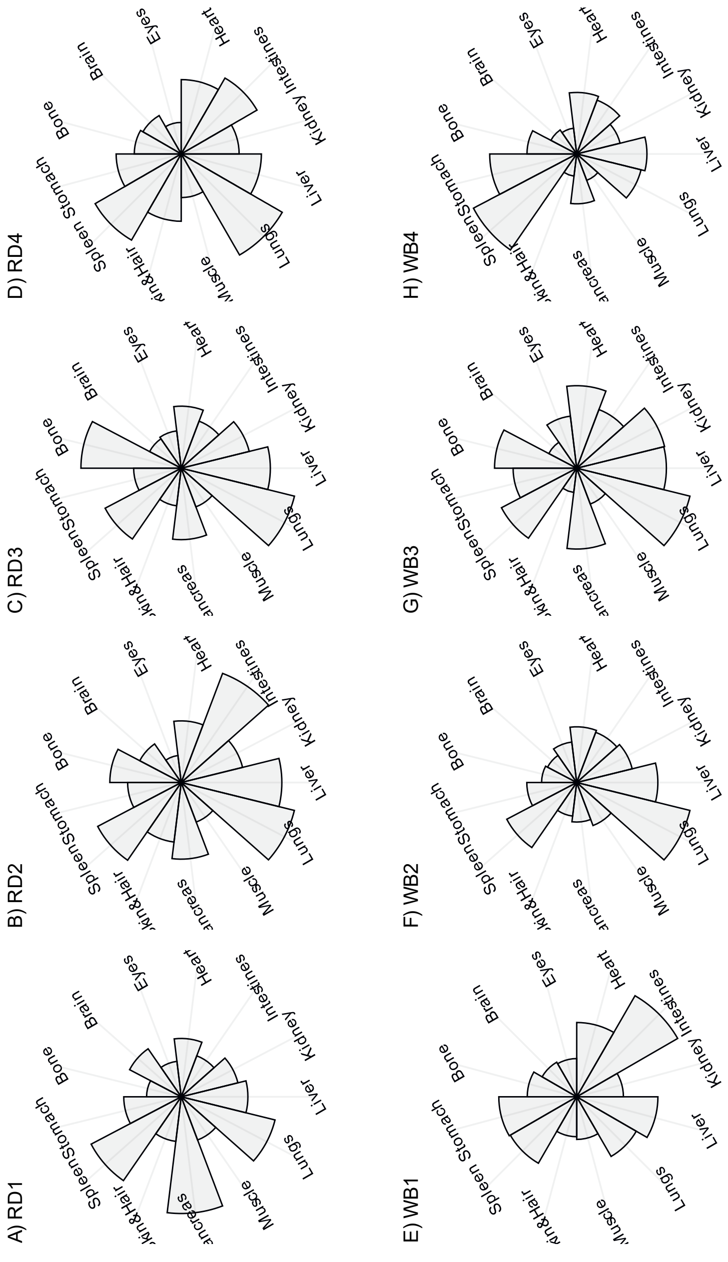

Figure 3.15 Distribution of nickel (Ni) per tissue per individual.

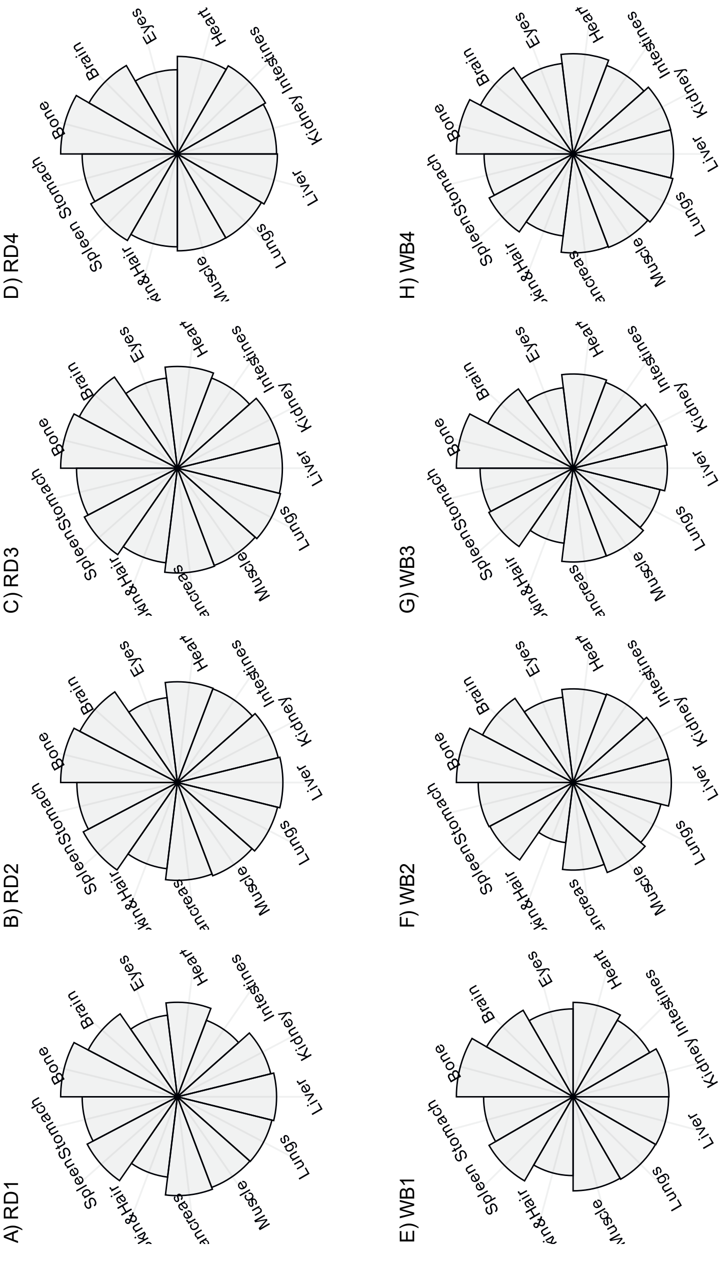

Figure 3.16 Distribution of phosphorous (P) per tissue per individual.

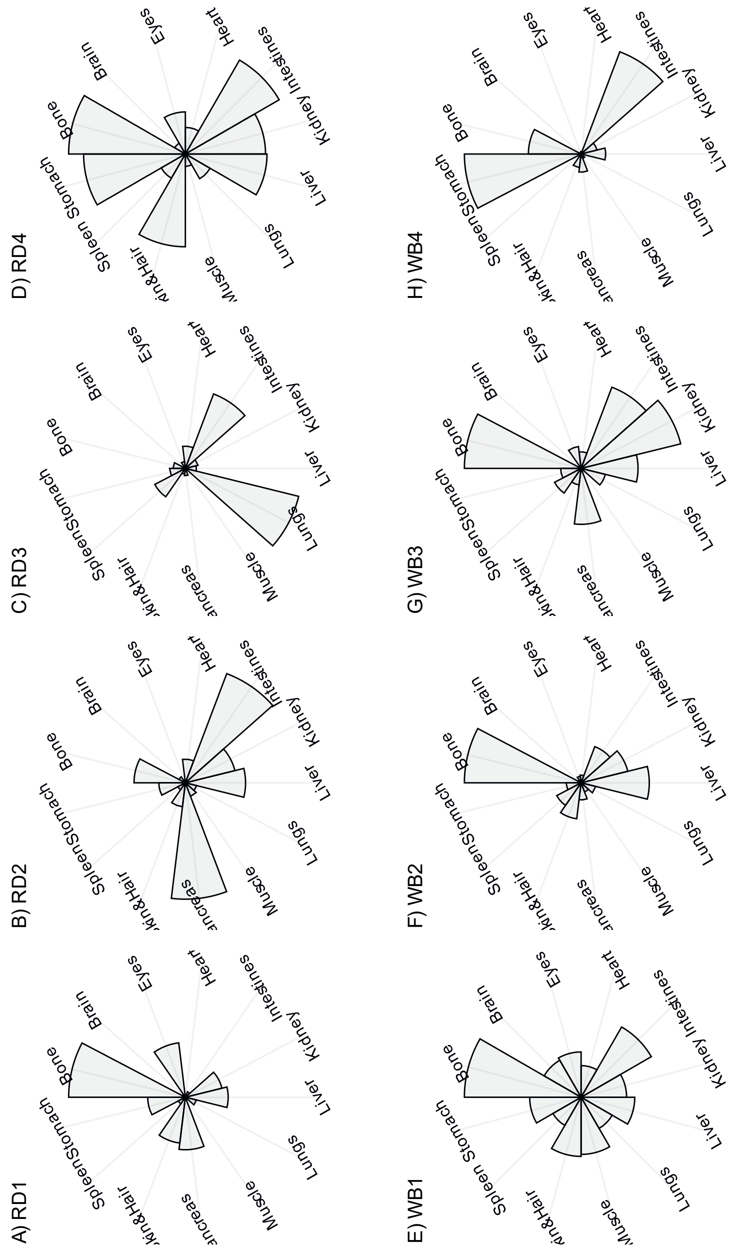

Figure 3.17 Distribution of lead (Pb) per tissue per individual.

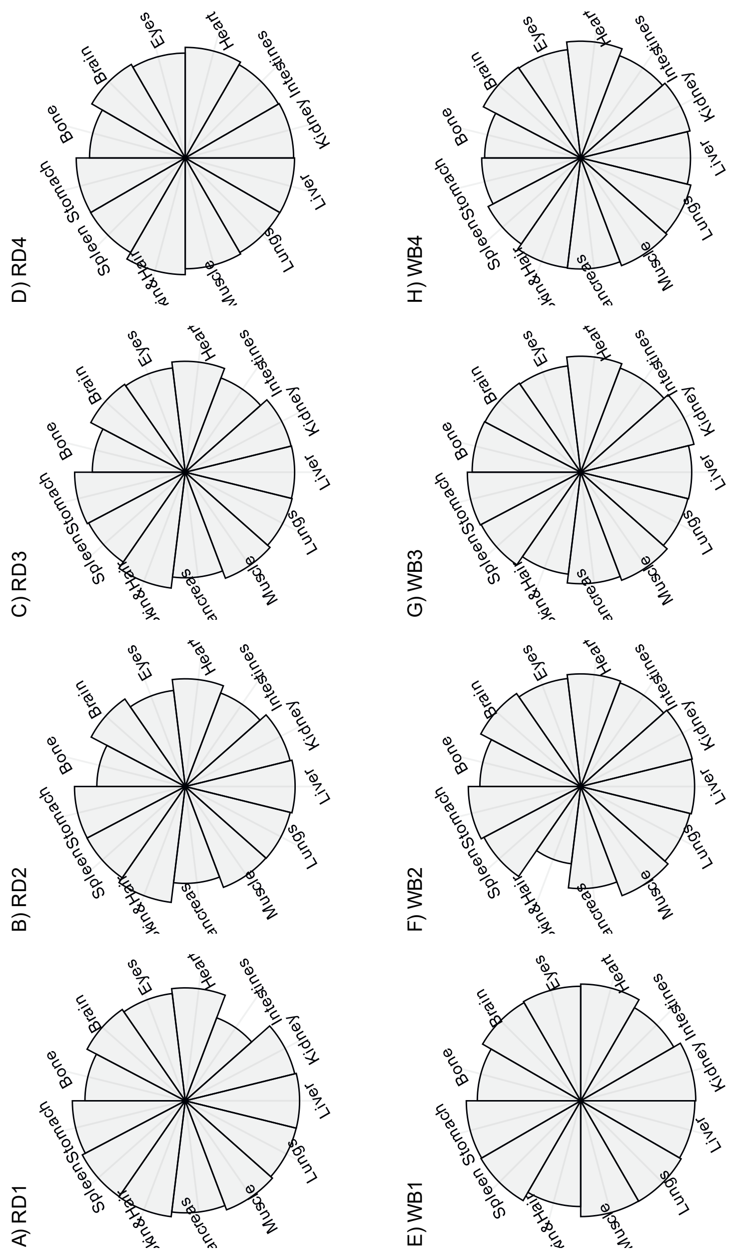

Figure 3.18 Distribution of sulfur (S) per tissue per individual.

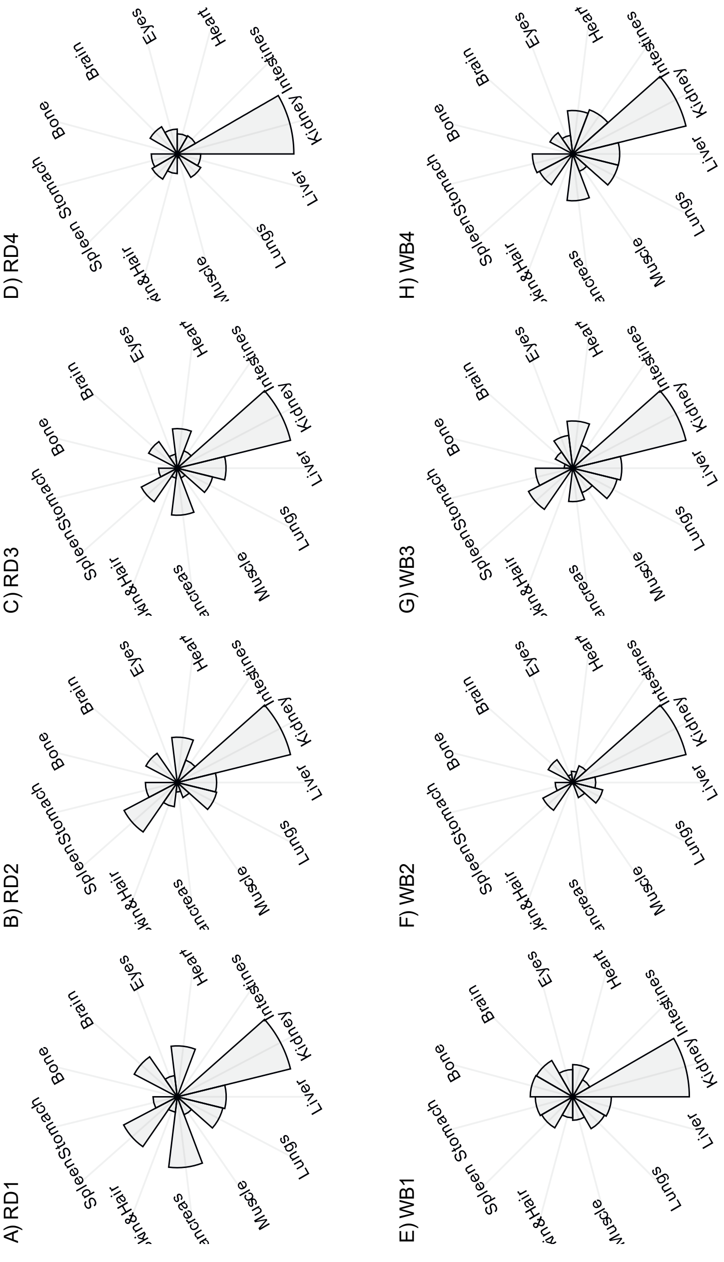

Figure 3.19 Distribution of selenium (Se) per tissue per individual.

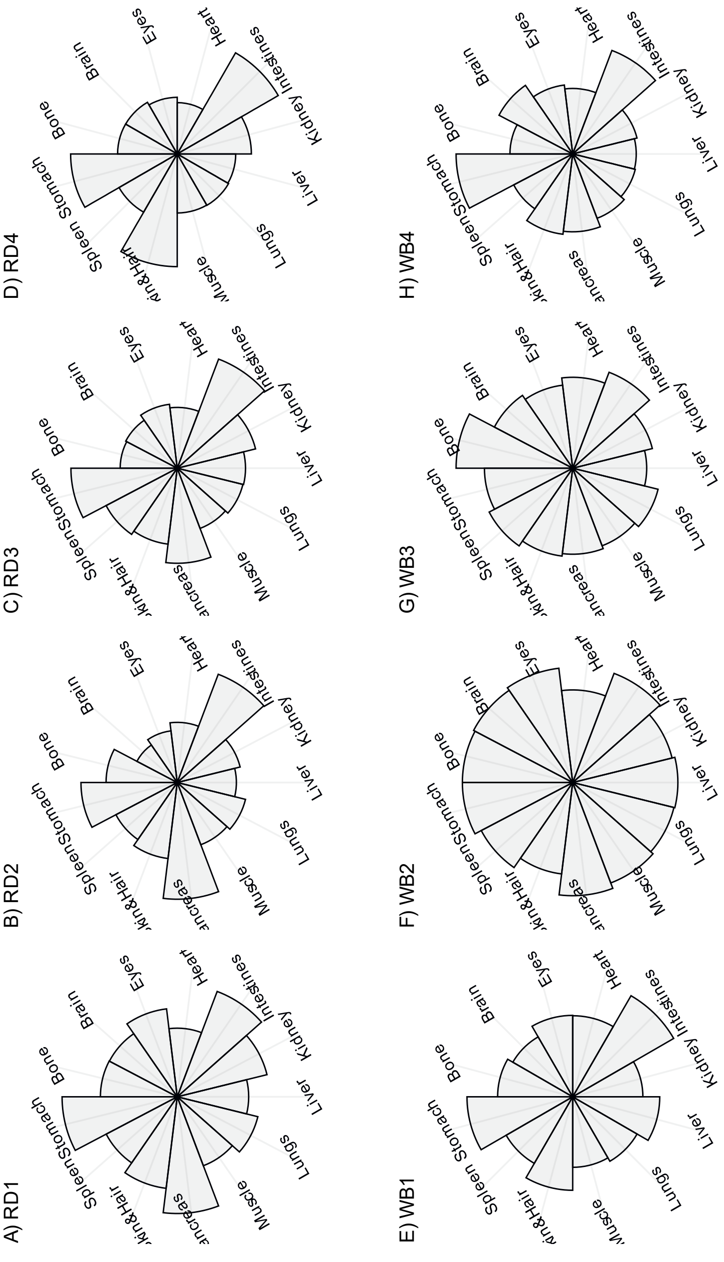

Figure 3.20 Distribution of silicon (Si) per tissue per individual.

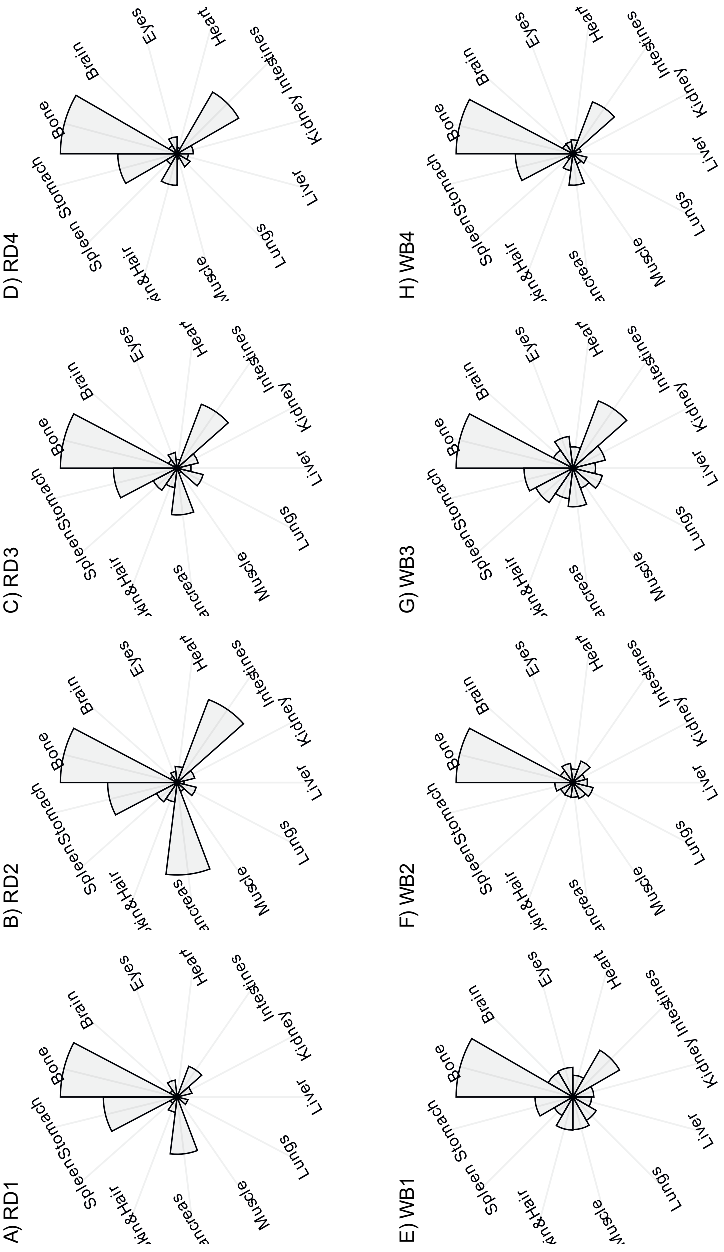

Figure 3.21 Distribution of strontium (Sr) per tissue per individual.

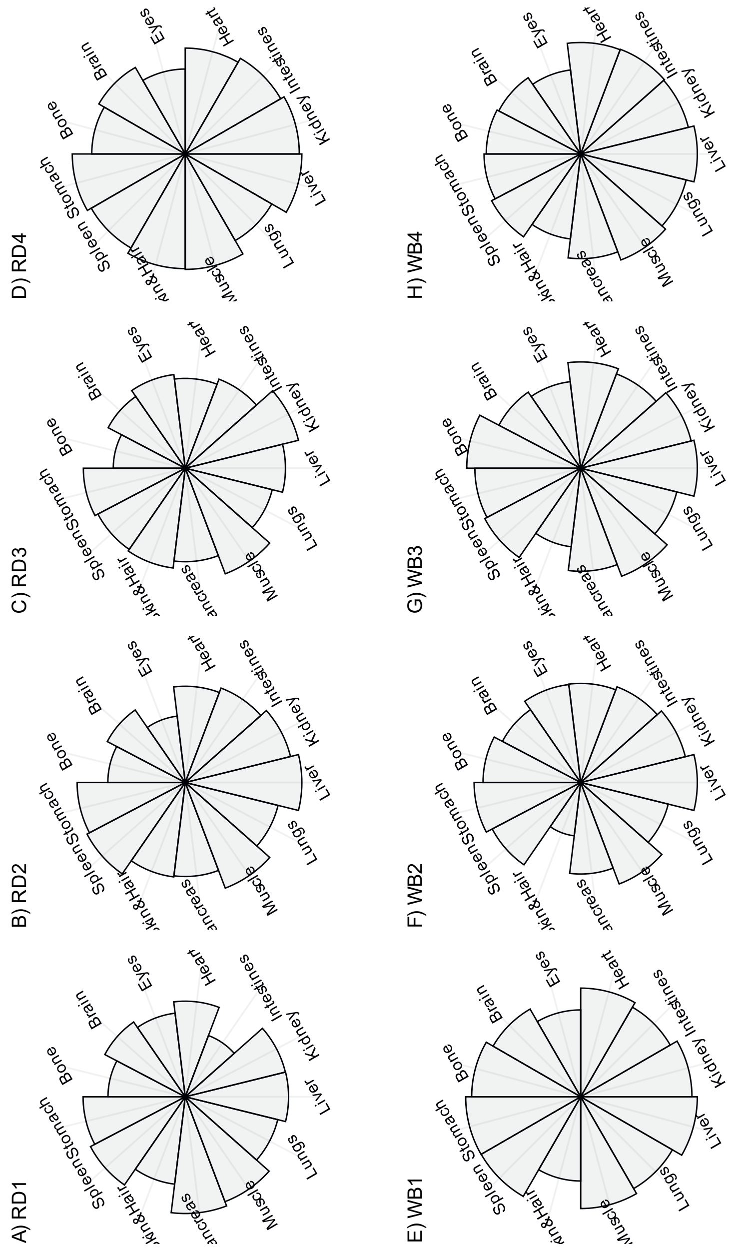

Figure 3.22 Distribution of zinc (Zn) per tissue per individual.

We found Ca in all body tissues for all individuals (Figure 3.4a-h). NRC (2002) described that about 98 percent of the total Ca pool is located in the bones. However, although we found the highest Ca concentration in bone for all individuals (Table 3.2+3.3), we found that the Ca concentrations in bone contributed to 86 to 94 percent of the total Ca of deer (Table 3.1+3.2), and 92 to 97 percent for boar (Table 3.1+3.3). WB4 had the lowest Ca concentration in its body (Table 3.1), of which the concentration in bone was about 92 percent of the total Ca (Table 3.1+3.3), and was the only boar with a total Ca concentration lower than 300,000 µg Kg^{-1} (Table 3.1). RD1 had the highest Ca concentration of the deer, followed by RD4 (Table 3.1). For these individuals, the percentage of Ca stored in bone was higher compared to RD2 and RD4 (Table 3.1+3.2).

3.3.5 Cadmium (Cd)

Cd is a highly toxic heavy metal that has no essential function in any physiological and biochemical process (e.g. NRC 2002; Patra et al. 2006; Yu et al. 2006; El-Sharaky et al. 2007; Swarup et al. 2007; Djukić-Ćosić et al. 2008). It accumulates in kidney, causing renal damage, from where it is very poorly and slowly excreted (NRC 2002). It is antagonistic to Zn, Cu and Fe (NRC 2002; Bridges & Zalups 2005). Acute Cd toxicity appears to be rare, but chronic Cd toxicity may be expressed by disturbed renal function, altered reproduction, and damaged lung function (e.g. Rami et al. 2014).

For all individuals, we indeed found the highest Cd concentrations in the kidney, but we also detected it in liver and, for some individuals, in pancreas (Figure 3.5a-h). In one wild boar (WB1), however, we found Cd more scattered throughout the body (Figure 3.5e), which might be due to a lower Se concentration (Table 3.1) as Se can act as antioxidant for both As and Cd toxicity (Zwolak 2020).

3.3.6 Cobalt (Co)

Co is a scarce but essential trace element that is an important component of vitamin B12 (NRC 2002). Although Co toxicity is less plausible due to its scarcity, symptoms of Co toxicity include reduced feed intake, hyperchromemia and eventually anemia (e.g. Ely et al. 1948; Keener et al. 1949). Co deficiency seems much more likely to occur and include symptoms as fatty degeneration of the liver, anemia with pale mucous membranes, and increased susceptibility to infections due to impaired neutrophil function (Underwood 1981; MacPherson et al. 1987; Paterson & MacPherson 1990).

Although ruminants seem to be more sensitive to Co deficiency than monogastric animals (NRC 2002), we found higher Co concentrations for deer than for boar (Table 3.1). For boar, we found comparable concentrations as found by Gasparik et al. (2012), although they only measured muscle, liver and kidney. For all deer, we found the highest concentrations in liver

(Figure 3.6a-d; Table 3.2), while the guts were more prominent for boar (Figure 3.6e-h; Table 3.3). Overall, we found Co more scattered throughout the body than expected.

3.3.7 Chromium (Cr)

Although some forms of Cr are known as ecotoxic metals, Cr is an important trace element for normal glucose metabolism, especially when animals experience physiologic stress (e.g. NRC 2002). Cr toxicity is primarily linked to hexavalent Cr (Cr^{6+}) exposure, that passes the cell walls faster and is at least five times more toxic than other Cr forms, eventually causing pathologic changes in the DNA (e.g. NRC 2002; Jennette 1979; Ryberg & Alexander 1990; Alexander 1993). Symptoms of Cr deficiency include reduced insulin sensitivity and reduced growth (e.g. Lindemann 1996). Other symptoms may include glucose intolerance, hunger hyperglycemia, neuropathy, and reduced muscle proportion (e.g. Anderson 1994; Pechova & Pavlata 2007).

Gasparik et al. (2004) reported Cr concentrations in liver, kidney, and muscle of Red deer in Slovakia, and found comparable Cr concentrations between these tissues. However, we found that these tissues did not store the major Cr pool in the body (Figure 3.7a-h). Cr was more randomly scattered throughout the whole body for RD1, RD3, RD4, WB1, WB2, and WB4 (Figure 3.7+c-f+h). Cr was predominantly found in the intestines of RD2 and WB3 (Figure 3.7b+g), the individuals with the highest Se concentrations (Table 3.1).

3.3.8 Copper (Cu)

Cu is an essential element that is a component of many enzymes in a wide range of biochemical processes that include cellular respiration, free radical detoxification and iron transport (NRC 2002). Cu toxicity has been most described for ruminants, especially cattle (e.g. NRC 2002; Pechova & Pavlata 2007). Commonly described symptoms include lethargy, recumbency, pale mucous membranes, excessive thirst, and jaundice (e.g. Suciu et al. 1981). Cu deficiency has been reported as ataxia in adult deer (e.g. Barlow et al. 1964; Terlecki et al. 1964; Reid et al. 1980). For ungulates in general, Cu deficiency may manifest subclinical with low Cu concentrations in liver and serum but without any other signs of poor health (e.g. Bingley & Anderson 1972; Thornton et al. 1972; Gogan et al. 1989).

Traditionally, Cu concentrations are measured in the liver and kidney (e.g. Handeland et al. 2017). Although we found the highest concentrations in liver for most of the deer (Table 3.2) and in kidney for some boar (Table 3.3), we found Cu present throughout the whole body (Figure 3.8a-h). It has been described in cattle that Cu accumulates in the liver before toxicosis becomes evident (NRC 2002). This seems unlikely for the deer and boar that we measured due to the scarcity of Cu in nature (e.g. López-Alonso & Miranda 2020). Furthermore, levels of Cu concentration that are considered as normal seem to be unclear or case specific as McCullough (1969), for instance, reported Cu levels in liver between 84 and 142 ppm and considered these to be normal, which is up to 600 times higher than the concentrations we found (Table 3.1-3.3).

3.3.9 Iron (Fe)

Fe functions as a component of heme in hemoglobin and myoglobin and is therefore very important for all vital organs in the body (NRC 2002). When Fe uptake exceeds the binding capacity, free Fe may increase in the body, which is very reactive and can cause increased free radical production and oxidative stress, which increases the need of anti-oxidants (Halliwell 1987). This is associated with symptoms like diarrhea, reduced feed intake and weight gain (NRC 2002). Fe levels in water are believed to be higher than those in food items and should not exceed 0.3 mg Fe per liter for humans, while animals may be able to cope with higher concentrations (NRC 2002). Fe deficiency seems to be more likely to occur, of which a major symptom is hypochromic microcytic anemia, which is the result of improper hemoglobin production (NRC 2002). Another symptom can be increased morbidity and mortality due to depressed immune responses (Möllerberg & Moreno-Lopez 1975). Generally, Fe deficiency is very rare due to the ubiquitous nature of Fe in the environment including soil contamination, and requirements decrease with increase of age (Underwood 1981).

We found Fe in all body tissues that we measured (Figure 3.9a-h). For both species, we found the highest concentrations in lungs - a vital organ (Reece et al. 201) - and the lowest concentrations in bones (Table 3.2+3.3). However, we cannot rule out that this is due to the gunshot.

3.3.10 Potassium (K)

K is one of the most abundant elements in the body and is important for many life functions including maintaining osmotic pressure, acid-base regulation, nerve impulse transport and muscle contraction (NRC 2002). Under natural conditions, K toxicity seems unlikely to occur (NRC 2002) and it is not well defined which dietary K concentration may lead to toxicity (Ward 1966a). It has been suggested that K toxicity can cause cardiac arrest (Ward 1966b). A daily intake of 0.06 to 0.15 percent K of the total food intake has been reported as too low for dairy cattle (Pradhan & Hemken 1968; Mallone et al. 1982). Signs of K deficiency include reduced feed and water intake, weight loss, loss of hair flossiness, and decreased pliability of the skin (e.g. NRC 2002).

K is thus needed in the entire body and, as such, we found it in all tissues that we measured (Figure 3.10a-h). For deer, spleen tended to store the highest concentrations of K (Table 3.2), while we did not identify a specific tissue for boar (Table 3.3). Bone, and skin and hair appeared to store the lowest K concentrations for both species (Table 3.2+3.3).

3.3.11 Magnesium (Mg)

Similar to K, Mg is an essential element that is needed for enzymatic reactions vital to every major metabolic pathway, normal nerve conduction, muscle function and bone mineral formation (NRC 2002). Animals may suffer from skeletal abnormalities when they consume excessive amounts of Mg in their diets (e.g. Lee et al. 1980), which is unlikely to occur since most animals are able to excrete large amounts of Mg via urine (NRC 2002). Symptoms of Mg deficiency - which is often described for livestock, despite being the fourth most abundant cation in the world - have been extensively described and include muscle twitches, tremors, osteoporosis, and cramps (e.g. Davis 1968; Rude & Gruber 2004; Rude et al. 2009).

We found Mg throughout the whole body, with no particular target tissue (Figure 3.11a-h). Mg was detectable in all tissues, except the intestines of RD3 (Figure 3.11d), which we would attribute to a measurement error since this is the only missing Mg concentration in our data. We found rather similar Mg concentrations for deer and boar, ranging from 19,319 to 36,420 µg Kg^{-1} (Table 3.1).

3.3.12 Manganese (Mn)

Mn is an essential trace element that is important for the forming of connective tissue, bones, blood clotting and sex hormones (NRC 2002). Mn toxicity has been widely described (e.g. Keen et al. 1984; Finley & Davis 1999), and can be associated with many symptoms including Parkinsonism, bradykinesia, tremor, impaired postural reflexes and dystonia (Calne et al. 1994; Finley & Davis 1999). Other symptoms include, especially for ruminants, reduced food intake and growth (Jenkins & Hidiroglou 1991). It has been shown that rats and humans that suffered from Fe deficiency experienced increased Mn absorption (e.g. Thomson et al. 1971), and vice versa (e.g. Davis et al. 1990; Davis et al. 1992). Although Mn deficiency is unlikely to occur since Mn is available in nearly all food items (Pennington et al. 1986), Mn deficiency reveals most likely in the form of skeletal abnormalities (e.g. Finley & Davis 1999; Nielson 2020), like enlarged joints, deformed legs with thickened and shortened long bones, and overall lameness in pigs, ruminants and poultry (Strause et al. 1986).

Mentioned target tissues for Mn include skeleton, liver and hair (Black et al. 1985), or brain and bone (e.g. Rahil-Khazen et al. 2002; O'Neal & Zheng 2015; Tajchman et al. 2021). Mn has been traditionally measured in tissues as liver, kidney and muscle (e.g. Gasparik et al. 2004; Malmsten et al. 2021). We found the highest concentrations in the guts for most individuals (Figure 3.12a-h). We found the highest concentration in bone only for WB3 (Table 3.1+3.3; Figure 3.12g). Bone stored the least Mn for most of the other individuals (Table

3.2+3.3). We found overall higher Mn concentrations for deer than for boar, respectively ranging from 2,785 to 5,462 µg Kg^{-1} and from 698 to 1,902 µg Kg^{-1} (Table 3.1).

3.3.13 Molybdenum (Mo)

As an essential trace element, Mo is a component of many enzymes throughout the body, including enzymes found in milk (Mills and Davis 1987, cited in NRC 2002). Mo is antagonistic to Cu, implying that Mo toxicity can occur in the form of Cu deficiency (e.g. Pitt et al. 1980; Vyskočil & Viau 1999; NRC 2002). Ruminants would be more sensitive to Mo toxicity than monogastric animals (Vyskočil & Viau 1999). Mo toxicity can cause diarrhea, anorexia, depigmentation of hair, neurological disturbances and premature death (Anke et al. 2010). Although naturally occurring Mo deficiency has never been demonstrated in free-living animals, Mo deficiency can be the result of low Mo levels in soil, plants, drinking water and other food items (e.g. Pandey et al. 2002). Mo is known for its anticarcinogen properties, low concentrations being associated with oesophageal cancer in particular (e.g. Luo et al. 1983; Komada et al. 1990; Pandey et al. 2002).

Mo concentrations are traditionally measured in liver and kidney (e.g. Pitt et al. 1980; Pandey et al. 2002; Grace et al. 2005). However, we found no specific tissue that stored the majority of the Mo pool in the body (Figure 3.13a-h). The eyes turned out to have the major content for WB3 (Figure 3.13g), while Mo had been hardly detected in this tissue for most of the other individuals (Figure 3.13a-d+f+h). We found slightly higher Mo concentrations in deer than in boar, ranging from 47.30 to 59.71 µg Kg^{-1} and 35.63 to 59.42 µg Kg^{-1}, respectively (Table 3.1).

3.3.14 Sodium (Na)

Na is an essential macro element that is important for life functions including controlling blood pressure, blood volume and water balance (NRC 2002). A proper Na and K balance is required for heart function and nerve impulse conduction (e.g. NRC 2002), and it is a major component of salts in saliva to buffer acid from ruminal fermentation (Blair-West et al. 1970). When the Na concentration in the blood is too high, which can be a result of dehydration, animals can suffer from hypernatremia, manifesting in symptoms like excessive thirst or lethargy (e.g. Hiyama et al. 2010; Noda & Hiyama 2015). Excessive Na levels would be first detectable in the brain (e.g. McKinley et al. 1978; Denton et al. 1996). Animals that suffer from Na deficiency are described to have an intense craving for salt, that they show by chewing and licking various objects (e.g. Babcock 1905; NRC 2002).

We detected Na in all tissues that we measured (Figure 3.14a-h). The highest Na concentrations were found in the eyes of boar (Table 3.3), and in the eyes and brain of deer (Table 3.2). We found the lowest concentrations in muscle, skin and hair (Table 3.2+3.3).

Overall, the deer tented to have slightly higher Na concentrations compared to the boar, ranging from 145,511 to 156,760 µg Kg^{-1} and 117,545 to 158,860 µg Kg^{-1}, respectively (Table 3.1).

3.3.15 Nickel (Ni)

The best described functions of the essential trace element Ni include increasing hormonal activity, lipid metabolism, and urease activity (e.g. NRC 2002; Hänsch & Mendel 2009; Kumar & Trivedi 2016). One of the best described forms for Ni toxicity is found in its carcinogenic effects (e.g. Das et al. 2019; Genchi et al. 2020). Other toxic effects are genotoxic, immunological, endocrine, neurogenic, cardiovascular, gastrointestinal, musculoskeletal, dermal and metabolic (Das et al. 2019), although it remains unclear when Ni accumulation would become a problem for wildlife (McIlveen & Negusanti 1994). Naturally occurring Ni deficiency is rare due to the extremely low intake requirements (Schroeder et al. 1974; Cartañá et al. 1991). Signs of Ni deficiency include depressed growth, lower reproduction, lower plasma glucose, or altered distribution of e.g. Fe, Cu, Ca, and Zn (Nielsen 1993).

Although it has been suggested that Ni is, once ingested, distributed mostly to kidney, bone and lungs (e.g. NRC 2002), we found it more randomly throughout the body (Figure 3.15a-h). For some individuals - RD2, RD3, RD4, WB2, and WB3 - we indeed found relatively high concentrations in lungs (Table 3.2+3.3; Figure 3.15b-d+f-g). We found remarkably high concentrations of Ni in the pancreas of RD1 (Table 3.2; Figure 3.15a), the intestines of RD2 and WB1 (Table 3.2+3.3; Figure 3.15b+e), and the spleen of WB4 (Table 3.3; Figure 3.15h).

3.3.16 Phosphorous (P)

P has more known functions in the animal body than any other element and is located in all body cells where it is involved in nearly all energy transactions (e.g. Davis 1968; NRC 2002). When P is excessive in the diet for a long period, it can cause problems of Ca metabolism (e.g. NRC 2002). This is most likely to occur in monogastric animals since ruminants can tolerate a wider Ca:P ratio (NRC 2002). P deficiency seems most likely to occur when animals forage on P poor soils (e.g. Brooks et al. 1984; Elliott et al. 2002). General signs of P deficiency include weight loss, stiff joints and muscular weakness (e.g. Fuller et al. 1976; NRC 2002). Other symptoms can be the desire to eat wood, bones, rocks and other materials (e.g. Theiler et al. 1924; Karn 2001).

We found P in every tissue and none of the tissues in particular (Figure 3.16a-h). Most individuals had the highest P concentrations in bone and the lowest concentrations in skin and hair, or eyes (Table 3.2+3.3). We found similar concentrations for deer and boar, ranging from 273,821 to 376,183 µg Kg^{-1} and from 223,948 to 399,327 µg Kg^{-1}, respectively (Table 3.1).

3.3.17 Lead (Pb)

Pb is the most common cause of toxicoses in animals (Neathery & Miller 1975). There is no evidence for its essentiality (e.g. Neathery & Miller 1975; NRC 2002). Often described symptoms of Pb toxicity include disturbed muscular coordination, reduced cognitive performance and anemia (e.g. Neathery & Miller 1975; Wani et al. 2015). Pb disturbs the balance between functions of other metals including Cu and Zn (Bąkowska et al. 2016).

Accordingly other heavy metals, the liver and kidney are traditionally considered as the target organs for Pb (e.g. Kottferová & Koréneková 1998; Bąkowska et al. 2016; Malmsten et al. 2021 30, 164-165]. It is also believed to accumulate in bone (e.g. Schroeder & Tipton 1968; Bridges & Zalups 2005). We found bone as the major storage pool of Pb for RD1, WB1, WB2 and WB3 (Figure 3.17a+e-g), whereas we found the highest concentrations in the digestive system for RD2 and WB4 (Figure 3.17b+h). For RD4 and WB3, Pb was more scattered throughout the body (Figure 3.17d+g), which was also the case to a lesser extent for WB1 (Figure 3.17e). RD3 was the only individual with the highest Pb concentration in the lungs (Figure 3.17c), with considerably higher concentrations compared to any of the other individuals (Table 3.1-3.3). This might be a sign of toxicosis, as the lungs are one of the vital organs (Reece et al. 2014), although reference values are missing to validate this presumption.

3.3.18 Sulfur (S)

S is an essential element that has many functions in the animal body, including forming several amino acids, and promoting DNA fixation and the antioxidant systems (e.g. NRC 2002; Mukwevho et al. 2014). It is present in every body cell (e.g. Qi et al. 1994). S toxicity is most likely to manifest neurologic transmissions, causing acute symptoms including blindness, muscle twitches and recumbency (Coghlin 1944). Other symptoms include severe enteritis, peritoneal effusion, and petechial haemorrhages in especially kidney (Bird 1970). It may occur that S toxicity can be smelled in the breath (NRC 2002). Symptoms of S deficiency in ruminants include reduced food intake, weight and hair loss, overall weakness and death, which are all signs of digestive tract or metabolism problems (Kincaid & Nash 1988; Qi et al. 1994). Since S deficiency has been mostly studied in ruminants, it is unclear whether monogastric animals experience similar symptoms.

Kierdorf et al. (2014) found S in higher concentrations in deer's antlers compared to pedicles. We found overall slightly higher S concentrations in deer compared to boar (Table 3.1). Skin and hair seems to contain the highest S concentrations for deer (Table 3.2), while we did not find any specific tissue for boar (Table 3.3). As S is part of every body cell, we found it distributed throughout the whole body with no particular target tissue (Figure 3.18a-h).

3.3.19 Selenium (Se)

Se has antioxidant properties when supplied in low concentrations (e.g. Zwolak 2020). It is important in several enzymes, helps to make DNA, and protects against cell damage and infections (e.g. NRC 2002). The soil is the best Se source for all life forms, although the Se concentration in the soil does not seem to be the best indicator of Se availability for animals (Elrashidi et al. 1989; Oldfield 2002). Se toxicity is most commonly known in the form of alkali disease - aka selenosis, severely damaged hooves (e.g. NRC 2002; Oldfield 2002). Se deficiency can cause white muscle disease or nutritional muscular dystrophy (e.g. NRC 2002), often resulting in death.

Kidney or liver are most commonly used as bioindicator of Se in the environment (e.g. Vikøren et al. 2005; Lazarus et al. 2008; Pilarczyk et al. 2009; Nowakowska et al. 2016). We found the highest Se concentration in kidney for all individuals (Table 3.2+3.3), while it was also found in nearly all other tissues that we analysed in lower concentrations (Table 3.2+3.3; Figure 3.19a-h). We found the highest concentrations in boar and the lowest concentrations in deer (Table 3.1).

3.3.20 Silicon (Si)

Si is only found in very trace amounts in animal bodies (NRC 2002). It has a role in connective tissue and healing from injuries (e.g. Carlisle 1986; Nielsen 1999; NRC 2002; Pérez-Granados & Vaquero 2002). Since Si is easily excreted, Si toxicity is unlikely to occur (Vasanthi et al. 2012). Si deficiency may lead to delays in growth, bone deformations and abnormal skeletal development (Carlisle 1986). It affects connective tissue metabolism and thus organic bone formation (Hott et al. 1993).

Although we found a lot of variation in the total Si concentration among individuals (Table 3.1) and it was scattered throughout all tissues we measured (Figure 3.20a-h), Si slightly tended to be most concentrated in the digestive system - intestines or pancreas - for some individuals (Table 3.2+3.3). Moreover, Bellés et al. (1998) described that Si prevents Al absorption and reduces Al concentrations in tissues including brain, liver, bone, kidney, and spleen. We found, however, the highest Al concentrations in the individuals with the highest Si concentrations - RD2, RD4, and WB4 -, while for none of these individuals the highest Si concentrations were measured in brain, liver, bone, kidney, or spleen (Figure 3.1b+d+h; Table 3.1-3.3).

3.3.21 Strontium (Sr)

Sr can be seen as the chemical analog of Ca, and as such, its major role is found in the formation and breakdown of bony material (e.g. Schönhofer et al. 1994; Standberg & Strandgaard 1995; Baeza et al. 2011). Bony material is most commonly used to measure the

Sr concentration in animals (Demesko et al. 2019). Sr toxicity seems unlikely to occur since dietary Sr can vary widely without any toxic symptoms (Nielsen 2004). It has been mentioned that high dietary Sr increased the risk of P deficiency (Jones 1938). Although Sr has never been shown to be an essential element, Sr has been shown to promote bone Ca and to reduce fracture rate in osteoporotic patients (Nielsen 2004). This might imply that osteoporosis may be related to Sr deficiency, although reference values to investigate this presumption are missing (Nielsen 2004).

We found indeed the highest Sr concentrations in the bones of all individuals (Table 3.2+3.3; Figure 3.21a-h). Skibniewski et al. (2015) used muscle as model tissue, however muscle belonged to the tissues with lowest Sr concentration in our analysis (Figure 3.21a-h), which was for RD3 and RD4 even the tissue with the lowest Sr concentration (Table 3.2).

3.3.22 Zinc (Zn)

As a component of many enzymes, Zn affects the metabolism of carbohydrates, proteins, lipids, and nucleic acids, and it helps in regulating hormones and the immune system (e.g. NRC 2002; Hänsch & Mendel 2009; Papachristodoulou et al. 2015). Zn salts have been shown to protect against different forms of toxicity, including Cu toxicity (Suttle & Mills 1966; Bremmer et al. 1976; Allen et al. 1983). Zn toxicity is most likely to occur in the form of Cu deficiency since Zn and Cu are antagonistic to each other (NRC 2002; Miller et al. 1989). Excessive Zn uptake can also give symptoms including epigastric pain, lethargy, and fatigue (Fosmire 1990). Zn deficiency include symptoms as reduced feed intake and reduced growth (NRC 2002).

Although most studies measure Zn in the liver, kidney, or muscle (e.g. Lazarus et al. 2008; Reimoser & Putmam 2011; Papachristodoulou et al. 2015; Skibniewski et al. 2015), we found Zn scattered throughout the whole body, with no particular tissue as major storage pool (Figure 3.24a-h). Overall, deer tended to have slightly higher Zn concentrations compared to boar (Table 3.1).

3.4 Discussion and conclusions

In this study, we aimed to gain insights in the ionome of large mammals, by measuring 22 chemical elements across 13 tissues of two ungulate species (deer and boar), and evaluating how these elements are distributed over the body. We used four individuals of deer and boar that lived in a nutrient-poor Dutch National Park (Veluwezoom), where deficiencies are most likely to occur. We found that the ionome was highly variable between and within the two species. For most elements, tissues having the highest and lowest concentration differed between individuals (Table 3.2+3.3). No single tissue accurately represented the accumulation of toxic or scarce elements in the bodies. These findings imply that analyses of elemental concentrations in single tissues do not necessarily reflect bioaccumulation of toxins or deficiencies of scarce elements.

We attempted to put our findings in the context of the biological and physiological role of the elements, and noticed that the lack of reference values per element per species indeed limited our understanding and the interpretation of the measured concentrations. Such reference values are required to determine any toxicities or deficiencies. The signs of toxicities or deficiencies are element-specific (e.g. Van Metre & Callan 2001; Wilson & Grace 2001; Barboza et al. 2003; Vikøren et al. 2005), and can be even species-specific (e.g. Barboza et al. 2003; Flynn et al. 1977; Arnhold et al. 2002). Since the margins per element for wild deer and boar remain unknown, we were unable to assess toxicities or deficiencies. Therefore, the elemental concentrations that we reported here must be seen as a first step to enlarge the comprehension of the elemental composition of wild living deer and boar.

Our study showed that at the individual level most elements are rather scattered throughout the whole body. Also elements that seem to have a target tissue, e.g. kidney for Se (Figure 3.19a-h), appeared to be more scattered than expected. Thus, when focusing on only a few samples of tissues traditionally mentioned as storage pools for particular elements, there is a high risk of underestimation or missing crucial information, which may lead to wrong conclusions. Moreover, we encourage further studies to collect samples of individuals in the shortest time interval, largest sample sizes, and most complete ionomes as possible.

We see four possible limitations of our study. First, the sample size per species was low given the high variability of elemental concentrations that we found within and between the species. Many more individuals would probably be needed to attain stable averages, if possible, of elemental concentrations. Second, the individuals we dissected were obtained from regular culling. This might introduce some unintended bias due to potential harvest bias in terms of sex, age and condition (e.g. Lombardini et al. 2014; Gürtler et al. 2018; Wenting et al. 2023c). Third, salt licks and corn - for the deer and boar, respectively - were provided occasionally to facilitate culling. Although we measured these salt licks and corn for the same elements as we did for the tissue samples that we analysed (Appendix 3.2), we are only able to speculate about the effect of these supplements on the elemental concentrations we that found. For instance, the mineral licks contained more Co and Cu than the corn (Appendix 3.2). Since we found higher Co and Cu concentrations in deer compared to boar (Table 3.1), it might be possible that the mineral licks contributed to these higher concentrations, although any evidence is missing (e.g. Tajchman et al. 2021). We assume that these supplements attracted the individuals equally. Fourth, we did not include the antlers of male deer in our analysis. Therefore, we cannot address their potential function of yearly shedding in reducing the ecotoxic burden in male deer. We propose to include this as an extra tissue in future studies examining the full ionome of deer. We do not believe, however, that any of the above-mentioned limitations affect the conclusions of our study.

We encourage other scientists to execute extensive surveys of the elemental composition of wild living animals, including as much information possible about these animals and the circumstances they encountered during their lives. This will not only improve the physiological understanding of trace elements in the animal body, but also enables us to link ionomic insights to ecological processes. We propose to install an international database where all the measured elemental concentrations can be uploaded per tissue and species combination, including the sex, status (e.g. pregnancy, lactating) and age of the individuals, to enlarge the current knowledge and to potentially approach reference values in the future.

3.5 End sections

3.5.1 Author contributions

Elke Wenting: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data Curation, Writing - Original Draft, Visualization, Project administration; Henk Siepel: Conceptualization, Methodology, Writing - Original Draft, Visualization; Patrick A. Jansen: Conceptualization, Writing - Original Draft.

3.5.2 Acknowledgements

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3.5.3 Conflicts of interest

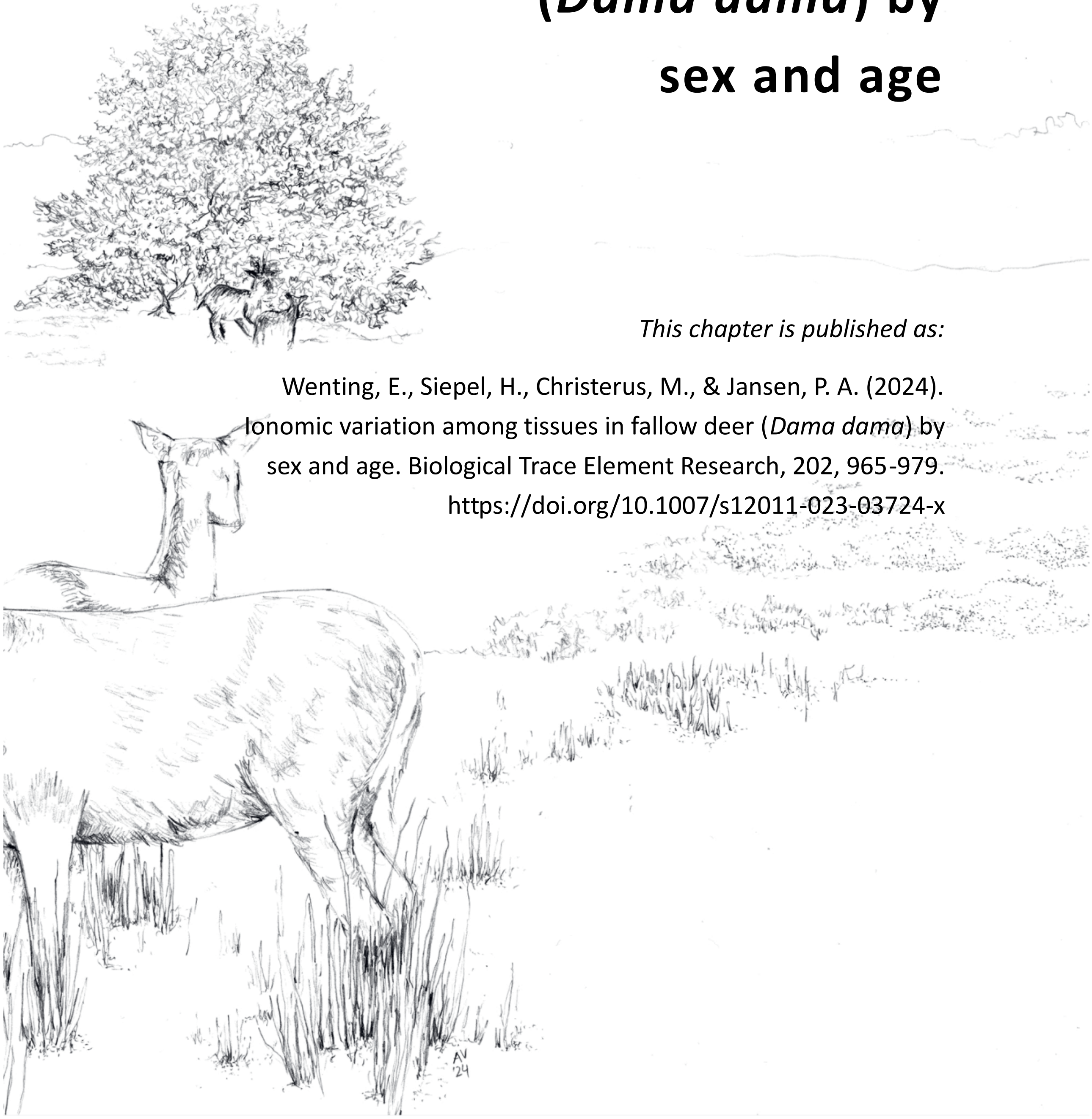
No actual or potential conflicts of interest are declared by the authors.

3.5.4 Data availability

The complete dataset is accessible through Figshare: https://doi.org/10.6084/m9.figshare.23633997.

Chapter 4

Ionomic variation among tissues in Fallow deer (*Dama dama*) by sex and age



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Abstract

(1) In mammals, the mineral nutrient and trace elemental composition of the body - the ionome - differs among individuals. It has been hypothesised that these differences may be related to age and sex, both for ecotoxic and essential elements.

(2) We investigated whether and how intraspecific ionomic variation is related to age and sex in Fallow deer (*Dama dama*). We tested the predictions that concentrations of ecotoxic elements increase with age, that ionomic variation is lower among young individuals than among older individuals, and that reproductive females (does) have the lowest concentrations of essential elements.

(3) Culled animals of different sex and age were obtained from a single protected area. The animals were dissected to collect 13 tissues, and concentrations of 22 different elements were measured in a sample of each tissue.

(4) We described substantial ionomic variation between individuals. Some of this variation was related to age and sex, as predicted. Based on the limited existing knowledge on chemical element allocation and metabolism in the body, sex-related differences were more difficult to interpret than age-related differences. Since reference values are absent, we could not judge about the consequences of the elemental values that we found.

(5) More extensive ionomic surveys, based on a wide range of elements and tissues, are needed to enlarge the understanding of within-species ionomic variation and potential biological, ecological, and metabolic consequences.

Keywords: ionomics, minerals, trace elements, Fallow deer, wildlife

4.1 Introduction

The mineral nutrient and trace elemental composition of organisms - the ionome (Lahner et al. 2003; Salt et al. 2008) - is an important expression of their physiological state (Lahner et al. 2003), relating to a variety of biological and ecological processes, including life history plasticity (Jeyasingh & Weider 2005; Jeyadingh et al. 2017), population growth (Ågren 2004; Vrede et al. 2004), foraging ecology (Ayotte et al. 2006), and carrion decomposition (Wenting et al. 2020). The ionome of animals - unlike plants - has long been assumed to be more or less constant, i.e. 'homeostatic' (Sterner & Elser 2002). This assumption was based on observations of nearly constant ratios of carbon (C) to nitrogen (N) to phosphorous (P) (Jeyasingh et al. 2017), three elements that are most often considered in studies considering ecological stoichiometry, i.e. the study of the balance of energy and multiple chemical elements in ecological interactions (Elser et al. 1996; Elser 2006; Raubenheimer et al. 2009).

However, recent studies have shown that the assumption of ionomic homeostasis does not hold for chemical elements in general, and that many elements are in fact much more scattered throughout the whole body. Wenting et al. (2020) examined the elemental composition of Fallow deer (*Dama dama*) and Eurasian otter (*Lutra lutra*) by measuring twelve elemental concentrations in twelve different organs and tissues and found differences in elemental concentration within and between the species. Ma et al. (2015) found, based on four tissues of 26 species and 18 elements, lineage-specific patterns and correlations between elements, tissues, and body mass. The causes of this variation within and among species remain unknown. Exploring drivers of ionomic variability requires extensive studies dealing with multiple chemical elements, tissues, and organs.

It has been suggested that age and sex are important drivers of intraspecific ionomic variation. Bioaccumulation of toxic elements - e.g. aluminium (Al), cadmium (Cd) and lead (Pb) - increases with exposure time and thus age (Domingo 1995). Young animals obtain essential elements from their mother via the placenta and milk (Campos et al. 2012), and may thus show less ionomic variation than adults, which must acquire these typically scarce elements through feeding (Crowe & Bradshaw 2014). Particularly reproductive females, which transfer elements to their young, may show more ionomic variation than younger animals. However, to our knowledge, these relationships have never been examined for a wide range of elements across multiple tissues. Thus, the role of sex and age as driver of ionomic variation in mammals remains vastly unexplored.

Some variation has been described for specific tissue-element combinations (e.g. Basparik et al. 2004; Sobańska 2005; Srebočan et al. 2012; Yarsan et al. 2014; Mulero et al. 2016; Giżejewska et al. 2017; Goos et al. 2017; Lazarus et al. 2018; Oropesa et al. 2022). For instance, Demesko et al. (2018) found that concentrations of manganese (Mn) and zinc (Zn) in the teeth of Roe deer (*Capreolus capreolus*) increased with age. Lazarus et al. (2008) found sex-related differences in Cd, iron (Fe), and Zn concentration in the kidney cortex, and for Pb in the jawbone, but did not report the magnitude of these differences. Cygan-Szczegielniak & Stasiak (2022) measured higher concentrations of heavy metals in the liver of Roe deer females compared to younger individuals. However, these studies all considered only the few tissues and elements that are commonly used as bioindicators, such as Cd and other ecotoxic heavy metals (e.g. Dobrowolska & Melosik 2002; Amici et al. 2012; Bąkowska et al. 2016; Squadrone et al. 2022). Thus, the overall magnitude of age- and sex-related variation, incorporating a wide range of elements and tissues, remains unknown.

The aim of this study was to describe whether and how intraspecific ionomic variation could be related to age and sex. Our approach was to measure the ionome, including a wide range of elements and tissues, of multiple individuals of Fallow deer, belonging to different sex and age groups, that were collected from a single protected area. We examined five predictions: (1) the total concentrations of essential elements are lower for reproductive females (does) than for younger females and males in general, while concentrations of toxic elements increase with age and are therefore highest for adults; (2) age-related differences are largest among females, with bioaccumulation of toxic elements increasing with age in tissues that excrete these elements (e.g. liver and kidney) and essential elements decreasing with age due to pregnancy; (3) age-related differences among males are more related to bioaccumulation of toxic elements than differences in essential elements; and (4) the least sex-related differences are found among calves, both for essential and toxic elements; resulting in (5) most sex-related differences being found among yearlings due to increasing age, predominantly as lower concentrations of essential elements for yearling females compared to males due to pregnancy. In addition, we considered other sex and age-related differences and speculated on cause of differences in the context of their biological and physiological role.

4.2 Material and methods

4.2.1 Study site and species

The Fallow deer is a terrestrial ungulate herbivore with an adult body weight of 40-80 kg and a non-nomadic lifestyle (Focardi et al. 2006). After a gestation period of 31 to 32 weeks, a doe gives birth to a single calf. Calves are born in May or June and are weaned after seven to nine months. Yearling females can be pregnant as most females give birth to their first calf in their second year of life (Armstrong et al. 1969; Clutton-Brock & McComb 1993). Being an intermediate feeder (Hofmann & Stewart 1972), Fallow deer is an ideal model species for this study as its browsing behaviour might compensate for the low amount of trace elements in the average vegetation.

The freshly culled individuals that we used were obtained from Deelerwoud (52°08’N, 5°89’E). Deelerwoud is a protected area at the Veluwe, the Netherlands, characterized by a gently rolling forest and heathland landscape (Van Belle 2006). It is situated on partly glacier deposits and on cover sands over these deposits (“mineral-poor cover sands”), causing the mineral availability to be limited to very scarce (Bobbink et al. 2010; Siepel et al. 2018; 2019). Kuiters (1996) found increasing levels of Cd and Pb with age in Red deer (*Cervus elaphus*) and Wild boar (*Sus scrofa*) from the Veluwe area, where concentrations varied over different food types (browse, grasses, dwarf shrubs, acorns, etc.) and over the season. Wolkers et al. (1994) also reported that levels of Cd and Pb at the Veluwe increased with age, even to such an extent that liver and kidney of Red deer and Wild boar were seen as unsuitable for human consumption.

4.2.2 Carcass collection and dissection

We obtained twenty fresh Fallow deer carcasses from regular culling in the hunting season 2021-2022 (between October and March). Twelve of these carcasses were females: four calves, four yearlings, and four does. The yearlings and does were pregnant, with different embryotic stages depending on the moment of culling. We were not able to age the does more precisely. The other eight carcasses were males: four calves and four yearlings. We did not include adult males (bucks) because none were culled during our study period. No animals were killed for the purpose of our study. According the Animal Welfare Officer of Wageningen University & Research, our study is not considered as experimentation on animals and thus permitted under Dutch law (Appendix 4.1).

In total, we dissected the carcasses to collect thirteen organs and tissues (henceforth ‘tissues’), belonging to different organ systems: skin and hair; muscle; brain; eyes; lungs; heart; spleen; kidney; liver; pancreas; stomach, including rumen; and intestines. In a shed at Deelerwoud, we dissected seven tissues: skin and hair, muscle, lungs, heart, spleen, kidney, and liver. We also dissected the entire guts - pancreas, stomach, and intestines -, the head - brain and eyes -, and the hind leg - bone -, that we further dissected in the dissection room of Wageningen Environmental Research. We dissected the hind leg into a bone sample by sawing a piece of bone from the lower leg and putting it in boiling water for a few minutes to loosen the remaining tissues, to retain a clean bone sample afterwards that we used in the next step of freeze-drying. We also further dissected the guts and head. All the collected tissues - frozen at minus 18 °C after dissection - were homogenized using a blender, except the bone sample, that was used in its entirety. Three tablespoons - 15-25 g each - of the homogeneous tissue samples were stored in plastic bags in the freezer before we prepared them for chemical analyses. After dissection all carcass remains were returned to the study area.

4.2.3 Measurements

Before the chemical analyses, we freeze-dried the tissue samples. The dry samples were transported to Radboud University in ice blocks to prevent defrosting. At Radboud University, we used a microwave destruction - aka digestion - method with 5 mL 65% nitric acid (HNO_3) and 2 mL 30% hydrogen peroxide (H_2O_2), after which the tissue samples were ready for measuring the elemental concentrations with Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) and Inductively Coupled Plasma Mass Spectroscopy (ICP-MS).

In total, we measured 22 elemental concentrations: Al, arsenic (As), boron (B), calcium (Ca), Cd, cobalt (Co), chromium (Cr), copper (Cu), Fe, potassium (K), magnesium (Mg), Mn, molybdenum (Mo), sodium (Na), nickel (Ni), P, Pb, sulfur (S), selenium (Se), silicon (Si), strontium (Sr), and Zn. Seven elements were measured using ICP-OES: Ca, K, Mg, Na, P, S, and Si. The other 15 elements were measured using ICP-MS. We used the same devices as Wenting et al. (2020). Correspondingly, the accuracy of these devices was guaranteed by using the following quality controls (QC): Multi element standard IV, Merck 1.11355; Phosphate standard, Merck 1.19898; Sulphate standard, Merck 1.19813; and Silicium standard, Merck 1.70236. The QC matrices were considered to correspond to the sample matrices since for both, any contamination of HNO_3 and H_2O_2 was eliminated by using blanks (see for more details Wenting et al. 2020).

4.2.4 Statistical analyses

All statistical analyses were done in R version 4.0.2 (R Core Team 2020). The statistical analyses should be considered as indicative rather than steadfastly; due to the low sample sizes, they have limited meaning. Yet, we believe that the indicative nature is helpful for determining the most notable differences, although we acknowledge that it should be considered as descriptive. For the first prediction - focusing on the total elemental concentrations -, we calculated and visualized the total elemental concentration per element per individual. We used Kruskal-Wallis tests to test for differences between the groups per element with a Bonferroni-corrected alpha of 0.00227. The second prediction - focusing on age-related differences among females - was analysed with Kruskal-Wallis tests per tissue-element combination. We used Mann-Whitney U tests to analyze each tissue-element combination for the third, fourth and fifth prediction - respectively focusing on age-related differences among males, sex-related differences among calves, and sex-related differences among yearlings. We used the step-up Benjamini and Hochberg procedure (Benjamini & Hochberg 1995) to correct for multiple testing using the p.discrete.adjust function of the discreteMTP package (Heller et al. 2012) in the analyses of the second to fifth prediction. We only visualised the element-tissue combinations that turned out significant for these predictions.

4.3 Results

The distribution of the elements over the body differed among the sex-age groups (Appendix 4.2). Although we found no differences in total elemental concentrations per sex-age group (prediction 1; Figure 4.1a-v), this is in line with the differences in tissue-element combinations that we found among the sex and age classes (Figure 4.2-4.5).

For 21 element-tissue combinations, concentrations differed significantly among age classes of females (prediction 2; Figure 4.2a-u). For example, the Cd concentration in eyes and kidney (Figure 4.2a-b) was higher for adults than for calves and yearlings. The majority of the other differences were found in essential elements, of which some had higher concentrations in adults (does) - including Fe in spleen (Figure 4.2e), Se in stomach (Figure 4.2p), and Zn in eyes (Figure 4.2t) -, and some had higher concentrations in calves - including K in brain (Figure 4.2f), Mg in brain (Figure 4.2h), and P in spleen (Figure 4.2m). Differences in Zn concentration were found four times, the most often of all elements: in bones (Figure 4.2r), muscle (Figure 4.2s), eyes (Figure 4.2t), and stomach (Figure 4.2u).

For 19 tissue-element combinations, concentrations differed significantly between calves and yearling males (prediction 3; Figure 4.3a-s). This included just one ecotoxic element: the Al concentration in kidneys was higher in yearlings than in calves (Figure 4.3a). For 9 of these tissue-element combinations, calves showed higher concentrations than the yearlings, e.g. Mn in eyes (Figure 4.3h), Si in muscle (Figure 4.3p), and Zn in muscle (Figure 4.3s). Yearlings had higher concentrations than calves in the other tissue-element combinations, e.g. Co in eyes (Figure 4.3b), Fe in muscle (Figure 4.3e), and Sr in kidney (Figure 4.3r).

For 18 element-tissue combinations, concentrations differed among sex classes for calves (prediction 4; Figure 4.4a-r). Concentrations in female calves were higher for 14 combinations - including Al in kidney (Figure 4.4a), Co in lungs (Figure 4.4c), P in lungs and spleen (Figure 4.4l+m), and Sr in heart (Figure 4.4p) -, compared to four combinations that were higher for male calves - As in muscle (Figure 4.4b), Co in stomach (Figure 4.4d), Fe in stomach (Figure 4.4g), and Se in brain (Figure 4.4o). Seven of the 18 differences were found in lungs: Co (Figure 4.4c), K (Figure 4.4h), Mg (Figure 4.4i), Ni (Figure 4.4k), P (Figure 4.4l), S (Figure 4.4n), and Zn (Figure 4.4q).

For 25 element-tissue combinations, concentrations differed among sex classes for yearlings (prediction 5; Figure 4.5a-y). Female yearlings had the highest concentrations for 11 of these

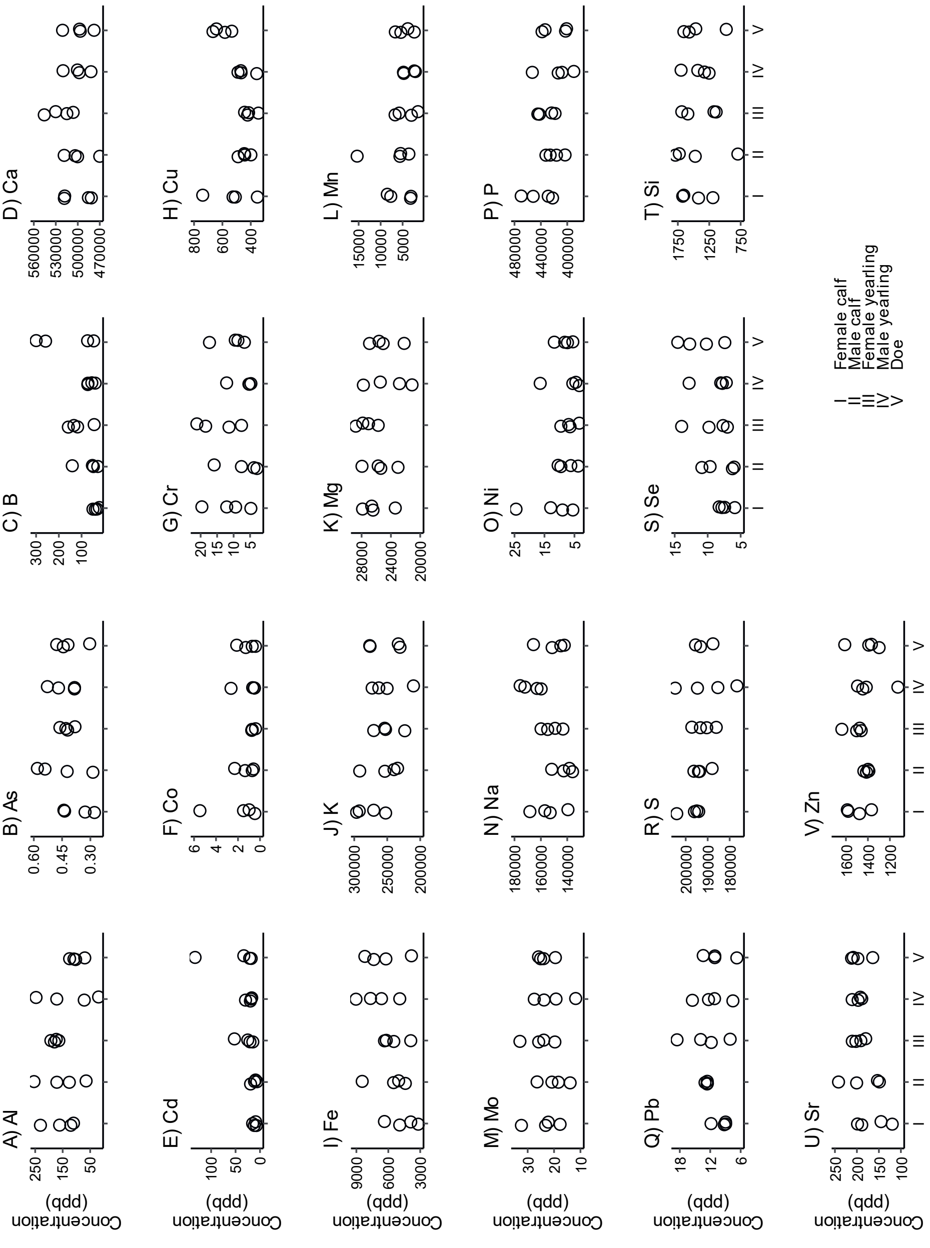


Figure 4.1 Total elemental concentrations per age-sex group of Fallow deer.

element-tissue combinations - including B in liver (Figure 4.5b), Cr in liver (Figure 4.5e), Mn in eyes (Figure 4.5m), and Zn in muscle (Figure 4.5y). Yearling males had higher concentrations for the other 14 significant element-tissue combinations - including Cu in heart (Figure 4.5g), Fe in pancreas (Figure 4.5i), K in intestines (Figure 4.5k), and Sr in kidney (Figure 4.5x). All these differences were for essential elements.

4.4 Discussion

This study aimed to determine whether and how ionomic variation, based on a wide range of elements and tissues, is influenced by age and sex. Fallow deer, collected from a single protected area, was used as a model species and we analysed multiple individuals belonging to different age and sex classes. We predicted the total concentrations of essential elements to be lowest and the accumulation of toxic elements to be highest for does (prediction 1). The distribution over the tissues seemed to differ among the groups (Appendix 4.2), but we found no differences in total concentrations per element between the age and sex classes (Figure 4.1a-v). As predicted, we indeed found differences in concentrations between sex and age classes for a substantial number of tissue-element combinations (Figure 4.2-5). We speculated about the biological and physiological role of the differences that we found.

4.4.1 Age-related differences in females

We predicted that most age-related differences occur within females, with bioaccumulation of toxic elements increasing with age, mostly in tissues that excrete these elements, and essential elements decreasing with age due to pregnancy (prediction 2). Some of the element-tissue combinations that turned out significant were in line with our prediction. For example, Cd increased with age in kidney (Figure 4.2b), which is in line with previous studies (Wolkers et al. 1994; Kuiters 1996).

We found higher Cd concentrations in eyes of older females (Figure 4.2a). An increase of Cd in eyes with age has been found in human retina as result of smoking, increasing risk of macular degeneration (Wills et al. 2008). Jamall & Roque (1989) found that a daily ingestion of 50 ppm Cd results in detectable accumulation after seven weeks in the eye of rats, implying that Cd in the diet can result in Cd accumulation in the eyes.

We found that K and Mg decreased with age in brain (Figure 4.2f+h). A decrease of K in brain with age has previously been found in humans (Duflou et al. 1989). It implies reduced brain function and lower responsiveness since both K and Mg are important for pulse transmission and oxygen levels in the brain (e.g. Somjen 2002). However, it remains unclear why female calves might be more variable in their brain K and Mg concentrations compared to yearling females and does. This larger variation might be caused by the limited amount does can transfer to their young in these mineral-poor environments.

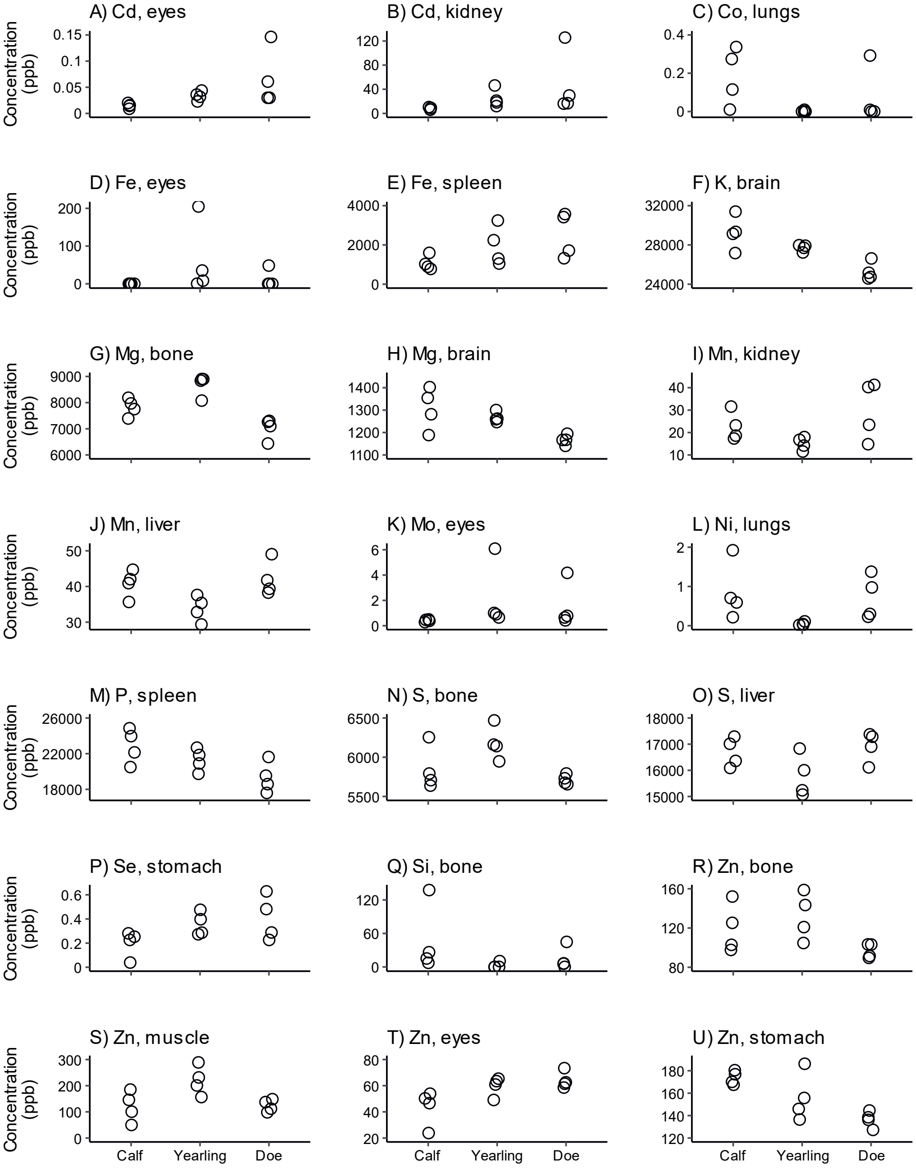


Figure 4.2 Significantly different tissue-element combinations (according to adjusted p-values using the step-up e njamini and Hochberg procedure (e njamini & Hochberg 1995)) between age groups in Fallow deer females. transfer to their young in these mineral-poor environments, although we do not have information on family relation of the culled animals.

Se functions as an important antioxidant, protecting against As and Cd toxicity, cancer, and heart disease (Tinggi 2008; Zwolak 2020). Dietary Se has been shown to affect the gut microbial colonisation (Pareira et al. 2020; Farreira et al. 2021), which might be in alignment with our finding that Se in stomach increased with age (Figure 4.2p). This increase might improve the uptake of other minerals when animals get older (Zwolak 2020).

We found four tissues that differed in Zn concentration among the female age groups (Figure 4.2r-u). First, Zn is essential for normal skeletal growth and bone homeostasis (O'Connor et al. 2020), and decreased with age (Figure 4.2r). This might be because bone mineral density tends to decline with age (Amin et al. 2020), causing the bone Zn concentration in younger animals more variable and lower in does. Second, Zn plays a role in muscle regeneration due to its effects on muscle cell activation (Hernández-Camacho et al. 2020). This might be most needed in young animals, although it remains unclear why we found higher Zn concentrations in muscle for yearling females compared to calves and does (Figure 4.2s). Third, Zn plays an integral role in maintaining a normal ocular function (Grahn et al. 2001). This might be more needed for older animals to slow down age-related macular degeneration (Vishwanathan et al. 2013), resulting in increasing Zn concentrations in eyes (Figure 4.2t). Last, Zn plays an important role in the production of digestive enzymes (Li et al. 2013). However, it remains unclear why we found decreasing Zn concentrations with age (Figure 4.2u).

Some of the other significant differences in element-tissue combinations were found in tissues where these elements play an important role. For example, the spleen has been mentioned to store the major Fe pool (Figure 4.2e) (Kolnagou et al. 2013). Ni in the lungs is associated with an increased risk of lung cancer (Figure 4.2l) (Grimsrud et al. 2002), and S is a constituent of bones and collagen, associated with an increased risk of osteoporosis (Figure 4.2n) (Whiting & Draper 1981). However, we were unable to interpret their potential relationship with age.

4.4.2 Age-related differences in males

We predicted age-related differences among males to be more related to bioaccumulation of toxic elements than differences in essential elements (prediction 3). However, the only toxic element that turned out significant was Al in the kidney (Figure 4.3a). Kidney's Al concentration was more variable, and for some much higher, for yearling males compared to calves, which was in alignment with previous studies (e.g. Yokel 19989). Free Al concentrations in the environment increase with decreasing pH due to anthropogenic acidification (De Graaf et al. 1997), which might be related to this finding.

We found higher Co concentrations and lower Mn concentrations in the eyes of yearling males compared to calves (Figure 4.3b+h). The Co concentration in eyes is

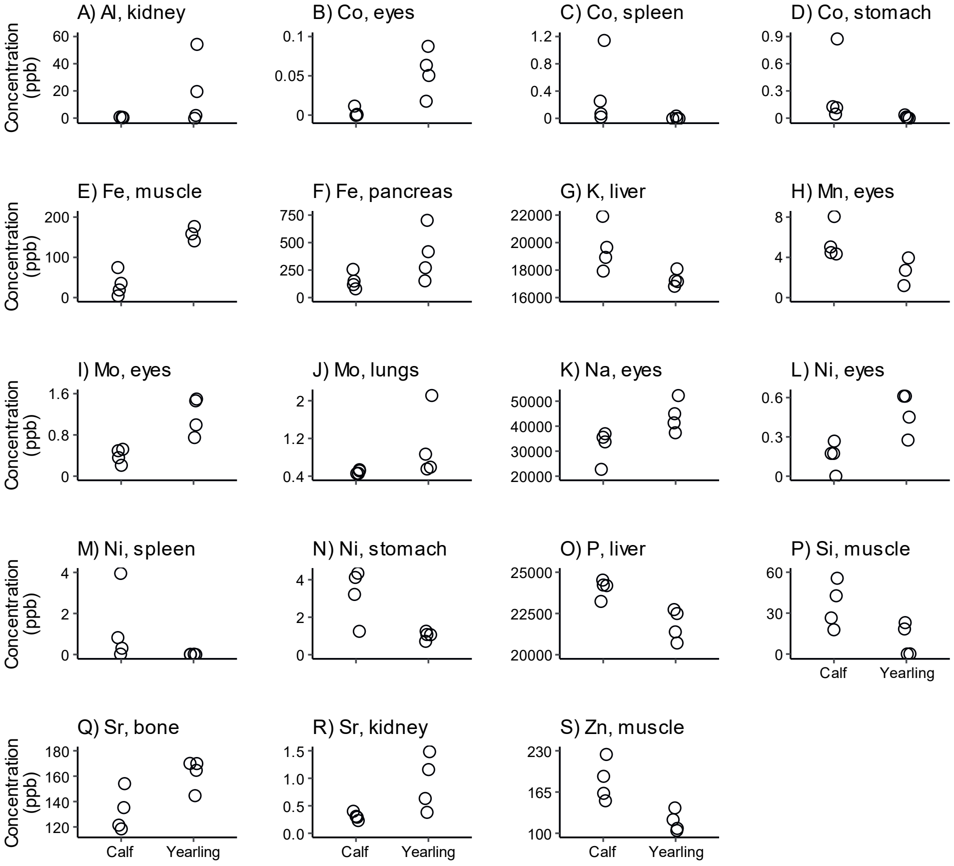


Figure 4.3 Significantly different tissue-element combinations (according to adjusted p-values using the step-up Benjamini and Hochberg procedure (Benjamini & Hochberg 1995)) between age groups in Fallow deer males.

associated with age-related macular degeneration in humans (Aberami et al. 2019), while senile cataractous has been associated with lower Mn levels in humans (Çekiç et al. 1999). However, it remains unknown whether this also applies to animals, specifically deer.

Fe in muscles is important for many metabolic functions and electron transfer during energy production (e.g. Hofer et al. 20008; Alves et al. 2022). DeRuisseau et al. (2013) reported that, in humans, the total concentration of Fe in muscles increases during growth but stabilises in senescence, which might be in line with our finding that yearling males have higher Fe concentrations in muscle compared to calves (Figure 4.3e).

We found higher Fe concentrations in pancreas for yearling males than for calves (Figure 4.3f). Fe in pancreas is associated with correct insulin synthesis and processing (Kimita & Petrov 2020; Marku et al. 2021). Increased Fe levels in pancreas have been associated with an increased risk of pancreatic cancer (Julián-Serrano et al. 2021), although this might not be relevant for wildlife. However, it seems unlikely that the animals in our study area, which is mineral poor, experienced an Fe overload that could lead to this difference in Fe concentration, especially when the acidification of this area is taken into account which set more Fe (and Al) free (Haynes & Swift 1985).

Male calves had higher Si concentrations compared to yearling males (Figure 4.3p). Si is needed for muscle building, and is found to decrease with age in rats (Jugdaohsingh et al. 2015). However, it remains unclear whether this also applies to deer or is relevant for wildlife.

We found higher Sr concentrations in bone and kidney for yearling males compared to calves (Figure 4.3q+r). Sr is considered as the chemical analog of Ca and has a major role in the formation and breakdown of bones and preventing against osteoporosis (Strandberg & Strandgaard 1995; Nielsen 2004; Baeza et al. 2011; Curtis et al. 2021). Sr overload has been associated with renal dysfunction (Schrooten et al. 1998; Cohen-Solal 2002). However, Sr toxicity seems very unlikely in our nutrient-poor study area.

Contrary to the higher Zn concentration in muscle of yearling females compared to female calves (Figure 4.2s), we found lower Zn concentrations in muscle for yearling males compared to male calves (Figure 4.3s). This might indicate that this difference is found by chance rather than driven by age or sex.

4.4.3 Sex-related differences in calves

As predicted, we indeed found the least significant tissue-element combinations when comparing sex-related differences among calves (prediction 4). However, we were unable to put most of our findings into the context of their biological and physiological role based on sex-related differences.

Female calves were more variable in their Al concentration in kidney and As concentration in muscle (Figure 4.4a-b). We expected accumulation of these elements to increase with age since Al is not transferred via milk (Muller et al. 1992) and thus should be taken up through the environment.

Higher Na concentrations in bones, as we found for female calves (Figure 4.4j), are associated with increased chance of osteoporosis at later age (Heaney 2006). Although females tend to have higher risks of osteoporosis in general (Clarke & Khosla 2020), it is unclear whether this might be associated with our finding.

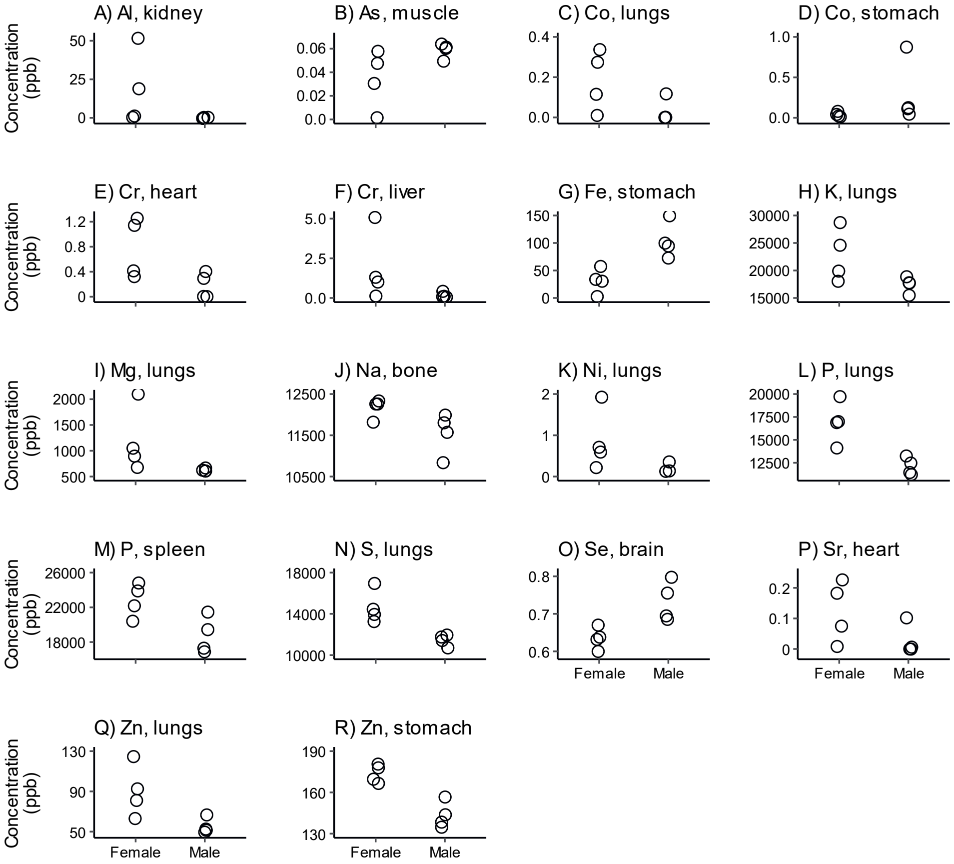


Figure 4.4 Significantly different tissue-element combinations (according to adjusted p-values using the step-up Benjamini and Hochberg procedure (Benjamini & Hochberg 1995)) between sex groups in Fallow deer calves.

We found higher Sr concentrations in the heart for females (Figure 4.4p + Figure 4.5w). Sr can protect the heart against heart infarct (Xing et al. 2021), although any sex-related differences remain unexplained, and it remains unknown whether this is applicable to deer as well.

Zn concentrations in the lungs and stomach were higher for females than for males (Figure 4.4q-r). Zn has anti-inflammatory, antioxidant and antiviral effects in lungs (Luan et al. 2022). It is also important for the production of digestive enzymes (Muralisankar et al. 2015). However, it is unknown how this might be associated with sex.

4.4.4 Sex-related differences in yearlings

We found most sex-related differences in element-tissue combinations when comparing the female and male yearlings (prediction 5). As expected, we found that for most of these element-tissue combinations, males had higher concentrations than females (14 and 11, respectively; Figure 4.5a-y). We were, however, unable to judge whether pregnancy was a major cause of these differences.

We found higher B concentrations in the liver of female yearlings compared to males (Figure 4.5b). Liver is the first tissue that will be affected by overexposure to B (Wilson & Ruszler 1997; Khaliq et al. 2018). However, this seems unlikely due to our nutrient-poor study area. Any sex-specific causes remain unknown.

For Co, we found higher concentrations in the eyes for males (Figure 4.5c) and in the liver of females (Figure 4.5d). Regarding the liver, it has been shown that supplemental Co did not increase the Co storage in the liver (Kincaid et al. 2003). This suggests that liver might not be the main target tissue for Co. Any sex-related differences in Co target tissues remain unknown.

We found higher Cr concentrations in the liver for females than for males (Figure 4.5e). This contradicts previous findings in ducks (Gochfeld & Burger 1987) and horses (Paßlack et al. 2014), where males had higher Cr liver concentrations than females.

We found higher Cu concentrations in the brain of females (Figure 4.5f) and in the heart of males (Figure 4.5g). Quinn et al. (2011) also found higher Cu concentrations in the brain of female rats and humans. This may be due to Cu-mediated pathological events in the brain. Besides, Cu deficiency might be a cause of ischaemic heart disease (DiNicolantonio et al. 2018) - of which symptoms are sex-specific (Aggarwal et al. 2018; Tomaszewski et al. 2019) -, although it is unclear whether this is directly related to Cu levels in heart tissue.

For Na, we found higher concentrations in the eyes, heart and liver of males (Figure 4.5p-r). Regarding the heart, Na in heart is associated with blood pressure (Patel & Joseph 2020), which is generally higher for males compared to females (Reckelhoff 2001).

We found higher S concentrations in the intestines of males than for females (Figure 4.5t). S amino acid metabolism is important for gut health (Shoveller et al. 2005). It has been suggested that sex-related differences could be due to sex-related differences of the gut microbiota (Hoeg et al. 2012; Wang et al. 2021), although we could only speculate whether this would result in higher S concentrations in males.

We found a higher Se concentration in the bones of males (Figure 4.5u) and in the spleen of females (Figure 4.5v). Se is crucial for bone development and bone mineral density

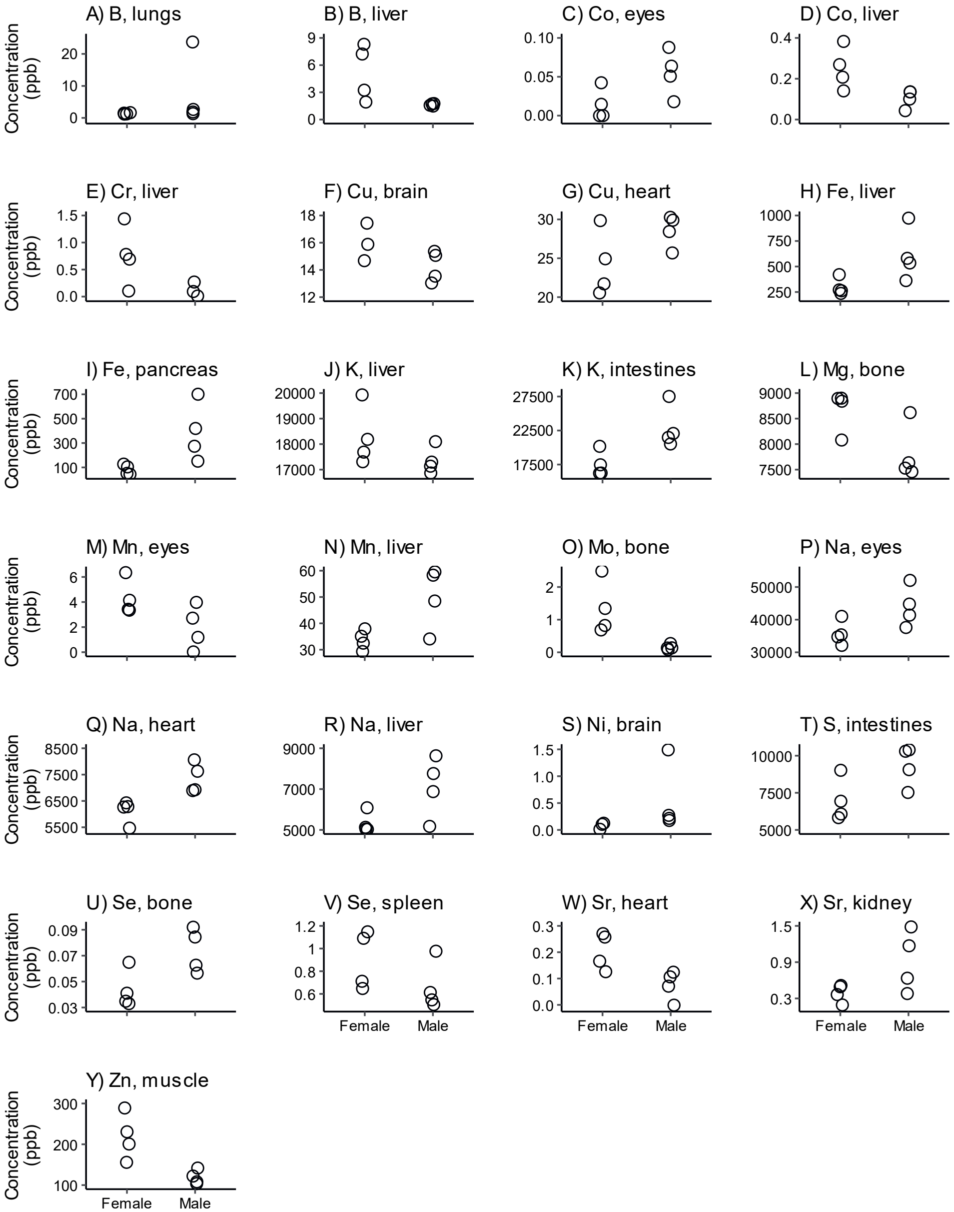


Figure 4.5 Significantly different tissue-element combinations (according to adjusted p-values using the step-up Benjamini and Hochberg procedure (Benjamini & Hochberg 1995)) between sex groups in Fallow deer yearlings.

maintenance (Hoeg et al. 2012; Yang et al. 2022). Also, both low and high Se concentrations can have negative effects on the immune function of the spleen (Zhang et al. 2020). However, although there are sex-related differences in Se metabolism (Barchielli et al. 2022), it is unclear how the differences that we found could be explained.

4.4.5 Limitations

A strength of this study was the use of individuals of a single population that roamed the same protected area and that were culled in the same hunting season. However, we also see three major limitations. First, we did not include bucks in our analysis since no bucks were culled in the area. Second, we used a small sample size, with four individuals in each group. Therefore, we cannot rule out that some differences that we found were due to outliers. Third, in mineral-poor areas such as our study area, certain elements are scarce and not distributed uniformly across the landscape. Thus, we cannot judge but only speculate whether some variation in elemental concentrations arose from differences between individuals in where they foraged, as differences in habitat and diet selection across age and sex classes of Fallow deer may occur (Apollonio et al. 1998). These limitations, however, do not invalidate our comparisons.

4.5 Conclusion and recommendations

We speculated on the biological and physiological role of chemical elements, focusing on age- and sex-related differences. In general, sex-related differences were more difficult to explain. This suggests that the current knowledge on chemical element allocation and metabolism in the body seems to be biased towards age-related patterns.

We found some ionomic differences between age and sex classes, as hypothesised, but the drivers of this variation remain unknown. Previous studies have suggested that diet might drive both sex-related (Gochfeld & Burger 1987) and age-related (Zhang et al. 2020) variation within species. Life-history traits and ontogeny are also mentioned as causes of ionomic variation (Prater et al. 2019). A number of decreasing elemental concentrations with age might refer to a decreased health condition of Fallow deer in this nutrient-poor environment (e.g. lower K in brains, lower Zn in muscles), although this is highly speculative. However, extensive studies in various species - including a wide range of elements and tissues - are missing to further explore these potential causes of ionomic variation. Therefore, we highly encourage the execution of such extensive ionomic surveys.

More extensive ionomic surveys are also needed to expand our understanding of physiological pathways underlying elemental allocation and metabolism. The limited available literature about the physiological role of elements in the body did not allow us to focus on Fallow deer only, accordingly we were not able to infer what the higher or lower values that we found would mean when observed in other species or humans. Moreover, reference values, that are needed to judge about any potential toxicities and deficiencies, are absent. Signs of toxicities and deficiencies are element-, or even species- specific (Arnhold et al. 2002; Barboza et al. 2003; Vikøren et al. 2005). Thus, we cannot judge about the consequences of the elemental values that we found without such reference values.

The lack of reference values, and the severe elemental variation within and among age and sex groups as we report in this paper, limit the use of wild-living animals for biomonitoring purposes, which is usually based on a limited number of elements in only a few tissues (e.g. Gaspariket al. 2004; Nowakowska et al. 2016; Squadrone et al. 2022). We believe that it is important to increase our understanding of this variation in more detail before this can be applied to biomonitoring purposes. It would be worthwhile to repeat our study in different areas, that differ in their mineral availability. Additionally, addressing ionomic variation in age and sex for other species would be valuable in order to investigate the general applicability of our study. Our study should thus been considered as a first step towards a more comprehensive understanding of the mammalian ionome.

In conclusion, we found that ionomic variation in a wide range of chemical elements and tissues within a single population was indeed partially related to age and sex. The limited existing knowledge about element-specific metabolic pathways and causes of ionomic variation did not allow us to put all these differences in a biological or physiological context. We encourage other scientists to conduct more extensive surveys of the ionome of different species, based on a wide range of elements and tissues, to enlarge our understanding of the mammalian ionome and potential biological, ecological, and metabolic consequences.

4.6 End sections

4.6.1 Author contributions

Elke Wenting: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing - Original Draft, Visualization, Supervision, Project administration, Funding acquisition. Henk Siepel: Conceptualization, Methodology, Resources, Writing - Original Draft, Visualization, Supervision. Melanie Christerus: Conceptualization, Methodology, Validation, Investigation. Patrick A. Jansen: Conceptualization, Writing - Original Draft, Funding acquisition.

4.6.2 Acknowledgements

We thank the managers and game wardens of Deelerwoud (Paul Jansen and Dirk Lieftink) for supplying us with the culled animals for analysis, and allowing us to dissect animals on-site. Furthermore we thank the technicians (Paul van der Ven and Sebastian Krosse) of the General Instruments of Radboud University for their help in the analysis of the elements.

4.6.3 Conflict of interest

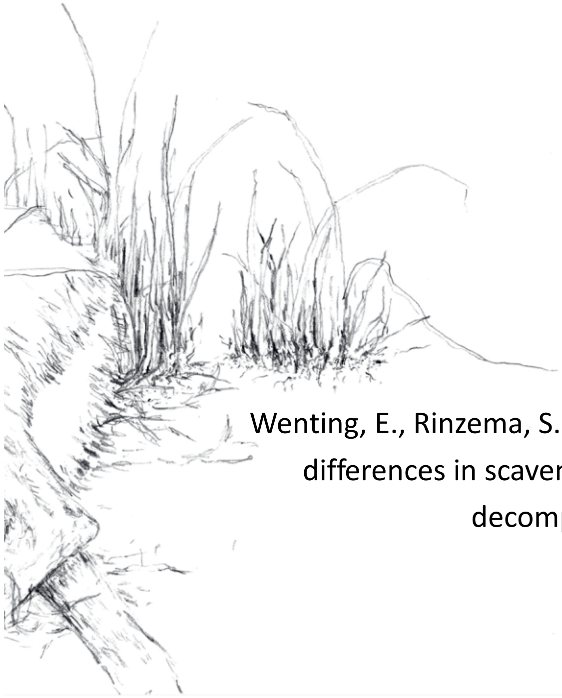
The authors have no relevant financial or non-financial interests to disclose.

4.6.4 Data availability

The data generated and analysed during this study is available via Figshare: https://doi.org/10.6084/m9.figshare.22140368.

Chapter 5

Functional differences in scavenger communities and the speed of carcass decomposition



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Abstract

Carcass decomposition largely depends on vertebrate scavengers. However, how behavioural differences between vertebrate scavenger species, the dominance of certain species, and the diversity of the vertebrate scavenger community affect the speed of carcass decomposition is poorly understood. As scavenging is an overlooked trophic interaction, studying the different functional roles of vertebrate species in the scavenging process increases our understanding about the effect of the vertebrate scavenger community on carcass decomposition. We used motion-triggered infrared camera trap footages to profile the behaviour and activity of vertebrate scavengers visiting carcasses in Dutch nature areas. We grouped vertebrate scavengers with similar functional roles. We found a clear distinction between occasional scavengers and more specialized scavengers, and we found Wild boar (*Sus scrofa*) to be the dominant scavenger species in our study system. We showed that these groups are functionally different within the scavenger community. We found that overall vertebrate scavenger diversity was positively correlated with carcass decomposition speed. With these findings, our study contributes to the understanding about the different functional roles scavengers can have in ecological communities.

Key words: scavenger community, vertebrate scavengers, carcass decomposition

5.1 Introduction

The decomposition of organic matter (i.e. detritus) is a crucial part of the cycling of energy and nutrients in all ecosystems (Swift et al. 1979; Moore et al. 2004). Up to 90% of the detritus pool consists of plant matter, which represents an enormous quantity in comparison to animal detritus (Swift et al. 1979; Barton et al. 2013). Despite being such a disproportionally small part of the overall detritus pool, animal detritus, predominantly in the form of carcasses, has been shown to largely affect ecological communities (e.g. Barton et al. 2013; Barton et al. 2019; Benbow et al. 2019), for instance as nutrient-rich food source for many organisms (Schoenly & Reid 1983), through its role in the nutrient cycle (e.g. Parmenter & MacMahon 2009; Benbow et al. 2019), and by stabilising food webs (Wilson & Wolkovich 2011).

The large impact that carcasses may have on ecosystem functioning can be attributed to the ubiquitous nature of the scavenging behaviour across species (DeVault et al. 2003; Wilson & Wolkovich 2011). We use the term ‘scavengers’ for all animal species that are involved in the process of carcass decomposition. Although scavenging is often overlooked in assessments of vertebrate diet composition (DeVault et al. 2003), vertebrate scavengers are often the primary consumers of carcasses in terrestrial ecosystems, consuming 35 to 75% of the total carrion pool (DeVault et al. 2003).

The majority of scavenger species consumes carcasses facultatively, meaning that carcasses occupy varying degrees of importance in these species’ diet in addition to other food sources and that these species could survive without it (DeVault et al. 2003; Wilson & Wolkovich 2011; Pereira et al. 2014). Although facultative scavenging is often considered to be an opportunistic feeding mechanism, it has recently been shown to be a highly regulated and constant behaviour for most species, governed by a variety of intrinsic and external factors (Selva et al. 2005), such as carcass type (Olson et al. 2016), habitat characteristics (Smith et al. 2017), as well as interguild and intraguild interactions (Selva & Fortuna 2007; Inagaki et al. 2020).

Different scavenger species may fulfil different functional roles in the carcass decomposition process (Sebastián-González et al. 2020; 2021), which would be reflected in differences in behaviour and preferences for tissues types. For example, Young et al. (2014) observed that Common buzzards (*Buteo buteo*) fed primarily on soft tissues in the early stages of decomposition, while Carrion crows (*Corvus corone*) increased their feeding as carcasses went into later stages of decomposition and exploited more different body parts. Due to such functional differences, the vertebrate species in the scavenger guild may complement each other in the removal of carcasses (Olson et al. 2016). Cortés-Avizanda et al. (2012), for instance, suggested that a diverse and species-rich scavenger community that functions synergistically may be the key to the stability and efficacy of carcass removal as an ecosystem service. Olson et al. (2012) found that the exclusion of an important scavenger species from the scavenger guild resulted in incomplete carrion depletion, even when the remaining species exhibited a compensatory response to the reduced competition. Accordingly, Hill et al. (2018) found that the exclusion of vultures resulted in decreased scavenging by facultative scavengers and overall slower decomposition rates. Additionally, Selva & Fortuna (2007) found that rare scavenger species were more likely to forage on carcasses that had already been frequently visited by more common scavengers, and suggested that these rare species matched their carcass choice with that of scavenger specialists like the Common raven (*Corvus corax*).

However, the different functional roles of vertebrate scavenger species and how these relate to the speed of carcass decomposition are still poorly understood (Barton et al. 2013). As scavenging is a frequently overlooked trophic interaction, studying the different functional roles of vertebrate species in the scavenging process increases our understanding of the effect of the vertebrate scavenger community on carcass decomposition. This study aimed to determine the different functional roles of the vertebrate scavengers, and how the diversity within the scavenger community relates to carcass decomposition speed. We predicted that higher vertebrate scavenger diversity would result in more efficient carcass exploitation, and therefore, in faster carcass decomposition (Hooper et al. 2005; Griffin et al. 2008).

5.2 Methods

5.2.1 Study system

We monitored the vertebrate animals that visited 49 carcasses in eight Dutch nature areas, in different periods between May 2012 and November 2020 (Figure 5.1). A minimum of two carcasses had been monitored in each of these areas. The carcasses were placed in heathlands or forested areas, whereby we avoided completely open or densely forested places. Different vertebrate scavenger communities were present in these areas, allowing us to study the effect of different scavenger guilds on the carcass decomposition speed.

5.2.2 Field methods

We used motion-triggered infrared camera traps to monitor the decomposition process of carcasses. Different models of camera traps were used throughout the years but all were part of the Bushnell TrophyCam product line. We attached the camera traps to trees, shrubs or actively placed poles at a distance of two meters from the carcass at one meter height

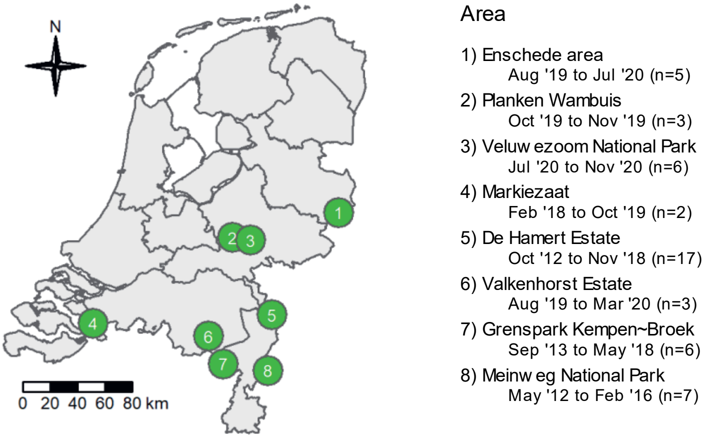


Figure 5.1 Map of the Netherlands showing the areas where we monitored carcasses until depletion. The period of monitoring and the number of monitored carcasses per area are indicated.

and slightly bended forward pointing towards the ground, depending on the local circumstances. All carcasses were positioned with the abdomen or back to the camera, and tied by the front and rear legs to trees or poles using natural ropes to prevent the carcasses from getting dragged out of view. The camera traps were set to videos of 60 seconds per trigger, with a two- or three-second interval between the triggers depending on the exact camera model. We visited the carcasses approximately every two weeks to replace the 32 or 64 GB SD card and to renew the batteries. We minimised the time spent and the number of people present at the carcass site as much as possible to reduce possible anthropogenic disturbance.

In our analyses, we only included carcasses of which the whole decomposition process was monitored, resulting in a total of 49 carcasses (Appendix 5.1). The carcasses were obtained from roadkills, except for Planken Wambuis and Veluwezoom National Park, where the carcasses were obtained from culling. No animals were killed for the purpose of this study. In total, we monitored the carcasses of 33 Roe deer (*Capreolus capreolus*), seven Wild boar (*Sus scrofa*), four European badger (*Meles meles*), three Red deer (*Cervus elaphus*), one sheep (*Ovis orientalis*), and one Fallow deer (*Dama dama*). Only complete carcasses were monitored, i.e. no guts only.

5.2.3 Annotation camera trapping videos

The collected camera trapping videos were uploaded to the online platform Agouti (Casaer et al. 2019), from which the footage was annotated. Per video, we annotated (1) the species and the number of individuals; (2) the behaviour of these animals (Table 5.1); (3) if applicable, the tissues that were eaten or collected; and (4) the stage of decomposition of the carcass. For the behaviour and tissues, we annotated the longest and second-longest shown behaviour or eaten tissue type, resulting in a maximum of two observations each. For simplicity, we did not distinguish between these in the further analyses, meaning that both the longest and second-longest shown behaviour or eaten tissue type were treated equally. In the case that two or more species visited the carcass in the same video, we annotated the video for each species separately.

Table 5.1 Overview of the definitions we used to annotate the behaviour of the scavengers that were recorded by the camera traps.

Behaviour	Abbreviation	Definition
Passing	PAS	Move in front of camera trap without moving body and/or head in the direction of the carcass.
Interest	INT	Body and/or head moves towards the carcass, or mouth/beak touches the carcass without any chewing/picking movements.
Eating	EAT	Mouth/beak touches the carcass, and removing carcass parts by chewing/picking movements.
Standing on carcass	STA	Touching the carcass with legs only, i.e. no other body parts than legs touch the carcass.
Intraspecific interaction	INTRA	Physical and non-physical contact between individuals of the same species.
Interspecific interaction	INTER	Physical and non-physical contact between individuals of a different species.
Collecting material	CM	Taking along carcass parts in the direct vicinity of the carcass.

Based on the quality of the videos, we were able to distinguish between seven tissue types and three stages of decomposition. For the tissue types, we distinguished between: (1) bones and hooves (hereafter ‘bones’); (2) hairs; (3) nose, ears, eyes, anus, and skin on the armpits and abdominal region (hereafter ‘soft tissues’); (4) skin on other parts of the body (hereafter ‘skin’); (5) muscle; (6) organs; and (7) insects and larvae that were present on the carcass (hereafter ‘insects’), i.e. indirect carcass consumption. Some behaviour and tissue observations were annotated as unknown. We excluded these observations from the analysis.

For the stages of decomposition, we distinguished between: (1) the bloated stage, in which the carcass is fresh and/or abdominal bloating occurs due to anaerobic microbial activity, and the carcass has no or only minor injuries that do not expose any entrails; (2) the active decay stage, characterized by rapid mass and volume loss due to increased scavenger activity, and during which at least some entrails are exposed; and (3) the advanced decay stage, characterized by a flat abdomen and only some parts of the skin and skeleton remains, possibly supplemented by some other tissue leftovers (Feddern et al. 2019). Twelve carcasses had such major injuries due to the cause of death that their decomposition started in the active decay stage.

Per carcass, we noted the day the carcass was placed and the day the carcass was fully decomposed to calculate the time to depletion per carcass. The carcass was considered as fully decomposed at the end of the advanced decay stage, when no carcass remains were visible anymore. The average daily temperature, based on the mean daily temperature from the nearest weather station (KNMI 2021), was calculated to include in the further analyses since the ambient temperature has been shown to be a primary determinant of carcass longevity (e.g. Parmenter & MacMahon 2009; Farwig et al. 2014; Ray et al. 2014). We also noted the start month, with January 2012 as month 1 - since the first carcasses were monitored in 2012 -, to correct for temporal autocorrelation.

5.3 Statistical analyses and results

5.3.1 Functional scavenger groups

All statistical analyses were done R version 4.0.2 (R Core Team 2020). In total, we annotated 6,805 videos of vertebrates visiting the carcasses. Below, we discuss the statistical analyses together with the results as further analyses were determined based on the foregoing results.

We started by determining the functional groups of scavengers in five steps. First, we selected the species that we included in the further analyses. This was done by selecting the species that showed eating behaviour, and from these species, we only selected the species with at least 30 observations. This resulted in a total of 17 species: Beech marten (*Martes foina*), Carrion crow, Cattle (*os taurus*), Common buzzard, Common raven, Domestic cat (*Felis catus*), Domestic dog (*Canis lupus familiaris*), European polecat (*Mustela putorius*), Fieldfare (*Turdus pilaris*), Great tit (*Parus major*), Horse (*Equus caballus*), Mistle thrush (*Turdus viscivorus*), Red fox (*Vulpes vulpes*), Roe deer, Song thrush (*Turdus philomelos*), Wild boar, and Wood mouse (*Apodemus sylvaticus*). After excluding the videos of the other species from the data, there were 6,548 videos left. In total, we had 9,100 observations of behavioural types and 6,752 observations of tissue types.

Second, for each species, we calculated the percentage of observations per decomposition stage, per behavioural type, and per tissue type (Table 5.2).

Third, we calculated for each species the average detection time - i.e. time until a species visited a carcass for the first time -, and the average time until first scavenging event - i.e. time until first annotation of EAT or CM behaviour (Table 5.2). Furthermore, we included the average adult body mass in grams for each species (Table 5.2; Jones et al. 2009), as a proxy of their capacity to tear open the carcass' skin, exposing more body parts of the carcass, thus enabling more carcass parts being consumed (e.g. Freeman & Lemen 2008).

Table 5.2 Percentage of observations per decomposition stage, behaviour, and tissue type, per selected species.

	Bloated stage	Active decay	Advanced decay	CM	EAT	INT	INTER	INTRA	PAS	STA
Bos taurus	51.56	26.56	21.88	0	4.76	27.38	0	1.19	66.67	0
E. caballus	44.12	54.41	1.47	0	5.22	46.27	0	16.42	32.09	0
A. sylvaticus	1.03	0	98.97	1.98	1.98	27.72	0	0	68.32	0
C. capreolus	8.47	22.03	69.49	0	3.07	26.15	0	0	70.77	0
Parus major	33.33	36.67	30	28.57	11.43	11.43	0	0	48.57	0
T. philomelos	3.13	3.13	93.75	2.7	19.92	5.41	0	0	62.16	10.81
T. pilaris	0	0	100	0	30.23	0	0	0	69.77	0
T. viscivorus	0	0	100	0	12.9	0	0	3.23	83.87	0
Buteo buteo	8.59	81.9	9.51	0.23	65.05	12.15	3.24	2.08	4.17	13.08
Corvus corax	1.61	45.86	52.53	4.48	67.88	3.59	0.91	16.1	4.73	2.41
C. corone	8.44	86.19	5.37	1.23	37.65	27.16	6.02	4.32	22.07	1.54
C. lupus familias	6.98	6.98	86.05	0	47.69	30.77	1.54	1.54	19.46	0
Felis catus	18.03	63.93	18.03	0	47.13	31.03	1.15	0	20.69	0
Martes foina	10.59	32.94	56.47	1.65	20.66	43.8	1.65	0.83	31.4	0
M. putorius	13.29	32.72	53.99	1.43	59.84	27.71	0.95	0.63	11.27	0.16
Vulpes vulpes	8.94	40.58	50.48	4.63	41.68	30.52	0.74	0.48	21.96	0
Sus scrofa	6.82	33.52	59.66	0.8	66.15	14.57	0.65	7.61	10.22	0

Table 5.2 Continued.

	Bones	Hairs	Insects	Muscle	Organs	Skin	Soft	Detection time	Time till scavenging	Adult body mass
Bos taurus	50	0	0	0	0	50	0	5550.6	836	613000
E. caballus	0	0	0	0	0	37.5	62.5	3	50	400000
A. sylvaticus	0	0	0	0	0	0	0	35449.7	45552	21.9
C. capreolus	0	50	0	0	0	50	.0.00	36969.7	28054	22502
Parus major	0	90.91	9.09	0	0	0	0	48780.4	41097.6	19.25
T. philomelos	0	20	80	0	0	0	0	35055	36147.5	67.75
T. pilaris	0	0	100	0	0	0	0	14348	17311	106
T. viscivorus	0	0	0	0	0	0	0	11291	14066	117.5
Buteo buteo	0.75	1.31	0	37.08	12.17	13.48	35.21	17895.6	12631.3	875
Corvus corax	2.38	4.67	0.25	44.65	17.66	21.31	9.08	7369.43	7513.75	1200
C. corone	1.52	4.04	3.54	59.1	3.54	6.57	21.72	12427.1	12319.3	375
C. lupus familias	14.29	0	0	4.76	4.76	76.19	0	23726.8	15216.5	35000
Felis catus	0	0	0	53.13	0	43.75	3.13	34149.3	6764	2884.8
Martes foina	62.5	12.5	0	0	0	25	0	30607.1	20530	1675
M. putorius	0.48	1.68	0	42.45	2.88	41.97	10.55	46149	15178.3	975.55
Vulpes vulpes	21.44	2.88	2.09	21.44	9.93	33.86	8.37	18921.6	25655.7	4820.36
Sus scrofa	5.55	1.46	0.24	49.12	7.64	33.11	2.87	8705.69	19933.9	84471.5

Fourth, the collected information per species as described in the second and third step (Table 5.2) were analysed with a principal component analysis (PCA) in order to group the species with the most similar scavenging habits (Figure 5.2a). This resulted in three groups: (1) the Grazers (G), consisting of Cattle and Horse; (2) the Occasionals (O), consisting of Fieldfare, Great tit, Mistle thrush, Roe deer, Song thrush, and Wood mouse; and (3) the more specialised scavengers. Based on this PCA (Figure 5.2a), Beech marten was located between the group Occasionals and Specialists, but due to its characteristics, especially low PAS behaviour (Table 5.2), we decided to group this species with the Specialists. The group Grazers was characterized by a low percentage of EAT behaviour (5% on average), and relatively high INT and PAS behaviour (36.5% and 49% on average, respectively). This group can be characterized by its high adult body mass. The group Occasionals was characterized by relatively low EAT and CM behaviour (12% and 5% on average, respectively) and a high percentage of PAS behaviour (67% on average). The only tissue types identified for this group were hairs, insects, and skin.

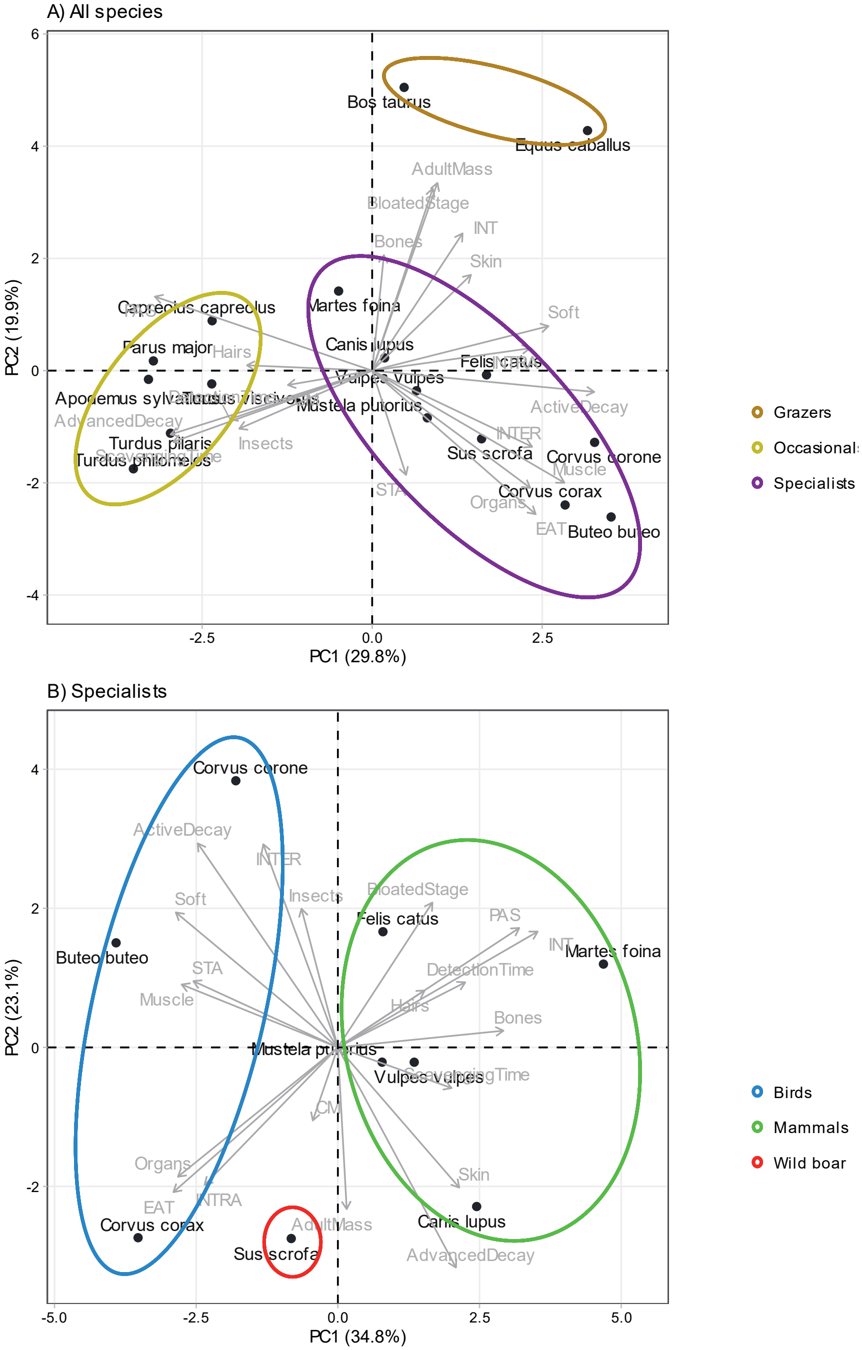


Figure 5.2 PCA biplots of (a) all the selected 17 species, and (b) the species defined as specialists. The circles indicate the scavenger groups we defined.

Last, we used an additional PCA to further analyse the species we grouped as Specialists (Figure 5.2b). Based on this analysis, we subdivided this group into three groups: (1) the Birds (B), consisting of Carrion crow, Common buzzard, and Common raven; (2) the Mammals (M), consisting of Beech marten, Domestic cat, Domestic dog, European polecat, and Red fox; and (3) the Wild boar (W). The group Birds was characterised by a prevalence for the active decay stage (71% on average) and the occurrence of STA behaviour for all species. The group Mammals had a larger body mass on average compared to the group Birds (9,071 and 817 grams respectively). Although species in the group Mammals were the most scattered and there was no single distinctive trait they all shared, this group was characterized by a percentage of PAS behaviour lower than 35%. Due to its high body mass, high percentage of EAT behaviour, and high percentage of INTRA behaviour (Table 5.2), which indicates a larger group size compared to the other species, we decided to treat Wild boar as a separate group. We used the FactoMineR (Le et al. 2008) and factoextra (Kassambara & Mundt 2017) packages to compute and visualise both PCAs.

Summarised, for further analysis, we divided the scavengers that visited the carcasses into five groups: (1) the Grazers (G); (2) the Occasional (O); (3) the Birds (B); (4) the Mammals (M); and (5) the Wild boar (W).

5.3.2 Carcass depletion time versus scavenger groups

We analysed in three steps how the time until carcass depletion was influenced by the presence of particular scavenger groups. First, we made an overview of which groups were present per carcass. In total, there were 15 combinations of scavenger groups observed (Appendix 5.2+5.3).

Second, since we noted that there were only six carcasses where the group Grazers was involved, spread over four combinations of groups, we tested whether the presence of the group Grazers influenced the depletion time, i.e. time until the end of the advanced decay stage. We selected the carcasses with the combination of groups with the group Grazers present and the carcasses with the same combination of groups without the group Grazers present. Using a linear mixed-effect model (Kuznetsova et al. 2017) with days to depletion as dependent variable, the group Grazers presence or absence and carcass initial state as fixed factors, mean daily temperature as covariate, and area, start month, and carcass species as random factors, we found no difference between the group Grazers presence or absence (LMM, df=3.676, F=0.388, p=0.570). Carcass initial state (LMM, df=17.975. F=0.982, p=0.335) and mean daily temperature (LMM, df=16.445, F=1.925, p=0.184) were not significant. Therefore, we decided to constitute the carcass groups without incorporating the presence or absence of the group Grazers. This resulted in 11 combinations of groups (Appendix 5.2).

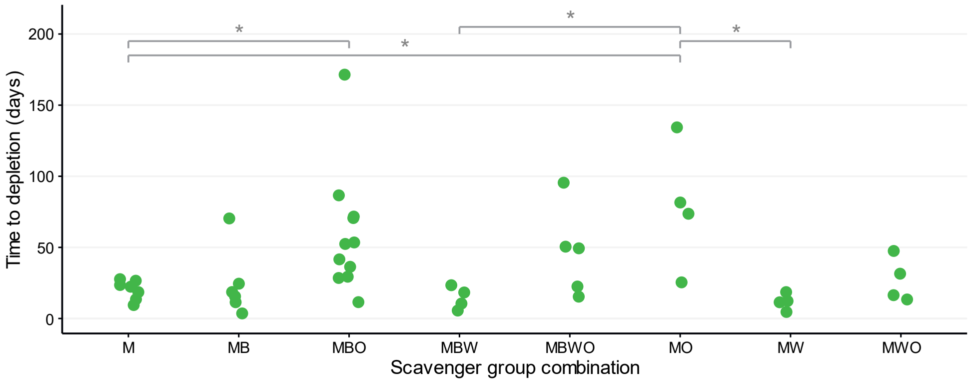


*Figure 5.3 The time until carcass depletion per scavenger group combination. The scavenger group combinations from left to right: M = Mammals; MB = Mammals and Birds; MBO = Mammals, Birds, and Occasionals; MBW = Mammals, Birds, and Wild boar; MBWO = Mammals, Birds, Wild boar, and Occasionals; MO = Mammals and Occasionals; MW = Mammals and Wild boar; and MWO = Mammals, Wild boar, and Occasionals. *p<0.05.*

Last, from these 11 combinations of groups, we selected the combinations that represented at least four carcasses (Appendix 5.2). This resulted in four carcasses being excluded from further analysis. In total, we analysed eight combinations of groups for differences in depletion time (Figure 5.3), using a linear mixed-effect model with days to depletion as dependent variable, the scavenger groups and carcass initial state as fixed factors, mean daily temperature as covariate, and area, start month, and carcass species as random factors. We found that carcasses with the groups Mammals and Occasionals present decomposed slower compared to three other groups: carcasses with only Mammals present; carcasses with Mammals, Birds, and Wild boar present; and carcasses with Mammals and Wild boar present. We also found that carcasses with only Mammals present decomposed faster than carcasses with Mammals, Birds, and Occasionals present (Figure 5.3; LMM, df=22.673 F=7.200; p<0.001). Again, carcass initial state (LMM, df=29.229, F=3.957, p=0.056) and mean daily temperature (LMM, df=32.558, F=2.295, p=0.139) were not significant.

5.3.3 Dominant scavenger group

Next, we tested whether the decomposition process was significantly sped up by the presence of a particular scavenger group. We used again linear mixed-effect models and included the same covariate, fixed factor and random factors as described before. We found that the presence of the group Wild boar accelerated the time to carcass depletion (Figure

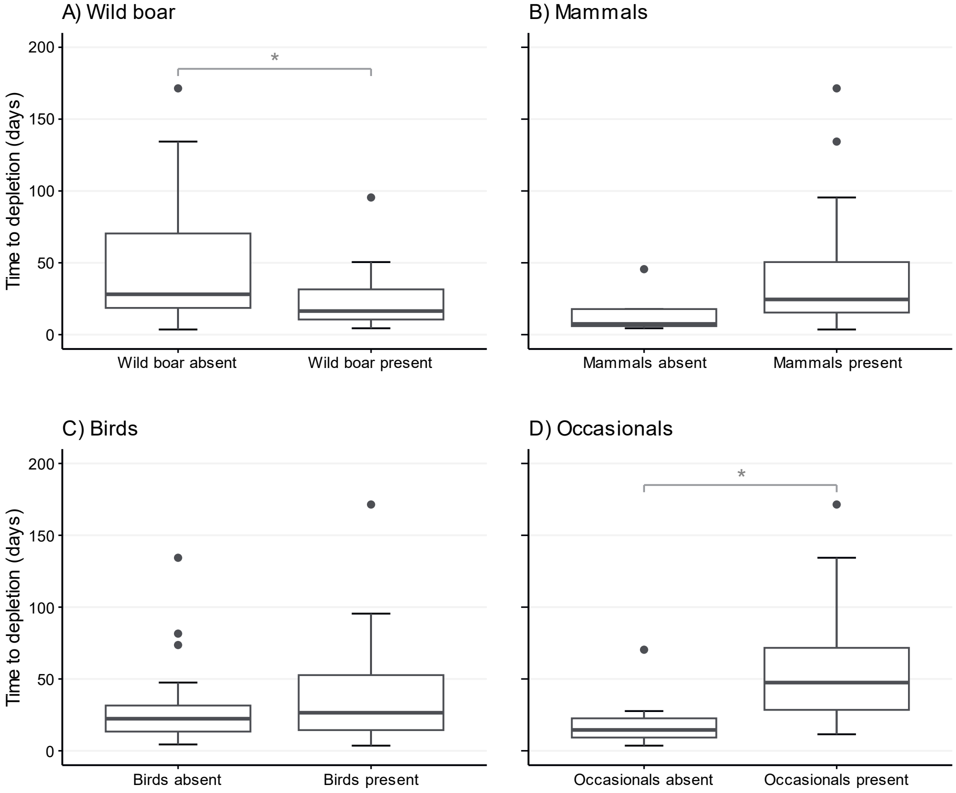


*Figure 5.4 The time until carcass depletion per scavenger group presence: (a) the Wild boar, (b) mammalian scavengers, (c) scavenging birds, and (d) occasionals. *p<0.05.*

5.4a; LMM, df=1, F=4.509; p=0.045). We did not find an effect for the presence of the group Mammals (Figure 5.4b; LMM, df=1, F=0.453, p=0.504), nor for the presence of the group Birds (Figure 5.4c; LMM, df=1, F=1.035, p=0.315). When the group Occasionals was present, we found that the time till depletion was longer compared to their absence (Figure 5.4d; LMM, df=1, F=14.373, p<0.001). Thus, we defined the Wild boar as the dominant scavenger group in our study system.

5.3.4 Scavenger diversity

We studied the effect of scavenger diversity on the speed of carcass decomposition in two ways. First, we calculated the Shannon's diversity index based on the vertebrate scavenger species per carcass (Oksanen et al. 2007). Using a linear mixed-effect model with depletion time as dependent variable, diversity index and carcass initial state as fixed factors, mean daily temperature as covariate, and area, start month, and carcass species as random

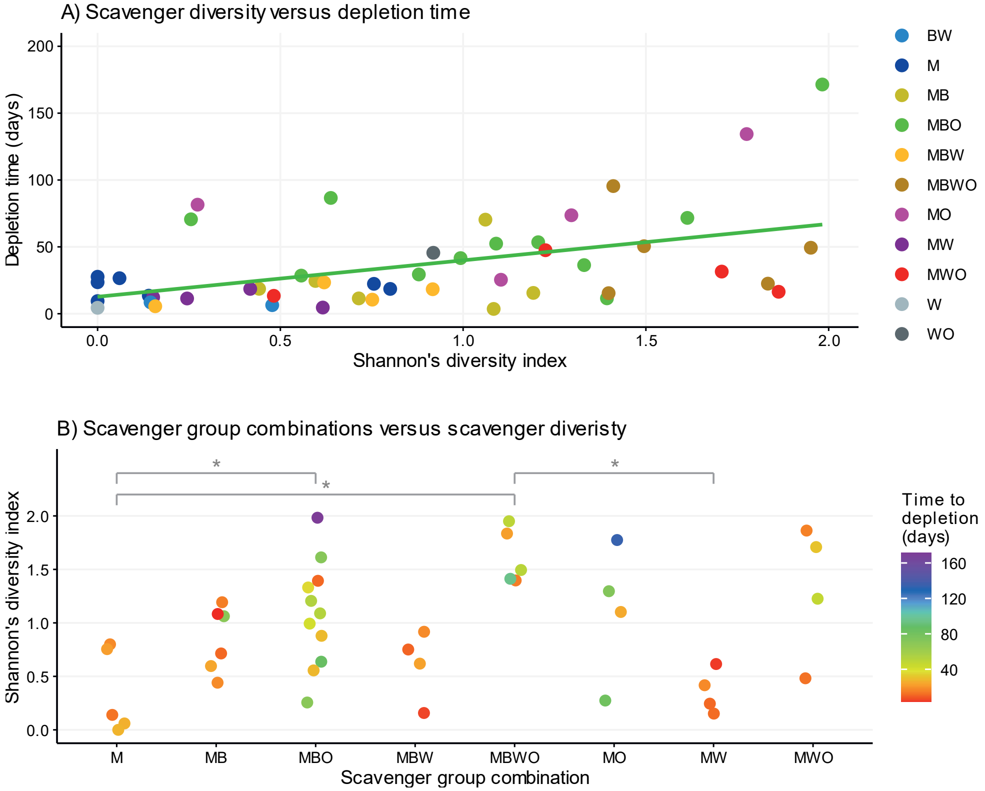


*Figure 5.5 The scavenger diversity - as Shannon's diversity index - versus (a) the time until carcass depletion, and (b) over the scavenger group combinations. The scavenger group combinations: BW = Birds and Wild boar; M = Mammals; MB = Mammals and Birds; MBO = Mammals, Birds, and Occasionals; MBW = Mammals, Birds, and Wild boar; MBWO = Mammals, Birds, Wild boar, and Occasionals; MO = Mammals and Occasionals; MW = Mammals and Wild Boar; MWO = Mammals, Wild boar, and Occasionals; W = Wild boar; and WO = Wild boar and Occasionals. *p<0.05.*

factors, we found a positive correlation between scavenger diversity and carcass depletion time (Figure 5.5a; LMM, df=42.533, F=11.408, p=0.002). Carcass initial state (LMM, df=42.572, F=0.192, p=0.664) and mean daily temperature (LMM, df=22.178, F=3.554, p=0.073) were not significant.

Second, we tested whether the diversity was higher when more scavenger groups were present on carcasses with a linear mixed-effect model with the diversity index as dependent variable, the carcass groups and carcass initial state as fixed factors, and the previous mentioned covariate and random factors. Although we found that most of the scavenger groups did not differ in the diversity index, we did find that the carcasses with all the scavenger groups - Mammals, Birds, Wild boar and Occasionals - present had a higher Shannon's diversity index compared to the carcasses with only the group Mammals and the groups Mammals and Wild boar present, and that the carcasses with Mammals, Birds, and Occasionals present had a higher Shannon's diversity index compared to carcasses with only Mammals present (Figure 5.5b; LMM, df=23.437, F=5.729, p<0.001). Again, carcass initial state (LMM, df=30.957, F=0.298, p=0.589) and mean daily temperature (LMM, df=25.048, F=0.302, p=0.587) were not significant.

5.4 Discussion

This study aimed to determine whether functional differences amongst vertebrate scavengers occurs, and how the diversity within the scavenger community relates to carcass decomposition speed. It became clear that the 17 selected vertebrate scavenger species benefited from the presence of carcasses in different ways (Figure 5.2a-b; Table 5.2). More specialistic bird and mammal species, e.g. Common raven and European polecat, mainly used the carcasses directly as a food source, while occasional scavengers, e.g. Great tit and Song thrush, used the carcasses rather indirectly by feeding on insects that they found on or close to the carcass. Great tit in particular often collected hair from the carcasses, which they presumably use for nest lining (Ondrušová & Adamík 2013).

Although the grazers - Cattle and Horse - and one occasional scavenger - Roe deer - are commonly known as obligate herbivores (Duncan & Poppi 2008; Rørvang et al. 2018), we observed them sometimes showing EAT behaviour (Table 5.2). Cattle was observed eating on bones and skin, Horse on skin and soft tissue, and Roe deer on hairs and skin (Table 5.2). These are all superficial, and thus easily accessible, tissues types, indicating that these obligate herbivores tend to only scavenge in a simple and casual way. Scavenging by mammalian herbivores is a well-documented phenomenon (Dudley et al. 2016), that is presumably a way for these species to ingest nutrients which are otherwise rare in their diet (Bazely 1989). This might imply that these species can find some nutrients that are beneficial to them in the tissues they consumed - e.g. sodium (Na), magnesium (Mg), phosphorus (P), and potassium (K) from the skin and hairs, and calcium (Ca) and P from the bones (Wenting et al. 2020).

Among the non-herbivore vertebrate species, the two domestic species in our data - Dog and Cat - contributed to the decomposition process of some carcasses. Most of these animals may be owned by humans in the surroundings of the study sites. For the Dogs, we observed that they all wore a dog collar. Although there were no visible indications that the Cats were owned by humans, we assumed that the observed individuals were suburban or farm cats since we only observed them on carcasses that had the nearest farm or house within a radius of 800 meters, which is a distance that could easily fall within a cat's home range (e.g. Barratt 1997; Meek 2003). Therefore, we presumed that these species did not have a real need to consume carcasses. Like obligate herbivores, their scavenging behaviour can be described as only a minor part in their total diet. For this reason, it is unlikely that the presence of these species would replace or overrule the role of other - probably more important - scavenger species in the community (Huijbers et al. 2015). Among the other specialised scavenger species, there were instances of direct competition between individuals for carcasses, which never occurred among the grazers or occasional scavengers, indicating that the carcasses were an important resource for these species.

The behaviour exhibited by the vertebrate species was consistent between our study areas, however we cannot conclude that the scavenging behaviour of these species would be the same across regions. The Wood mouse, for example, was characterised as an occasional scavenger with very little eating behaviour in our study (Table 5.2), while Young et al. (2014) found carrion to be a major part of their diet in the United Kingdom. This is probably a reflection of the local resource availability as Wood mouse prefers to forage on seeds but relies on carrion when their preferred food source is scarce (Montgomery & Montgomery 1990; Young et al. 2014). As another example, Carrion crow and European polecat were among the more prevalent scavengers - classified as scavenging bird and mammalian scavenger, respectively - in our study, while Selva et al. (2005) described these species as minor, occasional, scavengers in Bialowieza Primeval Forest, Poland. Since all the selected scavenger species in our study are facultative scavengers, resource availability presumably played an important role in determining their level of scavenging behaviour.

Olson et al. (2012) found that the exclusion of key scavenger species from a community resulted in a longer depletion process. Obligate scavengers as vultures are often considered as the strongest competitors in the terrestrial scavenger guild (Houston 1979), that can increase interspecific competition (e.g. Sebastián-González et al. 2013), and in turn speed up carcass decomposition speed (Cortés-Avizanda et al. 2012; Ogada et al. 2012; Hill et al. 2018). Although such obligate scavengers were absent from our study system, we found Wild boar to be the dominant scavenger, with its presence enhancing the carcass decomposition speed (Figure 5.4a). Wild boar belonged to the species with the highest intraspecific interaction behaviour (Table 5.2), indicating their social nature (e.g. Dardaillon 1988; Maselli et al. 2014; Sebastián-González et al. 2021). These results do not suggest that interspecific interactions between Wild boar and other species did not occur, but is presumably a limitation of the annotation protocol that we used since we only annotated the longest and second longest shown behavioural type. Thus, we cannot conclude that interspecific interactions were absent, but we can conclude that intraspecific interactions occur more frequently and for longer periods than interspecific interactions. Vultures are described as the most specialistic species, able to rapidly consume carcasses (e.g. Cortés-Avizanda et al. 2014; Mateo-Tomás et al. 2017), and potentially triple the carcass decomposition speed (Ogada et al. 2012). Although we found that Wild boar's presence did enhance the decomposition speed (Figure 5.4a), we cannot conclude that Wild boar had such a tremendous effect. Therefore, although we denote Wild boar as the dominant scavenger species in our study system, we cannot argue that this species had a comparable effect in our study system as vultures may have elsewhere.

Contrary to our expectation that a higher scavenger diversity would result in faster carcass decomposition, our results showed the opposite (Figure 5.5a). Probably our results can be explained by the longer monitoring periods when carcasses are decomposed at slower rates, resulting in longer windows of opportunity for species to detect and utilise the carcasses (Baruzzi et al. 2018). Accordingly, we found a higher diversity for carcasses with more scavenger groups involved in their decomposition process correlated with slow decomposition speed (Figure 5.5b). This implies that a slower carcass decomposition process would promote biodiversity the most, especially when taking into account the invertebrate species (e.g. Barton & Evans 2017), while fast carcass decomposition by only a few vertebrate scavenger species might more substantially promote other natural processes, e.g. the nutrient cycle, which is a key natural process for ecosystem functioning (e.g. Ngai & Srivastava 2006). We speculate that the existence of variety in both the carcass decomposition speed and the differences in scavenger diversity within an ecosystem would contribute most to biodiversity and overall ecosystem functioning simultaneously.

In conclusion, defining the most dominant scavenger species in an ecosystem, complemented with the scavenger specialists and occasional scavengers, provides more insights into the role that the scavenging process plays in the area, and how it would affect biodiversity and fundamental natural processes simultaneously.

5.5 End sections

5.5.1 Acknowledgements

We thank ARK Rewilding, the Netherlands, for the support by contributing to the camera traps that were used for this study.

5.5.2 Author contributions

EW: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, visualization, writing - original draft, writing - review & editing. SCYR: conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, writing - original draft. FvL: conceptualization, project administration, resources, supervision, writing - original draft.

5.5.3 Conflicts of interest

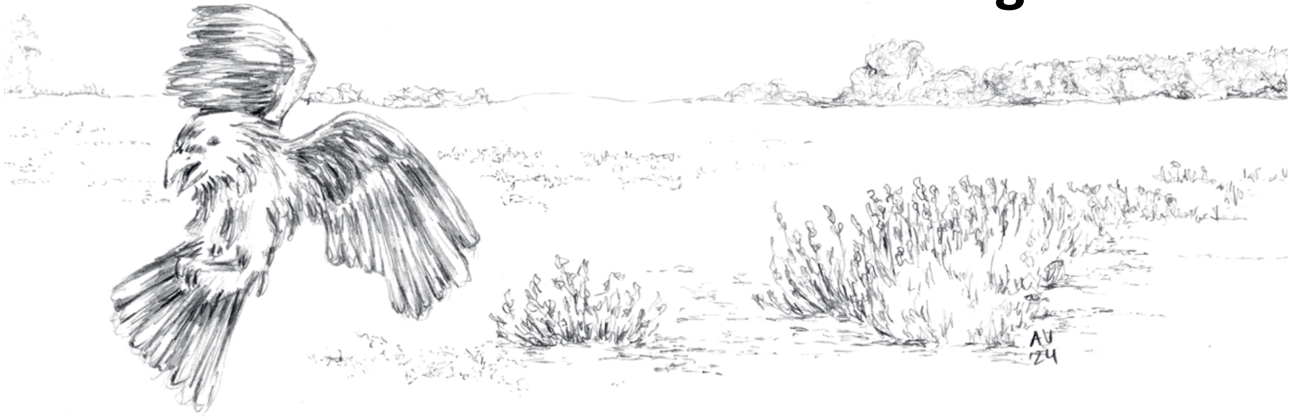
No actual or potential conflicts of interest are declared by the authors.

5.5.4 Data availability

The complete dataset will be accessible through Figshare: https://doi.org/10.6084/m9.figshare.14864850.

Chapter 6

Influence of tree cover on carcass detection and consumption by facultative vertebrate scavengers



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Abstract

Scavenging mammals and vultures can exploit and deplete carcasses much faster than other birds and invertebrates. Vultures are strongly influenced by habitat type, e.g. tree cover, since they rely on their eyesight to detect carcasses. It remains unclear whether and how facultative scavengers - both other birds and mammals - are influenced by tree cover and how that affect carcass decomposition time, which in turn affects biodiversity and ecological processes, including the cycle of energy and nutrients. We studied whether the carcass detection and consumption, hence carcass decomposition speed, by facultative avian and mammalian scavengers varies with tree cover in areas without vultures. Fresh mammal carcasses were placed in different landscapes across the Netherlands at locations that widely varied in tree cover. Camera traps were used to record carcass exploitation by facultative avian and mammalian scavengers and to estimate carcass decomposition time. We found that carcass detection and consumption by birds, wild boar and other mammals varied between locations. Carcass decomposition speed indeed increased with carcass detection and exploitation by mammals, especially by wild boar. However, this variation was not related to tree cover. We conclude that tree cover is not a major determinant of carcass exploitation by facultative scavengers in areas without obligate scavengers and large carnivores.

Keywords: carcass decomposition, facultative scavengers, carrion, wild boar, tree cover

6.1 Introduction

The decomposition of dead animal bodies - carcasses - has potentially far-reaching effects on biodiversity and ecological processes, including the cycle of energy and nutrients (Swift et al. 1979; Moore et al. 2004) - a key natural process for ecosystem functioning (Ngai & Srivastava 2006). Several studies have shown that carcasses can alter local nutrient dynamics (e.g. Macdonald et al. 2014; Barton et al. 2019; Quaggiotto et al. 2019). In addition, carcass availability in general subsidises facultative scavengers and can be important for species survival under harsh conditions, e.g. during winter (e.g. Wilson & Wolkovich 2011).

The species assemblages involved in the carcass decomposition process can vary enormously, with a wide spectrum of possible assemblages (e.g. Olson et al. 2016; Wenting et al. 2022). At the one extreme, carcasses are entirely decomposed by invertebrate scavengers and microbial decomposers (Carter et al. 2007; Bump et al. 2009). At the other extreme, carcasses are almost exclusively consumed by vertebrate scavengers - i.e. birds and mammals (DeVault et al. 2003). The first extreme would proceed slower, providing a longer time window for scavenger species to arrive, hence strongly promote biodiversity (e.g. Blázquez et al. 2009; Barton & Evans 2017; Wenting et al. 2022). The second extreme would strongly promote the nutrient cycle by faster redistributing carcass-derived nutrients over large areas via scavengers (e.g. Bump et al. 2009; Parmenter & MacMahon 2009; Wenting et al. 2023). Most carcasses, however, decompose through an in-between scenario resulting in highly variable decomposition speeds (Olson et al. 2016; Wenting et al. 2022).

The carcass decomposition process is influenced by many biotic and abiotic factors, including ambient temperature (e.g. Selva et al. 2005; Parmenter & MacMahon 2009), carcass type (Olson et al. 2016), carcass size (e.g. Ogada et al. 2012; Moleón et al, 2015; Turner et al. 2017), and local species assemblage (e.g. Farwig et al. 2014; Wenting et al. 2022). Also habitat type may be relevant as, for example, Arrondo et al. (2019) found that carcasses located in open areas were detected and consumed earlier compared to carcasses located in more heterogeneous and forested areas. Similarly, Pardo-Barquín et al. (2019) found that the amount of tree cover lowered scavenger richness and diversity, particularly hindering avian scavengers to access carcasses. The amount of tree cover, therefore, is expected to be an important driving factor for scavengers in detecting and consuming carcasses, hence affecting carcass depletion speed.

However, studies focussing on the effects of habitat type are predominantly biased towards invertebrate scavengers (e.g. Barton & Evans 2007; Farwig et al. 2014). Moreover, understanding the effect of habitat type on the carcass decomposition process is biased towards areas where vultures are present (e.g. Houston 1986; Gavashelishvili & McGrady 2006; Hill et al. 2018; Byrne et al. 2019; Oliva-Vidal et al. 2022). Since vultures are obligate scavengers and are known to have an enormous effect on carcass removal from ecosystems (e.g. Ogada et al. 2012; Cortés-Avizanda et al. 2014; Mateo-Tomàs et al. 2017; Sebastián-González et al. 2021), these findings might not necessarily apply to areas where vultures are absent. Facultative scavengers are the most prominent consumers of carcasses in areas without vultures (DeVault et al. 2003), but the importance of habitat type on facultative avian and mammalian scavengers in areas without vultures remains unclear.

Facultative avian and mammalian scavengers differ in their adaptations and abilities to detect and consume carcasses (e.g. Selva et al. 2005; Wenting et al. 2022). In general, avian scavengers seem to be better adapted to carcass detection in open areas as they are mainly guided by their eyesight (e.g. Wilmers et al. 2003; Selva et al. 2005), while carcass detection of mammalian scavengers is mainly driven by olfactory cues (e.g. Ruxton and Houston 2004; Stahler et al. 2002; Selva et al. 2005). Also, as a result of their morphology, the average maximum intake rate of mammals is generally larger compared to birds (Van Gils et al. 2007). Consequently, carcasses that are dominantly exploited by mammals - wild boar (*Sus scrofa*, henceforth ‘boar’) in particular (Wenting et al. 2022) - would faster decompose than when birds are dominantly present.

Thus, whether avian or mammalian scavengers dominate the carcass decomposition process matters because it may greatly affect the decomposition speed. We expect that the carcass decomposition process is strongly influenced by the amount of tree cover. We tested five predictions: (1) the denser the tree cover, the longer it takes before carcasses are first detected and first scavenged by birds, but the faster carcasses are first detected and first scavenged by boars or other mammals; (2) the denser the tree cover, the lower the proportion of carcass consumed by birds, but the higher the proportion consumed by boars or other mammals; (3) the sooner carcasses are first detected or first scavenged by birds, boars or other mammals, the higher the proportion of carcass consumed by these groups; (4) the carcass decomposition speed is not influenced by time to first detection or first scavenging by birds, but is accelerated by time to first detection or first scavenging by boars or other mammals; and (5) the carcass decomposition speed is not influenced by the proportion of carcass consumed by birds, but is accelerated when the proportions consumed by boars or other mammals increase.

6.2 Methods

6.2.1 Study area

We monitored the vertebrate animals that visited 59 carcasses in eight Dutch protected areas, in different periods between May 2012 and July 2021 (Figure 6.1). We selected

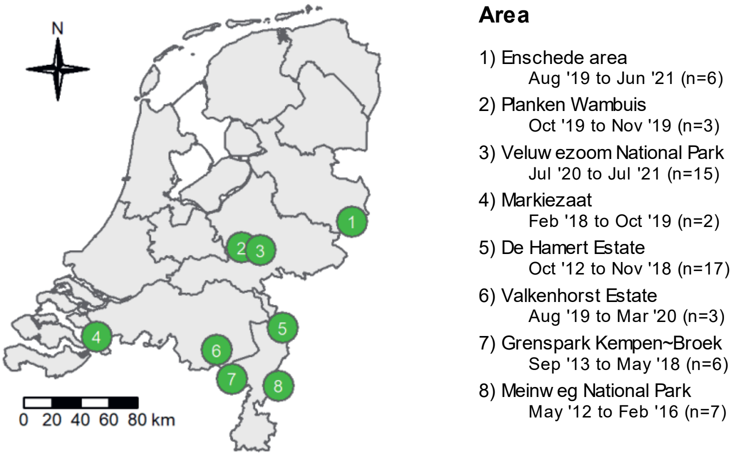

Figure 6.1 Map of the Netherlands showing the areas where we monitored carcasses until depletion. The period of monitoring and the number of monitored carcasses per area are indicated.

locations within these areas that represented the variation of tree cover, e.g. heathlands with barely any tree cover, forest edges with intermediate tree cover, and dense forest with high tree cover. Some areas occupied all gradations of tree cover, e.g. Veluwezoom National Park and Meinweg National Park. Other areas occupied different gradations as, for instance, Planken Wambuis only occupied higher tree cover, while Valkenhorst Estate only occupied intermediate tree cover. The forest types were similar among the areas, consisting of a mix of deciduous and coniferous forest.

The majority of the monitored carcasses - 49 out of 59 - were also used to analyse the effect of functional differences in scavenger communities on the carcass decomposition speed (Wenting et al. 2022). Not all areas inhabited the same species (Appendix 6.1), hence we included area as random factor in the statistical analyses. Most noteworthy, avian and mammalian scavengers were present in all areas, while boar was absent in Markiezaat and De Hamert Estate but present in the other areas. In all study areas, the species with the highest capacity to consume carcasses - large carnivores and vultures (Sebastián-Gonzalez et al. 2021) - were absent at the time of monitoring.

6.2.2 Field methods

We used the same protocol as described in Wenting et al. (2022), using motion-triggered infrared camera traps - all part of the Bushnell TrophyCam product line - to monitor fresh carcasses until depletion. We attached the camera traps to trees, shrubs or actively placed poles at a distance of two meters from the carcass, about one-meter height and slightly bent forward pointing toward the ground, depending on the local circumstances. All carcasses were positioned with the abdomen or back to the camera, and tied at the front and rear legs to trees or poles using natural ropes to prevent dragging out of view. The camera traps were set to videos of 60 seconds per trigger, with a two- or three-second interval - depending on the exact camera model - between the triggers. We visited the carcasses every two weeks on average to replace the 32 or 64 GB SD card and to renew the batteries. We minimised the time spent and the number of persons present at the carcass site as much as possible to reduce possible anthropogenic disturbance.

We only included carcasses of which we monitored the whole decomposition process in the analyses, resulting in a total of 59 carcasses. Only entire carcasses were monitored, i.e. no guts only. The carcasses were obtained from roadkills, except for Planken Wambuis and Veluwezoom National Park, where we obtained the carcasses from regular culling. No animals were killed for the purpose of this study. In total, we monitored carcasses of 34 roe deer (*Capreolus capreolus*), 3 red deer (*Cervus elaphus*), 7 fallow deer (*Dama dama*), 4 European badgers (*Meles meles*), one domestic sheep (*Ovis orientalis aries*), and 10 boars. For practical reasons, the carcass placement was on a non-randomised stratified case-by-case basis. The stratification was based on the variation in tree cover among and within the areas, to ensure that we monitored carcasses over the entire gradient of tree cover present in the areas. We included carcass species as random factor in the statistical analyses since not all carcass species might attract the same scavenger species (e.g. Butler-Valverde et al. 2022). For instance, carnivore carcasses in particular - European badger in our case - might attract other scavenger species than herbivore carcasses (Moleón et al. 2017). However, the scavenger communities of the carcasses used in our study did not differ between carcasses or areas (Wenting et al. 2022).

6.2.3 Annotation camera trapping videos

The collected videos were uploaded to the online platform Agouti (Casaer et al. 2019), from which the footage was annotated, as described in Wenting et al. (2022). For this study specifically, we annotated per video (1) the species that visited the carcass; (2) the number of individuals on each video; (3) whether direct scavenging behaviour, i.e. eating or collecting carcass materials, was shown; and (4) the scavenger group to which the species belonged. We focused on the three scavenger groups, as defined by Wenting et al. (2022), that were most relevant for our study: (1) Birds; (2) Mammals, and (3) Wild boar. The group Birds consisted of common raven (Corvus corax), common buzzard (Buteo buteo) and carrion crow (Corvus corone). The group Mammals (henceforth ‘other mammals'), consisted of beech marten (Martes foina), domestic cat (Felis catus), domestic dog (Canis lupus familiaris), European polecat (Mustela putorius) and red fox (Vulpes vulpes). The birds group was characterised by a prevalence for carcasses in the active stage of decomposition, while the other mammals group was present in all stages of decomposition. The other mammals group was characterised by their overall larger body size compared to the birds. The third group, wild boar, was characterised by large body size, high percentage of eating behaviour, and high amount of intraspecific behaviour, indicating larger group sizes and more social behaviour compared to any of the other groups (Wenting et al. 2022).

We expanded the carcasses monitored in Wenting et al. (2022) with ten additional carcasses. Some of these ten carcasses were visited by species that were not yet included in the scavenger groups defined by Wenting et al. (2022). We assessed these species based on the same criteria of e.g. at least 30 observations and showing direct scavenging behaviour. We noticed that we had to add three scavenger species to the scavenger groups: European badger, pine marten (Martes martes) and European robin (Erithacus rubecula). Based on their behaviour towards the carcasses and their prevalence, we classified European badger and pine marten as other mammals. European robin was classified as occasional scavenger (Wenting et al. 2022) and hence not relevant for the purpose of our study.

Per carcass, we noted (1) the time to carcass depletion, based on the date of carcass placement and the date of depletion; (2) the percentage of tree cover (see below); (3) the mean daily temperature, based on the nearest weather station (KNMI 2023); (4) the area the carcass was located; (5) the carcass species; (6) the start month calculated from the first monitored carcass, enabling us to correct for temporal autocorrelation; and (7) carcass initial state, i.e. whether the skin was such severely damaged due to the cause of death that we considered it opened at the moment of carcass placement. Carcasses were considered as fully decomposed when none of the carcass remains were visible anymore.

Furthermore, we calculated per carcass (1) the time until first detection and (2) first scavenging event per scavenger group; and (3) the proportion of carcass consumption per scavenger group. The proportion of carcass consumption per scavenger group was based on the total number of observations per carcass, thus calculated as the proportion of consuming scavengers per group. The calculated proportions were rescaled to avoid zeros and ones in the analyses (Smithson & Verkuilen 2006).

6.2.4 Tree cover calculation

We retroactively calculated the percentage of tree cover (henceforth ‘tree cover’) for the monitored carcasses. We loaded the shapefiles in Google Earth, where we used aerial photos to calculate the tree cover. This calculation was based on 100 random sampling points using a 30 m radius circle around each carcass location, of which we created shapefiles with the sf package (Pebesma et al. 2020). The 30 m corresponds with approximately 0.3 ha. This was chosen to reflect the properties of the location of the carcass as well as the properties of the landscape immediately around it, accounting for the sightlines of overflying birds from different angles (Ilangakoon et al. 2015). We divided the year into four periods to take into account the annual change of leaf area index in temperate forests: (1) the leaf production period in May; (2) the leaf constant period from June through September; (3) the leaf senescent period from October through November; and (4) the leaf dormant period from December through April (Blackburn & Milton 1995; Gond et al. 1999; Wang et al. 2005; Croft et al. 2014). We based the photographic analysis on the time of the year a carcass was monitored. However, due to infrequent adequate satellite photos, it was not possible to calculate the tree cover for all sites in the production and senescent period. In those cases, we calculated the average tree cover of the constant and dormant period. We always ensured that the vegetation type did not change between the used aerial photo and the monitoring period.

6.2.5 Statistical analyses

We used mixed-effects Cox models (Therneau & Therneau 2015) to analyse how the time till first detection or first scavenging depended on tree cover. We tested this per scavenger group, with ambient temperature and carcass initial state as covariates, and area, carcass species, and start month as random factors. For boar, we excluded the carcasses monitored in Markiezaat and De Hamert Estate since this species did not occur in these areas.

We used Beta-distributed generalized linear mixed models with a logit link (Brooks et al. 2023) to analyse the proportion of carcass consumed per scavenger group. We tested whether tree cover was related to the proportion consumed with three models - one for each group - with the proportion consumed as dependent variable, the percentage of tree cover as independent variable, ambient temperature and carcass initial state as covariates, and area, carcass species, and start month as random factors. We tested whether the time to first detected or first scavenged was related to the proportion consumed with two models per group (six in total), with the proportion consumed as dependent variable, the time to first detection or first scavenging as independent variable, ambient temperature and carcass initial state as covariates, and area, carcass species, and start month as random factors. We only included the carcasses that were visited by the corresponding scavenger group in the analyses.

We used linear mixed-effects models (Kuznetsova et al. 2017) to analyse the carcass decomposition speed. We used two models per scavenger group (six in total) to test whether the time until first detection or first scavenging was related to the depletion time, with depletion time as dependent variable, time to first detection or first scavenging as independent variable, ambient temperature and carcass initial state as covariates, and area, carcass species, and start month as random factors. We analysed whether the carcass decomposition speed was influenced by proportion of carcass consumed with three models - one per scavenger group -, with depletion time as dependent variable, proportion of carcass consumed as independent variable, ambient temperature and carcass initial state as covariates, and area, carcass species, and start month as random factors. Again, we only included the carcasses that were visited by the corresponding scavenger group in the analyses.

All statistical analyses were done in R version 4.3.1 (R Core Team 2023). See Appendix 6.2 for an overview of all test statistics.

6.3 Results

The camera traps recorded a total of 13,122 videos of vertebrates visiting the 59 carcasses that we monitored, of which 11,570 videos belonged to the scavenger groups Birds, Other mammals and Wild boar, that we included in the analyses. Direct scavenger behaviour - i.e. eating or collecting carcass materials (Wenting et al. 2022) - was annotated in 9,488 of these videos. After multiplying with the number of individuals counted per video, there were 15,142 observations of direct scavenging behaviour, that we used to calculate the proportion of carcass consumption per scavenger group. One of the carcasses, monitored at Veluwezoom National Park, was visited by occasional scavengers only, and was therefore excluded from further analyses.

The monitored carcasses were placed under tree cover varying from 0 to 99 percent. The time till first detection and first scavenging varied from less than a day to 43 days, and the depletion time varied from 3.5 to 140 days.

6.3.1 First detection and first scavenging

We found that tree cover did not explain time of first carcass detection by any of the scavenger groups (Figure 6.2a-c), neither for birds (β=-0.837, SE=0.584, p=0.150), boars (β=1.292, SE=0.663, p=0.52), nor other mammals (β=0.913, SE=0.560, p=0.100). The same applied to time to first scavenging (Figure 6.2a-c; birds: β=-0.684, SE=0.619, p=0.270; boar: β=0.126, SE=0.644, p=0.840; other mammals: β=0.866, SE=0.601, p=0.150). We found, however, that increasing mean daily temperature reduced the predicted relative hazard of first detection (β=-0.117, SE=0.044, p=0.007) and first scavenging (β=-0.153, SE=0.051, p=0.003) by birds, implying that carcasses were later detected with increasing ambient temperature. Initially opened carcasses had a lower predicted relative hazard of first scavenged by boar compared to initially closed carcasses, implying that it took longer before opened carcasses were scavenged for the first time by boars compared to initially closed carcasses (β=-1.555, SE=0.776, p=0.045).

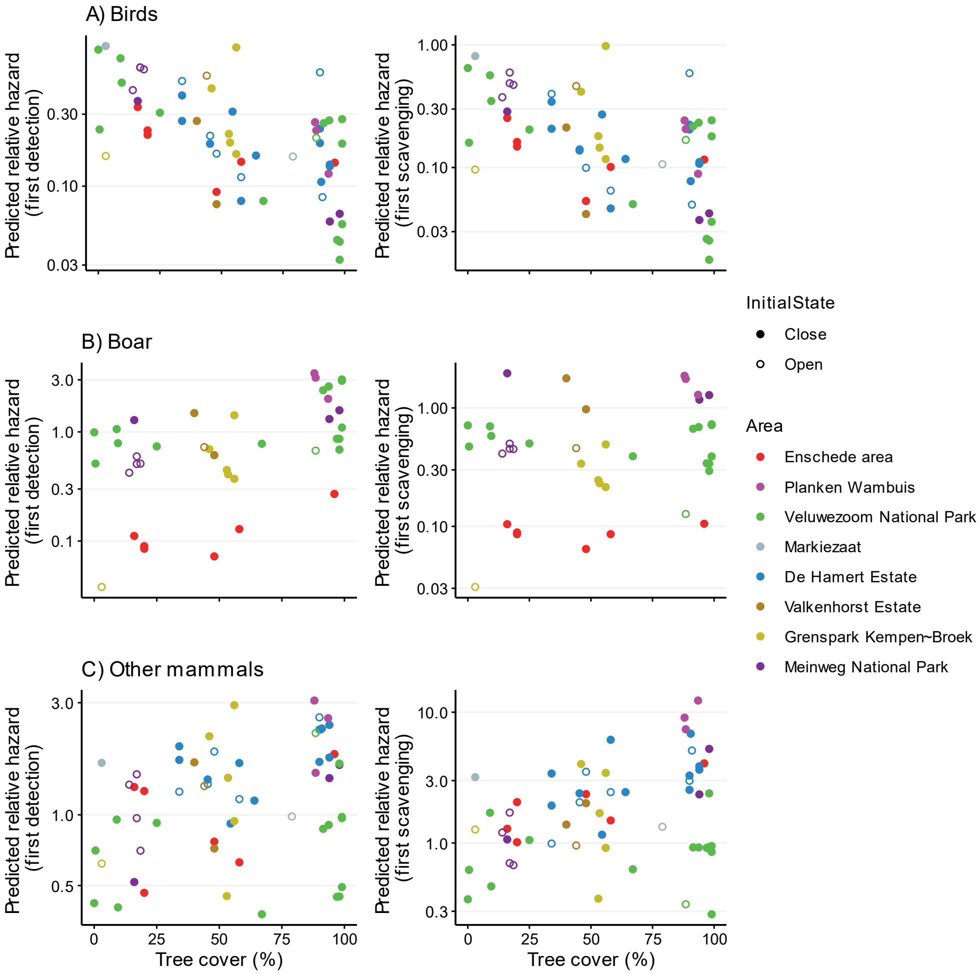

Figure 6.2 Predicted relative hazard of being first detection or first scavenging event of birds (a), boar (b), or other mammals (c) versus the percentage of tree cover.

6.3.2 Carcass consumption

Tree cover was not related to the proportion of carcass consumed by birds (Figure 6.3a; β=-0.889, SE=0.952, p=0.350), boar (Figure 6.3b; β=-0.379, SE=0.846, p=0.654), or other mammals (Figure 6.3c; β=0.337, SE=0.637, p=0.597). Also, none of the covariates - mean daily temperature (birds: β=-0.012, SE=0.090, p=0.895; boar: β=0.008, SE=0.057, p=0.887; other mammals: β=0.062, SE=0.041, p=0.130) and carcass initial state (birds: β=-0.147, SE=0.658, p=0.823; boar: β=0.437, SE=0.995, p=0.661; other mammals: β=0.154, SE=0.501, p=0.758) - was significant for any scavenger group.

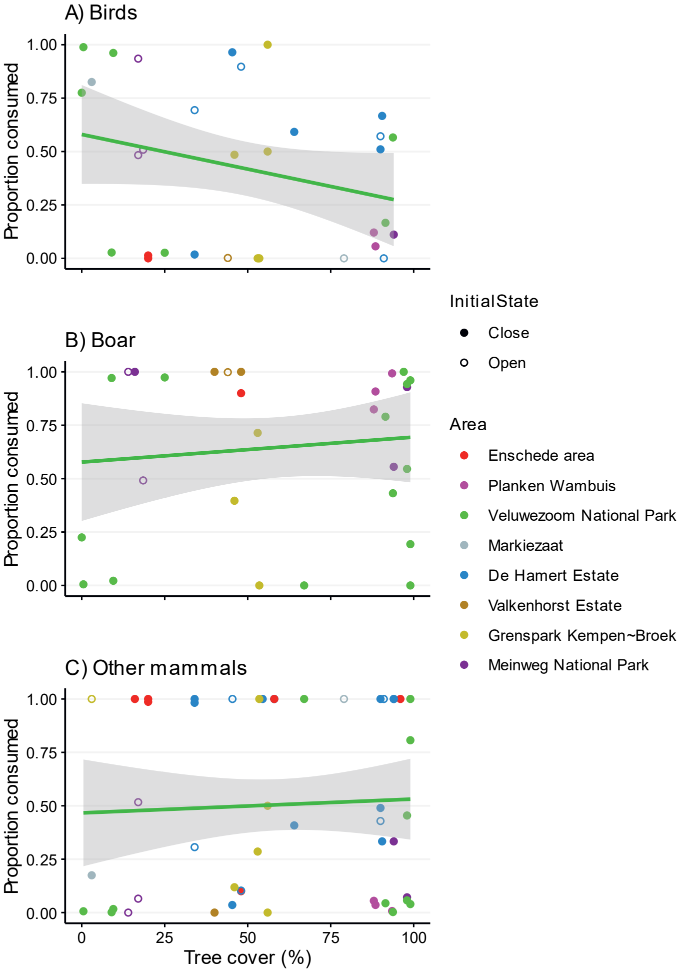

Figure 6.3 The proportion of carcass consumption attributed to birds (a), boar (b), or other mammals (c) versus the percentage of tree cover.

The proportion of carcass consumed by birds was higher when birds detected the carcasses sooner (Figure 6.4a; β=-0.125, SE=0.037, p<0.001). Boar consumed a larger proportion when they sooner scavenged a carcass for the first time (Figure 6.4b; β=-0.038, SE=0.012, p=0.001). The proportion of carcass consumed by other mammals was not influenced by the time of first detection or first scavenging (Figure 6.4c; β=0.008, SE=0.016, p=0.625; β=0.002, SE=0.011, p=0.840, respectively). Also, neither for days to first detection nor first scavenging, any of the covariates - mean daily temperate (birds: β=0.005, SE=0.066, p=0.938; β=0.059,

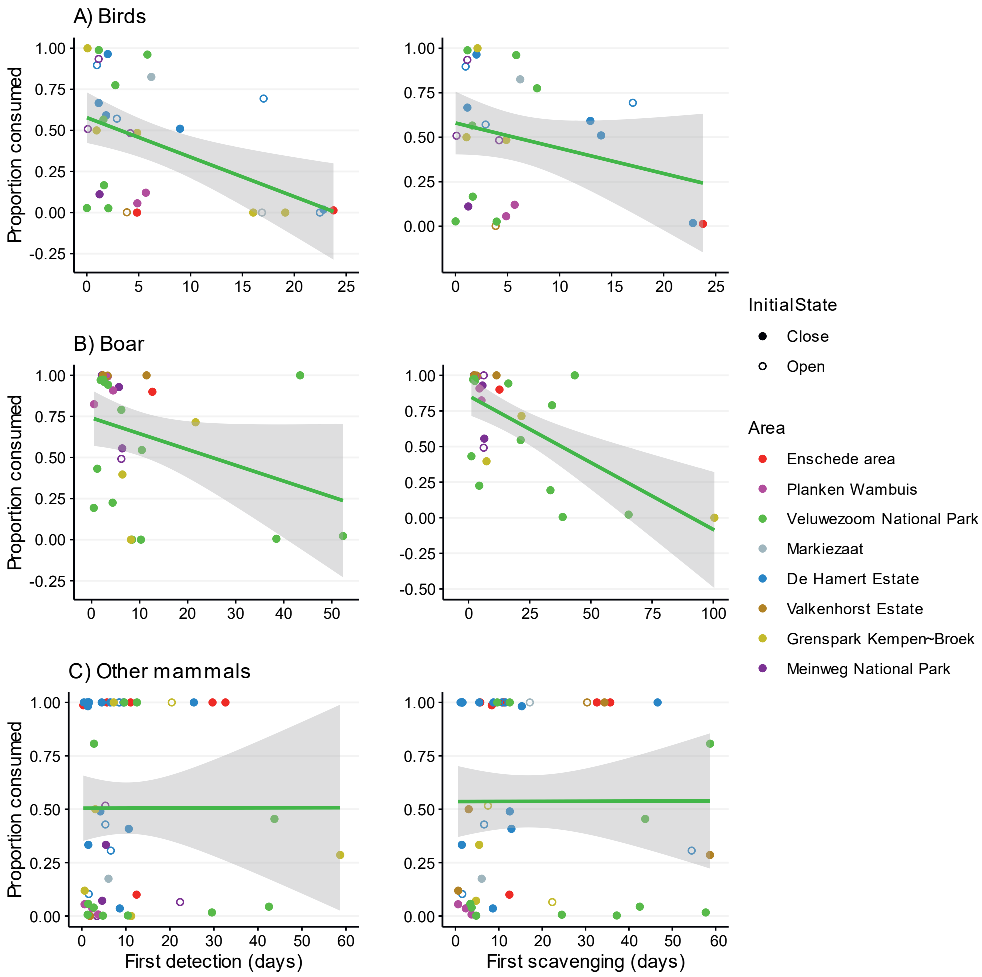

Figure 6.4 The proportion of carcass consumption attributed to birds (a), boar (b), or other mammals (c) versus the days until first detection or first scavenging event.

SE=0.071, p=0.412, respectively; boar: β=-0.006, SE=0.054, p=0.910; β=0.093, SE=0.053, p=0.080, respectively; other mammals: β=0.066, SE=0.044, p=0.100; β=0.073, SE=0.044, p=0.100, respectively) or carcass initial state (β=0.027, SE=0.569, p=0.962; β=-0.159, SE=0.668, p=0.812, respectively; boar: β=0.433, SE=1.011, p=0.668; β=0.874, SE=0.789, p=0.268, respectively; other mammals: β=0.075, SE=0.475, p=0.874; β=-0.106, SE=0.516, p=0.837, respectively) - was significant.

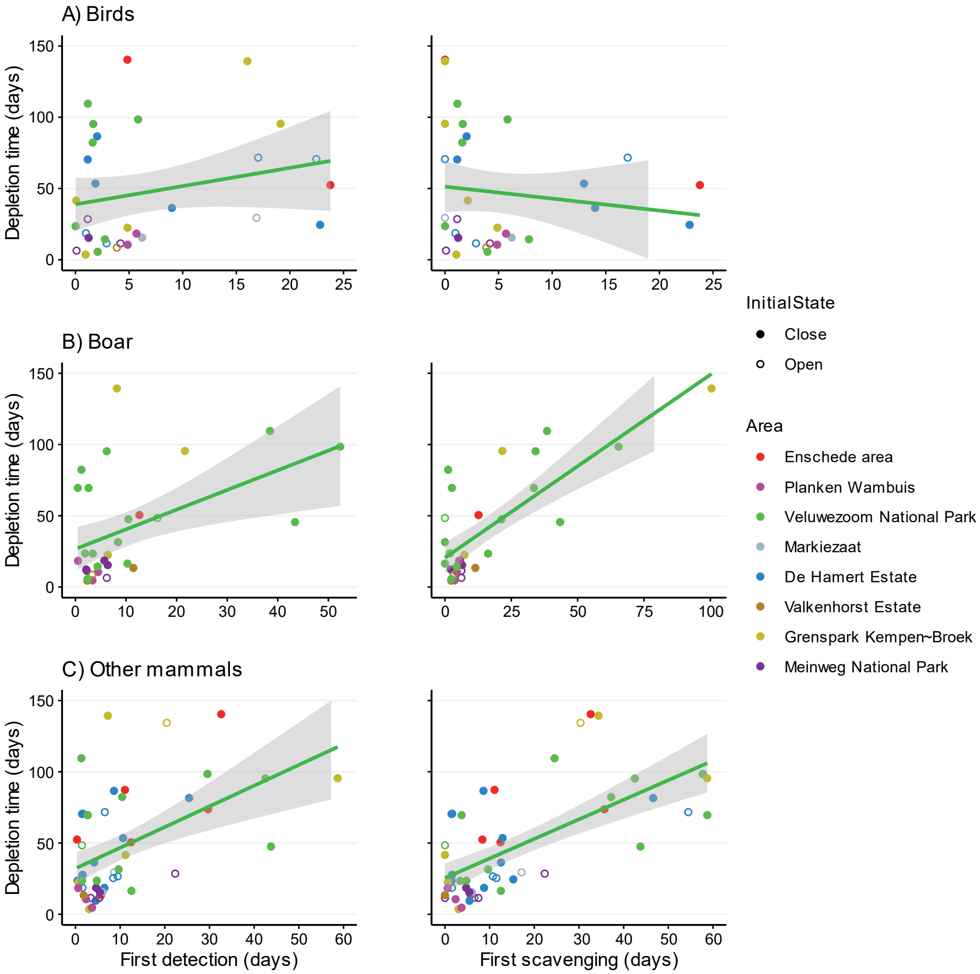

Figure 6.5 The days until first detection or first scavenging event of birds (a), boar (b), or other mammals (c) versus the days until carcass depletion.

6.3.3 Depletion time

The time to first detection or first scavenging by birds was not related to the carcass depletion time (Figure 6.5a; Appendix 6.2). We found that carcasses faster decomposed when they were sooner first detected (β=1.130, SE=0.409, df=12.975, p=0.016) or first scavenged (β=1.230, SE=0.190, df=23.867, p<0.001) by boars (Figure 6.5b). We also found that carcasses faster decomposed when they were sooner first detected (β=1.231, SE=0.364, df=45.649, p=0.002) or first scavenged (β=1.304, SE=0.228, df=44.993, p<0.001) by other mammals (Figure 6.5c). Mean daily temperature was not, neither for first detection (birds:

β=2.814, SE=1.558, df=21.378, p=0.085; boar: β=-0.478, SE=1.325, df=13.114, p=0.724; other mammals: β=0.716, SE=0.944, df=36.434, p=0.453) nor first scavenging (birds: β=3.089. SE=1.542, df=19.716, p=0.059; boar: β=-1.444, SE=0.952, df=25.421, p=0.142; other mammals: β=1.174, SE=0.780, df=33.161, p=0.151), related to the time to depletion. The same applied to carcass initial state, which was not, nor for first detection (birds: β=-27.709, SE=14.706, df=26.118, p=0.071; boar: β=-8.307, SE=17.753, df=24.324, p=0.644; other mammals: β=-0.869, SE=11.016, df=46.855, p=0.938) nor first scavenging (birds: β=-27.946, SE=14.981, df=24.716, p=0.074; boar: β=-11.289, SE=13.843, df=25.945, p=0.422; other mammals: β=-3.036, SE=9.433, df=46.822, p=0.749), related to the time to depletion.

We found a longer time to carcass depletion when a larger proportion was consumed by birds (Figure 6.6a; β=40.897, SE=13.459, df=22.418, p=0.013). When boar consumed a larger proportion, the time to depletion was shorter (Figure 6.6b; β=-65.706, SE=18.163, df=22.566, p=0.001). We found no effect of proportion consumed by other mammals on the time to depletion (Figure 6.6c; β=-3.509, SE=13.876, df=43.614, p=0.802). None of the covariates - mean daily temperature (birds: β=2.060, SE=1.265, df=13.316, p=0.127; boar: β=0.147, SE=1.242, df=21.002, p=0.907; other mammals: β=0.973, SE=1.090, df=33.620, p=0.378) or carcass initial state (birds: β=-19.262, SE=13.145, df=19.072, p=0.159; boar: β=-23.791, SE=20.716, df=19.138, p=0.265; other mammals: β=0.039, SE=13.726, df=41.816, p=0.998) - was significant for any of the scavenger groups.

6.4 Discussion

In this study, we aimed to examine how tree cover and carcass detection by facultative avian and mammalian scavengers, in areas without vultures and without top predators, influenced carcass consumption by different scavenger groups, hence time to carcass depletion. In general, we found tree cover not to be the dominant factor determining carcass exploitation by the different scavenger groups. Our results showed, for instance, that mean daily temperature was a better predictor for the time of first detection and first scavenging by birds than tree cover (Appendix 6.2). The carcass decomposition process is intrinsically linked to temperature-dependent biochemical processes (e.g. DeVault et al. 2003; Carter et al. 2007; Matuszewski et al. 2010) but the effect of ambient temperature on vertebrate scavenger activity seems contradictory. On the one hand, decreasing ambient temperature has been shown to increase scavenger activity (e.g. Selva et al. 2005; Gomo et al. 2020), while on the other hand, enhanced ambient temperatures might facilitate earlier carcass detection due to increased olfactory cues (e.g. Peers et al. 2020; Inagaki et al. 2022). However, it remains unclear why we only found an effect of ambient temperature on first detection and first scavenging by birds, while we did not find an effect for boars or other mammals.

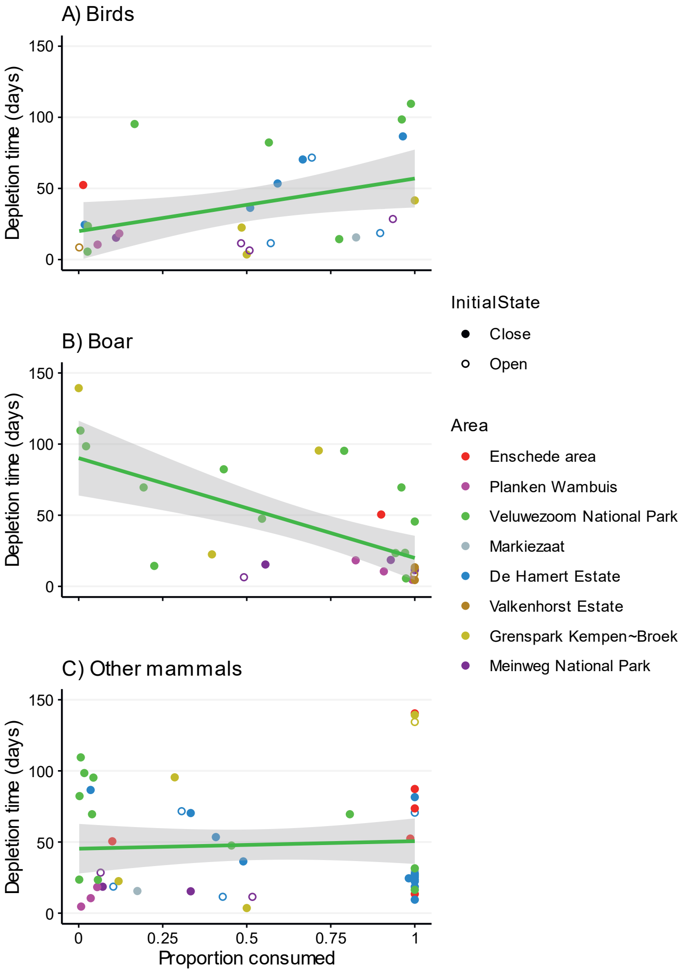

Figure 6.6 The proportion of carcass consumption attributed to birds (a), boar (b), or other mammals (c) versus the days until carcass depletion.

Despite that first detection or first scavenging by boars was not influenced by tree cover, it took longer before initially opened carcasses were first scavenged by boars than initially closed carcasses, while the other scavenger groups were not influenced by carcass initial state (Appendix 6.1). Boars are known to change their behaviour to avoid anthropogenic disturbances - in our case manually opened carcasses (e.g. Johann et al. 2020; Fradin & Chamaillé-Jammes 2023). Moreover, wild boar is extremely sensitive to olfactory cues (e.g. Lavelle et al. 2017). It remains, however, unclear why birds and other mammals were not influenced by carcass initial state. Common ravens, for example, quickly learn the potential danger of humans (e.g. Blum et al. 2022), and it has been described that red foxes change their daily activity patterns when human disturbance is high (e.g. Díaz-Ruiz et al. 2016).

We assumed that birds would exploit carcasses more in open habitats due to their use of eye-sight (e.g. Ruxton & Houston 2004; Selva et al. 2005). However, common ravens - the most abundant bird species during our study, contributing to 70 percent of the bird observations (Appendix 6.1) - can locate carcasses even in densely forested areas (Rösner et al. 2005). In boreal forests, ravens can even become forest specialists, breeding and foraging inside large natural forests (Andren 1992). It has also been described how ravens build their nests in forest edges, from where they can reach both open and forested areas, although they would slightly prefer to forage in open habitats (e.g. Dunk et al. 1997). Common buzzards - with 21 percent of observations the second most abundant bird species in our study (Appendix 6.1) - strongly prefer to forage in rugged areas (e.g. Sergio et al. 2005) or open habitats like meadows (e.g. Kitowski 2000; Wuczyński 2005; Wikar et al. 2008). Therefore, it does not seem evident that the scavenging birds in our study were driven by tree cover, as has been described for vultures (e.g. Arrondo et al. 2019; Oliva-Vidal et al. 2022). Although we can only speculate about this, it might be that facultative avian scavengers rely on vultures for locating carcasses, and respond to tree cover only when vultures are present.

Mammalian scavengers would be mostly driven by olfactory cues when detecting carcasses (e.g. Ruxton and Houston 2004; Stahler et al. 2002; Selva et al. 2005), which does not automatically mean that they would sooner detect carcasses under denser tree cover. Red fox - with 66 percent of the observations the most abundant mammal in our study (Appendix 6.1) - generally prefers cover-rich habitats but might shift towards more open areas when proved to be beneficial (e.g. Lucherini et al. 1995; White et al. 2006). Pine martens are predominantly active in forested areas and even avoid open habitats, while beech martens use both open and forested areas, even visiting man-made objects and inhabit buildings (e.g. Goszczyński et al. 2007). Boars would mostly forage in open habitats close to forest edges, enabling them to escape into the forest in case of danger (e.g. Meriggi & Sacchi 2001; Geisser & Reyer 2004). Moreover, in general, mammals are typically more vulnerable to predation in open areas compared to birds, but when large carnivores are absent, the overall mammalian willingness to scavenge in open areas might increase (e.g. Allen et al. 2015), which might have contributed to our findings. In addition, the bird species in our study are mostly diurnal (e.g. Butet et al. 2010; Loretto et al. 2016), while the mammals are mostly nocturnal or crepuscular (e.g. Keuling et al. 2008; Díaz-Ruiz et al. 2016). We recommend studying this in more detail in future studies since we were not able to analyse this in our study due to technical limitations.

We found that a larger proportion of carcasses was consumed by birds when birds sooner detected them for the first time (Figure 6.4a) and by boars when boars sooner scavenged for the first time (Figure 6.4b). Birds are more active in the early stages of decomposition

(Wenting et al. 2022). Corvids - common ravens and carrion crow in our study - are known to forage in large flocks (e.g. Marzlufi & Heinrich 1991; Rösner et al. 2005), although larger flocks do not necessarily represent larger feeding rates (Marzlufi & Heinrich 1991). Boars are known for their social behaviour and tend to scavenge in large groups (e.g. Dardaillon 1988; Maselli et al. 2014). These aspects might, however, have caused some unintended bias due to the method we used to calculate the proportion of carcass consumed per scavenger group. The number of observations was multiplied by the number of individuals, meaning that the number of observations of birds and boar might be overestimated compared to the observations of other mammals. The other mammals did not typically forage in large groups, were generally more active during the later stages of decomposition, and detected carcasses later compared to birds and boars (Wenting et al. 2022), which might explain why time to first detection or first scavenging by other mammals did not affect the proportion of carcass consumed by other mammals (Figure 6.4c).

In general, mammals - both boar and other mammals in our study - have larger bite sizes than birds (e.g. Van Gils et al. 2007). This might explain why the time to carcass depletion was not influenced by time to first detection or first scavenging by birds (Figure 6.5a), and that a larger proportion of carcass consumed by birds even resulted in a longer depletion time (Figure 6.6a). Thus, when boars or other mammals detected or scavenged from carcasses for the first time, this might have had a larger effect on carcass depletion time, as our results suggest, both for boars (Figure 6.5b + Figure 6.6b) and other mammals (Figure 6.5c). It remains unknown, however, why a larger proportion of carcass consumed by other mammals did not speed up the time to depletion (Figure 6.6c).

In conclusion, our results showed that tree cover may not be the dominant factor driving carcass exploitation by facultative vertebrate scavengers in areas without obligate scavengers or large predators. Carcasses decomposed faster when they were sooner detected or scavenged for the first time by boars or other mammals, and when boars consumed higher proportions of the carcasses. This is in line with idea that wild boar plays a key role in areas without obligate scavengers (Wenting et al. 2022), although their behaviour might be less predictable compared to vultures. Wild boars' presence does not automatically result in faster carcass decomposition, but carcass consumption by wild boar does. As a result, we speculate that scavenging by wild boar might have a larger accelerating effect on nutrient cycles compared to other vertebrate facultative scavengers. Thus, when obligate scavengers or large predators are absent, carcass exploitation by facultative scavengers, particularly wild boar, determines the carcass decomposition process, which is not related to a habitat characteristic like tree cover.

6.5 End sections

6.5.1 Author contributions

Elke Wenting: Conceptualization (lead), Data Curation (lead), Formal Analysis (lead), Funding Acquisition (equal), Investigation (lead), Methodology (equal), Project Administration (lead), Resources (lead), Supervision (equal), Validation (lead), Visualization (lead), Writing - Original Draft (lead). Patrick A. Jansen: Conceptualization (supporting); Funding Acquisition (equal); Methodology (supporting); Writing - Original Draft (supporting). Luke Pattipeilohy: Conceptualization (supporting); Formal Analysis (supporting); Investigation (supporting); Writing - Original Draft (supporting). Peter van Lunteren: Conceptualization (supporting); Formal Analysis (supporting); Investigation (supporting); Writing - Original Draft (supporting). Henk Siepel: Conceptualization (supporting); Methodology (supporting); Supervision (equal); Writing - Original Draft (supporting). Frank van Langevelde: Conceptualization (supporting); Funding Acquisition (equal); Methodology (supporting); Supervision (equal); Writing - Original Draft (supporting).

6.5.2 Acknowledgements

We thank ARK Rewilding Netherlands for their support by contributing to the camera traps that were used for this study. We also thank everyone involved in the carcass provisioning.

6.5.3 Conflicts of interest

No actual or potential conflicts of interest are declared by the authors.

6.5.4 Data availability

The complete dataset used in this study, including details about the monitored carcasses, is available through Figshare: https://doi.org/10.6084/m9.figshare.23634000.

Chapter 7

Leakage of nutrients into the soil due to carrion decomposition can enhance plant growth

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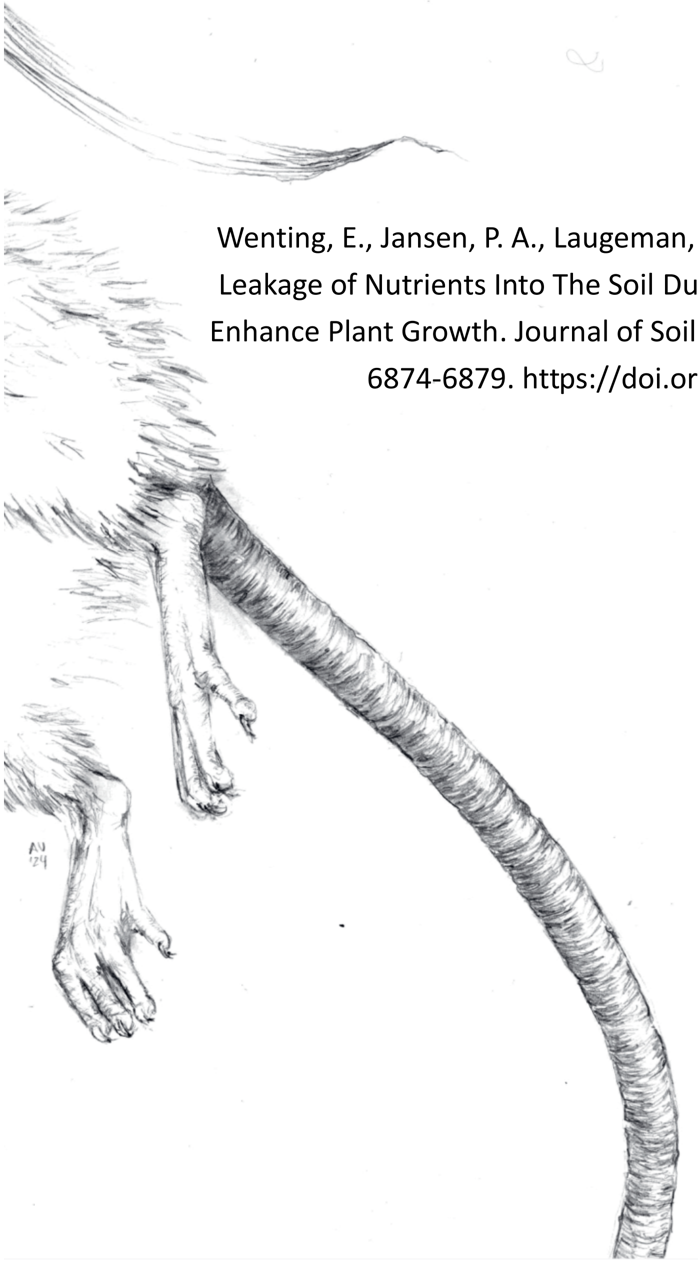

Abstract

Purpose: Carrion decomposition has potentially far-reaching effects on nutrient cycles. Recent studies have demonstrated changes in soil-nutrient dynamics and enhanced plant growth because of nutrient leakage from decomposing carrion. However, only macronutrients have been evaluated so far, overlooking effects on a wide range of other essential or ecotoxic elements. This study aimed to examine how leakage affects the chemical composition of soil below decomposing carrion for a wide range of chemical elements, and how this in turn affects plant growth.

Methods: We performed an experiment in which we let carrion fluid leak from dead mice for different periods of time and measured 22 elemental concentrations in the soils underneath. Then, we grew F1 maize plants on these soils and measured plant biomass.

Results: We found that leakage elevated concentrations of 13 essential elements (C, Ca, Co, Fe, K, Mg, Mn, Mo, Na, Ni, P, Se, and Zn) beneath the carrion. None of the potential ecotoxic elements turned out significant. Plant growth was up to nine times higher in soils enriched by carrion fluid.

Conclusion: Our results demonstrate that a wide range of chemical elements leak into the soil as a result of carrion decomposition, in concentrations that enhanced net plant growth. Our study must be considered as a first step towards a more comprehensive approach for investigating elemental leakage into the soil due to carrion decomposition. Further research may consider larger carcasses, more comprehensively examine the effects of multiple elements on plant growth, and examine how factors like scavenger activity, which may intercept carrion before elemental leakage can happen, affects leakage into the soil.

Keywords: carrion; decomposition; soil chemistry; nutrient cycle

7.1 Introduction

Carrion is an ephemeral but highly nutritious resource for many organisms, so-called scavengers (e.g. Barton et al. 2013; DeVault et al. 2003; Wilson & Wolkovich 2011). Carrion decomposition has potentially far-reaching effects on nutrient cycling, a key driver of ecosystem functioning (Ngai & Srivastava 2006). Animals play a crucial role in this cycle by accumulating large amounts of nutrients in their bodies, collected over long timespans and large areas (Doughty et al. 2016). These include essential elements such as cobalt (Co) and selenium (Se), which are scarce and hard to gather for all lifeforms (Crowe & Bradshaw 2014). When animals die, their bodies - including all the accumulated elements - enter the detritus pool in the form of carrion at a single point in time and space (e.g. Barton et al. 2013), marking the start of the decomposition process.

While large parts of carrion decompose through consumption by scavengers and decomposers, some parts may leak into the soil in the form of 'carrion fluid'; a mixture of bodily fluids, decomposition products and microbial byproducts that drain from the carrion. This has been shown to alter local soil concentrations of macronutrients (e.g. Bump et al. 2009; Barton et al. 2016; Keenan et al. 2019; Macdonald et al. 2014; Parmenter & MacMahon 2009; Quaggiotto et al. 2019). For instance, Benninger et al. (2008) found increased nitrogen (N) and phosphorus (P) concentrations in the soil beneath decomposing Wild boar (*Sus scrofa*) carcasses. Towne (2000) found higher N concentrations at carcass sites after one year of decomposition. Likewise, Melis et al. (2007) found increased calcium (Ca) concentrations in the soil underneath European bison (*is on bonasus*) carcasses. Such leakage of carrion fluid may positively affect plant growth in the close vicinity of decomposing carrion (Carter et al. 2007; Towne 2000).

However, animal bodies also contain a wide range of non-macro-elements, including elements that are essential for plants and animals, and some that are ecotoxic and may impede plant growth (Robinson et al. 2009). It has not been comprehensively examined how leakage of these elements influences soil elemental concentrations beneath carrion (Perrault & Forbes 2016). As a result, how multiple elements may contribute to carrion-driven local soil fertility and subsequent plant growth remain unknown.

In this study, we examined the leakage from carrion of a wide range of chemical elements, including essential and ecotoxic elements, and how this affected plant growth. We performed a controlled lab experiment in which we manipulated the duration of fluid leakage from the exact same carrion type - i.e. lab-raised mice (*Mus musculus*) -, measured a wide range of elemental concentrations (both essential and ecotoxic elements) in initially mineral-poor substrate below the carrion, and grew identical plants (F1 maize) in these substrates that were only affected by carrion fluid leakage to assess effects on plant growth, in the exact same habitat. We excluded scavenger activity to ensure that the source of leakage was controlled.

7.2 Material and methods

We performed the experiment in three steps. First, we established 378 transparent plastic containers that were half filled with bare sand (“silver sand”), which is mineral-poor and does not by itself represent a suitable growing substrate to plants. Containers were covered with fine-meshed perforated plastic lids to exclude insects, and placed on worktables in a tunnel greenhouse at Wageningen University & Research Campus (see Appendix 7.1 for a schematic overview of the experimental design). We randomly assigned 360 of these containers to one of six treatments, in which carrion was placed and left to decompose for 14 to 24 days (six treatments with 60 replicates each). As carrion, we used identical frozen mice that were purchased at an online pet food store (www.animalfoodexpress.nl) in a single batch. The remaining 18 containers served as a control, i.e. received no carrion. Carrion was individually weighed before and after the decomposition period to calculate the weight decline per replicate, analysed using a linear model.

Second, we took soil samples - 5 cm of top soil directly beneath the carrion - from a selection of containers that we used in the previous step, for measuring the elemental concentrations in the substrate. The selection included, for each treatment, the eight containers with the highest and the eight with the lowest carrion weight loss. We also sampled 13 of the 18 control containers. We measured 23 elemental concentrations in total. N and carbon (C) were analysed using the Dumas method (Shea & Watts 1939). For the other elements, samples were prepared with microwave digestion with 5 mL 65% nitric acid (HNO3) and 2 mL 30% hydrogen peroxide (H2O2). We used inductively coupled plasma optical emission spectrometry (ICP-OES) to measure concentrations of nine elements (Van de Wiel 2003): aluminum (Al), Ca, iron (Fe), potassium (K), magnesium (Mg), sodium (Na), P, sulfur (S), and silicon (Si). We used inductively coupled plasma mass spectrometry (ICP-MS) to measure the concentration of twelve more elements (Van de Wiel 2003): chromium (Cr), manganese (Mn), Co, nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), Se, strontium (Sr), molybdenum (Mo), cadmium (Cd), and lead (Pb). Four of these elements - Al, As, Cd, and Pb - are commonly assessed ecotoxic elements, while the other 18 elements are essential to plant and animal growth (e.g. Gasparik et al. 2004; Wenting et al. 2023a). We used Mann-Whitney U tests to compare concentrations between carrion-treated and control substrates, and reported the median elemental concentrations and 25% and 75% quantiles, and test statistics per element.

Last, we tested whether soil enriched by elemental leakage from carrion enhanced plant growth. This was done in a greenhouse at Radboud University in Aug-Oct 2021. We pregerminated F1 Zea maize seeds on vermiculite and sorted the sprouts by size after six days. Sprouts of the same size were potted in the carrion-treated and control substrates. The plants were watered every other day with rainwater until we harvested them after 44 days. Rainwater in Europe is rich in N (Stevenazzi et al. 2019), a commonly limiting element for plant growth (Ågren et al. 2012), hence we excluded N from further analysis and focused on the other elements. Insects entered the greenhouse and fed on some plants. To account for this source of variation, we scored the amount of insect damage for every plant before harvesting by five levels: plants (1) that died prematurely; (2) without insect damage; (3) with 0-30% insect damage; (4) with 30-70% insect damage; and (5) with more than 70% insect damage. We dried the harvested plants at 70 degrees Celsius for 48 hours, and weighed them with a precision of four decimals to determine the plant biomass. We used a linear mixed-effects model with damage level as random factor to compare the biomass of the plants that grew on the carrion-treated substrates with the controls. We used R version 4.0.2 for all statistical analyses (R Core Team 2020).

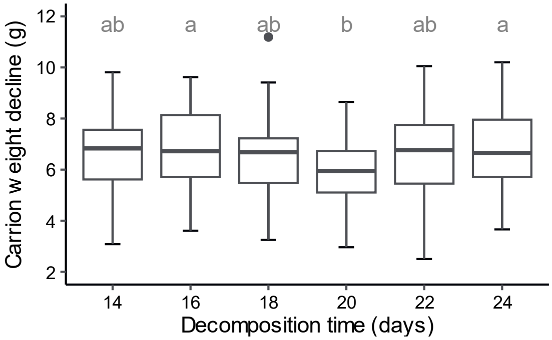

Figure 7.1 Carrion weight decline after 14 to 24 days of decomposition time in a controlled experiment. The letters indicate significant differences between the median weight decline (see text for statistics).

7.3 Results and discussion

We found no systematic decline in carrion weight loss over time (Figure 7.1; linear model, df=5, F=2.903, p=0.014). Likewise, there were no trends in elemental concentrations in the soil over time, implying that most leakage happened during the preceding 14 days. Therefore, we pooled the leakage-duration treatments and only focused on comparing the leakage versus control substrates. We found that the elemental concentrations were higher in soils with leakage than in the controls (Table 7.1). The median Mo concentration was even up to 16 times higher with leakage compared to the control (0.000175 and 0.000011 µg Kg-1, respectively). All the 13 elements for which leakage elevated concentrations are essential and are moderately or highly mobile (Jigyasu et al. 2020). For none of the ecotoxic elements differences were significant. These findings extend those of previous studies to a much wider range of elements than considered so far (e.g. Barton et al. 2019; Quaggiotto et al. 2019). Our study should be considered as a first step towards a more comprehensive approach to investigate carrion-related nutrient fluxes.

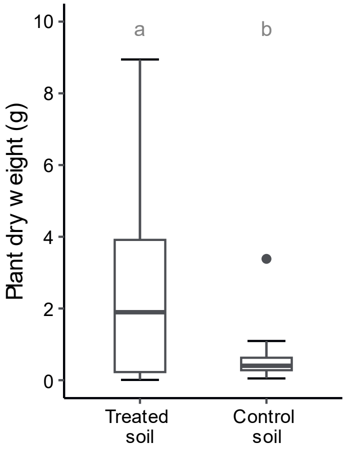

Figure 7.2 Plant growth on substrates subject to nutrient leakage from carrion versus control substrates. The letters indicate significant differences between the median plant biomass (see text for test statistics).

*Table 7.1 Elemental concentrations (µg Kg-1) in soils with and without leakage of chemical elements from carrion, reported as 25% quantile - median - 75% quantile. Test statistics from Mann-Whitney U tests are reported. Elements are in alphabetical order and p-values are adjusted using the step-down procedure of Heller & Gur (2011). * indicates the significant elements.*

	Carrion-treated substrate					Control substrate					Test statistics
Al	1.909	-	2.154	-	2.401	1.97	-	2.025	-	2.187	W=569, p=0.129
As	0.0014	-	0.0016	-	0.0018	0.00146	-	0.00151	-	0.00175	W=602, p=0.129
C	1882	-	2700	-	3787	1030	-	1520	-	2540	W=427, p=0.040*
Ca	0.28	-	0.334	-	0.413	0.276	-	0.286	-	0.321	W=394, p=0.026*
Cd	0.000027	-	0.000029	-	0.000034	0.000024	-	0.000027	-	0.000031	W=467, p=0.064
Co	0.0039	-	0.0044	-	0.005	0.0035	-	0.004	-	0.0044	W=411, p=0.034*
Cr	0.005	-	0.0058	-	0.0067	0.0051	-	0.0052	-	0.006	W=519, p=0.129
Cu	0.0027	-	0.0033	-	0.0046	0.0031	-	0.0035	-	0.0038	W=643, p=0.129
Fe	2.203	-	2.416	-	2.648	2.122	-	2.159	-	2.466	W=436, p=0.043*
K	0.293	-	0.338	-	0.392	0.266	-	0.29	-	0.334	W=359, p=0.012*
Mg	0.622	-	0.685	-	0.783	0.582	-	0.606	-	0.667	W=409, p=0.034*
Mn	0.02	-	0.023	-	0.026	0.018	-	0.0192	-	0.0231	W=413, p=0.034*
Mo	0.000082	-	0.000175	-	0.00026	0.00001	-	0.000011	-	0.00002	W=141, p<0.001*
Na	0.242	-	0.253	-	0.261	0.203	-	0.22	-	0.227	W=179, p<0.001*
Ni	0.009	-	0.0102	-	0.012	0.0084	-	0.0087	-	0.0095	W=356, p=0.012*
P	0.691	-	0.71	-	0.724	0.67	-	0.688	-	0.692	W=297, p=0.002*
Pb	0.0035	-	0.0039	-	0.0042	0.0035	-	0.0038	-	0.0042	W=619, p=0.129
S	0.086	-	0.104	-	0.13	0.083	-	0.095	-	0.123	W=529, p=0.129
Se	0.0025	-	0.0028	-	0.0032	0.00236	-	0.00254	-	0.00273	W=445, p=0.047*
Si	0.589	-	0.647	-	0.722	0.622	-	0.655	-	0.68	W=690, p=0.532
Sr	0.0021	-	0.0023	-	0.0028	0.00206	-	0.00221	-	0.00239	W=528, p=0.129
Zn	0.021	-	0.028	-	0.033	0.0196	-	0.0213	-	0.0243	W=376, p=0.018*

Growth of maize was significantly higher on soils enriched with carrion fluid than on control soils (Figure 7.2; linear mixed-effects model, df=1, F=11.489, p<0.001), with plant biomass being up to nine times higher. Plant growth was not related to concentrations of any elements in particular (multiple regression with backward deletion, F=2.593, p=0.057). These results indicate that leakage of carrion fluid can enhance net local plant growth.

We presume that the enhanced plant growth on carrion-treated soils (Figure 7.2) was the result of the net positive effect of all the evaluated elements combined (Table 7.1). Our experimental design, however, did not allow us to specify the magnitude of the impact on plants per element. We encourage future studies to comprehensively investigate which elements have strongest effects on plant growth.

By using rainwater, which is N-rich (Stevenazzi et al. 2019), we were able to eliminate N as a potential limiting factor for plant growth. It has been suggested that N is not likely to be the limiting factor for plant growth in many regions due to long-lasting N deposition (e.g. Siepel et al. 2019). Excessive N deposition results in skewed proportions between N and scarce essential elements, increasing the importance of carrion decomposition as potential high-quality source of these elements. We therefore consider our results to be relevant in natural systems, especially in systems with high N deposition.

Scavengers are a potential key determinant of elemental leakage from carrion, with the capacity to consume carrion before leakage can even occur (e.g. Gutiérrez-Cánovas et al. 2020; Wenting et al. 2022). However, in this controlled experiment, we did not include the effect of scavenger consumption, nor other important aspects of carrion ecology such as carrion type and size (e.g. Moleón et al. 2015; 2017). Our results particularly apply to small carcasses such as mice but the effect might be different for larger carcasses, or under more natural circumstances. Moreover, our experimental design did not allow us to examine how long the elevated elemental concentrations in the soil will last. We encourage future studies to explore the importance of such aspects on elemental leakage of carrion fluid for a wide range of elements as we assessed here.

7.4 Conclusions

In conclusion, leakage of carrion fluid affects at least 13 essential elemental concentrations in the soil underneath decomposing carrion. In the case of the mice carrion - as we used here -, these concentrations can enhance net plant growth. Our study must be considered as a first step towards a more comprehensive approach for investigating elemental leakage into the soil due to carrion decomposition.

7.5 End sections

7.5.1 Author contributions

Elke Wenting: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Project administration, Funding acquisition. Patrick A. Jansen: Conceptualization, Methodology, Writing - Original Draft, Funding acquisition. Mathijs J.B. Laugeman: Methodology, Validation, Formal analysis, Investigation. Frank van Langevelde: Conceptualization, Methodology, Resources, Writing - Original Draft.

7.5.2 Acknowledgements

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7.5.3 Competing interests

The authors have no competing interest to declare that are relevant to the content of this article.

7.5.4 Data accessibility

The data used for this manuscript is available via Figshare: https://doi.org/10.6084/m9.figshare.22058870.

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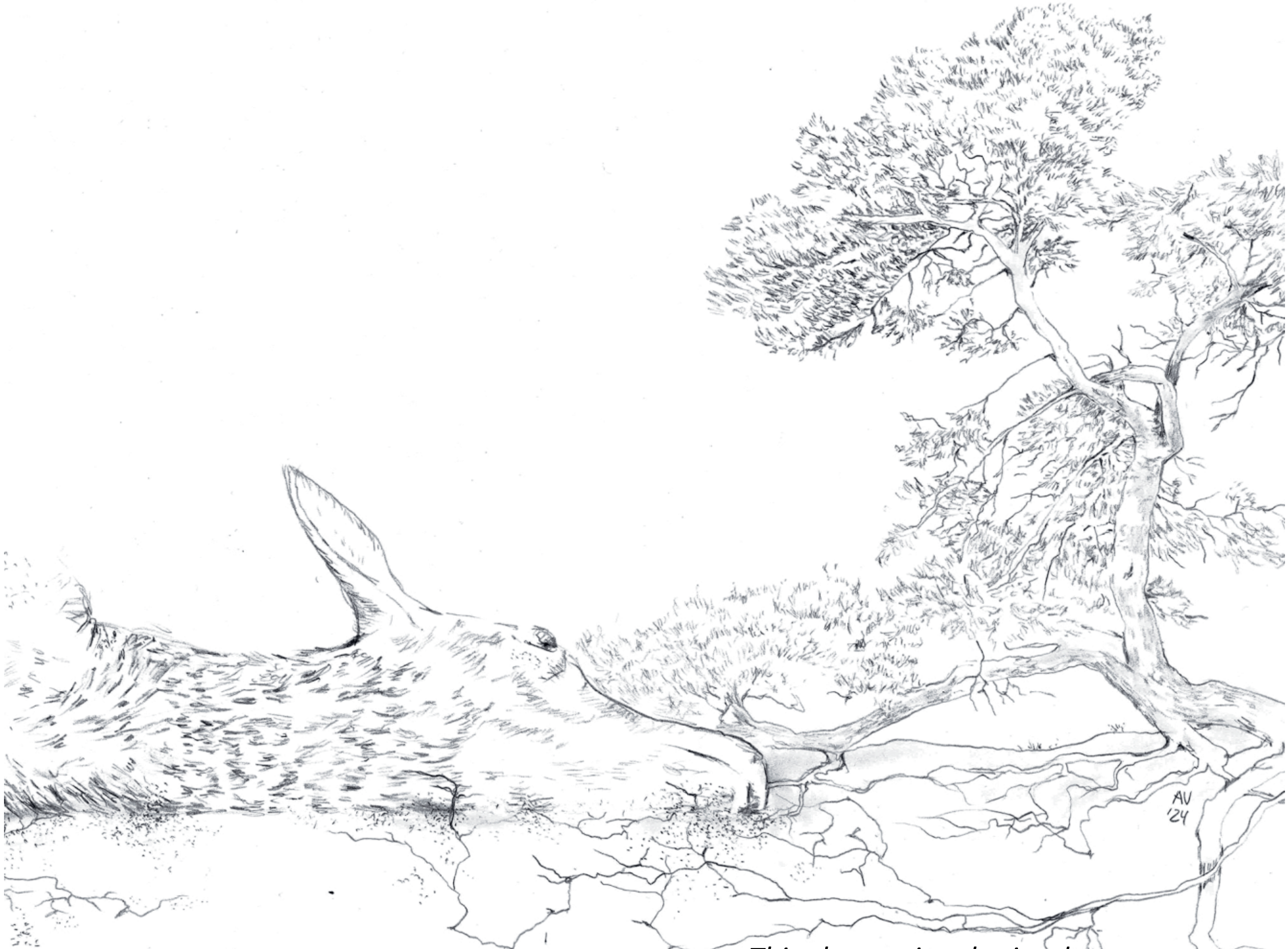
Chapter 8

The influence of vertebrate scavengers on leakage of nutrients from carcasses



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Abstract

The decomposition of carcasses by scavengers and microbial decomposers is an important component of the biochemical cycle that can strongly alter the chemical composition of soils locally. Different scavenger guilds are assumed to have a different influence on the chemical elements that leak into the soil, although this assumption has not been empirically tested. Here, we experimentally determine how different guilds of vertebrate scavengers influence local nutrient dynamics. We performed a field experiment in which we systematically excluded different subsets of vertebrate scavengers from decomposing carcasses of fallow deer (*Dama dama*), and compared elemental concentrations in the soil beneath and in the vegetation next to the carcasses over time throughout the decomposition process. We used four exclusion treatments: excluding (1) no scavengers, thus allowing them all; (2) wild boar (*Sus scrofa*); (3) all mammals; and (4) all mammals and birds. We found that fluxes of several elements into the soil showed distinct peaks when all vertebrates were excluded. Especially trace elements (Cu and Zn) seemed to be influenced by carcass decomposition. However, we found no differences in fluxes between partial exclusion treatments. Thus, vertebrate scavengers indeed reduce leakage of elements from carcasses into the soil, hence influencing local biochemical cycles, but did so independent of which vertebrate scavenger guild had access. Our results suggest that carcass-derived elements are dispersed over larger areas rather than locally leak into the soil when vertebrate scavengers dominate the decomposition process.

Keywords: carrion, decay, nutrient cycle, scavengers, decomposition

8.1 Introduction

The decomposition of dead animal bodies - carcasses - can severely alter the local soil chemical composition and biochemical cycles (e.g. Carter et al. 2007; Barton et al. 2019; Quaggiotto et al. 2019). Despite its relatively small contribution of approximately 1% to the total detritus pool, carcass decomposition is pivotal in imparting limiting nutrients within ecosystems (e.g. Parmenter & MacMahon 2009; Barton et al. 2016; Wenger et al. 2019). We use the term 'nutrients' when the chemical elements serve as food particles, the term 'minerals' when elements are still bound in soil chemical compounds and the term '(chemical) elements' in a general more neutral way. Existing studies have predominantly focused on a restricted number of chemical elements in relation to carcass decomposition (e.g. Barton et al. 2013; Monk & Schmitz 2022). Consequently, our understanding of the role of carcass decomposition in nutrient cycles, based on a wide variety of essential nutrients, is limited.

The extent to which carcass decomposition alters the local biochemical cycles highly depends on the interplay between scavengers - both vertebrates and invertebrates - and microbial decomposers (Carter et al. 2007; Stiegler et al. 2020). The outcome of the interplay itself is subject to a range of abiotic factors - e.g. ambient temperature - and biotic factors - e.g. the composition of the scavenger guild (e.g. Meyer et al. 2013; Farwig et al. 2014; Feddern et al. 2019; Olea et al. 2019; Wenting et al. 2022). Carcass consumption by vertebrate scavengers, in particular, would play a key role in dispersing carcass-derived nutrients over larger areas, resulting in a reduced impact on local nutrient dynamics (e.g. DeVault et al. 2003; Melis et al. 2007; Benbow et al. 2019; Subalusky & Post 2019). Contrary, carcass decomposition dominated by microbial decomposers and invertebrate scavengers would have enlarged effects on local nutrient dynamics (Janzen 1977).

Different scavenger species, particularly vertebrates, are assumed to differently influence elemental fluxes because they differently influence the carcass decomposition speed (Wenting et al. 2022). This is mainly because birds and mammals exploit carcasses in different ways (e.g. Moleón et al. 2014; Patterson et al. 2022). In the absence of obligate scavengers such as vultures, wild boar (*Sus scrofa*), in particular, can greatly speed up the carcass decomposition process (Wenting et al. 2022). Vertebrates can, in general, consume larger amounts in short time periods compared to invertebrates and can so disperse carcass-derived elements over larger areas (e.g. DeVault et al. 2003; Sebastián-González et al. 2016; Morales-Reyes et al. 2018). Carcass decomposition dominated by invertebrates might proceed slower (e.g. Zanetti et al. 2015), although delayed insect access can decrease decomposition rates as well (Pechal et al. 2014). However, the impact that different scavenger guilds can have on local nutrient dynamics remains unknown.

It has been demonstrated that carcass decomposition can lead to increased elemental leakage into the soil (e.g. Barton et al. 2019; Wenting et al. 2023a). However, so far, studies have focused on a limited number of chemical elements. For example, carcass decomposition can alter the soil chemical composition by increasing concentrations of C and N, coupled with changes in pH (Macdonald et al. 2014; Keenan et al. 2018; Quaggiotto et al. 2019). Concentrations of P can slightly increase as soft tissues decompose, but a long-lasting substantial increase, along with Ca, can occur due to slow decomposition of skeletal remains (e.g. Melis et al. 2007; Barton et al. 2016; Wenger et al. 2019; Heo et al. 2021).

Carcass decomposition is believed to cause local nutrient pulses (Yang et al. 2008). However, comprehensive overviews of elemental changes during the decomposition process are scarce. Some studies measured electrical conductivity (EC) below decomposing carcasses, as a proxy for multiple elemental changes, e.g. Na and K (e.g. Keenan et al. 2018; Quaggiotto et al. 2019). Yet, EC does not provide any insights into the changes of individual elemental concentrations. Others considered Ca, K, Na, Mg, and S below carcasses. They found pulses in K, Na, and S concentrations that gradually dissipated over time, whereas Ca and Mg showed a continuous release over longer periods (e.g. Parmenter & MacMahon 2009). Wenting et al. (2023a) provided the most comprehensive overview to date, measuring 22 chemical elements, but did not measure changes over time. Thus, the magnitude of alleged pulses of elements leakage from decomposing carcasses over time remains unknown.

Carcass-derived elements can leak into the soil in concentrations that enhance plant growth (Barton et al. 2016; Wenting et al. 2023a). Such nutrient pulses can result in the formation of islands of fertility (Zaady et al. 1996; Carter et al. 2007). When plants grow on the edges of decomposing carcasses and take up the available nutrients, plant quantity or plant quality is expected to increase. Investment in quantity results in increased plant growth, i.e. biomass (e.g. Towne 2000; Danell et al. 2002). Investment in quality results in increased elemental concentrations in plant tissues (e.g. Pilon-Smits et al. 2009). Melis et al. (2007) described that the effects on plant nutrient concentrations are not easily detectable in temperate ecosystems. To date, however, there are no studies examining the elemental concentrations in plants close to carcasses, including a wide range of chemical elements.

This study aimed to experimentally determine how different guilds of vertebrate scavengers influence local nutrient dynamics in a protected area without obligate vertebrate scavengers. We hypothesised that vertebrate scavengers, particularly wild boar, can take up the majority of the carcass-stored elements in their bodies, and that local leakage of elements will thus be greater as more vertebrate scavengers are excluded. We investigated this with a field experiment in which we systematically excluded different subsets of vertebrate scavengers from decomposing carcasses, and compared elemental concentrations in the soil beneath and in the vegetation next to the carcasses over time throughout the decomposition process.

8.2 Methods

8.2.1 Study area

The experiment was performed at Veluwezoom National Park (henceforth 'Veluwezoom'), the Netherlands (52°02'N, 6°01'E). Veluwezoom, a former agro-silvopastoral landscape (Kuiters 2005), is a protected area of 5,000 ha situated partly on glacier deposits and partly on cover sands. This characterises the natural mineral availability as limited to very scarce. The area contains a mosaic of dry grass-heathlands, pastures, abandoned crop fields and woodland. Natural processes with minimal human interference are of major importance in the management strategy (Kuiters 2005), in which a key role is played by free-ranging Scottish highland cattle (*Bos taurus*), Icelandic horses (*Equus ferus caballus*), fallow deer (*Dama dama*), red deer (*Cervus elaphus*) and wild boar (Bruinderink & Lammertsma 2001). Veluwezoom inhabits several facultative vertebrate scavengers, including wild boar, European pine marten (*Martes martes*), European badger (*Melis melis*), common raven (*Corvus corax*), and common buzzard (*Buteo buteo*) (Wenting et al. 2022).

8.2.2 Experimental design

We performed the experiment at two locations in the southern part of Veluwezoom: Beekhuizen (52°00'35.1"N, 5°59'34.8"E) and Herikhuizen (52°01'03.7"N, 6°00'25.3"E), in which we followed the decomposition of fresh carcasses of fallow deer. The carcasses were obtained from regular culling, i.e. no animals were killed for the purpose of our study. The carcasses were originated from the same areas as described by Wenting et al. (2023b; 2023c). Fallow deer is a terrestrial herbivorous ungulate with an adult body weight of 40-80 kg and a non-nomadic lifestyle (Focardi et al. 2006).

Each experimental run consisted of four treatments, i.e. we needed four carcasses per run. These treatments differed in the subset of scavenger guilds that got access to the carcasses: (1) no scavengers excluded; (2) wild boar excluded; (3) all mammalian scavengers excluded; and (4) all vertebrates excluded, i.e. mammals as well as birds. The carcasses in the first treatment were unprotected, i.e. allowing all scavengers. For the second treatment, we used a firm fence to exclude wild boar from the carcasses. The carcasses in the third and fourth treatment were placed in exclosures with electric fences, with a voltage of at least 5.5 volt. Cages excluded avian scavengers in the fourth treatment (Appendix 8.1). These treatments were based on the scavenger guilds described by Wenting et al. (2022), in which Veluwezoom was one of the study areas. In a pilot study we also tried to exclude all invertebrate scavengers as a fifth treatment, but this appeared impossible for practical reasons.

Each run started at the same day, meaning that we needed four carcasses simultaneously. Carcasses were randomly assigned to one of the treatments. The carcasses were placed about 30 m apart from each other at one of the two locations. All carcasses were placed with their abdomen to the south. For the first three treatments, carcasses were placed on top of a net (to lift them, see data collection section) and tied by the front and rear legs to poles of 50 cm above the ground to prevent dragging. Carcasses in the fourth treatment were placed in a cage made of 1x1 cm chicken wire. All carcasses were placed in direct contact with the soil.

Using two locations allowed us to run two experimental runs at the same time. We repeated the experiment eight times in total (Table 8.1) between 15 October 2019 and 13 September 2022. The last carcasses were sampled on 20th of June 2023.

Table 8.1 Overview of the experimental runs.

Run	Location	Start date	Sampling weeks	Notes
(1) VZ2	Beekhuizen	15th Oct ‘19	0-4-8-12-16-20	Sampling not continued due to pandemic
(2) VZ3	Herikhuizen	4th Feb ‘20	0	Destroyed by wild boar, excluded from analyses
(3) VZ4	Herikhuizen	8th Sept ‘20	0-3-8-12-17-21-24-27-32-36-40	
(4) VZ5	Beekhuizen	3rd Nov ‘20	0-4-9-13-16-19- 24-28-32	
(5) VZ6	Beekhuizen	14th Sept ‘21	0-4-9-13-17-21- 25-29-33-37-40	
(6) VZ7	Herikhuizen	26th Oct ‘21	0-3-7-11-15-19- 23-27-31-34-38	
(7) VZ8	Beekhuizen	8th March ‘22	0-4-8-12-15-19-23-28-32-36-40	Only treatment 2-4 due to management change
(8) VZ9	Herikhuizen	23th Aug ‘22	0-4-7-12-16-20-24-27-32-36-40	
(9) VZ10	Beekhuizen	13th Sept ‘22	0-4-9-13-17-21-24-29-33-37-40	Only treatment 2-4 due to management change

8.2.3 Data collection and measurements

We followed the decomposition process of each run for about 40 weeks, with sampling intervals of 4 ± 1 weeks. We collected three sample types per moment of sampling: (1) the first 5 cm of the soil; (2) roots, i.e. below-ground plant parts; and (3) shoots, i.e. above-ground plant parts. The root and shoot samples were taken as a combined vegetation sample. The soil samples were taken directly beneath the carcasses and the vegetation samples were taken at the direct edges of the carcasses. To access the soil, we lifted the carcass using a transportable scaffolding (Appendix 8.1). After sampling, the scaffolding allowed us to put the carcasses back in exactly the same position as before.

For each sample type, a control sample was taken at approximately 1 m distance from the carcass. This distance was based on previous studies, which found no or only very limited lateral spread beyond 30 cm from carcasses (Melis et al. 2007; Keenan et al. 2019; Barton et al. 2020).

All samples were dried for at least 48 hours starting on the day of sampling: the soil samples at 40°C and the vegetation samples at 70°C. After drying, we separated the roots and shoots from the vegetation samples using sieves and tweezers. The soil samples were homogenised using sieves. All the samples were stored in paper bags until chemical analysis in the laboratories of Radboud University.

We used a microwave digestion method with 5 ml 65% nitric acid (HNO3) and 2 ml hydrogen peroxide (H2O2) to prepare all the samples for measuring the elemental concentrations. We used Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) to measure nine elements: Al, Ca, Fe, K, Mg, Na, P, S, and Si. Another 12 elements were measured using Inductively Coupled Plasma Mass Spectroscopy (ICP-MS): As, Cd, Co, Cr, Cu, Mn, Mo, Ni, Pb, Se, Sr, and Zn.

8.2.4 Statistical analyses

All statistical analyses were done in R version 4.3.1 (R Core Team 2023) in four steps. First, the control samples were used to test whether the local conditions at our study sites changed over time. We used linear mixed-effects (LMM) models to check whether the corresponding element showed a different trend over time, depending on the scavenger exclusion treatments. We used the elemental concentration as depended variable, the interaction between sampling week and treatment as independent variable, and the experimental run as random factor. The step-up procedure (Benjamini & Hochberg 1995) was then used to account for multiple testing.

Second, elements were selected for further analyses. Therefore, we calculated the average concentrations of elements in the non-significant soil control samples. We also estimated the average elemental concentrations in fallow deer, summing up the concentrations reported by Wenting et al. (2023c). Elements with higher concentrations in the soil than in fallow deer were excluded. Then, we selected elements with a described function for both plants and animals, based on the literature. The remaining elements were classified as trace or macro element based on Robinson et al. (2009) and Kaspari (2021).

Third, we used Mann-Whitney U tests to determine whether the initial concentrations of the selected elements differed between the control samples and the samples taken at the carcass site just before placing the carcasses. This enabled us to determine whether the elemental concentrations beneath the carcasses differed from the control sites or not, and how to interpret the potential patterns over time as described in the next step.

Last, we used linear mixed-effects models (LMMs) to test whether the concentrations of the selected elements showed different trends over time, depending on the different scavenger exclusion treatments. The analyses were done for each of the three sample types - soil, root and shoot - separately. Standardised concentrations were used in these analyses, with the values of week 0 set to 1. Values lower than 1 thus indicated a decrease in elemental concentration over time, values higher than 1 indicated an increase. We used scatterplots with trendlines to visualise the trends for each element over time.

We reported only the most relevant test statistics in the text (see Appendices 2-4 for a full overview of the test statistics). In general, the statistical analyses should be considered as indicative rather than steadfastly due to the relatively low number of replicates. Also, we only tested for linear trends, even though non-linear relations might exist. Additional to the statistical analyses, we therefore described whether we observed any patterns over time, although these might not be statistically significant.

8.3 Results

8.3.1 Control samples

The control samples showed no significant pattern over time for any of the sample types, nor for any of the scavenger exclusion treatments in the control samples (Appendix 8.2). Therefore, we were able to use the control samples of the soil to calculate the average elemental concentrations in the soil at our study sites. No elements were thus removed from further analyses based on the analyses of the control samples.

8.3.2 Selection of elements

Comparing the elemental concentration in the soil, based on the control samples, to the elemental concentration in fallow deer, we found that the elemental concentration in the soil was higher than the concentration in fallow deer for seven of the elements: Al, As, Co, Cr, Fe, Pb, and Si (Table 8.2). For all the other elements, the concentration in fallow deer was higher than the concentration in the soil.

Table 8.2 Selection of the elements for further analyses. The elemental concentrations in the soil are based on the control soil samples. The elemental concentrations in fallow deer ("deer" in table) are based on the concentrations reported in Wenting et al. (2023b). Mean concentrations with standard error are reported. The division in trace and macro elements is based on Robinson et al. (2009) and Kaspari (2021).

	Deer	Soil	Comment	Selected
Al	143	7,057	Fallow deer < Soil	No
As	0.42	4.75	Fallow deer < Soil	No
B	89	4.18	Essential for plants and animals (Robinson et al. 2009; Pizzorno 2015; Abdelnour et al. 2018)	Yes, trace
Ca	504,309	991	Essential for plants and animals (Kaspari 2021)	Yes, macro
Cd	24.6	0.31	Highly toxic heavy metal (e.g. Patra et al. 2006)	No
Co	1.24	5.1	Fallow deer < Soil	No
Cr	10.22	15.76	Fallow deer < Soil	No
Cu	487.2	14.6	Essential for plants and animals (Robinson et al. 2009)	Yes, trace
Fe	5,879	7,609	Fallow deer < Soil	No
K	257,217	1,545	Essential for plants and animals (Kaspari 2021)	Yes, macro
Mg	25,639	915	Essential for plants and animals (Kaspari 2021)	Yes, macro
Mn	5,087	424	Essential for plants and animals (Robinson et al. 2009)	Yes, trace
Mo	22.7	1.27	Essential for plants and animals (Robinson et al. 2009)	Yes, trace
Na	153,425	141	Essential for animals, no function for plants (Kaspari 2021)	No
Ni	8.54	6.29	Essential for plants and animals (Robinson et al. 2009)	Yes, trace
P	426,028	1,429	Essential for plants and animals (Kaspari 2021)	Yes, macro
Pb	11.5	43.6	Fallow deer < Soil	No
S	193,661	628	Essential for plants and animals (Kaspari 2021)	Yes, macro
Se	9.06	2.87	Essential for animals, no function for plants (Robinson et al. 2009)	No
Si	1,435	3,359	Fallow deer < Soil	No
Sr	187.6	6.87	Not essential for plants or animals (Robinson et al. 2009)	No
Zn	1,444	58.5	Essential for plants and animals (Robinson et al. 2009)	Yes, trace

According to Robinson et al. (2009), five of the remaining elements could be classified as essential trace elements for both plants and animals: Cu, Mn, Mo, Ni, and Zn. They classified B as essential for plants only (Robinson et al. 2009), but recent studies proved B to be essential for animals as well (Pizzorno 2015; Abdelnour et al. 2018). Another five of the remaining elements could be classified as essential macro elements for both plants and animals: Ca, K, Mg, P, and S (Kaspari 2021).

Summarising, we selected eleven elements on which we focused in the further analyses (Table 8.2). These included six trace elements (B, Cu, Mn, Mo, Ni, and Zn) and five macro elements (Ca, K, Mg, P, and S).

8.3.3 Initial concentrations

None of the initial elemental concentrations (at week 0) significantly differed from the elemental concentration in the control sample, for none of the sample types (Appendix 8.3). For that reason, we assumed that all (potential) trends over time that we found in the next sections could be attributed to the different scavenger exclusion treatments. In the following sections, we used the standardised concentrations per experimental run (week 0 as value 1).

8.3.4 Soil

For all trace elements, the treatment that excluded all vertebrate scavengers showed the highest concentrations (Figure 8.1a-f). For Cu, soil concentrations peaked at approximately 25 weeks (Figure 8.1b; LMM, df=3, F=5.917, p=0.004). The same applied to Zn (Figure 8.1f; LMM, df=3, F=7.795, p<0.001). For both elements, the treatment excluding all vertebrates differed from the other treatments (Appendix 8.4). This treatment also showed the highest values for Mn (Figure 8.1c; LMM, df=3, F=0.032, p=0.993), Mo (Figure 8.1d; LMM, df=3, F=0.051, p=0.993), and - to a lesser extent - B (Figure 8.1a; LMM, df=3, F=0.587, p=0.993). We did not observe any pattern for Ni (Figure 8.1e; LMM, df=3, F=1.357, p=0.940). Moreover, we did not find any pattern for the other treatments that excluded different subsets of vertebrate scavengers.

Similarly, for all macro elements, the treatment excluding all vertebrate scavengers showed the highest values over time (Figure 8.2a-e). This was most clear for K (Figure 8.2b; LMM, df=3, F=0.040, p=0.993), P (Figure 8.2d; LMM, df=3, F=0.550, p=0.993), and S (Figure 8.2e; LMM, df=3, F=0.537, p=0.993). The highest values of Mg were attributed to the treatments excluding no scavengers and excluding all vertebrate scavengers (Figure 8.2c; LMM, df=3, F=0.029, p=0.993). The trendline of the treatment excluding all vertebrates tended to be higher for Ca, although we did not observe a clear pattern (Figure 8.2a; LMM, df=3, F=0.420, p=0.993). However, none turned out significant.

8.3.5 Roots

For all the root samples, the trendline of the treatment excluding all vertebrate scavengers was generally higher for B (Figure 8.3a; LMM, df=3, F=0.303, p=0.906), Cu (Figure 8.3b; LMM, df=3, F=7.234, p<0.001), Mn (Figure 8.3c; LMM, df=3, F=0.007, p=0.999), and Zn (Figure 8.3f; LMM, df=3, F=0.347, p=0.906). Especially for B and Mo, the highest values were attributed to this treatment. We observed most fluctuations for the treatment excluding wild boar for Mo (Figure 8.3d; LMM, df=3, F=0.690, p=0.769). There was no clear pattern for

Ni (Figure 8.3e; LMM, df=3, F=1.310, p=0.575). Only Cu turned out significant, with the treatment excluding all vertebrate scavengers being different from the other treatments (Appendix 8.4).

Overall, the treatment excluding all mammals tended to be slightly higher compared to the other treatments (Figure 8.4a-e), especially for S (Figure 8.4e; LMM, df=3, F=1.197, p=0.575) and, to a lesser extent, P (Figure 8.4d; LMM, df=3, F=2.015, p=0.575). We did not observe any potential pattern for Ca (Figure 8.4a; LMM, df=3, F=1.191, p=0.575), K (Figure 8.4b; LMM, df=3, F=1.609, p=0.575), or Mg (Figure 8.4c; LMM, df=3, F=0.948, p=0.657). Accordingly, none of the macro elements showed a significant pattern over time in the root samples.

8.3.6 Shoots

We observed a pulse in Cu (Figure 8.5b; LMM, df=3, F=0.709, p=0.602) and Zn (Figure 8.5f; LMM, df=3, F=0.050, p=0.985) in the shoot samples for the treatment excluding all vertebrate scavengers, reaching a peak at approximately 22 weeks. Only for Mo, all the trendlines seemed to slightly decrease, with the treatment excluding no scavengers approaching its start level again at the end of the sampling period (Figure 8.5d; LMM, df=3, F=3.896, p=0.053). We did not observe any potential pattern for B (Figure 8.5a; LMM, df=3, F=1.631, p=0.287), Mn (Figure 8.5c; LMM, df=3, F=1.051, p=0.453), or Ni (Figure 8.5e; LMM, df=3, F=1.207, p=0.423).

The trendlines of the macro elements tended to end higher at the end of the sampling period for the treatment excluding all mammals for K (Figure 8.6b; LMM, df=3, F=2.418, p=0.184), Mg (Figure 8.6c; LMM, df=3, F=3.570, p=0.054), and P (Figure 8.6d; LMM, df=3, F=4.062, p=0.053). We did not observe any potential pattern for Ca (Figure 8.6a; LMM, df=3, F=1.717, p=0.287) or S (Figure 8.6e; LMM, df=3, F=1.714, p=0.287). Although for the latter, the treatment excluding wild boar and the treatment excluding all mammals tended to end slightly higher compared to the other two treatments.

8.4 Discussion

Vertebrate scavengers are believed to accelerate elemental cycles by preventing leakage of elements from carcasses into the soil, but this idea had never been empirically tested. We determined how exclusion of different scavenger guilds influenced local nutrient dynamics during carcass decomposition. We found that experimental exclusion of vertebrate scavengers indeed resulted in higher nutrient pulses below carcasses and thus changed local nutrient dynamics. This is also in line with previous studies (e.g. Parmenter & MacMahon 2009; Quaggiotto et al. 2019; Barton et al. 2020). For example, Quaggiotto et al. (2019) and

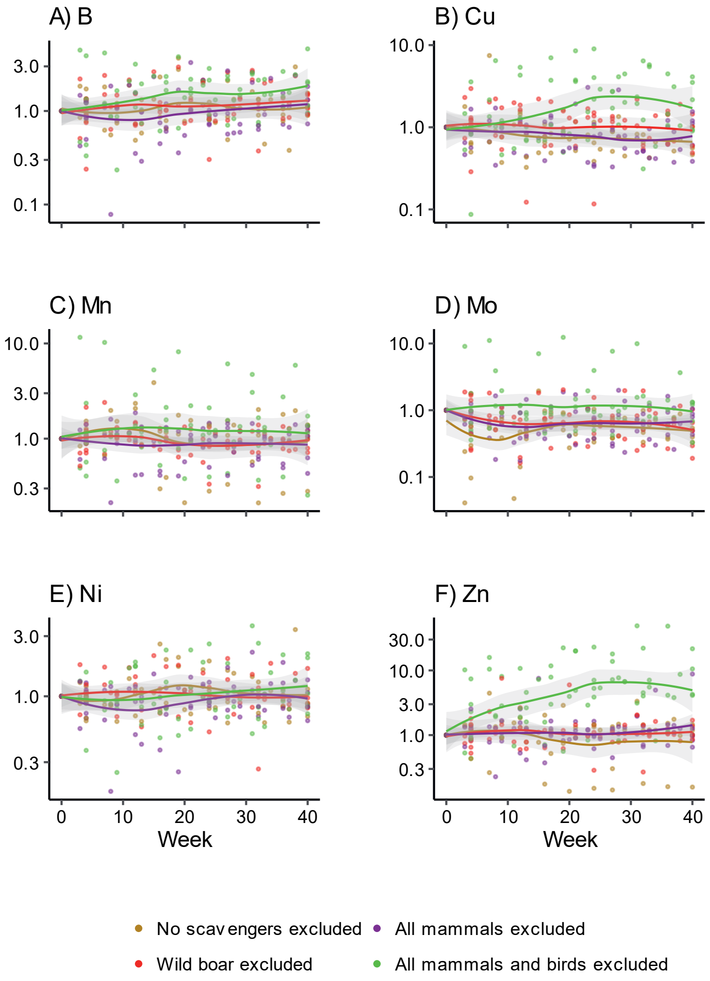


Figure 8.1 Leakage of trace elements over time into the soil beneath decomposing carcasses, when different subsets of scavengers are excluded. Visualised as the standardised concentrations, with week 0 as value 1.

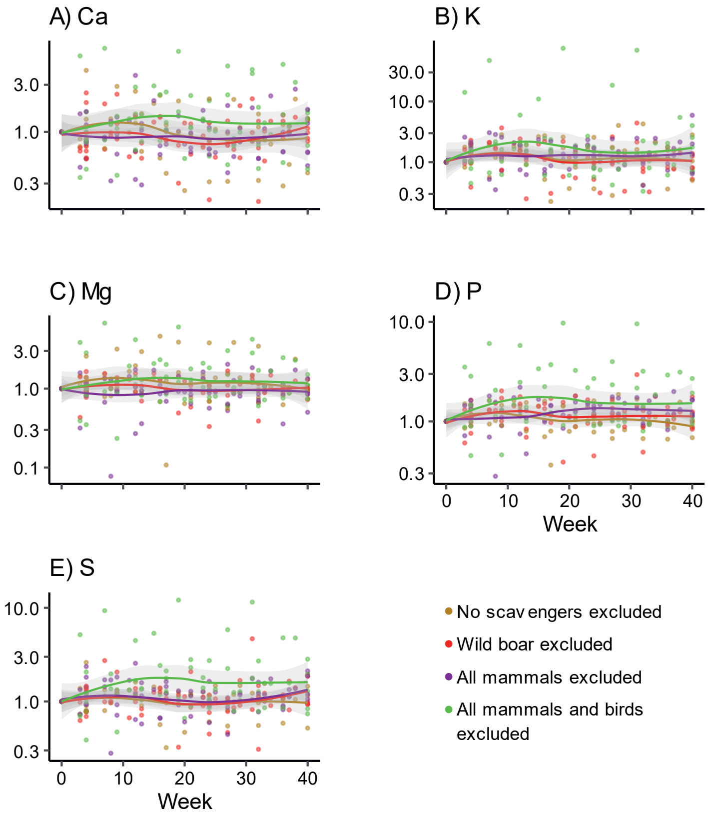


Figure 8.2 Leakage of macro elements over time into the soil beneath decomposing carcasses, when different subsets of scavengers are excluded. Visualised as the standardised concentrations, with week 0 as value 1.

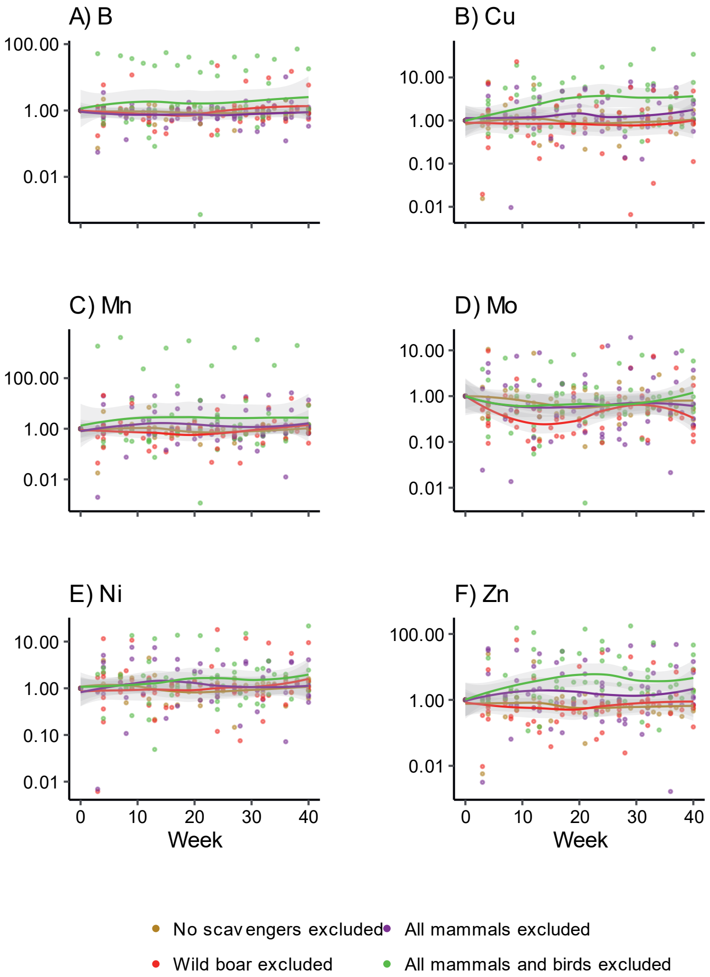


Figure 8.3 Uptake of trace elements over time in below-ground vegetation parts (roots) on the edge of decomposing carcasses, when different subsets of scavengers are excluded. Visualised as the standardised concentrations, with week 0 as value 1.

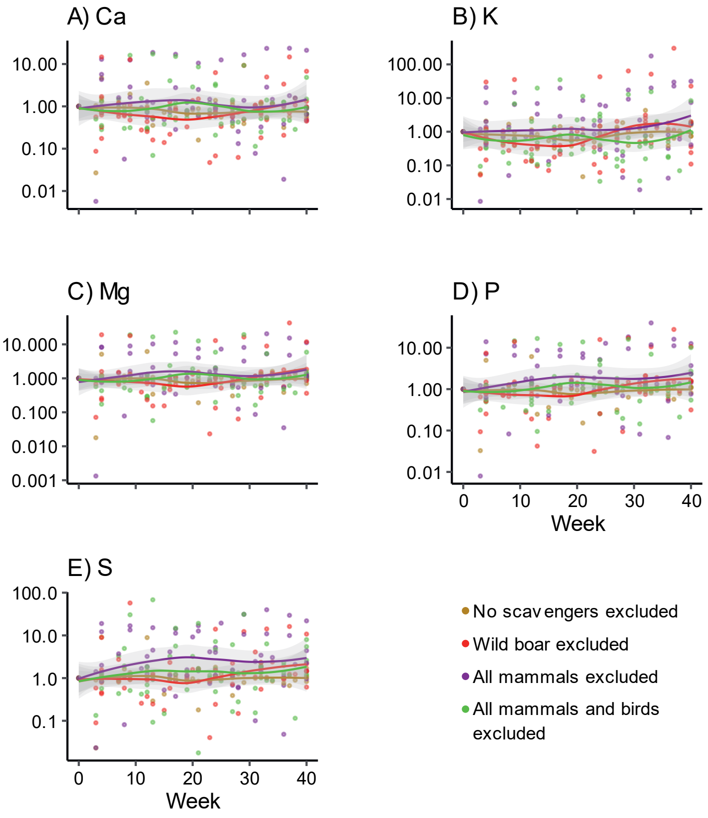


Figure 8.4 Uptake of macro elements over time in below-ground vegetation parts (roots) on the edge of decomposing carcasses, when different subsets of scavengers are excluded. Visualised as the standardised concentrations, with week 0 as value 1.

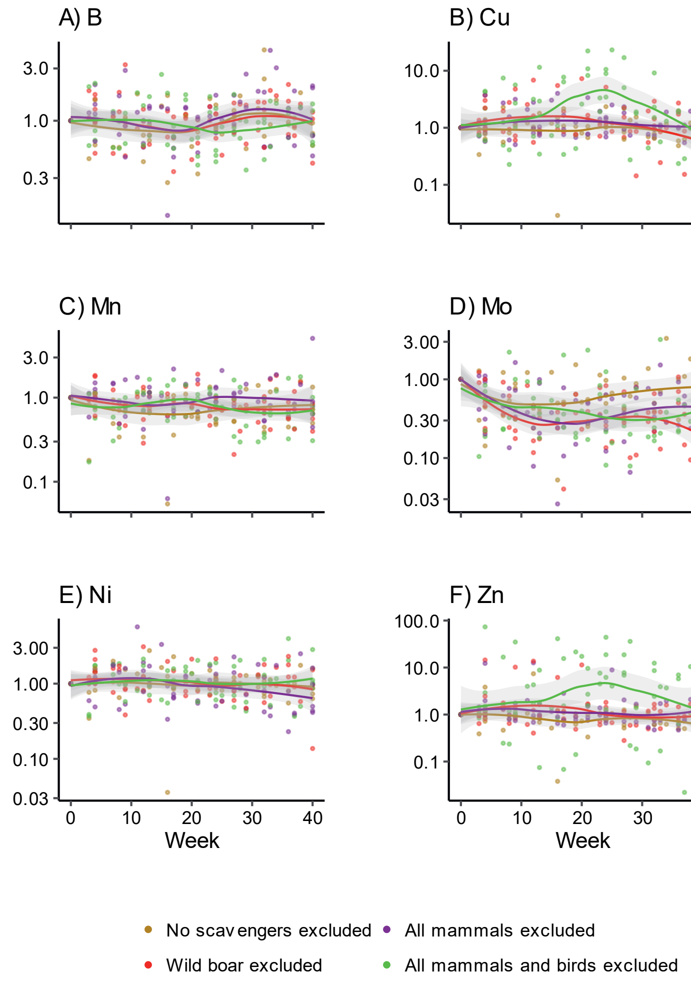


Figure 8.5 Uptake of trace elements over time in above-ground vegetation parts (shoots) on the edge of decomposing carcasses, when different subsets of scavengers are excluded. Visualised as the standardised concentrations, with week 0 as value 1.

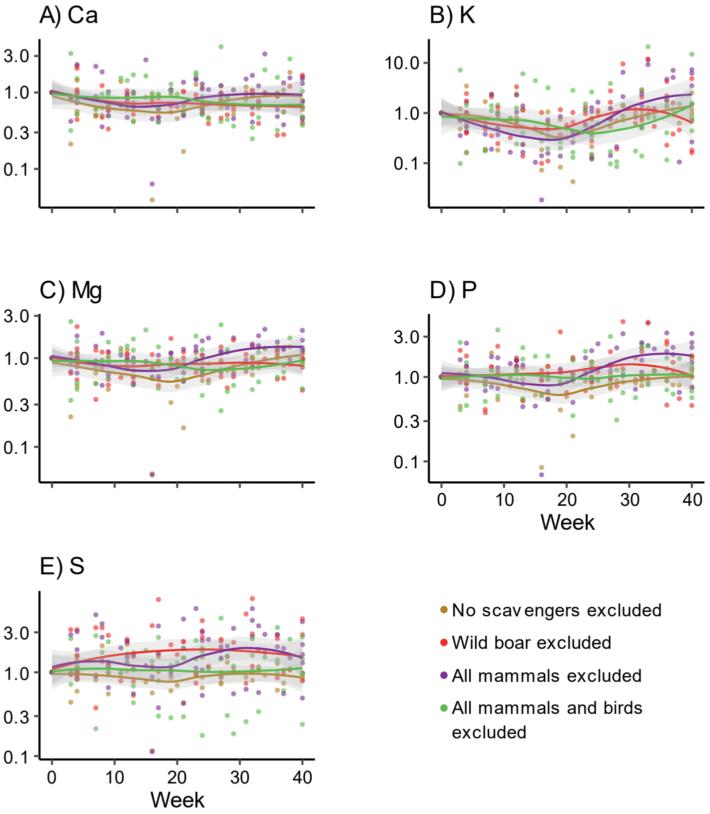


Figure 8.6 Uptake of macro elements over time in above-ground vegetation parts (shoots) on the edge of decomposing carcasses, when different subsets of scavengers are excluded. Visualised as the standardised concentrations, with week 0 as value 1.

Barton et al. (2020) excluded vertebrate scavengers but allowed invertebrates, and found increased EC, pH, and total N and P. Increased EC indicates increasing elemental concentrations but does not give information for any element separately (e.g. Wilson 1938; Heiniger et al. 2003; Selway 2014). In this paper, we followed eleven elements underneath carcasses over time.

We did not observe any clear differences among the three treatments that allowed different subsets of vertebrate scavengers. Also the treatments allowing vertebrates but no wild boar did not differ from the treatment allowing all scavengers, in disagreement with our expectation that wild boar would have a stronger influence than other vertebrates. Yet our findings are in line with Bump et al. (2009), who allowed all vertebrate scavengers and found no or only minor changes in local biochemical dynamics. Melis et al. (2007) also allowed all scavengers and concluded that large proportions of carcass-derived nutrients are recycled via these scavengers rather than locally leaking into the soil. Macdonald et al. (2014), in contrast, found increased EC beneath carcasses while allowing all scavengers, but did not elaborate on the degree to which vertebrates contributed to the decomposition process, hence it is not clear to which extent vertebrates consumed the carcasses.

Overall, fluxes of trace elements below carcasses seemed to be more influenced by invertebrate scavengers than by vertebrates. Vertebrate scavengers seem to limit leakage of elements from carcasses into the soil. Particularly, we found that Cu levels increased in the soil and in the below-ground vegetation parts when only invertebrate scavengers were present (Figure 8.1b + Figure 8.3b). Cu is an essential trace element (Robinson et al. 2009; Kaspari 2021). It is a component in many enzymes in animals (e.g. NRC 2001) and plays a key role in photosynthesis in plants (e.g. Yruela 2009). Our results suggest that plants can immediately take up increased levels of Cu when it is supplied to the soil. The peak in Cu levels in the shoots (Figure 8.5b) suggests that those plants can invest it in the above-ground vegetation parts as well. This seems to also apply for Zn (Figure 8.1f + Figure 8.3f + Figure 8.5f), another essential element in both plants and animals (e.g. Broadley et al. 2007; Papachristodoulou et al. 2015).

Fluxes of macro elements were not significantly different between exclusion treatments. Yet the highest values in the soil for some of these elements, e.g. K, P and S (Figure 8.2b+d-e), were found in the treatment excluding all vertebrates. This implies that these elements can also leak into the soil during decomposition processes dominated by invertebrates but that our sample size might be too low to statistically confirm. This potential leakage was also found in controlled experiments (Wenting et al. 2023a).

We did not find any significant pattern for macro elements and most of the trace elements in any of the sample types. Apart from no elements leaking from carcasses, which is highly unlikely (Wenting et al. 2023a), a likely explanation for our results could be that essential elements are rapidly taken up by plants (Hobbie 1992). This could have resulted in an increase in biomass, rather than an increase of elemental concentrations in plants (e.g. Ingestad & Agren 1991; Pilon-Smits et al. 2009). This explanation seems to be in line with previous studies that described that elemental leakage due to carcass decomposition can enhance plant growth (e.g. Towne 2000; Wenting et al. 2023a). We were, however, unable to measure this alleged rapid uptake by plants and investment in biomass since we decided not to sample plant biomass in our experiment for several reasons. For instance, we sampled the carcasses repeatedly and chose not to disrupt the experiment drastically by clipping vegetation. Also, our experimental setup did not allow us to prevent herbivory. Herbivory - both by vertebrates (e.g. Bigger & Marvier 1998; Ford & Grace 1998) and invertebrates (e.g. Carson & Root 1999; Belovsky & Slade 2000; Throop 2005; La Pierre et al. 2015) - can reduce plant biomass enormously. We believe it is worthwhile to examine this concept of plant biomass increase due to carcass-driven elemental fluxes and enhanced herbivory in a more controlled experiment.

Some essential scarce trace elements, e.g. Co (NRC 2001), showed higher concentrations in the control soil samples than in fallow deer bodies (Table 8.2). We could therefore not measure local changes for these elements that could be attributed to carcass decomposition. However, it has been demonstrated in a controlled experiment that such trace elements can also leak into the soil due to carcass decomposition (Wenting et al. 2023a). In a more natural setting, like the experiment we report here, Co might be hardly detectable due to its scarcity (e.g. Pratt & Fonstad 2009).

Most ecotoxic elements - Al, As, Cd, and Pb - were measured in higher concentrations in the control soil samples than in fallow deer bodies (Table 8.2). Al is poorly absorbed in animal bodies (NRC 2001; Pérez-Granados & Vaquero 2002), which might be reflected by our finding. Contrary, As is well absorbed by animals and toxicity is likely to occur (NRC 2001; Ventura-Lima et al. 2011). Pb is the most common cause of toxicoses in animals (Neathery & Miller 1975). Our findings imply that As and Pb toxicity might not be of great importance in our study area. Cd, a heavy metal without any function but causing severe renal damage (e.g. Patra et al. 2006; Yu et al. 2006; Swarup et al. 2007), was the only ecotoxic element that we measured in lower concentrations in the soil control samples than in fallow deer. Although we can only speculate about the consequences of these findings, this might imply a non-excessive ecotoxic load in our study area. Therefore, the decomposition of carcasses is unlikely to worsen the overall toxicity in the landscape.

In conclusion, we found that vertebrate scavengers limited the influence of elemental leakage from the carcasses into the soil, and hence did not influence local nutrient dynamics. When vertebrate scavengers were excluded, fluxes of several nutrients into the soil showed distinct peaks, which was also reflected in the vegetation. Especially essential trace elements (Cu and Zn) seemed to be influenced by carcass decomposition. However, we did not find any significant differences between treatments with partial exclusion of vertebrate scavengers. Although wild boar can significantly speed up the carcass decomposition process compared to other vertebrates (e.g. Wenting et al. 2022), this species does not seem to differently influence elemental leakage during the process. Our results suggest that carcass-derived nutrients are dispersed over larger areas rather than locally leak into the soil when vertebrate scavengers dominate the decomposition process. Hence, vertebrate scavengers spatially homogenise soil chemical elements in the landscape, in contrast to carcasses that are not consumed by vertebrate scavengers, that result in islands of soil fertility.

8.5 End sections

8.5.1 Author contributions

Author Contributions: EW, PAJ, HS and FvL designed the experiment. EW, SB and DFD performed the experiment in the field. EW and SB prepared the chemical analyses in the laboratory. EW analysed the data and wrote the manuscript. PAJ, SB, DFD, HS and FvL assisted in writing the manuscript.

8.5.2 Acknowledgements

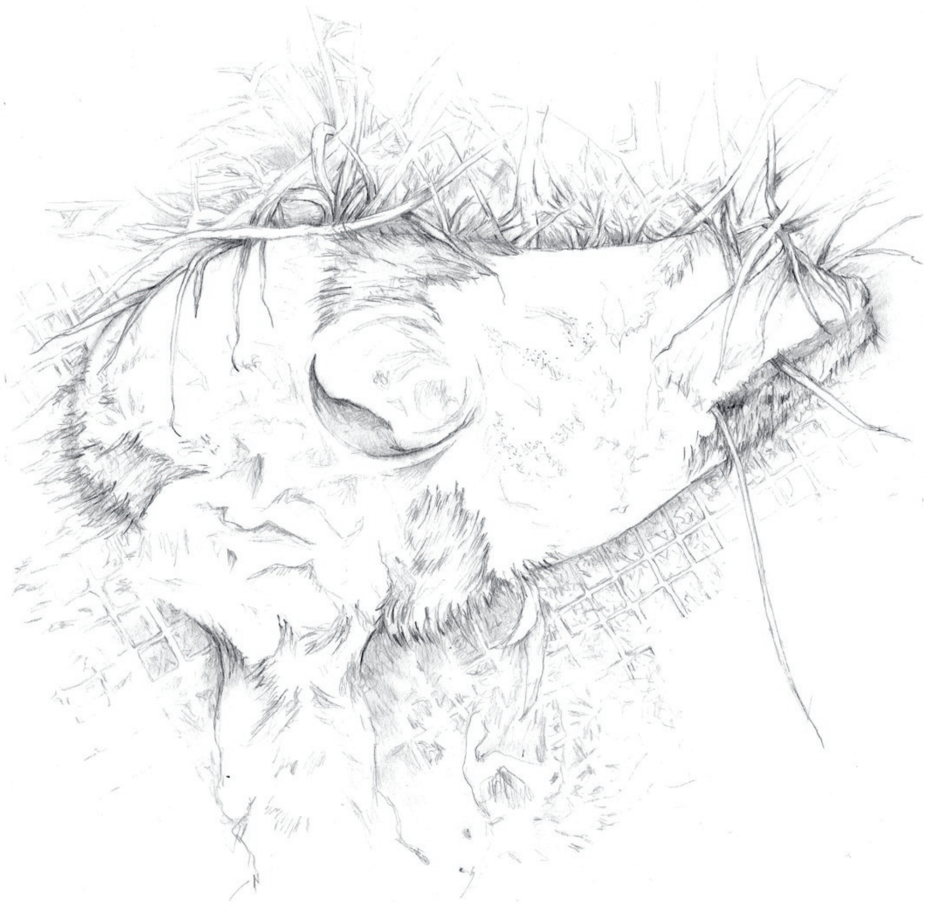
We are indebted to the managers and hunters of Veluwezoom National Park, who allowed us to perform the experiment on their properties and transport the carcasses to the study locations. Special thanks to all the dedicated volunteers that assisted in preparing the experiment in the field, assisted in the sampling procedure and did the maintenance of the fences. Furthermore we thank the technicians of the General Instruments of Radboud University (Paul van der Ven and Sebastian Krosse) for their help in measuring the elemental concentrations.

8.5.3 Conflicts of interest

No actual or potential conflicts of interest are declared by the authors.

8.5.4 Data accessibility

The complete dataset used in this study is available through Figshare: https://dpo.org/10.6084/m9.figshare.24865638.



Chapter 9

Synthesis

Wenting, E.

9.1 Introduction

In this thesis, I tested the idea that animals, especially larger-sized mammals, play a key role in the terrestrial nutrient cycle by accumulating a wide range of (scarce) chemical elements in their bodies over their entire lifespan. These elements are eventually released, very locally and during a short time period, in the form of carrion when animals die. The interplay between scavengers and detritivores would then determine the fate of the carrion-stored elements: being redistributed over large areas via scavengers, or locally leak into the soil beneath decomposing carrion via decomposition by detritivores or carrion fluids. The scavenger-dominated pathway would result in a more patchy distribution of elements among the landscape, which positively contributes to the production of above-ground biomass when elements are taken up by plants. Contrary, the detritivore-dominated pathway would locally result in high concentrations of elements, which increases the chance that elements leach to deeper soil layers where they become unavailable for plants. It thus matters which pathway dominates. However, it had never been investigated how carrion and scavengers influence biochemical cycles of a wide range of chemical elements, which I did in this thesis.

This thesis is the first step in determining the role of larger-sized animals in biochemical cycles, via carrion decomposition and scavenging. In particular, I focused on Dutch protected areas, that are characterised by imbalanced nutrient cycles and the absence of obligate scavengers and large carnivores. I focused on three major knowledge gaps that must be addressed at first: (1) the elemental composition, i.e. ionome, of larger-sized mammals that are an importance source of carrion (Chapter 2-4); (2) the structure of local scavenger communities (Chapter 5-6); and (3) the leakage of elements into the soil due to carrion decomposition (Chapter 7-8).

In this final chapter, I start with evaluating the findings of each knowledge gap, based on the insights obtained from Chapter 2-8, and I discuss their relevance and coherence. After that, I link the results and obtained insights to - missing links of - the carrion-centred nutrient cycle (Chapter 1, Figure 1.1), and discuss emerging knowledge gaps. Subsequently, I evaluate how the obtained insights relate to implications for nature management by discussing some current relevant subjects. Then, I discuss some implications for nature conservation and rewilding practices. I end this chapter with some concluding remarks.

9.2 Evaluating identified knowledge gaps

9.2.1 The mammalian ionome

The mammalian ionome - reflecting the physiological state of organisms (Lahner et al. 2003) - was believed to be under relative tight homeostatic control and therefore approximately constant (e.g. Sterner & Elser 2002). The reasoning behind this widely-held assumption is that animals, unlike plants (e.g. Robinson 1994; Sabehat et al. 1998; De Graaf et al. 2009), are mobile and can so obtain all the chemical elements they require from their foraging habitat (e.g. Daufresne 2013; Doughty et al. 2016). The assumption of ionomic homeostasis has been based on C:N:P ratios - the main focus of ecological stoichiometric studies (Sterner & Elser 2002; Raubenheimer et al. 2009; Jeyasingh et al. 2017) -, although a much wider range of chemical elements is essential for both plants and animals (Robinson et al. 2009; Kaspari 2021). In Chapter 2, we disproved the assumption of ionomic homeostasis by evaluating a wider range of elements in tissues of species that differ tremendously in diet and prevalence, i.e. fallow deer (*Dama dama*) and Eurasian otter (*Lutra lutra*). We found substantial variation between and within the species (Figure 2.2-2.3; Table 2.1). This finding is in line with some other recent studies that indicated ionomic variation in mammals to be much more prevailing when including a wider range of elements rather than C:N:P ratios (e.g. Ma et al. 2015; Jeyasingh et al. 2017), although none of these studies measured the ionome as extensive as we did.

Specific tissues of wild-living ungulates are commonly used as bioindicators for ecotoxicity and potential deficiencies (e.g. Ellen et al. 1989; Yarsan et al. 2014; Nowakowska et al. 2016; Oropesa et al. 2022). These tissues, e.g. liver and kidney, are assumed to be the target tissue for multiple - potentially ecotoxic - elements (e.g. Vahter & Concha 2001; Gasparik et al. 2004; Malmsten et al. 2021). Chapter 3, however, showed that elements are much more scattered over the entire body than previously assumed (Figure 3.1-3.22). There was no single tissue that accurately represented the accumulation of particular elements in the bodies (Table 3.2-3.3). Also, the example of Cd that was most scattered over the body in the individual with the lowest Se concentration (Figure 3.5e; Table 3.1) - Se acts as antioxidant for e.g. Cd toxicity (Zwolak 2020) - highlights the importance of context that measuring multiple elements in multiple tissues can provide. Thus, only focusing on the assumed target tissues might cause unintended bias that limits the use of such tissues as bioindicators for environmental pollution or potential deficiencies.

Existing ionomic variation can be species-, or even tissue- or area-specific (e.g. Wilson & Grace 2001; Barboza et al. 2003). This is in line with our findings in Chapter 2-3. Moreover, as shown in Chapter 4, some of the ionomic variation seems to be related to differences in sex and age (Figure 4.2-4.5). This implies that the selection of specific individuals for biomonitoring purposes can be an additional source of unintended bias and can thus increase the chance of - unintended - potential misinterpretation.

The knowledge gap that I aimed to address with Chapter 2-4 was the unknown elemental composition - i.e. ionome - of free-ranging larger-sized mammals. This was important to verify the assumption that carcasses of these mammals contain high-valuable (scarce) elements. The ionome was much more variable than expected. That indicates that these larger-sized animals are - individually - more influenced by their foraging habitat than previously assumed. The high variability of the mammalian ionome has potentially far-reaching consequences for understanding the role of animals in the terrestrial nutrient cycle. Since the elements that are stored in animals - hence carcasses after their death - can significantly vary, the elements that leak into the soil due to carrion decomposition can significantly vary as well. Also, there might be some underlying - yet unknown - physiological mechanisms causing ionomic variation, that is sex and age related. This emphasises that, in general, we need to be more careful with such assumptions.

9.2.2 Scavenger community interactions

Scavengers are assumed to play a key role in redistributing carrion-derived elements over the landscape (e.g. Selva et al. 2003; 2005; Turner et al. 2017; Patterson et al. 2022), especially when they are fast enough to localise and consume the carrion before detritivores can colonise it (Janzen 1977; Ruxton et al. 2014). There is increasing evidence that different scavenger species have specific traits and abilities to locate and exploit carcasses (e.g. Moreno-Opo et al. 2016; Kane et al. 2017; Stiegler et al. 2020), and therefore that different scavenger species fulfil different functional roles in the process of carrion decomposition (Sebastián-González et al. 2020; 2021). The local scavenger guild thus strongly influences the decomposition process (e.g. Selva & Fortuna 2007; Killengreen et al. 2012). Existing studies focussing on the local scavenger community were predominantly carried out in areas with obligate scavengers and/or large carnivores present (e.g. Selva & Fortuna 2007; Olson et al. 2012; 2016). Chapter 5 provided new insights into the Dutch scavenger community - characterised by the absence of obligate scavengers and/or large carnivores - by defining functional groups of facultative vertebrate scavengers, that we linked to the carrion decomposition speed (Figure 5.4).

In accordance with previous studies, more specialised species such as common raven (*Corvus corax*), common buzzard (*Buteo buteo*) and red fox (*Vulpes vulpes*) mainly used carcasses as a food source when exploiting them (Table 5.2; e.g. Cagnacci et al. 2003; Rösner et al. 2005; Grubb et al. 2018; Forbes et al. 2022). Wild boar (*Sus scrofa*) was an important scavenger in our study system (e.g. Figure 5.4a; Figure 6.6b), as it has been reported elsewhere (e.g. Forsyth et al. 2014; Newsome et al. 2023). Wild boar was able to exploit carcasses much more rapidly than other mammalian scavengers (Chapter 5; e.g. Figure 5.4a; Table 5.2), which reinforces their distinct role in the local scavenger guild (Chapter 6; e.g. Figure 6.5b).

Some scavenger species were classified as occasional scavengers, while others described them as major scavengers, or the other way around. For example, wood mouse (*Apodemus sylvaticus*) has been described as a major scavenger species in the United Kingdom (Young et al. 2014), but we classified it as occasional scavenger in Dutch protected areas (Figure 5.2b; Table 5.2). Carrion crow (*Corvus corone*) and European polecat (*Mustela putorius*) in Bialowieza Primeval Forest Poland were mentioned as only minor scavengers (Selva et al. 2005), while we classified them among the scavenger specialists (Figure 5.2b; Table 5.2). More notably, a widely observed pattern that contradicts our results is that a more diverse (vertebrate) scavenger community and higher species richness result in more complete and faster carrion removal from ecosystems (e.g. Cortés-Avizanda et al. 2012; Olson et al. 2016; Hill et al. 2018). We observed the opposite: the faster the carrion was removed from the ecosystem, the lower the species richness that was involved in the decomposition process (Figure 5.5a). Slower decomposition, on the contrary, provided longer time windows for species to detect and exploit the carrion.

The amount of tree cover was not correlated to carcass exploitation by vertebrate scavengers (Chapter 6; Figure 6.2), which contradicts with findings in other regions (e.g. Hiraldo et al. 1991; Oliva-Vidal et al. 2022). Tree cover has been shown to be particularly important for obligate scavengers, i.e. vultures (e.g. Pardo-Barquín et al. 2019), that were absent in Dutch protected areas. Therefore, the examples of contradictory findings emphasise that functional roles of facultative scavengers and their way of carrion exploitation can considerably differ between areas.

Another important finding was the high variability of the carcass decomposition speed (Chapter 5-6). In general, the carcass decomposition speed can be influenced by many factors, including ambient temperature, moisture, and carcass type and size (e.g. Carter et al. 2008; 2010; Benbow et al. 2019; Moraleda et al. 2022). Obligate scavengers - i.e. vultures - are generally described as the most efficient species in locating and consuming carcasses, contributing the most to rapid carrion removal from ecosystems (e.g. Moreno-Opo et al. 2016; Sebastián-González et al. 2016). Wild boar - as defined the “dominant scavenger species” in Dutch protected areas (Chapter 5-6) - does not seem to fulfil exactly the same role due to its opportunistic and omnivorous nature (e.g. Schley & Roper 2003; Cuevas et al. 2013; Ballari & Barrios-García 2014). When wild boar was involved in the carrion decomposition process, that could result in faster decomposition than without wild boar (Figure 5.4a; Figure 6.6b), but their presence in a particular area does not automatically mean that wild boar exploits every single carcass.

The high variation of the carrion decomposition speed - ranging from 3.5 to 140 days (Chapter 5-6) - reflects the unpredictability whether the carrion-stored elements will be recycled via the scavenger-dominated or the detritivore-dominated pathway. This is even reinforced by the different functional roles that scavenger species can fulfil in different ecosystems, which emphasises the importance of understanding the local scavenger community. The elemental leakage into the soil beneath carrion might therefore be even more unpredictable and variable in the absence of obligate scavengers - e.g. in Dutch protected areas -, but also more substantial when the decomposition time takes longer.

9.2.3 Carcass-related elemental leakage

Based on the assumption of ionomic homeostasis, it was also assumed that the elements stored in carcasses were relatively constant. However, because the assumption of ionomic homeostasis was disproved (Chapter 2-4), I also expected the elemental leakage due to carrion decomposition to be highly variable. Indeed, we found overall increased elemental concentrations, with considerable variation, that leaked into the soil beneath decomposing carrion (Chapter 7-8).

A wide range of elements leak into the soil during carrion decomposition. We proved this empirically in a controlled experiment (Table 7.1) and under more natural conditions (e.g. Figure 8.1). Both experiments were done on nutrient-poor soils. Vertebrate scavengers, fulfilling different functional roles (Chapter 5), did not seem to influence the leakage of elements (Figure 8.1-8.6). Even the distinct ability of wild boar to exploit carcasses (Chapter 5-6) was not reflected in the elements that leaked into the soil during the decomposition.

We were not able to detect any influence of carrion decomposition on macro elements (Figure 8.2+8.4+8.6), while previous studies did report changes in these elements (e.g. Barton et al. 2016; Quaggiotto et al. 2019; Heo et al. 2021). Most of these studies were conducted in areas where macro elements, e.g. N and P, are known as the limiting factors of primary production, e.g. Artic tundra (Danell et al. 2002), semiarid shrub-steppe environment in Wyoming (Parmenter & MacMahon 2009), or temperate woodland in Australia (Macdonald et al. 2014). Such macro elements are not limiting factors in Dutch protected areas anymore due to the long-lasting effects of acidification and excessive N deposition (e.g. Vogels et al. 2023).

We found a distinct peak in scarce trace elements, e.g. Cu and Zn (e.g. Figure 8.1b+f), which suggests that such trace elements are more limiting than macro elements in Dutch protected areas (Melis et al. 2007). This might correspond with the alleged availability - or imbalance - of chemical elements in Dutch protected areas. Elemental leakage into the soil indicates, in general, that the elements were not taken up by vertebrate scavengers. Reversely, elements that did not leak into the soil were in all probability taken up by vertebrate scavengers, and so redistributed over larger areas. This implies that vertebrate scavengers would indeed play an important role in the cycling of particularly trace elements in protected areas.

9.3 Recapitulation

The results of my thesis showed that vertebrate scavengers, as hypothesised, seem to prevent the leakage of chemical elements into the soil during the carrion decomposition process. I demonstrated that larger-sized mammals - the major carrion pool in terrestrial ecosystems (Beasley et al. 2019) - vary considerably in their elemental composition (Chapter 2-4), which reflects the highly variable landscape they encounter. This has important implications for biomonitoring of toxicities and potential deficiencies in protected areas since there is no single tissue that accurately represents the elemental load of animals. It also adds complexity to the elemental leakage of carrion fluids into the soil, which seems to be most distinct for (scarce) trace elements and when only invertebrate scavengers are involved in the decomposition process (Chapter 7-8). Furthermore, I showed that interactions among vertebrate scavengers can influence the carrion decomposition speed but that scavenger behaviour is unpredictable (Chapter 5-6). Consequently, the potential and actual influences of vertebrate scavengers on the leakage of elements is hard to predict and not inherently related to their functional roles. Thus, I conclude that any aspect of the role of larger-sized animals in biochemical cycles, via the natural circle of life and death, is characterised by substantial variation.

9.4 Emerging knowledge gaps

Until now, I have discussed how the insights obtained in Chapter 2-8 contribute to the knowledge gaps that I identified in Chapter 1. In the next sections, I delve into other knowledge gaps that are open and/or emerging based on the obtained insights. These can be seen as the next knowledge gaps that are of interest to address, in order to further increase the understanding of the carrion-centred nutrient cycle (Figure 1.1).

9.4.1 Selective foraging of animals for chemical elements

Animals take up chemical elements from their environment (Figure 1.1, arrow i) and are so assumed to alter biochemical - elemental - cycles. Herbivores and omnivores would be, more than carnivores, influenced by the elements that are available in their foraging habitat (e.g. Galef Jr. 1996; Toft 1999; Kooijman et al. 2004). The uptake of elements from the environment might contribute to the variation of the mammalian ionome (Chapter 2-4), and thus influence the elements that eventually enter the carrion pool (Figure 1.1, arrow ii).

Selective foraging behaviour of larger-sized animals is believed to alter elemental cycles (e.g. Augustine & McNaughton 1998; Bardgett & Wardle 2003; Wang et al. 2016; Andriuzzi & Wall 2017). However, the underlying mechanisms, effect size, and directions are not well-understood and can vary on a local scale (e.g. Pringle et al. 2023). In general, it has been assumed that grazers accelerate elemental cycles, while browsers decelerate, but that appears to be too generalising and not applicable in all ecosystems (e.g. Sitters & Andriuzzi 2019). Especially in nutrient-poor or imbalanced ecosystems, such as Dutch protected areas, selective foraging strategies - including the impact of grazing or browsing - is hardly studied but might be even more relevant since animals would be even more in need to balance their elemental uptake (Proulx & Mazumder 1998).

Foraging behaviour by carnivores, once presumed to primarily optimise energy intake, is increasingly recognised as a complex interplay involving the selective search for essential elements (e.g. Westoby 1978; Belovsky 1984). The concept of nutritional geometry - i.e. the study of complex interactions between organisms and their dietary choices in the context of multiple nutritional components (e.g. Raubenheimer et al. 2009; Raubenheimer & Simpson 2016) - has been extensively applied to herbivores and omnivores (e.g. Rothman et al. 2011; Johnson et al. 2013; Nie et al. 2015). There is more and more evidence that challenges the idea that carnivores solely focus on prey availability to optimize energy intake (e.g. Charnov 1976; Pyke et al. 1977). Instead, carnivores demonstrated a capacity for selective foraging to optimise macronutrient uptake (Kohl et al. 2015). Surplus killing may be an expression of selective foraging for elements by carnivores (Herbers 1981; Jeschke 2007). Controlled experiments have revealed carnivores' capacity for selective feeding strategies for macronutrients, yet studies exploring foraging for micronutrient are scarce or even absent (Mayntz et al. 2009; Hewson-Hughes et al. 2011; 2013; Jensen et al. 2014). Moreover, existing research primarily focuses on captive settings, leaving a gap in understanding the selective foraging behaviour of free-ranging carnivores. As facultative scavenging is common among carnivores (e.g. DeVault et al. 2003; Wilson & Wolkovich 2011; Pareira et al. 2014), selective foraging strategies for specific essential (trace) elements might play a crucial role in shaping their tendency to exploit carrion.

Scavenging behaviour by obligate herbivores (Chapter 5) - "osteophagy" (e.g. Deokar 1983; Hutson et al. 2013; Mori et al. 2018) - might be another example of selective foraging behaviour to optimise elemental uptake (e.g. Cáceres et al. 2013; Dudley et al. 2016). Osteophagy implies that carrion provides a unique set of nutrients that is beneficial for these animals (Bazely 1989). It can be hypothesised that animals select food items that they otherwise do not consume, e.g. carrion, when it is nutritionally beneficial to them. This would be even more prominent in nutrient-poor or imbalanced environments. However, elemental intake is not equivalent to elemental uptake (McDowell 1992). The physical form in which food items are presented greatly determines the digestibility and potential uptake of elements (e.g. Sampelayo et al. 1998; Giacco et al. 2016; Zewdie 2019). For example, hard seeds pass through the digestive tract without any elements taken up in the body if animals incompletely chew (McDowell 1992). This is in line with, for example, Bredin et al. (2008), that reported that giraffes (*Giraffa camelopardalis*) were not capable of taking up macro elements from bones, that they did select as a resource. Osteophagy is an unexplored type of behaviour. Therefore, it remains unclear whether, or to which extent, other herbivores are physiologically capable to derive (trace) elements via carrion consumption. Moreover, carrion consumption poses risks for herbivores since their gastrointestinal tract is not adapted to prevent disease transmission or poisoning from animal-based food (e.g. Reece et al. 2014). Therefore, osteophagy would be the result from the trade-off between herbivores' needs to balance their elemental uptake and the risk of death.

9.4.2 Redistribution of elements via animals (including scavengers)

When scavengers consume carrion (Figure 1.1, arrow iii), they are assumed to transport the carrion-derived elements over longer distances (Figure 1.1, arrow iv). Especially larger-sized animals are considered as long-distance transporters of nutrients (Doughty et al. 2016; Pringle et al. 2023). These animals transport elements in different ways than abiotic mechanisms (e.g. Subalusky et al. 2017; McInturf et al. 2019; Subalusky & Post 2019). However, although large animals would disperse large amounts of elements over large areas, the effects on vegetation and biochemistry are still poorly understood (Pringle et al. 2023). The studies that tried to disentangle this knowledge gap only focused on C:N:P stoichiometry (e.g. Wrench et al. 1997; Subalusky et al. 2018; Daufresne 2021; Li et al. 2021; Sitters & Olde Venterink 2021a; 2021b). Therefore, the role of animals in the redistribution of trace elements over the landscape remains underexposed.

Animals might play a key role in restoring disturbed or imbalanced nutrient cycles because their influence might be even more prominent under such circumstances (e.g. Hettelingh et al. 2017; Vogels et al. 2023). Riesch et al. (2022), for example, described that grazing by red deer (*Cervus elaphus*) can have mitigating effects in areas with excessive N deposition. It remains unknown, however, whether grazing by animals, or animals in general, can have similar effects on restoring cycles of trace elements.

Movement patterns of larger-sized animals, including all trophic levels, would eventually determine how animals disperse the consumed elements over the landscape (e.g. Kie et al. 2005; Ganskopp & Bohnert 2009). These movement patterns might be driven by the heterogeneity of the landscape, e.g. animals' search for food. Increasing the understanding of movement patterns of such animals would provide important new insights on the landscape scale at which animals take up and disperse elements from their environment.

Another way in which animals can influence elemental cycles is via drinking surface water (e.g. Shirley et al. 1951; Leppard 2013). All essential elements can occur in water (McDowell 1992). Chapman Jr. et al. (1962) demonstrated that Cu was much more available to cattle from their drinking water than from their food. The potential influence of trace elements taken up from drinking water by wild-living animals has been overlooked in protected areas, despite that it might have far-reaching consequences for the role of animals in redistributing elements over the landscape.

Deliberated soil consumption can be a coping mechanism of animals in nutrient-poor or imbalanced environments (e.g. Field & Purves 1964; Healy 1972; Beyer et al. 1994; Ayotte et al. 2006). Jones & Hanson (1985) suggested that Mg, Ca, and Na are the most important elements that can be consumed by deliberated soil consumption. However, it has never been studied whether animals can take up considerable concentrations of scarce trace elements in this way, and so disperse these elements over larger distances.

9.4.3 Animal excreta

When animals, including scavengers, take up chemical elements from the environment, they release some via their excreta, i.e. faeces and urine (Figure 1.1, arrow iv). I hypothesise that elements enter the soil nutrient pool in lower concentrations, compared to via carrion decomposition, when they are released via animal excreta. However, this has never been studied yet. Existing studies about chemical elements in animal excreta have predominantly focused on macro elements, mainly N and P, and fiber contents (e.g. Howery & Pfister 1990; Hodgman et al. 1996; Asada & Ochiai 1999; Sitters et al. 2017; Čupić et al. 2021; 2023; Barbero-Palacios et al. 2023). Some studies included other macro elements, e.g. K, Na and Mg (Moe & Wegge 2008), but studies that included trace elements are absent.

Elemental concentrations in animal excreta are used to indicate the quality of the diet of animals (e.g. Leslie Jr. & Starkey 1987; Showers et al. 2006; Miyashita et al. 2007; Monteith et al. 2014). Mubanga et al. (1985), for instance, suggested that N concentrations in deer faeces can be used to monitor trends in diet quality. However, again, existing studies have only focused on macro elements. Ramírez et al. (1996) included Cu, Fe, Mn, and Zn in their analyses, but concluded that the lack of reference values limit the understanding of reported concentrations. Since then, there are only few studies that reported concentrations of some trace elements in faeces (e.g. Wang et al. 2012; Yu et al. 2019; Li et al. 2022) but extensive surveys are missing.

Moreover, it has never been studied how the absorption of trace elements via food consumption is related to elemental concentrations in the faeces. Also, it remains unknown how the physical form in which food items are presented influence the digestibility and subsequently the elemental concentrations in faeces (McDowell 1992). Thus, in general, it remains unknown how the absorption of elements relates to the availability in the environment.

Trace elements in animal urine have been studied even less than in faeces (e.g. Gustafson & Olsson 2004; Mochizuki et al. 2011). This is probably due to the limited applicability in biomonitoring - as have been evaluated in humans (e.g. Kučera et al. 1995) - and associated difficulties in acquiring adequate sample sizes (Alonso et al. 2004). That also means that the impact that animal urine might have on the natural redistribution of trace elements is neglected.

Thus, studies that examined the elemental composition of animal excreta are scarce and insights concerning trace elements are even scarcer. I hypothesise that animal excreta contain lower concentrations of essential trace elements compared to carrion, because animals need these elements in their bodies to maintain their life functions. Some of the collected trace elements might be excreted due to physiological and chemical processes in the body (e.g. Crowe & Bradshaw 2014), but the majority of the collected trace elements would be invested in the body itself. This implies that carrion would have a more prominent role in the cycle of trace elements compared to macro elements, although this hypothesis has never been studied.

9.4.4 Leaching of elements and/or uptake by plants

Chemical elements that enter the soil nutrient pool in high concentrations might leach to deeper soil layers (Figure 1.1, arrow viii), where they become unavailable for plants (Lehmann & Schroth 2002; Rashmi et al. 2017). If that happens, the elements are - at least temporarily - lost from the ecosystem (Havlin et al. 1999). The process of leaching has been well-studied for macro elements, especially N and P, since leaching of these elements can considerably contribute to soil acidification, increasing the overall imbalance of nutrients (e.g. Lehmann & Schroth 2002; Yadav et al. 2020). However, leaching of trace elements is less well-studied (e.g. Banuelos & Ajwa 1999; Vollpracht & Brameshuber 2016).

Also, the uptake of macro elements by plants is much better understood than the uptake of trace elements (e.g. Graham & Stangoulis 2003; Kaspari 2021; Rizwan et al. 2021). It is unknown which enhanced concentrations of trace elements in the soil due to carrion decomposition can contribute to enhanced plant growth (Chapter 7). The role of trace elements for plants can be considerably different than for animals (e.g. Cataldo & Wildung 1983; Soetan et al. 2010; Kaspari & Welti 2023). Therefore, more extensive single- and multi-dimensional experimental manipulations, including a wide range of elements, should be executed. I suggest investigating this initially within the first 5-6 cm of the soil, since most root biomass is found at that depth (De Kroon et al. 2012).

In general, there is a higher chance of elements leaching when they are more soluble in water (McBride et al. 1999). But more soluble elements are also easier taken up by plants (e.g. Reid & Hayes 2003). Examples of water soluble elements include Cu, Fe, Mn, Mo, and Zn (e.g. Naozuka et al. 2010; Crowe & Bradshaw 2014). Although this corresponds with the elements that were affected by carrion decomposition (Chapter 7-8), I can only speculate whether water solubility would be one of the underlying mechanisms that also determines elemental leakage during carrion decomposition. I expect decomposition time to be important with this respect: the longer the decomposition time, the more time there is for elements to leak into the soil, while the longer it takes before plants can establish on the location the carrion had decomposed, in turn increasing the chance of leaching. This might be linked to the amount of carrion consumption by scavenger, especially during the first stages of decay. It would be worthwhile to empirically investigate this hypothesis.

9.4.5 Influence of re-establishing large carnivores

So far, my focus was on Dutch protected areas, that were characterised by disturbed nutrient balance, no obligate scavengers and no large carnivores. However, large carnivores are rapidly re-establishing to their historical ranges across Europe (e.g. Chapron et al. 2014; Galaverni et al. 2016). The grey wolf (*Canis lupus*), for example, is currently re-establishing in the Netherlands (Lelieveld et al. 2016; BIJ12 2023). This raises questions about the extent to which the insights remain applicable or might change in the coming years. It is unclear whether the main source of carrion, derived from human hunting - as it is nowadays - or wolf predation, might change when human hunting strategies need to be reconsidered.

The presence of the wolf can have cascading effects on ecosystem functioning, which is well-studied in North American wolf habitats (e.g. Lesmerises et al. 2012; Ripple & Beschta 2012; Gantchoff et al. 2022). The European situation is considerably less well-studied (e.g. Nowak et al. 2017; Reinhardt et al. 2019), despite the essential differences compared to the North American continent. It is generally harder to predict trophic cascades in more human-dominated landscapes such as European systems (e.g. Hebblewhite et al. 2005; Dorresteijn et al. 2015). Insights obtained from North American wolf habitats might not be equally relevant in European wolf habitats (Focardi et al. 2017). The current situation of re-establishing large predators in Dutch protected areas provides a unique study system to examine the cascading effects. This includes the influence of large carnivores in the carrion-driven elemental cycle, in European ecosystems.

9.5 Implications for nature management

9.5.1 Biomonitoring of toxicities and potential deficiencies

Biomonitoring of (eco)toxicity and potential deficiencies in European ecosystems is commonly done by measuring the elemental concentrations in specific tissues - e.g. liver, kidney, hair and bone - of free-living ungulates (e.g. Ellen et al. 1989; Yarsan et al. 2014; Nowakowsha et al. 2016). Based on the results of Chapter 2-4, I emphasise the importance of interpreting such measurements with caution. It is extremely important to acknowledge the shortcomings and uncertainties of these commonly used biomonitoring practices, given the high variation of the mammalian ionome and the lack of species- and tissue-specific reference values. Moreover, the elemental concentrations in mammals do not automatically reflect their presence in the environment and vice versa, since elements can differ in function and absorption in plants and animals (Kaspari & Welti 2023). However, results of common biomonitoring methods are not always treated with the necessary caution, which may unintendedly lead to wrong conclusions.

Commonly used tissues can be highly variable in their elemental concentrations and may not be the best bioindicators. For example, bones are frequently used as bioindicator (e.g. Tataruch & Kierdorf 2003; Jota Baptista et al. 2022) but we found the highest ionomic variation in bones (Chapter 2). Such variation can considerably vary between species and individuals (e.g. Mn in red deer and wild boar; Chapter 3), although ionomic variation in bones might slightly reduce with age (Chapter 4). Also, not all studies used the same bone types (e.g. Windisch et al. 2002; Ludolphy et al. 2021; Skoko et al. 2023) but the ionomic variation among bone types remains unknown.

The use of hairs is considered to be a non-invasive and non-lethal method of biomonitoring, and is therefore frequently used (e.g. Cygan-Szczegielniak et al. 2018; Jota Baptista et al. 2022; Oropesa et al. 2022; Squadrone et al. 2022). It is the only tissue that can be used to examine the influence of seasonal or yearly circumstances, especially when individual recognition of animals is possible. Hairs might thus provide insights into the influence of seasonal or annually changing circumstances on the ionomic variation, although I emphasise - again - the caution with which measured concentrations must be treated.

I did not aim to generate a new method for biomonitoring by advocating that it should be common practice to measure the ionome as complete as possible, as we did (Chapter 2-4). But I do advocate that it is important to acknowledge the uncertainties and limitations of biomonitoring practices based on only a few tissues and/or elements.

9.5.2 Supplementary feeding of wildlife and mineral licks

Supplementary feeding is a common management practice in mineral-poor areas (e.g. Abraham et al. 2023; Duvall et al. 2023). It is generally used to prevent malnutrition and potential deficiencies (Murray et al. 2016). There are several types of supplementary feeding practices, including additional food provisioning (e.g. hay and corn) and providing mineral licks. It is widely observed that supplementary feeding, in all forms, attracts wildlife and can so alter their movement patterns (e.g. Orams 2002; Dubois & Fraser 2013; Milner et al. 2014; Selva et al. 2014; Sánchez-García et al. 2015).

In Veluwezoom National Park - the main study area of this thesis - mineral licks and corn are occasionally provided to facilitate regular culling of red deer and wild boar, respectively (Chapter 3). The mineral licks contained more Co and Cu than the corn, and the Co and Cu concentrations in red deer were higher than in wild boar (Chapter 3). This supplementary feeding might have influenced the ionome of these animals. However, potential correlations between supplementary feeding and the mammalian ionome have never been studied. If supplementary feeding would indeed change the ionome of animals, it might have far-reaching consequences on the cycle of elements throughout the ecosystem. Moreover, it may add elements that are foreign to the area, of which the potential consequences are unknown. Therefore, I recommend that supplementary feeding, in all forms, should be done with severe caution until the potential consequences on overall ecosystem functioning are better understood.

9.5.3 Removing carcasses depletes ecosystems

Animals - carcasses after death - contain considerable concentrations of scarce trace elements in their bodies (Chapter 2-4). Some of these concentrations seem to be higher in the animals than in the soil (Table 8.2). The most scarce elements, e.g. Cu and Zn, are among the elements that are most influenced by carrion decomposition (Figure 8.1+8.3+8.5). Carcasses are thus important in the cycle of particularly trace elements. That implies that systematically removing carcasses from ecosystems, for example through hunting bags, would deplete the ecosystem more and more. This is likely to be even more substantial in areas with disturbed or imbalanced nutrient cycles. Thus, it is essential to leave - the majority of the - carcasses in nature to ensure that they stimulate the biochemical cycle of scarce trace elements.

9.6 Implications for nature conservation and restoration

9.6.1 Carrion provisioning to restore imbalanced nutrient cycles

In recent years, management practices are more and more (experimentally) applied to restore imbalanced nutrient cycles in protected areas (e.g. Weijters et al. 2018; Desie et al. 2020; Chakraborty et al. 2023). The role of (vertebrate) animals in such nutrient cycles is more and more recognised (e.g. Beard et al. 2002; Vanni 2002; Abraham 2021) but still underexposed. I hypothesise that - particularly larger-sized - animals have a disproportionately large influence on the cycle of trace elements in nature. These animals contain substantial concentrations of trace elements in their bodies (Chapter 2-4), that seem to be higher compared to the soil (Chapter 8). Consequently, promoting the natural circle of life and death considerably contributes to the restoration of disturbed and imbalanced nutrient cycles, thereby fostering ecosystem functioning.

The turnover of macro elements in ecosystems is significantly influenced by ungulates as the major consumers of vegetation and their excreta (McKendrigk et al. 1980; Hobbs 1996). But the most striking impact of herbivores occur after their death, when they enter the detritus pool in the form of carrion (Selva et al. 2003; 2005). Whereas previous studies found significant impact of carcasses on macro elements (e.g. Parmenter & MacMahon 2009; Macdonald et al. 2014), we only measured weak responses (Chapter 7-8). We did find, however, some distinct peaks in scarce trace elements, i.e. Cu and Zn (Chapter 8). This implies that availability of macro elements does not limit primary production in Dutch protected areas (Melis et al. 2007). This aligns with the expectation due to excessive N deposition and decades of acidification (Vogels et al. 2023).

I expect the availability of carrion in the landscape to be decisive for its influence on nutrient cycles, particularly of trace elements, not its spatiotemporal distribution. Habitat characteristics that are important for obligate scavengers, e.g. tree cover, do not seem to be equally important to facultative scavengers (Chapter 6). Also, vertebrate facultative scavengers did not differently influence elemental leakage into the soil (Chapter 8), despite their different functional roles (Chapter 5). That implies that the involvement of vertebrate scavengers in carrion decomposition, in general, considerably contributes to the cycle of nutrients - including trace elements - through ecosystems.

9.6.2 Quantifying the elements stored in carrion

Carrion can be seen as a unique set of chemical elements, including elements that are scarce in nature (e.g. Crowe & Bradshaw 2014). This raises questions about quantifying the total amounts of elements that are stored in carrion. However, I argue that our current understanding of the mammalian ionome, including the causes and magnitude of its high variability, is insufficient yet to calculate such total amounts of elements. Based on the current knowledge, rough estimates are too uncertain to yield meaningful insights that can be used to quantify carrion-stored elements on an ecosystem scale. Moreover, using rough estimates may suggest that the current knowledge is adequate for this purpose. This appears unjustified yet.

Nevertheless, I do advocate that leaving carcasses in nature, as providing a unique set of chemical elements in relatively high concentrations, can be seen as a mitigating measure that substantially contributes to restore disturbed (trace) elemental cycles.

9.6.3 Carrion provisioning to promote biodiversity

It has been shown that the presence of carrion can contribute to biodiversity by providing a critical or valuable food source for numerous species. That can be either vertebrates (e.g. Selva & Fortuna 2007; Blázquez et al. 2009; Olson et al. 2012; Mateo-Tomás et al. 2015; Sebastián-González et al. 2016) or invertebrates (e.g. Gu et al. 2014; Barton et al. 2017; Barton & Evans 2017). The most specialistic species - that contribute the most to the overall species diversity - are invertebrates (e.g. Melis et al. 2004; Muñoz-Lozano et al. 2019; Van Klink et al. 2020). These species are mostly associated with the later stages of decomposition (Braig & Perotti 2009). I argued that slow decomposition, by providing a longer time window for species to detect and exploit carrion, would be more beneficial for the effect on biodiversity (Chapter 5). Fast decomposition due to vertebrate scavengers stimulates the redistribution of elements over the landscape, and would therefore be more beneficial for nutrient cycles. Both fast and slow decomposition occur naturally, regardless of the species assemblages that are present in specific areas (Chapter 5-6). Based on these insights, I state that the presence of carcasses contributes to both biodiversity and nutrient cycles on an ecosystem scale, but not on the individual carcass scale.

9.6.4 Animals cause and maintain the heterogeneous landscape

Carrion decomposition is believed to be more important in less complex - more homogenous - landscapes, such as tundra and prairie, compared to more complex and heterogeneous landscapes, such as temperate ecosystems (Melis et al. 2007). However, this reasoning is based on macro elements (e.g. Towne 2000; Danell et al. 2002). I doubt this reasoning and argue that carrion decomposition is of equal importance among all landscapes. In temperate ecosystems, such as Dutch protected areas, trace elements are of major importance for all animals and thus ecosystem functioning. Carrion provides a unique set of trace elements. Scavengers have, via carrion consumption, a major impact on the redistribution of trace elements over the landscape.

I acknowledge that the impact of carrion decomposition is likely to be easier detectable in homogeneous landscapes compared to heterogeneous landscapes. Heterogeneous landscapes are associated with more biodiversity compared to homogeneous landscapes (e.g. Loreau et al. 2003; Morelli et al. 2013; Katayama et al. 2014; Harlio et al. 2019), and are considered as more complex in general (e.g. Johnson et al. 1992; Pe'er et al. 2006; Emanuel et al. 2011). In areas where macro elements are not limiting primary production, for example due to imbalanced nutrient cycles, it is reasonable that only minor effects can be detected when focussing on such macro elements (Melis et al. 2007). In heterogeneous landscapes, especially when nutrient cycles are disturbed or imbalanced, carrion decomposition would be of great importance in the cycle of, in particular, trace elements.

I hypothesise that the natural circle of life and death of larger-sized animals is the major cause of variation - "heterogeneity" - in the landscape. Animals themselves are driven by the heterogeneity of the landscape, for example in their movement patterns to optimise the elements that they take up through their food. When foraging, animals try to reach ionomic homeostasis in their bodies which is most optimal for maintaining their life functions. However, animals do not fully succeed in reaching ionomic homeostasis in the heterogeneous - and therefore unpredictable - landscape that they inhabit. It is unpredictable for animals where they can find the most optimal food sources in terms of trace elements. This ionomic imbalance is expressed in the ionomic variation within and between species (Chapter 2-4). When animals die, the trace elements that they collected during their life become, very locally and during a short time period, available for scavengers and other decomposers. Carrion decomposition is then the course via which trace elements are redistributed across the landscape. The unpredictable behaviour of facultative scavengers - resulting in unpredictable carrion decomposition speed (Chapter 5-6) - eventually results in highly variable decomposition patterns and unpredictable and variable leakage of elements beneath decomposing carrion (Chapter 7-8). Thus, the circle of life and death of animals create and maintain the heterogeneity of the landscape. Accordingly, heterogeneous landscapes can persist and be maintained because there are free-living larger-sized animals that eventually die and decompose.

9.6.5 Circle of life and death of ungulates should be central in rewilding practices

Large ungulates are, via their natural circle of life and death, critical for ecosystem functioning by creating and maintaining heterogeneity in the landscape. I argue that stimulating natural processes - a common theme in rewilding practices (e.g. Perino et al. 2019) - without substantial populations of large ungulates are condemned to fail on the long term because these animals are the key drivers of the redistribution of essential trace elements throughout ecosystems. Thus, before it is possible to focus on the protection and restoration of populations of large carnivores and other threatened species, the focus should be first and foremost on stimulating natural processes via the natural circle of life and death. Therefore, stimulating the existence of substantial populations of large ungulates and other common species is crucial, and would create robust and resilient ecosystems. Accordingly, I argue that species conservation and stimulation of biodiversity can only be done in a sustainable way in such robust ecosystems, in which large ungulates and other common species are key.

9.7 Conclusions

I conclude that the overarching theme of my thesis is substantial variation: variation in the mammalian ionome (Chapter 2-4), variation in the carrion decomposition speed and scavenger behaviour (Chapter 5-6), and variation in the elemental leakage into the soil (Chapter 7-8). The natural circle of life and death of larger-sized animals creates and maintains the variation - "heterogeneity” - of the landscape. Thus, the landscape is heterogeneous, causing variability of the ionome, which in turn reinforces the heterogeneity of the landscape when animals die.

Below, I repeat the most noticeable conclusions point by point.

- The mammalian ionome is highly variable, corresponding to the heterogeneous landscape faced by animals.
- The carrion decomposition speed is highly variable and hard to predict. While wild boar can notably accelerate the decomposition process, their scavenging behaviour differs from that of obligate scavengers.
- Vertebrate scavengers can prevent carrion-stored elements to leak into the soil, and so crucially contribute in the redistribution of carrion-stored elements over large areas.
- The presence of carrion can promote both biodiversity and nutrient cycles on an ecosystem scale, but not on the individual carcass scale.
- Larger-sized animals create and maintain the heterogeneity of the landscape through their natural circle of life and death by redistributing essential trace elements through ecosystems.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Supplementary materials

Appendix 2.1 Spike-and-recovery experiments

	ppb	ppb	ppb	ppb	ppb	ppb	ppb	ppb	ppb	ppb	ppb	ppb
	Ca	Co	Cu	Fe	K	Mg	Mn	Na	P	Pb	Se	Zn
1	869.7	-0.4344	12.82	2176	16490	800.9	21.06	11140	15100	-7.242	1.993	87.89
1B	2768	2040	2001	3975	17370	2767	2042	12320	15240	2052	2051	2007
2	728.1	-0.8252	16.75	181.7	24080	1215	7.704	13240	28740	-5.562	2.124	98.84
2 B	2929	2163	2147	2355	28250	3454	2168	16650	30720	2165	2020	2160
3	424.8	0.9403	16.64	165.1	22590	1915	4.534	3969	15610	0.3063	2.251	152.9
3 B	2367	2017	1989	2185	25970	3978	2009	6146	16320	2047	2051	2066
4	477.1	1.518	297.4	349.5	16010	1091	62.95	5848	24910	2.153	3.755	187.8
4B	2465	2024	2271	2373	18020	3080	2063	7862	24620	2057	2010	2112
5	1024	0.1541	20.56	2465	20080	1451	238.9	13080	20590	-2.998	3.004	190.4
5b	3024	1988	1984	4486	21830	3387	2220	14980	20140	2011	1992	2072
6	942	1.232	38.57	290.3	18760	1369	90.26	12510	18130	-0.5995	5.438	157.5
6b	3114	2068	2068	2392	22390	3544	2144	15300	20440	2102	2123	2134
7	458.4	1.389	15.11	174.6	21310	1813	6.785	3563	14860	-7.93	2.235	143.4
7b	2507	1992	2004	2245	27120	4110	2007	6200	17470	2007	1982	2064
8	969.6	1.406	23.01	196.6	28830	1462	9.699	15930	31470	-0.6368	7.17	121.6
8b	3431	2121	2132	2332	28540	3480	2124	16770	30550	2140	2132	2138
9	1074	1.034	24.26	1306	16830	980.1	65.41	12810	18480	-3.434	2.747	102.8
9b	14900	2018	2003	3343	18470	3160	2088	14890	23760	2040	2015	2020
10	478.9	0.1247	262.3	531.5	15120	1062	50.41	5584	21890	-8.114	0.6084	181.8
10b	2508	1999	2257	2594	17700	3093	2043	7875	23560	2030	2013	2104
11	497.9	-0.0508	270.1	348	15990	1109	56.13	5805	24100	0.5069	0.782	188.3
11b	2612	2086	2364	2473	18730	3206	2131	8172	25870	2120	2012	2190
12	3711	1.794	30.48	285.6	19590	1933	384.1	11880	14070	2.705	1.217	171.2
12b	6060	2054	2058	2346	21720	4004	2436	14230	14640	2075	2058	2115
13	987	0.3024	14.34	4561	21320	1384	183.6	10190	19410	-7.695	2.202	198.5
13b	2913	1916	1923	6014	21580	3159	2075	11390	17150	1936	1954	2016
BL	106.7	0.531	-0.9872	15.38	28.45	25.53	1.854	-250.3	16.16	-7.561	1.186	9.487
BL b	2034	1937	1897	1952	1792	1933	1913	1643	12.86	1962	1988	1855
14	1015	1.151	19.57	1804	14520	815	42.89	10020	12530	-3.015	-0.6468	89.8
14b	2973	1906	1920	3565	15680	2651	1945	11420	11800	1929	2035	1899
15	624.5	0.3917	29.77	754.4	15440	1274	29.6	8084	14860	-2.814	1.739	117.2
15b	3515	2008	2024	2820	18690	3377	2034	10800	16850	2046	2030	2037
16	349.2	0.0217	14.56	225.3	23470	2027	4.785	4163	16450	-1.052	1.227	149.7
16b	2392	2038	2034	2293	26460	4147	2027	6373	17430	2062	2117	2105
17	452.1	-0.32	526.8	484.1	10690	757.5	44.92	4609	16540	4.315	5.212	199.9
17b	2491	1979	2539	2570	13350	2783	2015	6989	18530	2012	1998	2078
18	1188	0.1878	65.08	370	23800	1867	71.19	16900	26350	-1.005	3.298	272.6
18b	3171	2100	2165	2519	23390	3761	2160	17360	24030	2127	2060	2269
19	1582	-0.4446	17.97	2281	19400	1488	668.6	12590	18380	-3.233	1.387	219
19b	3789	2135	2171	4684	22800	3643	2790	15430	18660	2157	2191	2288
20	515.1	0.5566	40.97	613	19620	1571	19.22	7598	17410	-6.303	1.097	175.7
20b	2545	2040	2075	2777	23430	3748	2051	10260	19310	2079	2045	2097
21	661.6	0.7907	442.4	421.8	17720	1182	87.83	6100	24910	1.561	3.918	223
21b	2707	2072	2469	2521	17220	3136	2149	7309	22580	2103	1997	2189
22	1388	1.556	23.7	311.7	21960	1225	10.58	13850	28110	0.3171	1.355	139.1
22b	3820	2077	2095	2367	24160	3289	2082	16060	29260	2100	2086	2100
23	4502	4.072	25.3	245.8	19350	1992	765	11100	13640	-2.159	-0.8276	238.8
23b	5939	2150	2192	2429	20390	3909	2852	13020	12880	2165	2190	2261
24	283.8	2.352	18.22	203.4	19820	1785	7.744	2947	14590	-2.73	4.17	218.1
24b	2218	1937	1948	2158	19310	3523	1927	4492	12870	1971	1962	1992

	% Ca	% Co	% Cu	% Fe	% K	% Mg	% Mn	% Na	% P	% Pb	% Se	% Zn
1												
1B	0.989	1.020	0.995	0.993	1.012	1.011	1.011	1.006	0.959	1.029	1.025	0.965
2												
2 B	1.055	1.082	1.064	1.073	1.019	1.047	1.080	1.032	0.940	1.086	1.009	1.026
3												
3 B	0.979	1.008	0.986	1.010	1.072	1.024	1.002	1.041	0.940	1.023	1.024	0.961
4												
4B	0.993	1.011	0.987	1.008	0.990	0.992	1.000	0.993	0.905	1.027	1.003	0.964
5												
5b	0.995	0.994	0.982	0.997	0.977	0.976	0.990	0.982	0.881	1.007	0.994	0.945
6												
6b	1.007	1.033	1.011	1.024	0.942	0.988	1.019	0.927	0.888	1.051	1.058	0.978
7												
7b	1.010	0.995	0.994	1.028	1.113	1.053	1.000	1.080	0.993	1.008	0.990	0.960
8												
8b	1.166	1.060	1.054	1.064	0.949	1.017	1.057	0.958	0.936	1.070	1.062	1.009
9												
9b	4.856	1.008	0.990	1.013	0.985	1.062	1.011	1.010	1.166	1.022	1.006	0.961
10												
10b	0.996	0.999	0.988	1.007	0.963	0.982	0.994	0.978	0.916	1.019	1.006	0.958
11												
11b	1.028	1.043	1.031	1.040	0.969	1.001	1.034	0.986	0.920	1.060	1.006	0.994
12												
12b	1.076	1.026	1.014	1.029	1.026	1.029	1.025	1.044	0.928	1.036	1.028	0.976
13												
13b	0.978	0.958	0.955	0.923	0.934	0.937	0.951	0.942	0.808	0.972	0.976	0.918
BL												
BL b	0.965	0.968	0.949	0.969	0.883	0.954	0.956	0.939	0.006	0.985	0.993	0.923
14												
14b	0.991	0.952	0.951	0.944	0.961	0.946	0.952	0.962	0.822	0.966	1.018	0.909
15												
15b	1.325	1.004	0.996	1.011	1.029	1.013	1.001	1.032	0.960	1.025	1.014	0.960
16												
16b	1.004	1.019	1.009	1.021	0.957	0.984	1.011	0.973	0.873	1.032	1.058	0.973
17												
17b	0.993	0.990	0.980	1.010	0.953	0.976	0.983	0.974	0.900	1.004	0.996	0.934
18												
18b	1.014	1.050	1.050	1.072	0.952	0.998	1.045	0.963	0.891	1.064	1.028	1.005
19												
19b	1.061	1.068	1.076	1.098	1.072	1.048	1.047	1.064	0.921	1.080	1.095	1.032
20												
20b	0.997	1.020	1.015	1.045	1.017	1.017	1.015	1.011	0.935	1.043	1.022	0.958
21												
21b	1.029	1.036	1.019	1.049	0.911	1.003	1.031	0.935	0.877	1.051	0.997	0.989
22												
22b	1.095	1.038	1.034	1.014	0.945	0.992	1.035	0.953	0.910	1.050	1.042	0.977
23												
23b	0.902	1.073	1.082	1.079	0.939	0.970	1.026	0.978	0.810	1.084	1.095	1.008
24												
24b	0.986	0.967	0.966	0.990	0.994	0.987	0.960	0.978	0.868	0.987	0.979	0.909
	Mean recovery percentage											
	1.180	1.017	1.007	1.021	0.983	1.000	1.009	0.990	0.878	1.031	1.021	0.968

Appendix 3.1 Letter of approval of the Animal Welfare Centre

WAGENINGEN UNIVERSITY
WAGENINGEN UR

For quality of life

Memo

Corporate Education and Research

DATE
20/01/2023

POSTAL ADDRESS
Bode 120
The Netherlands

VISITORS' ADDRESS
Bornse Weilanden 5
6708 WG Wageningen

INTERNET
www.wageningenuniversity.nl

HANDLED BY

TELEPHONE

EMAIL

TO
Elke Wenting

FROM
Janneke Arts, Animal Welfare Officer

Dear Elke,

The Animal Welfare Body assessed the work protocol**: "Variability of the ionome of Wild boar (Sus scrofa) and Red deer (Cervus elaphus) in a Dutch national park, with implications for biomonitoring**, received on **16/01/2023**.

It is the opinion of the AWO that this is not an animal experiment as referred to in the Dutch Act on Animal Experiments, since the experimental procedures described in present protocol will not make use of live animals or animals that were killed for the purpose of tissue sampling.

In any unforeseen circumstances, or intended deviations from the aforementioned protocol, please contact the animal welfare officer.

Yours sincerely,
Dr. Ir. Ing. J.W.M. Arts

Animal Welfare Officer

Wageningen UR (Wageningen University and various research institutes) is specialised in the domain of healthy food and living environment.

Appendix 3.2 Salt licks

Elemental concentrations (µg Kg^{-1}) of the salt licks - for deer - and corn - for boar - used to lure animals to cull sites in Veluwezoom National Park.

	Corn	Salt lick 1	Salt lick 2
Al	-	-	1.79
As	0.024	-	0.0375
B	4.65	-	-
Ca	67.15	-	-
Cd	0.0089	-	-
Co	-	0.0157	0.153
Cr	-	-	-
Cu	5.29	338	2842
Fe	18.29	-	1,067
K	8,259	-	-
Mg	2,695	1,541	1,527
Mn	20.29	-	129
Mo	0.235	-	0.217
Na	-	658,178	640,362
Ni	0.549	1.907	1.402
P	7429	-	-
Pb	-	-	-
S	2,013	-	-
Se	0.021	35	16.561
Si	10.23	37.33	3.667
Sr	-	0.159	0.319
Zn	43.93	1,255	519

Appendix 4.1 Letter of approval of the Animal Welfare Centre

WAGENINGEN UNIVERSITY
WAGENINGEN UR

For quality of life

Memo

Corporate Education
and Research

DATE
02/03/2023

POSTAL ADDRESS
Bode 120
The Netherlands

VISITORS' ADDRESS
Bornse Weilanden 5
6708 WG Wageningen

INTERNET
www.wageningenuniversity.nl

HANDLED BY

TELEPHONE

EMAIL

TO
Elke Wenting

FROM

Janneke Arts, Animal Welfare Officer

Dear Elke,

The Animal Welfare Body assessed the work protocol**: "Ionomic variation among tissues in Fallow deer (Dama dama) by sex and age"**, received on **02/03/2023**.

It is the opinion of the AWO that this is not an animal experiment as referred to in the Dutch Act on Animal Experiments, since the experimental procedures described in present protocol will not make use of live animals or animals that were killed for the purpose of tissue sampling.

Your application has been registered by IvD-WU with number **NAE_2023.W-010**

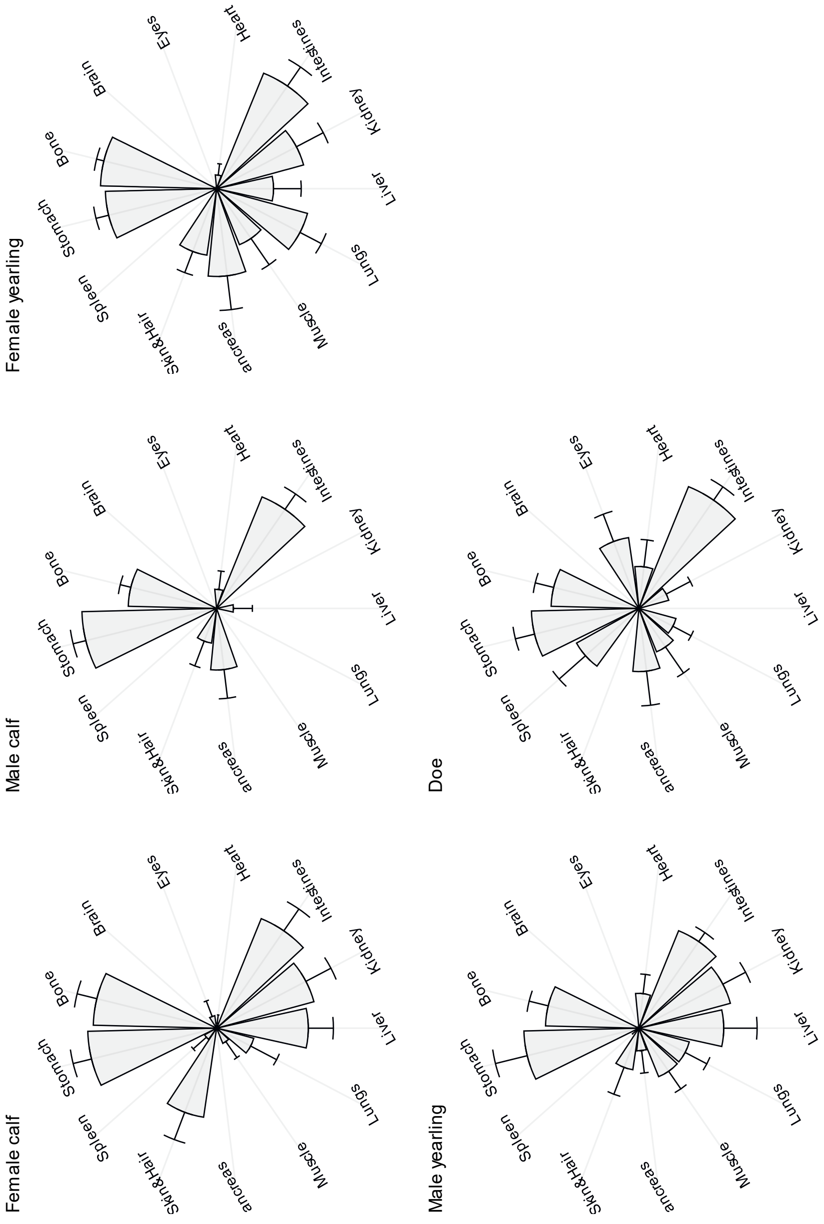
In any unforeseen circumstances, or intended deviations from the aforementioned protocol, please contact the animal welfare officer.

Yours sincerely,
Dr. Ir. Ing. J.W.M. Arts

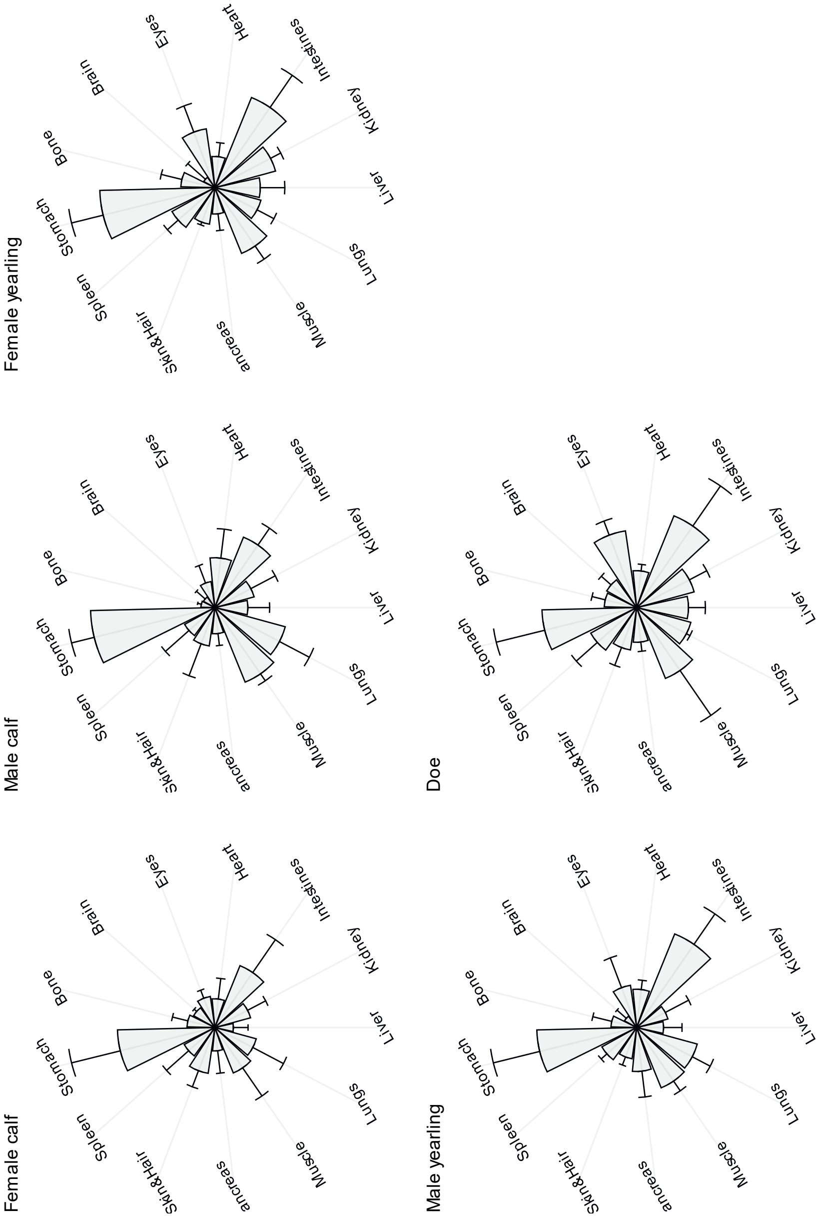
Animal Welfare Officer

Wageningen UR (Wageningen University and various research institutes) is specialised in the domain of healthy food and living environment.

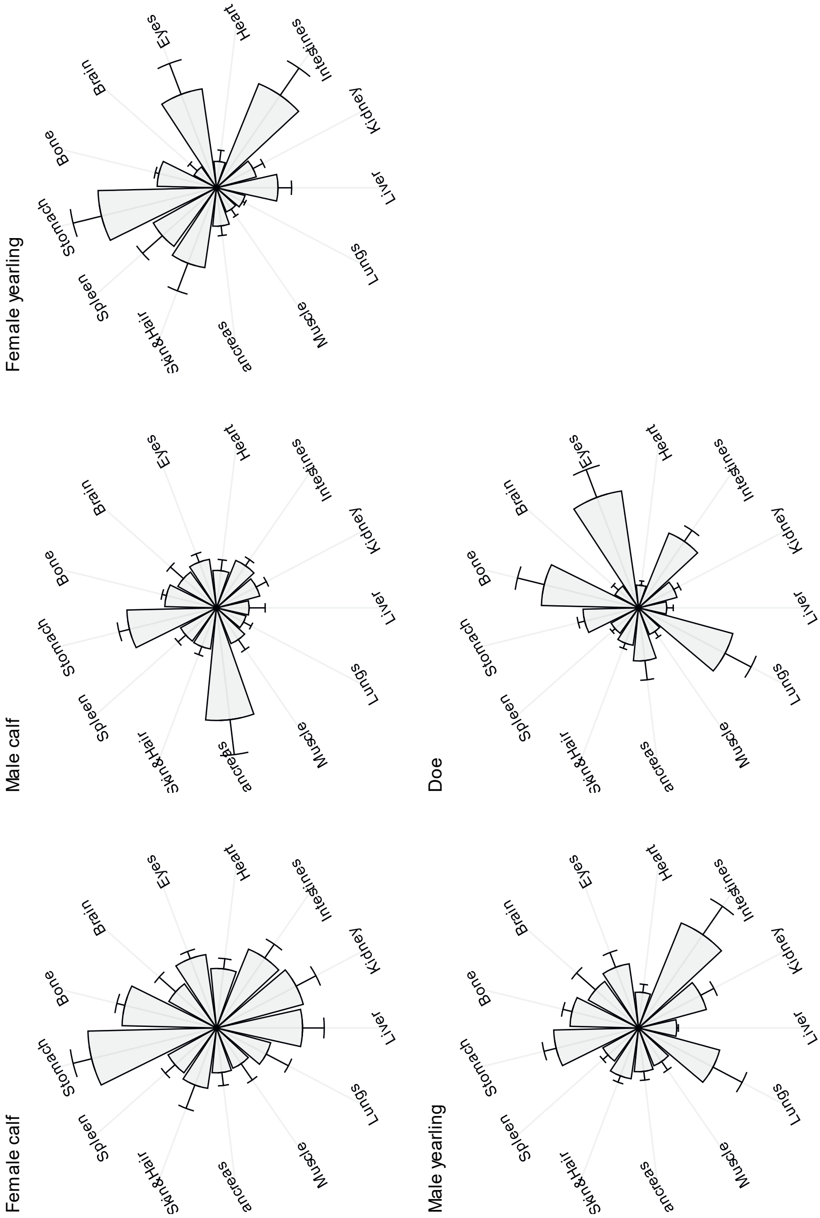
Appendix 4.2 Distribution of the elements over the body per sex-age groups



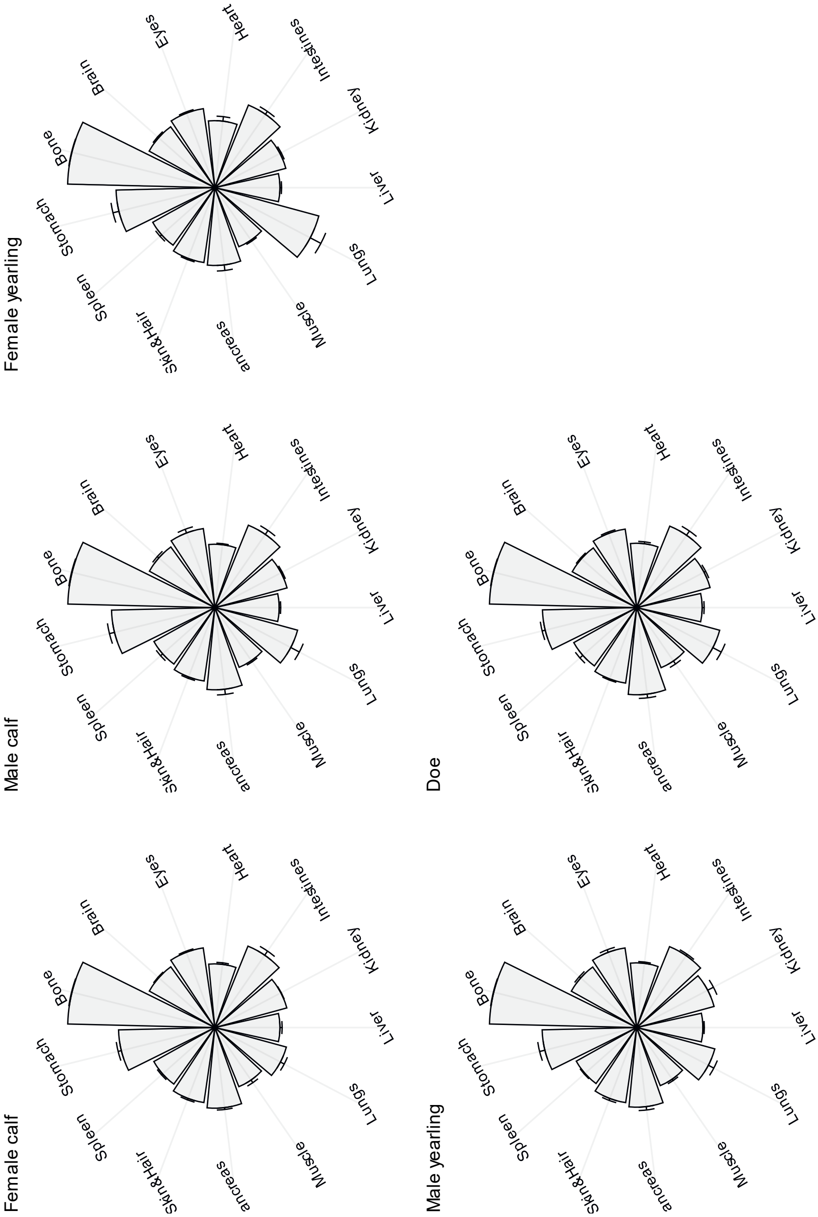
Distribution of aluminium (Al) per tissue per group.



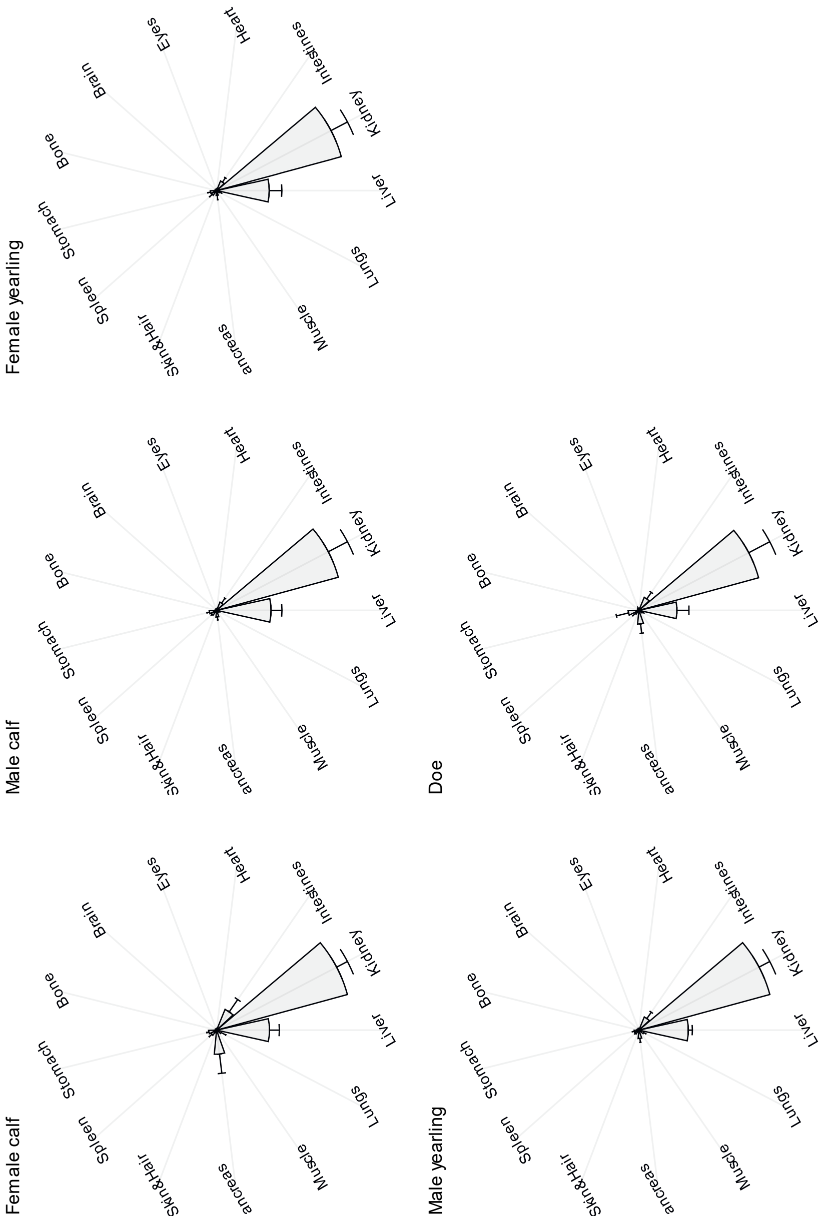
Distribution of arsenic (As) per tissue per group.



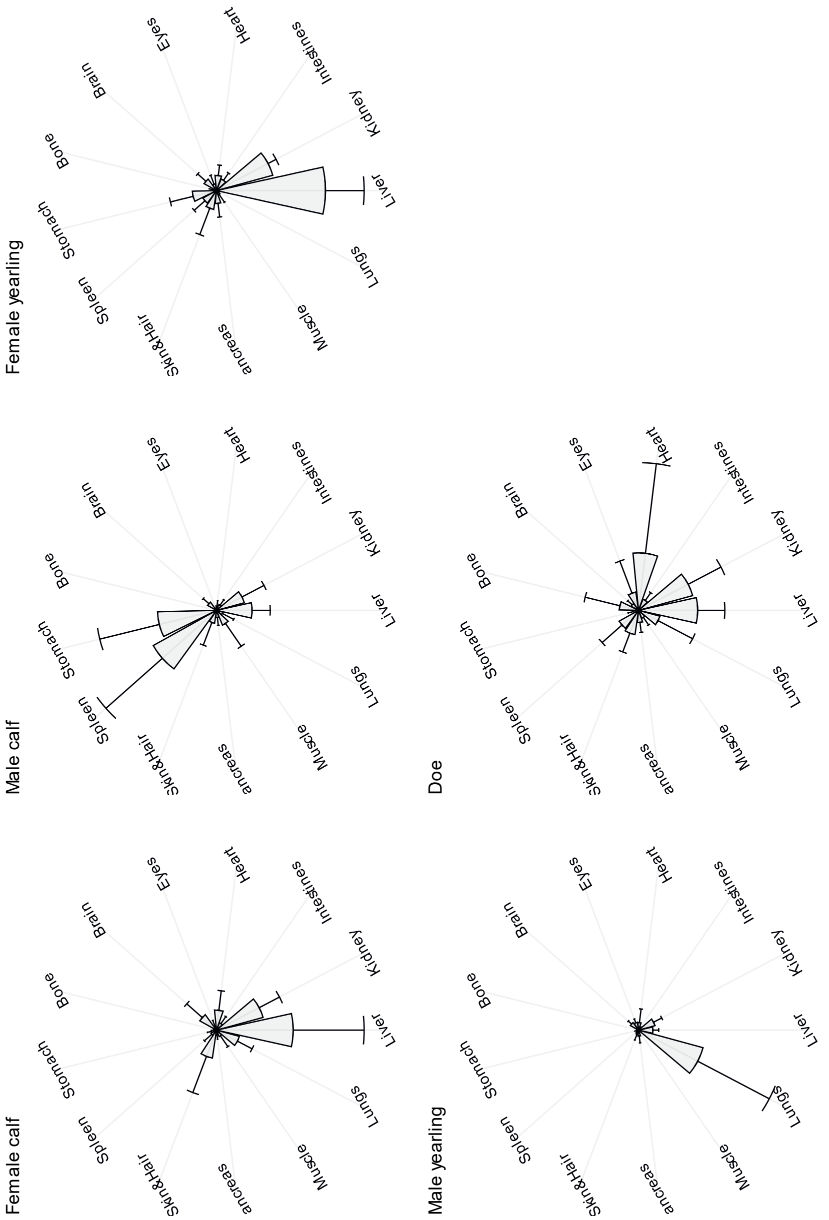
Distribution of boron ( ) p er tissue per group.



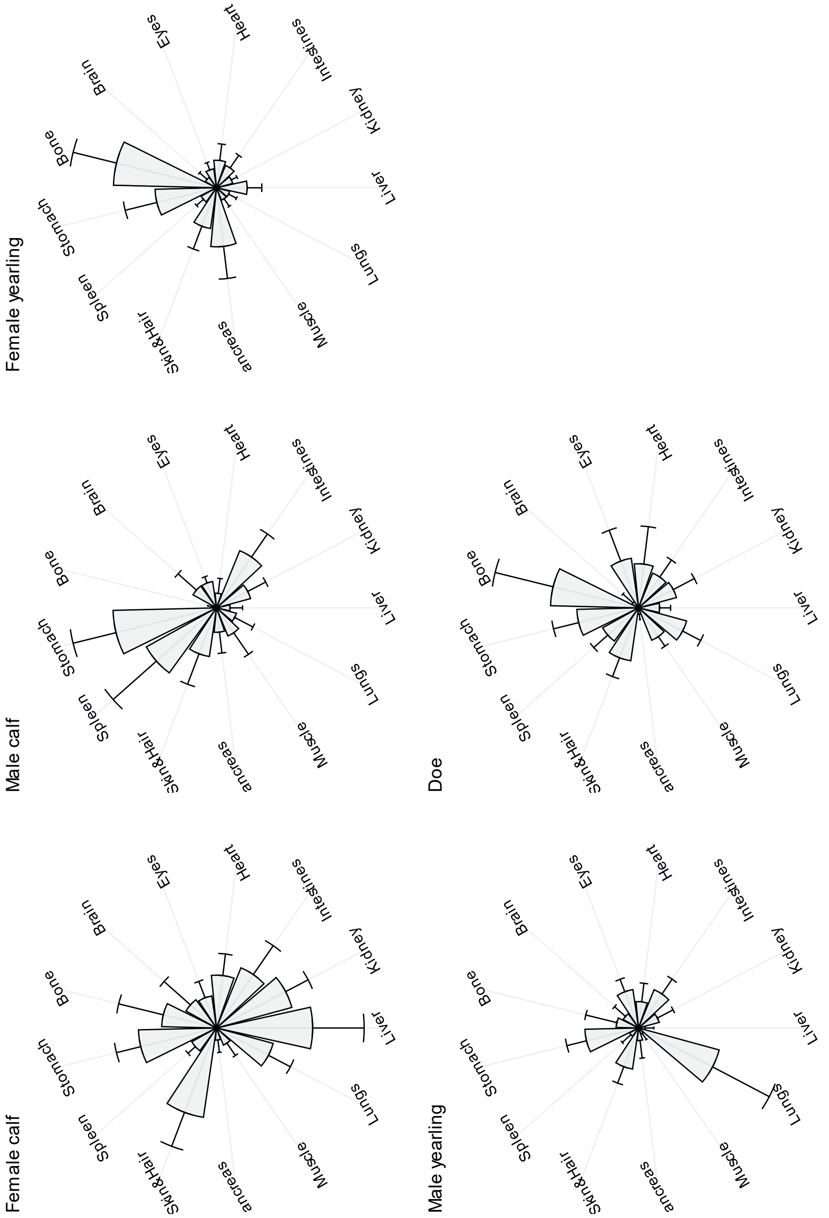
Distribution of calcium (Ca) per tissue per group.



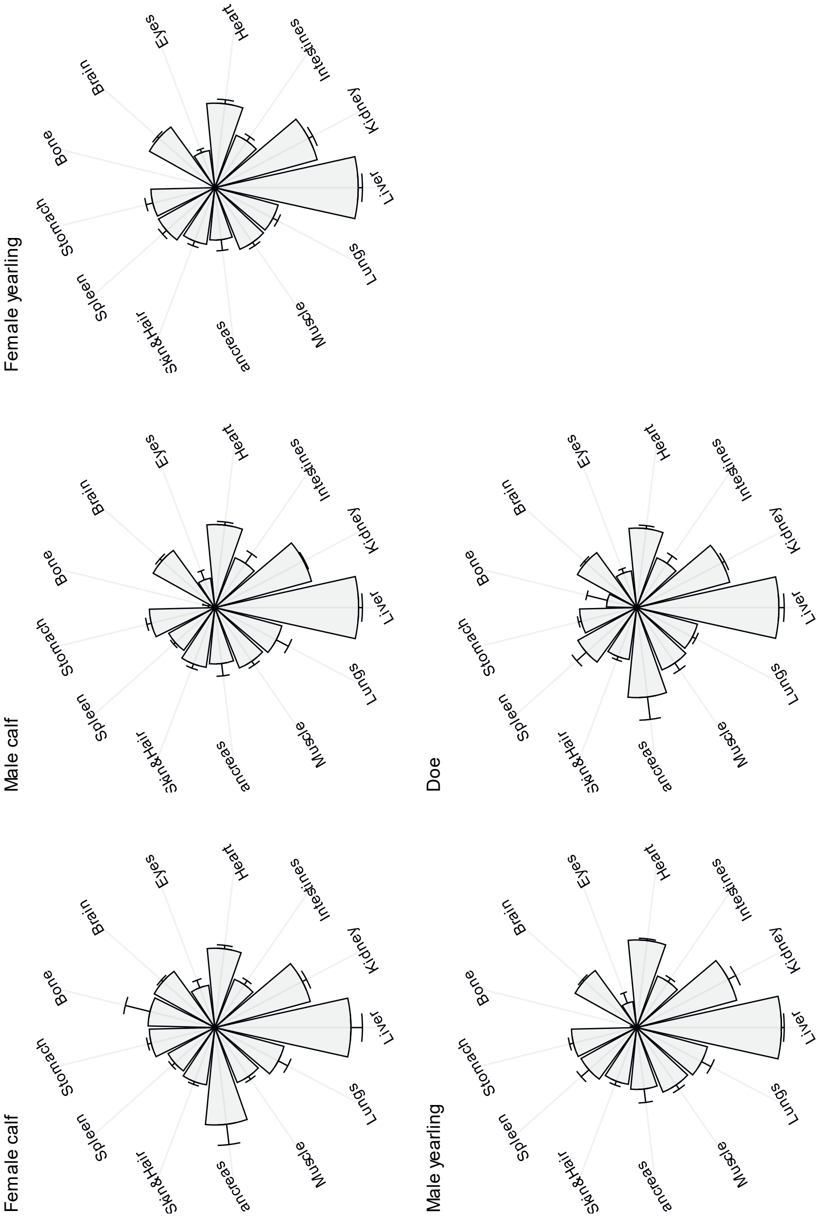
Distribution of cadmium (Cd) per tissue per group.



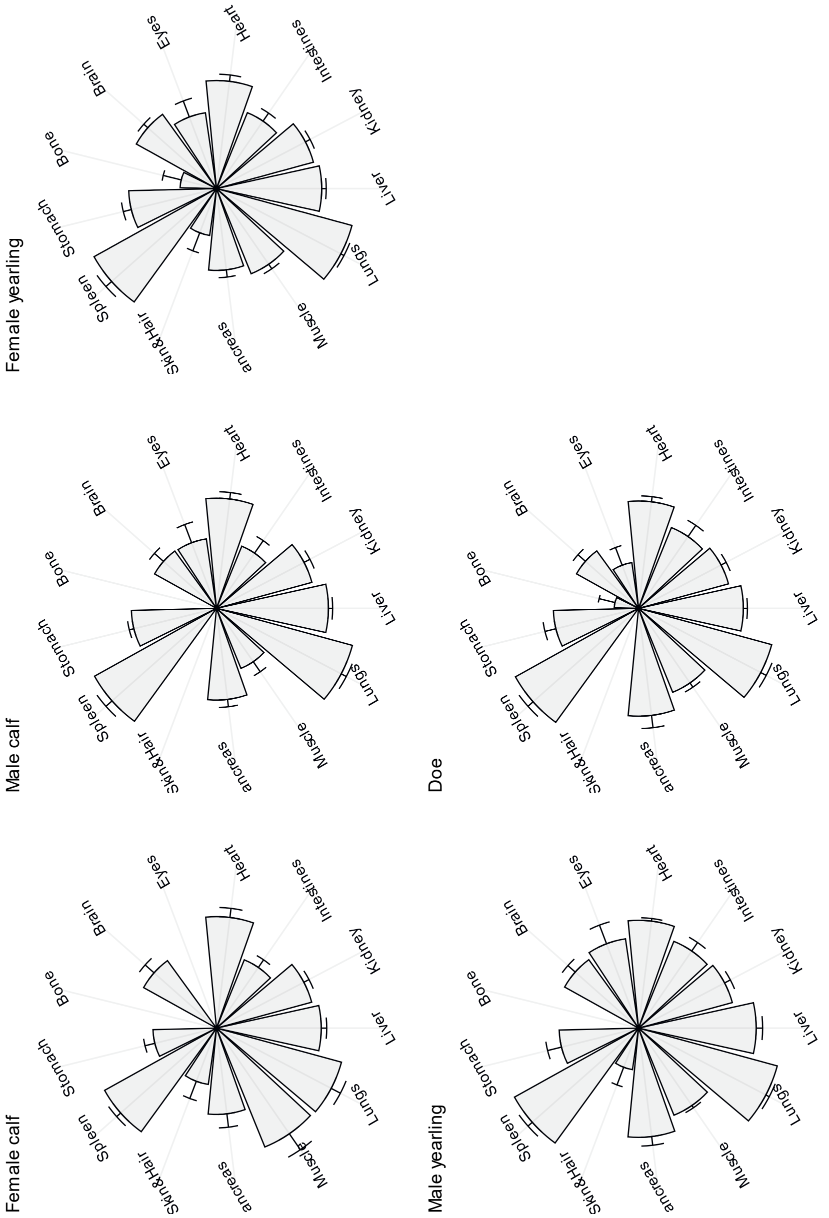
Distribution of cobalt (Co) per tissue per group.



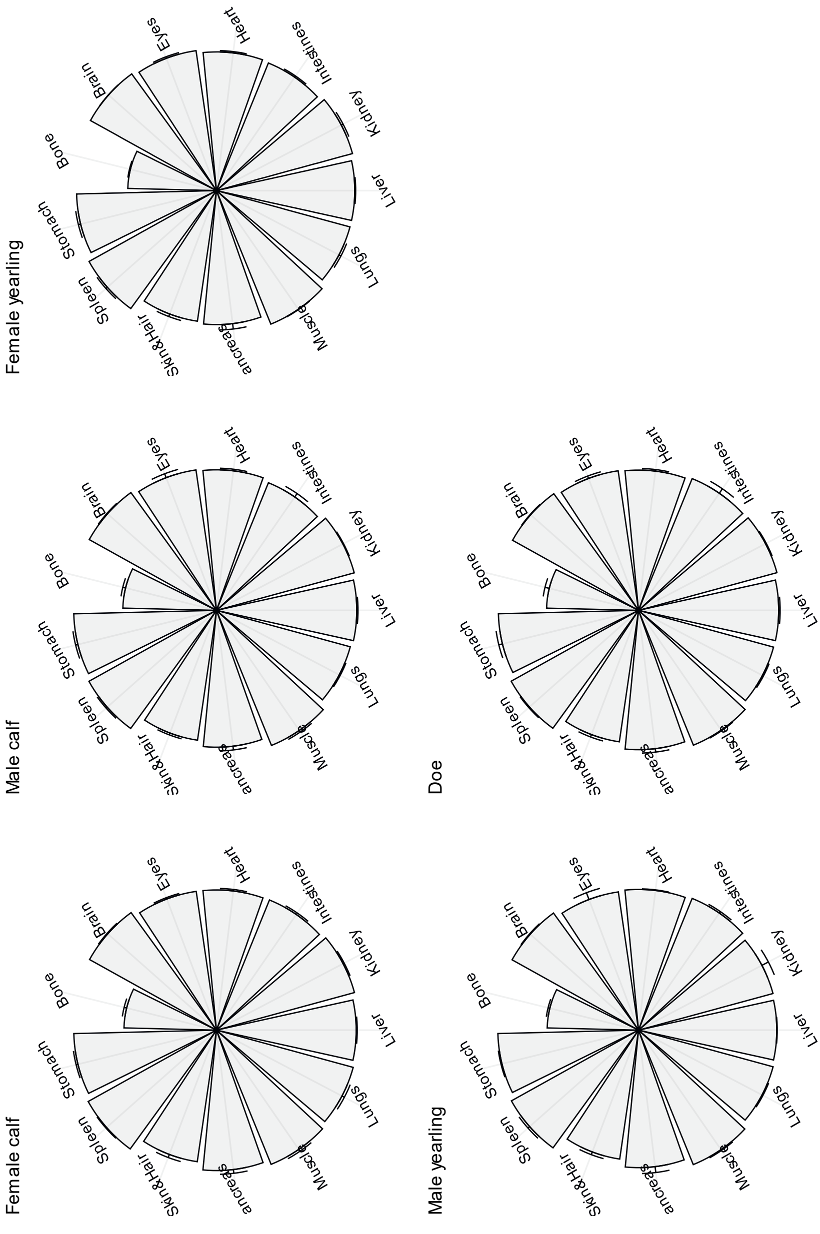
Distribution of chromium (Cr) per tissue per group.



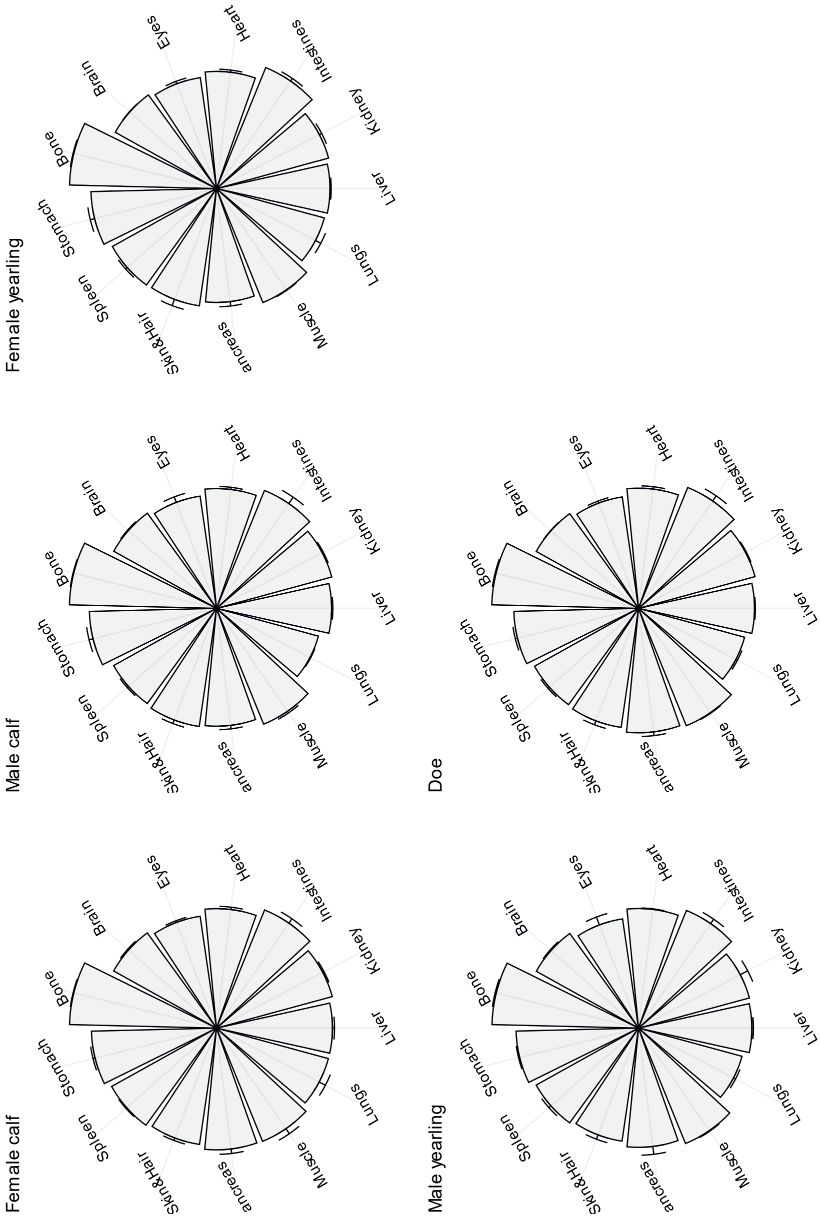
Distribution of copper (Cu) per tissue per group.



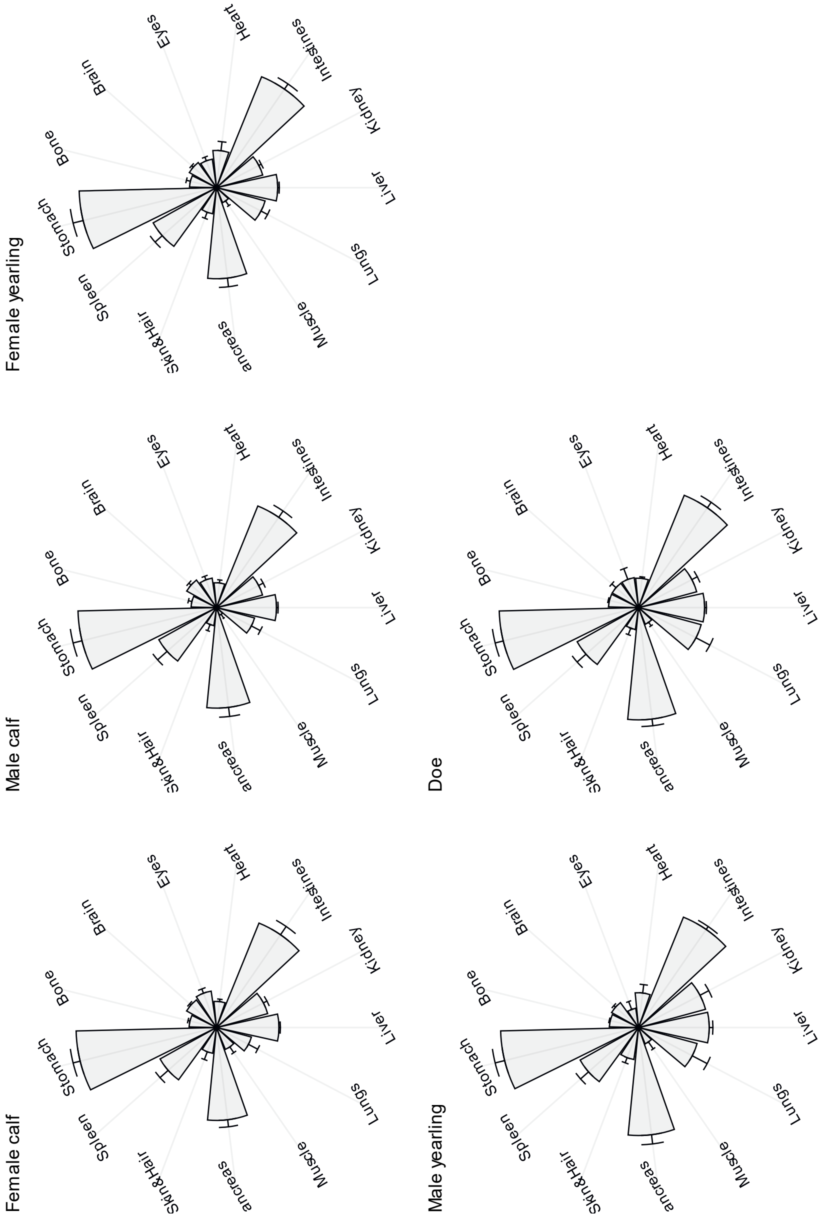
Distribution of iron (Fe) per tissue per group.



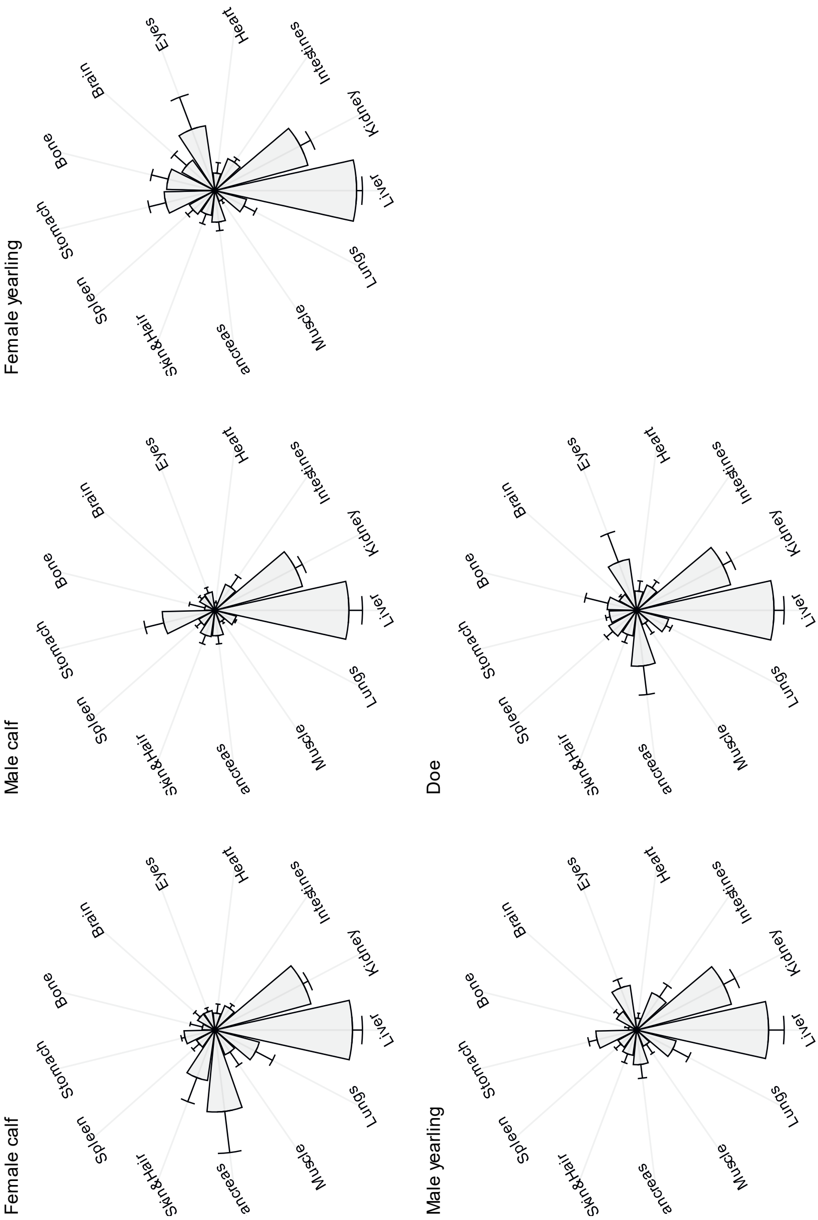
Distribution of potassium (K) per tissue per group.



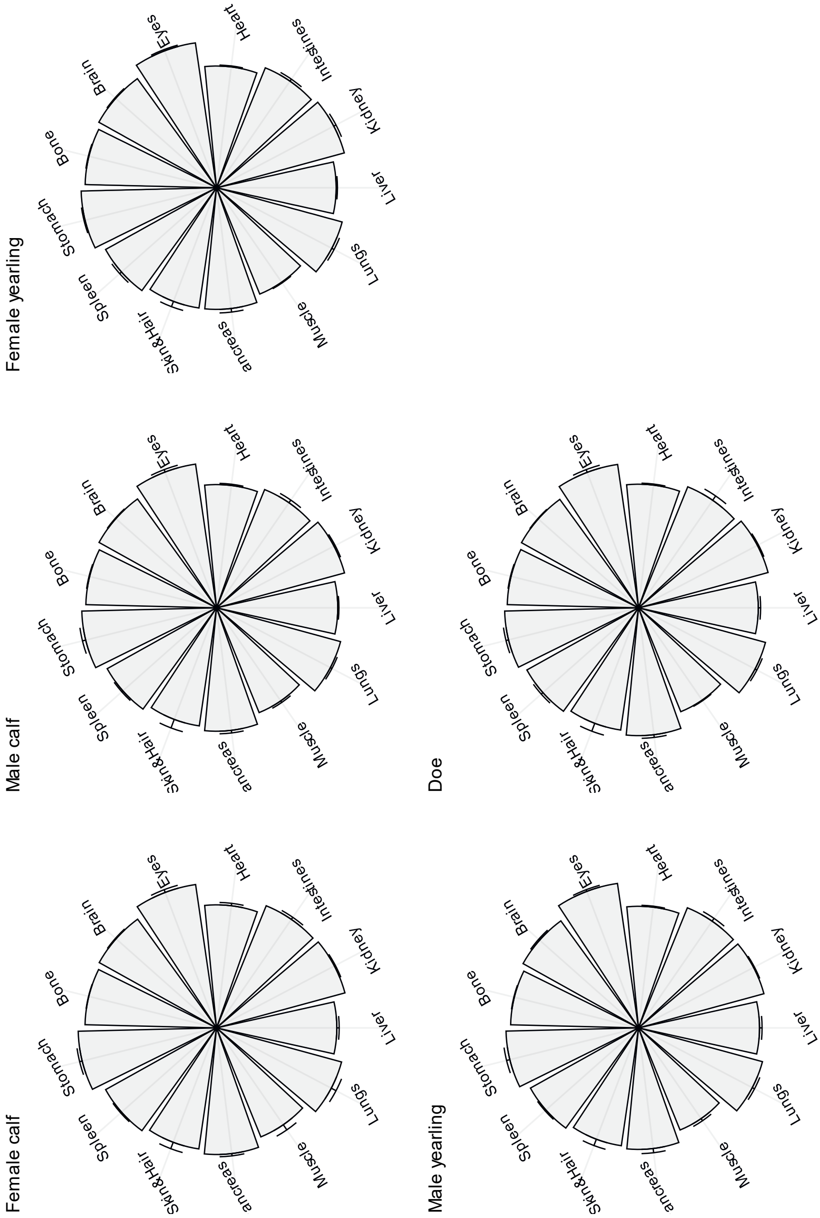
Distribution of magnesium (Mg) per tissue per group.



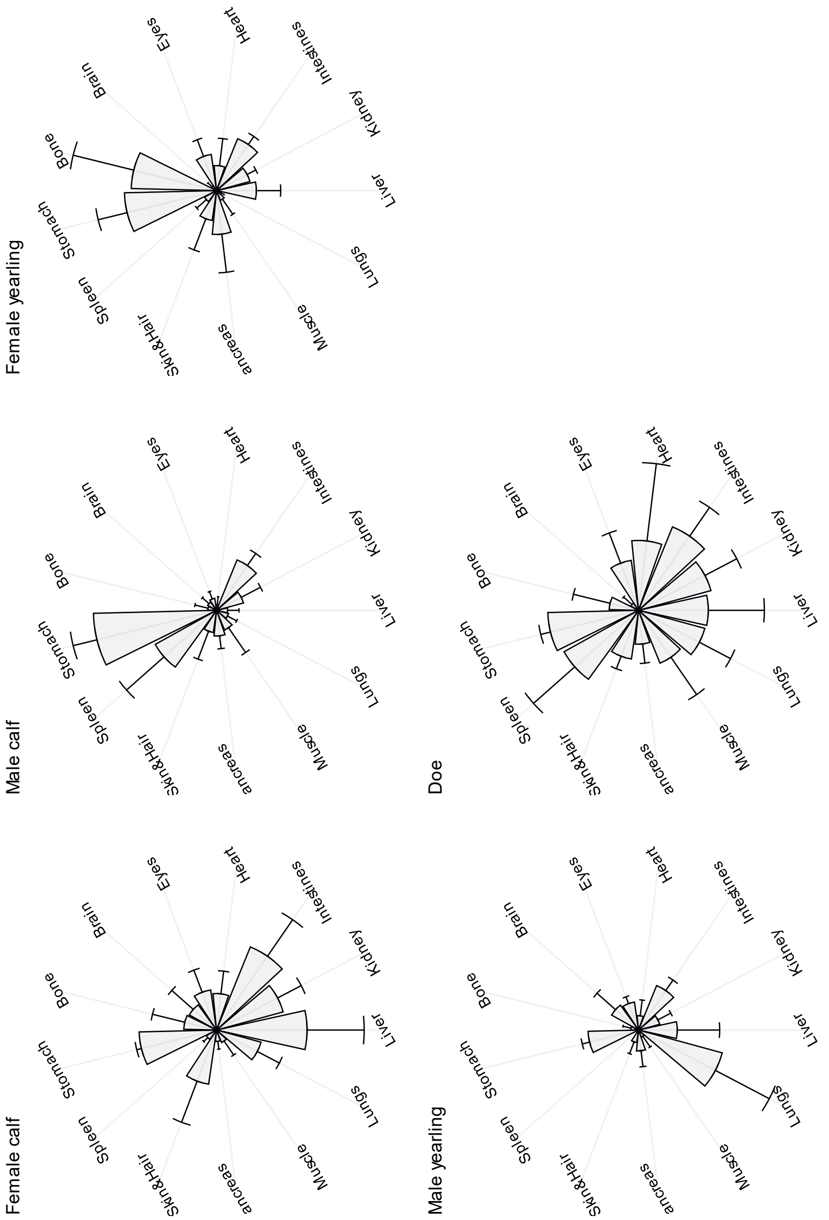
Distribution of manganese (Mn) per tissue per group.



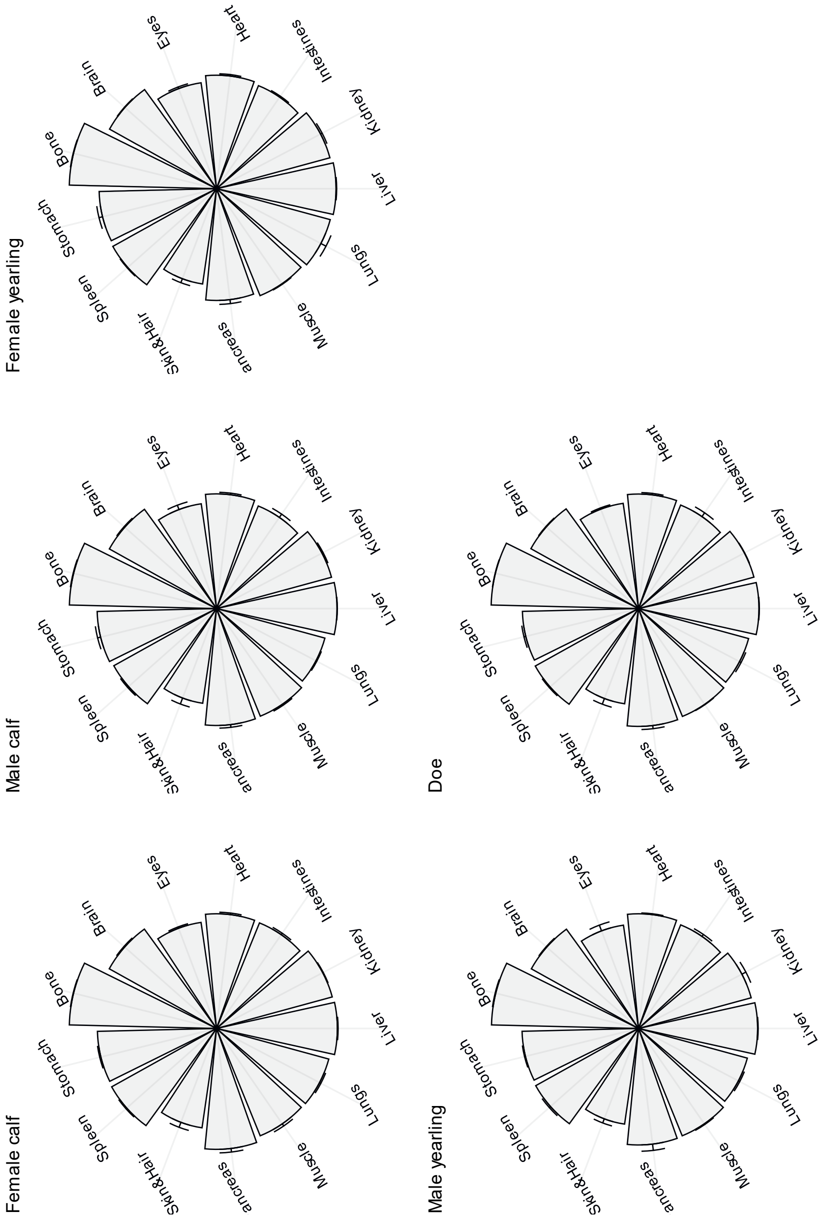
Distribution of molybdenum (Mo) per tissue per group.



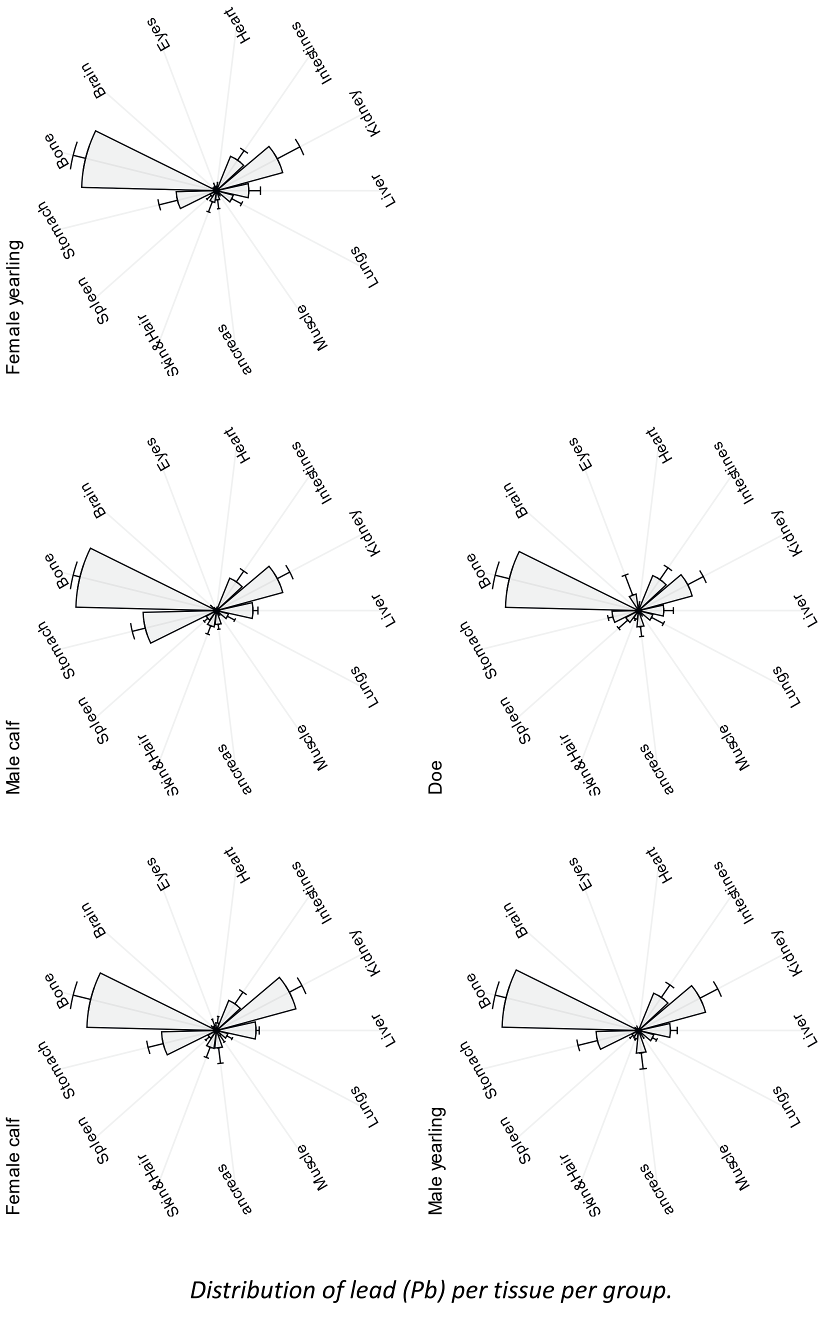
Distribution of sodium (Na) per tissue per group.



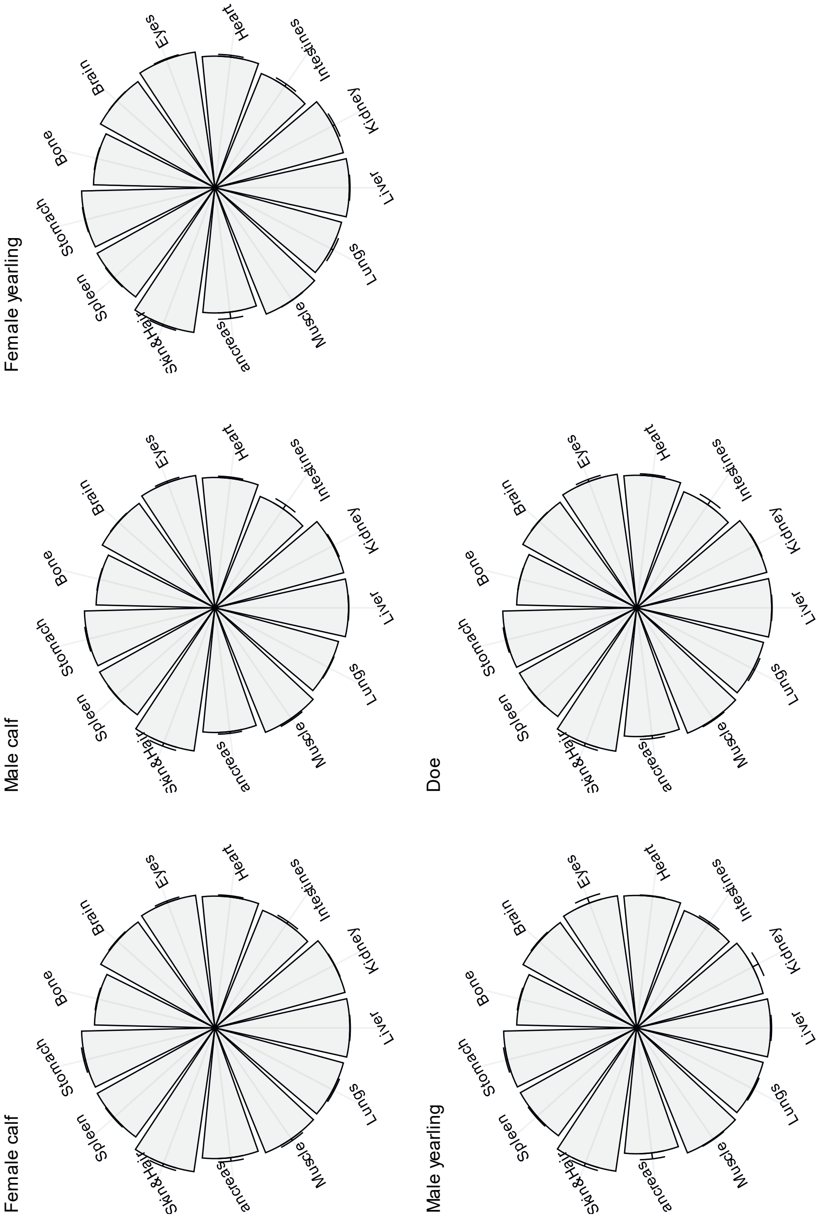
Distribution of nickel (Ni) per tissue per group.



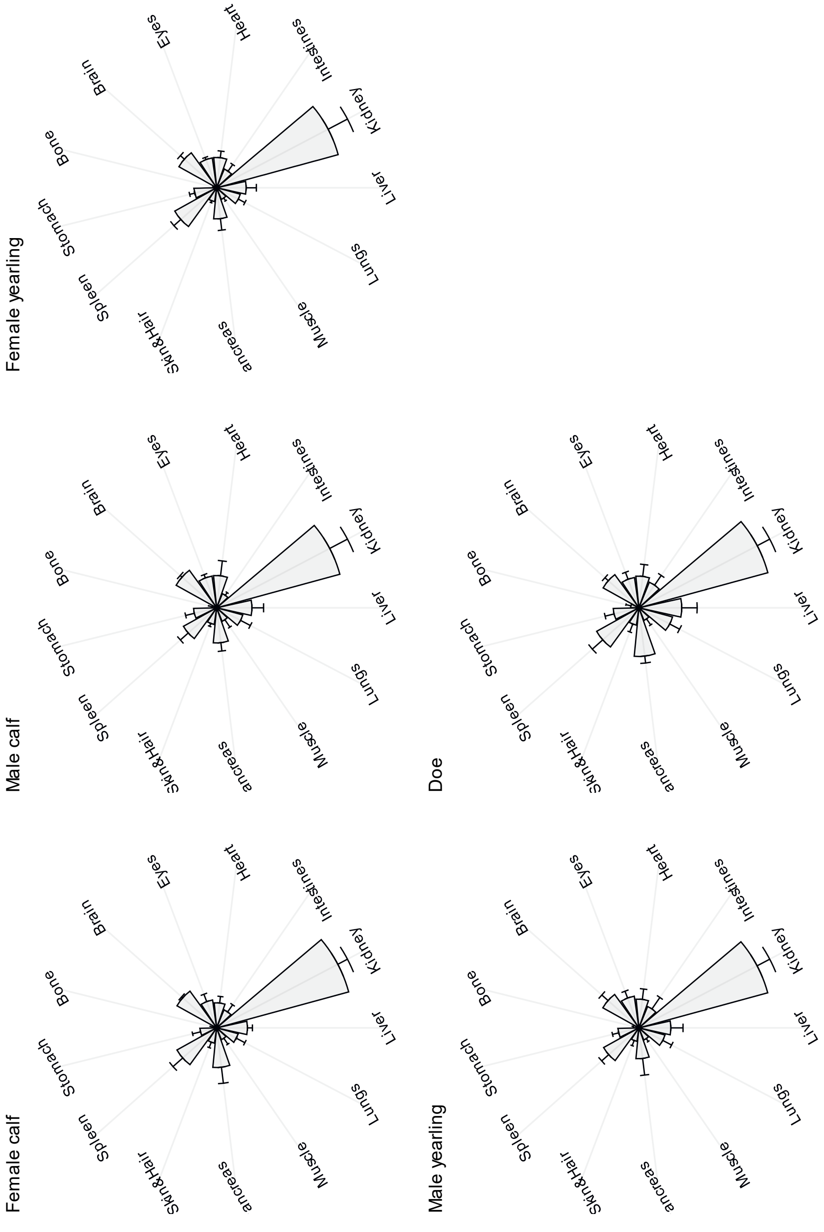
Distribution of phosphorous (P) per tissue per group.



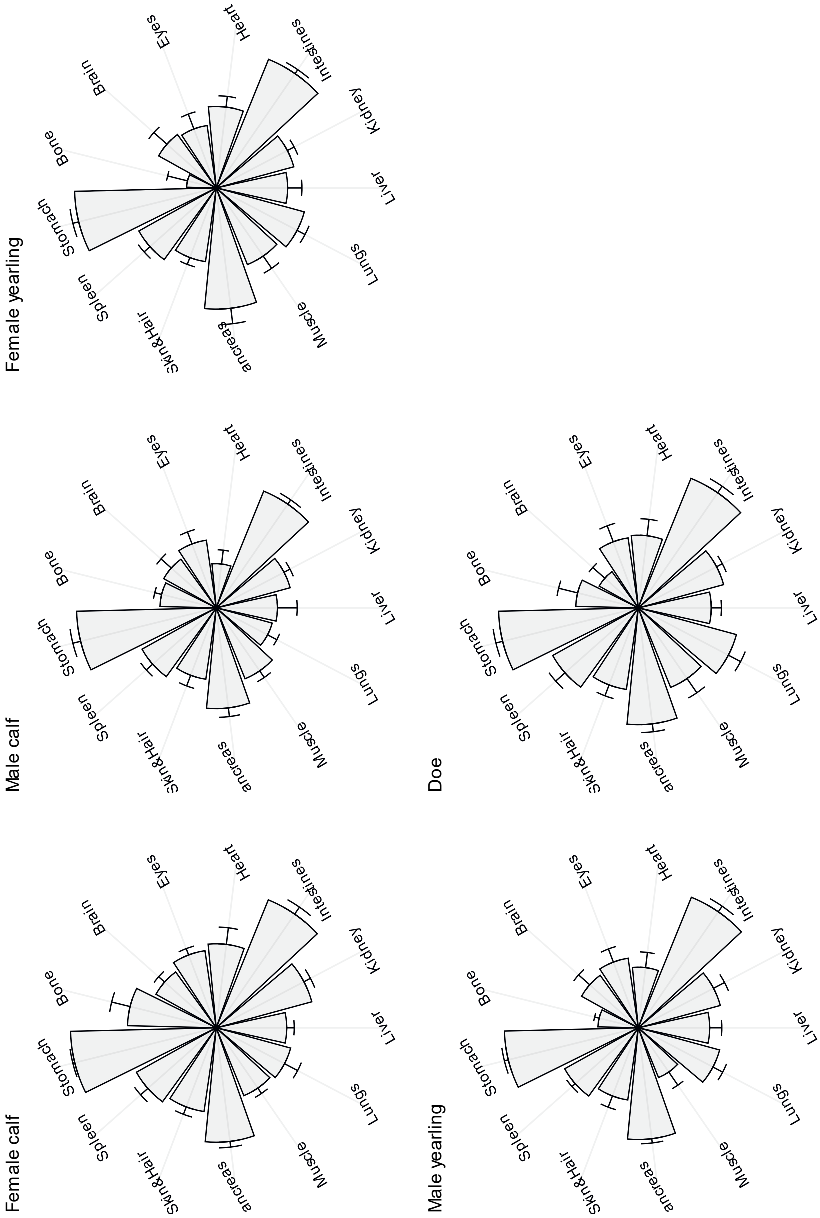
Distribution of lead (Pb) per tissue per group.



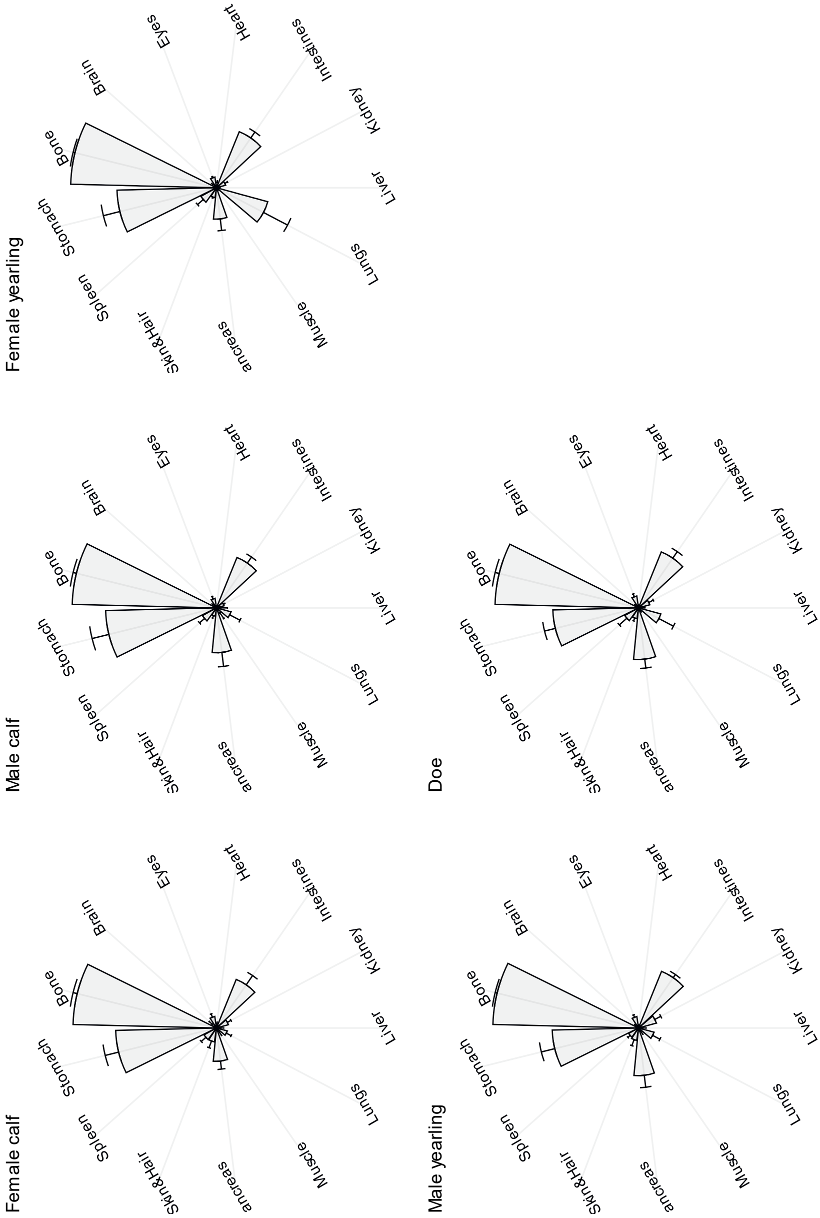
Distribution of sulfur (S) per tissue per group.



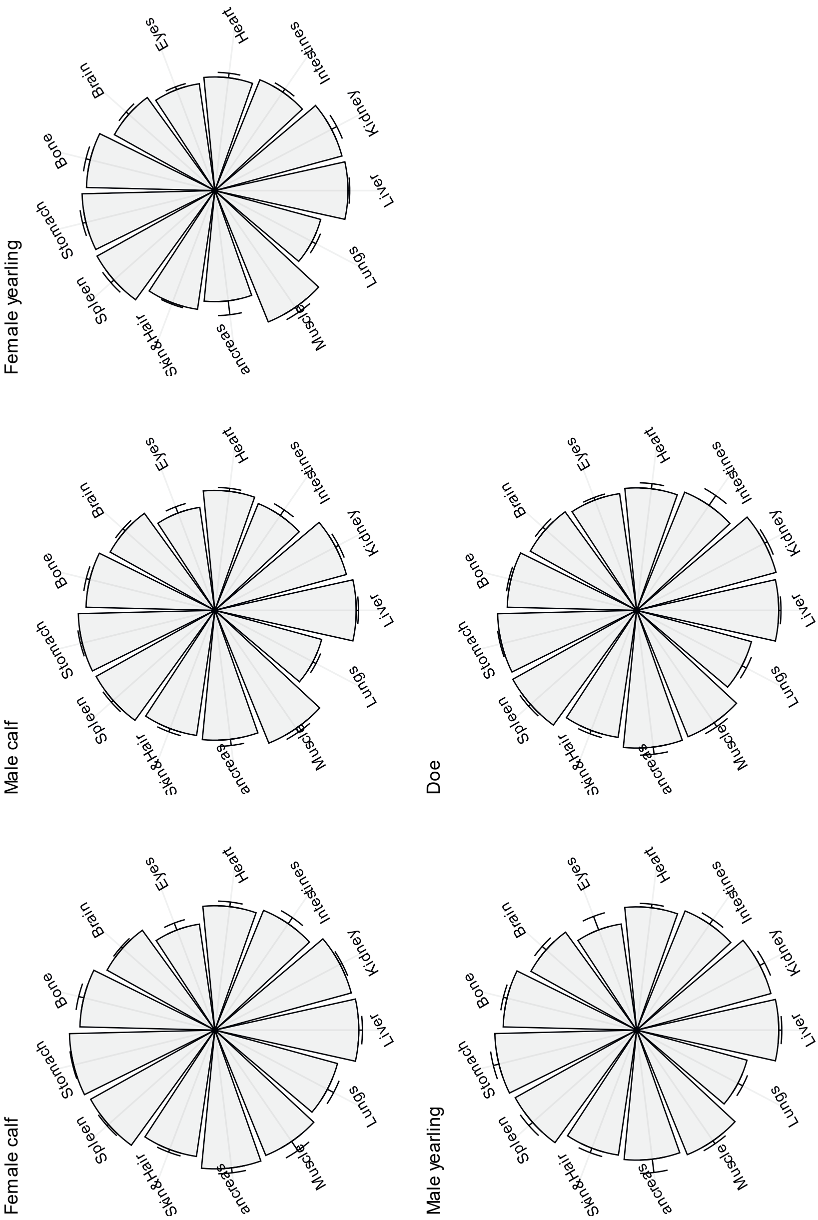
Distribution of selenium (Se) per tissue per group.



Distribution of silicon (Si) per tissue per group.



Distribution of strontium (Sr) per tissue per group.



Distribution of zinc (Zn) per tissue per group.

Appendix 5.1 Overview of the carcasses

ID	Individual carcass code	Area	Carcass species	Initial state*	Carcass placement	Carcass depletion	Days to depletion	Scavenger group codes**
1	1e_Hogerwaard-dwarsweg_1	Markiezaat	Roe deer	O	30-9-2019 15:02	30-10-2019	29.374	MBO
2	Hamert_1_ Werkschuur_1	De Hamert Estate	Roe deer	C	17-10-2012 11:00	5-11-2012	18.542	M
3	Hamert_1_ Werkschuur_2	De Hamert Estate	Roe deer	C	27-11-2012 15:26	3-1-2013	36.357	MBO
4	Hamert_1_ Werkschuur_3	De Hamert Estate	Sheep	O	15-3-2013 11:56	27-3-2013	11.503	MBO
5	Hamert_1_ Werkschuur_4	De Hamert Estate	Roe deer	C	3-4-2013 15:26	13-6-2013	70.357	MB
6	Hamert_3_ Werkschuur_1	De Hamert Estate	Roe deer	O	20-7-2015 9:14	29-9-2015	70.615	MBO
7	Hamert_3_ Werkschuur_2	De Hamert Estate	Roe deer	C	6-10-2015 8:18	3-11-2015	27.654	M
8	Hamert_3_ Werkschuur_3	De Hamert Estate	European badger	C	5-1-2016 13:07	15-1-2016	9.453	M
9	Hamert_2_ Heereven_1	De Hamert Estate	Roe deer	C	8-6-2013 10:35	2-7-2013	23.559	M
10	Hamert_2_ Heereven_2	De Hamert Estate	Roe deer	C	9-1-2014 15:47	1-2-2014	22.342	M
11	Hamert_2_ Heereven_3	De Hamert Estate	Roe deer	C	13-2-2014 9:56	11-5-2014	86.586	MBO

* C = closed carcass; decomposition started in bloated stage. O = opened carcass; decomposition started in active decay. ** Scavenger groups involved in the decomposition process: M = mammalian scavengers; B = scavenging birds; W = Wild boar; O = occasionals; and G = grazers.

ID	Individual carcass code	Area	Carcass species	Initial state*	Carcass placement	Carcass depletion	Days to depletion	Scavenger group codes**
12	Hamert_2_Heereven_4	De Hamert Estate	Roe deer	O	12-5-2014 9:12	31-5-2014	18.617	MB
13	Hamert_2_Heereven_5	De Hamert Estate	Roe deer	O	13-6-2014 10:25	10-7-2014	26.566	M
14	Hamert_2_Heereven_6	De Hamert Estate	Roe deer	C	6-11-2014 10:40	27-1-2015	81.556	MO
15	Hamert_2_Heereven_7	De Hamert Estate	Roe deer	C	21-3-2015 12:12	15-4-2015	24.492	MB
16	Hamert_2_Heereven_8	De Hamert Estate	Roe deer	O	17-4-2015 14:18	13-5-2015	25.404	MO
17	Hamert_2_Heereven_9	De Hamert Estate	Roe deer	O	27-1-2016 8:17	8-4-2016	71.655	MBO
18	Hamert_4_Bosrand_1	De Hamert Estate	Roe deer	C	28-9-2018 12:49	21-11-2018	53.466	MBO
19	KempenBroek_Loozerheide_1	Grenspark Kempen~Broek	Roe deer	C	27-2-2018 10:00	3-3-2018	3.583	MBG
20	KempenBroek_Loozerheide_2	Grenspark Kempen~Broek	Roe deer	C	20-3-2018 10:00	1-5-2018	41.583	MBOG
21	KempenBroek_Stramproy_1	Grenspark Kempen~Broek	Roe deer	C	22-2-2018 12:25	17-3-2018	22.483	MBWOG
22	Markiezaat_1_DeDuintjes_1	Markiezaat	Roe deer	C	19-2-2018 10:28	7-3-2018	15.564	MBG

* C = closed carcass; decomposition started in bloated stage. O = opened carcass; decomposition started in active decay. ** Scavenger groups involved in the decomposition process: M = mammalian scavengers; B = scavenging birds; W = Wild boar; O = occasionals; and G = grazers.

ID	Individual carcass code	Area	Carcass species	Initial state*	Carcass placement	Carcass depletion	Days to depletion	Scavenger group codes**
23	Melickerheide2_1	Meinweg National Park	Wild boar	C	21-5-2012 15:10	6-6-2012	15.368	MBWO
24	Melickerheide2_2	Meinweg National Park	Roe deer	C	7-6-2012 10:31	26-6-2012	18.562	MW
25	Wijffelterbroek_ Graus2_1	Grenspark Kempen~Broek	Roe deer	C	8-10-2015 15:52	27-11-2015	49.339	MBWO
26	Meinweg_1_ Eikelerveld_1	Meinweg National Park	Roe deer	C	6-11-2013 16:54	19-11-2013	12.296	MW
27	Meinweg_1_ Eikelerveld_2	Meinweg National Park	Roe deer	O	25-11-2013 14:29	2-12-2013	6.397	BW
28	Meinweg_1_ Eikelerveld_3	Meinweg National Park	European badger	O	6-2-2014 14:19	18-2-2014	11.403	MW
29	Meinweg_2_ Slenk_1	Meinweg National Park	Roe deer	O	14-1-2016 12:00	12-2-2016	28.5	MBO
30	Meinweg_2_ Slenk_2	Meinweg National Park	European badger	O	12-2-2016 13:09	24-2-2016	11.452	MB
31	Valkenhorst_ Wildviaduct_1	Valkenhorst Estate	Roe deer	C	9-8-2019 13:39	14-8-2019	4.431	W
32	Valkenhorst_ Wildviaduct_2	Valkenhorst Estate	Roe deer	O	11-11-2019 11:23	20-11-2019	8.526	BW
33	Valkenhorst_ Wildviaduct_3	Valkenhorst Estate	Roe deer	C	28-2-2020 14:43	13-3-2020	13.387	MWO

* C = closed carcass; decomposition started in bloated stage. O = opened carcass; decomposition started in active decay. ** Scavenger groups involved in the decomposition process: M = mammalian scavengers; B = scavenging birds; W = Wild boar; O = occasionals; and G = grazers.

ID	Individual carcass code	Area	Carcass species	Initial state*	Carcass placement	Carcass depletion	Days to depletion	Scavenger group codes**
34	Wijffelterbroek_Graus1_1	Grenspark Kempen~Broek	Wild boar	C	27-9-2013 12:5	1-1-2014	95.463	MBWO
35	Wijffelterbroek_Graus3_1	Grenspark Kempen~Broek	European badger	O	24-6-2014 15:15	6-11-2014	134.365	MO
36	Veluwezoom_1_1	Veluwezoom National Park	Wild boar	C	3-7-2020 10:22	18-8-2020	45.568	WO
37	Veluwezoom_2_1	Veluwezoom National Park	Wild boar	C	3-7-2020 11:49	4-8-2020	31.508	MWO
38	Veluwezoom_3_1	Veluwezoom National Park	Wild boar	C	3-7-2020 12:21	20-8-2020	47.485	MWOG
39	Veluwezoom_4_1	Veluwezoom National Park	Wild boar	C	3-7-2020 14:22	20-7-2020	16.401	MWOG
40	Veluwezoom_6_1	Veluwezoom National Park	Wild boar	C	5-8-2020 14:09	29-8-2020	23.41	MBW
41	Veluwezoom_11_1	Veluwezoom National Park	Fallow deer	C	30-10-2020 10:04	5-11-2020	5.581	MBW
42	Brunink_1	Overijssel	Roe deer	C	6-8-2019 13:52	25-1-2020	171.422	MBO
43	Brunink_2	Overijssel	Roe deer	C	8-2-2020 12:18	22-2-2020	13.488	M
44	Brunink_3	Overijssel	Roe deer	C	30-3-2020 14:04	22-5-2020	52.414	MBO

* C = closed carcass; decomposition started in bloated stage. O = opened carcass; decomposition started in active decay. ** Scavenger groups involved in the decomposition process: M = mammalian scavengers; B = scavenging birds; W = Wild boar; O = occasionals; and G = grazers.

ID	Individual carcass code	Area	Carcass species	Initial state*	Carcass placement	Carcass depletion	Days to depletion	Scavenger group codes**
45	Hegeveld_1	Overijssel	Roe deer	C	3-9-2019 8:55	16-11-2019	73.628	MO
46	Aamsveen_1	Overijssel	Roe deer	C	12-5-2020 12:00	2-7-2020	50.5	MBWO
47	PlankenWambuis_4_1	Planken Wambuis	Red deer	C	15-10-2019 9:15	20-10-2019	4.615	MW
48	PlankenWambuis_7_1	Planken Wambuis	Red deer	C	28-10-2019 16:30	16-11-2019	18.313	MBW
49	PlankenWambuis_8_1	Planken Wambuis	Red deer	C	1-11-2019 11:28	12-11-2019	10.522	MBW

* C = closed carcass; decomposition started in bloated stage. O = opened carcass; decomposition started in active decay. ** Scavenger groups involved in the decomposition process: M = mammalian scavengers; B = scavenging birds; W = Wild boar; O = occasionals; and G = grazers.

Appendix 5.2 Number of carcasses per combination of scavenger groups

(A) with Grazers included; ( ) without Grazers; and (C) the selected combinations.

A)		B)		C)	
Code	Count	Code	Count	Code	Count
BW	2	BW	2	M	7
M	7	M	7	MB	6
MB	4	MB	6	MBO	11
MBG	2	MBO	11	MBW	4
MBO	10	MBW	4	MBWO	5
MBOG	1	MBWO	5	MO	4
MBW	4	MO	4	MW	4
MBWO	4	MW	4	MWO	4
MBWOG	1	MWO	4		
MO	4	W	1		
MW	4	WO	1		
MWO	2				
MWOG	2				
W	1				
WO	1				
Total	49	Total	49	Total	45

Appendix 5.3 Overview of the scavenger species that were present per study area

X = with showing scavenging behaviour; + = without showing scavenging behaviour.

	Enschede area	Planken Wambuis	Veluwezoom National Park	Markiezaat	De Hamert Estate	Valkenhorst Estate	Grenspark Kempen~Broek	Meinweg National Park
Bos taurus			+				X	
E. caballus				X				
A. sylvaticus	X		+			+		+
C. capreolus	X		+	+	+	+	+	X
Parus major	X			X	X		X	
T. philomelos	X		X		+			
T. pilaris			X					
T. viscivorus			X					
Buteo buteo	X	X	X	X	X	X	X	X
Corvus corax		X	+		X			
C. corone				X	X		X	
C. lupus familias					X	+	+	
Felis catus					X		X	
Martes foina	X				X		X	X
M. putorius	X				X		X	+
Vulpes vulpes	X	X	X	X	X		X	X
Sus scrofa	X	X	X			X	X	X

Appendix 6.1 Number of observations per area of facultative vertebrate scavenger species

Area	Birds			Boar	Other mammals						
	B. buteo	C. corax	C. corone	S. scrofa	C. lupus familias	F. catus	M. foina	M. martes	M. meles	M. putorius	V. vulpes
Enschede area	5			9			130		2	392	41
Planken Wambuis	119	11		1670							279
Veluwezoom National Park	1006	4390	16	3970	1			52	34		421
Markiezaat	27		479								122
De Hamert Estate	235	1163	11		50	5	47		9	117	821
Valkenhorst Estate	3			1076	2						
Grenspark Kempen~Broek	91		224	285	2	56	26			1	58
Meinweg National Park	194			344			8			1	88
Total observations	*1680*	*5564*	*730*	*7291*	*55*	*61*	*211*	*52*	*45*	*511*	*1830*
% of scavenger group	*21%*	*70%*	*9%*	*100%*	*2%*	*2%*	*8%*	*2%*	*1.50%*	*18.50%*	*66%*

Appendix 6.2 Overview of test statistics

Test statistics belonging to prediction 1: the denser the tree cover, the longer it takes before carcasses are first detected and first scavenged by birds, but the faster carcasses are first detected and first scavenged by boars or other mammals.

Mixed effects Cox model (fixed factors)	Coefficient (β)	SE	z	p	
Birds - first detection, tree cover					
- *Tree cover*	-0.837	0.584	-1.43	0.15	
- *Temperature*	-0.117	0.044	-2.69	0.007	*
- *Carcass initial state*	0.349	0.414	0.84	0.4	
Birds - first scavenging, tree cover					
- *Tree cover*	-0.684	0.619	-1.11	0.27	
- *Temperature*	-0.153	0.051	-3.02	0.003	*
- *Carcass initial state*	0.317	0.459	0.69	0.49	
Boar - first detection, tree cover					
- *Tree cover*	1.292	0.663	1.95	0.052	
- *Temperature*	-0.096	0.05	-1.94	0.053	
- *Carcass initial state*	-1.078	0.667	-1.62	0.11	
Boar - first scavenging, tree cover					
- *Tree cover*	0.126	0.644	0.2	0.84	
- *Temperature*	-0.06	0.05	-1.2	0.23	
- *Carcass initial state*	-1.555	0.776	-2	0.045	*
Other mammals - first detection, tree cover					
- *Tree cover*	0.913	0.56	1.63	0.1	
- *Temperature*	-0.035	0.042	-0.85	0.4	
- *Carcass initial state*	0.238	0.429	0.55	0.58	
Other mammals - first scavenging, tree cover					
- *Tree cover*	0.866	0.601	1.44	0.15	
- *Temperature*	0.04	0.048	0.83	0.41	
- *Carcass initial state*	-0.423	0.484	-0.87	0.38	

Mixed effects Cox model (random effects)	Std. dev.	Variance		Std. dev.	Variance
Birds - first detection, tree cover			- first scavenging, tree cover		
- *Area*	0.017	<0.001		0.02	<0.001
- *Carcass species*	0.384	0.148		0.348	0.121
- *Start month*	0.017	<0.001		0.02	<0.001
Boar - first detection, tree cover			- first scavenging, tree cover		
- *Area*	1.115	1.244		1.336	1.786
- *Carcass species*	0.02	<0.001		0.02	<0.001
- *Start month*	0.02	<0.001		0.02	<0.001
Other mammals - first detection, tree cover			- first scavenging, tree cover		
- *Area*	0.171	0.029		0.372	0.138
- *Carcass species*	0.019	<0.001		0.823	0.677
- *Start month*	0.692	0.479		0.772	0.596

Test statistics belonging to prediction 2: the denser the tree cover, the lower the proportion of carcass consumed by birds, but the higher the proportion consumed by boars or other mammals.

Generalized linear mixed-effects model (GLMM) (fixed factors)	Coefficient (β)	SE	z	p
Birds - proportion consumed, tree cover				
Intercept	-0.041	0.845	-0.048	0.962
- Tree cover	-0.889	0.952	-0.934	0.35
- Temperature	-0.012	0.09	-0.132	0.895
- Carcass initial state	-0.147	0.658	-0.224	0.823
Boar - proportion consumed, tree cover				
Intercept	0.56	0.74	0.757	0.449
- Tree cover	-0.379	0.846	-0.448	0.654
- Temperature	0.008	0.057	0.142	0.887
- Carcass initial state	0.437	0.995	0.439	0.661
Other mammals - proportion consumed, tree cover				
Intercept	-0.407	0.579	-0.704	0.482
- Tree cover	0.337	0.637	0.529	0.597
- Temperature	0.062	0.041	1.513	0.13
- Carcass initial state	0.154	0.501	0.308	0.758

Generalized linear mixed-effects model (GLMM) (random factors)	Variance ($\sigma2$)	Std. dev.
Birds - proportion consumed, tree cover		
- *Area*	<0.001	<0.001
- *Carcass species*	<0.001	<0.001
- *Start month*	<0.001	<0.001
Boar - proportion consumed, tree cover		
- *Area*	<0.001	<0.001
- *Carcass species*	<0.001	<0.001
- *Start month*	<0.001	<0.001
Other mammals proportion consumed, tree cover		
- *Area*	<0.001	<0.001
- *Carcass species*	<0.001	<0.001
- *Start month*	<0.001	<0.001

Test statistics belonging to prediction 3: the sooner carcasses are first detected or first scavenged by birds, boars or other mammals, the higher the proportion of carcass consumed by these groups.

Linear mixed-effects model (LMM) (fixed factors)	Coefficient (β)	SE	z	p	
Birds - first detection, proportion consumed					
Intercept	0.261	0.638	0.409	0.683	
- Days first detection	-0.125	0.037	-3.36	<0.001	*
- Temperature	0.005	0.066	0.078	0.938	
- Carcass initial state	0.027	0.569	0.047	0.962	
Birds - first scavenging, proportion consumed					
Intercept	0.084	0.742	0.122	0.91	
- Days first scavenging	-0.068	0.041	-1.655	0.098	
- Temperature	0.059	0.071	0.82	0.412	
- Carcass initial state	-0.159	0.668	-0.238	0.812	
Boar - first detection, proportion consumed					
Intercept	0.463	0.713	0.65	0.516	
- Days first detection	0.005	0.023	0.22	0.826	
- Temperature	-0.006	0.054	-0.112	0.91	
- Carcass initial state	0.433	1.011	0.428	0.668	
Boar - first scavenging, proportion consumed					
Intercept	0.717	0.699	1.026	0.305	
- Days first scavenging	-0.038	0.012	-3.176	0.001	*
- Temperature	0.093	0.053	1.749	0.08	
- Carcass initial state	0.874	0.789	1.107	0.268	
Other mammals - first det., proportion consumed					
Intercept	-0.312	0.504	-0.618	0.537	
- Days first detection	0.008	0.016	0.489	0.625	
- Temperature	0.066	0.04	1.663	0.1	
- Carcass initial state	0.075	0.475	0.159	0.874	
Other mammals - first scav., proportion consumed					
Intercept	-0.176	0.611	-0.288	0.774	
- Days first scavenging	0.002	0.011	0.202	0.84	
- Temperature	0.073	0.044	1.66	0.1	
- Carcass initial state	-0.106	0.516	-0.206	0.837	

Linear mixed-effects model (LMM) (random factors)	Variance (σ2)	Std. dev.		Variance	Std. dev.
Birds - first detection, proportion consumed			- first scavenging, proportion consumed		
- *Area*	<0.001	<0.001		<0.001	<0.001
- *Carcass species*	<0.001	<0.001		<0.001	<0.001
- *Start month*	<0.001	<0.001		<0.001	<0.001
Boar - first detection, proportion consumed			- first scavenging, proportion consumed		
- *Area*	<0.001	<0.001		<0.001	<0.001
- *Carcass species*	<0.001	<0.001		<0.001	<0.001
- *Start month*	<0.001	<0.001		<0.001	<0.001
Other mammals - first det., proportion consumed			- first scavenging, proportion consumed		
- *Area*	<0.001	<0.001		<0.001	<0.001
- *Carcass species*	<0.001	<0.001		<0.001	<0.001
- *Start month*	<0.001	<0.001		<0.001	<0.001

Test statistics belonging to prediction 4: the carcass decomposition speed is not influenced by time to first detection or first scavenging by birds, but is accelerated by time to first detection or first scavenging by boars or other mammals.

Generalized linear mixed-effects model (GLMM) (fixed factors)	Coefficient (β)	SE	df	t	p	
Birds - depletion, first detection						
Intercept	25.671	14.715	14.572	1.745	0.102	
- Days first detection	1.079	0.908	27.871	1.188	0.245	
- Temperature	2.814	1.558	21.378	1.807	0.085	
- Carcass initial state	-27.709	14.706	26.118	-1.884	0.071	
Birds - depletion, first scavenging						
Intercept	35.238	16.186	13.452	2.177	0.048	
- Days first scavenging	-0.9	1.059	27.793	-0.85	0.403	
- Temperature	3.089	1.542	19.716	2.003	0.059	
- Carcass initial state	-27.946	14.981	24.716	-1.865	0.074	
Boar - depletion, first detection						
Intercept	32.41	18.003	11.985	1.8	0.097	
- Days first detection	1.13	0.409	12.975	2.765	0.016	*
- Temperature	-0.478	1.325	13.114	-0.36	0.724	
- Carcass initial state	-8.307	17.753	24.324	-0.468	0.644	
Boar - depletion, first scavenging						
Intercept	35.708	13.107	14.924	2.724	0.016	
- Days first scavenging	1.23	0.19	23.867	6.466	<0.001	*
- Temperature	-1.444	0.952	25.421	-1.516	0.142	
- Carcass initial state	-11.289	13.843	25.945	-0.815	0.422	
Other mammals - depletion first detection						
Intercept	25.068	11.974	22.766	2.093	0.048	
- Days first detection	1.231	0.364	45.649	3.379	0.002	*
- Temperature	0.716	0.944	36.434	0.759	0.453	
- Carcass initial state	-0.869	11.016	46.855	-0.079	0.938	

Generalized linear mixed-effects model (GLMM) (fixed factors)	Coefficient (β)	SE	df	t	p
Other mammals - depletion first scavenging					
Intercept	14.993	10.509	13.974	1.427	0.176
- Days first scavenging	1.304	0.228	44.993	5.731	<0.001 *
- Temperature	1.174	0.78	33.161	1.468	0.151
- Carcass initial state	-3.036	9.433	46.822	-0.322	0.749

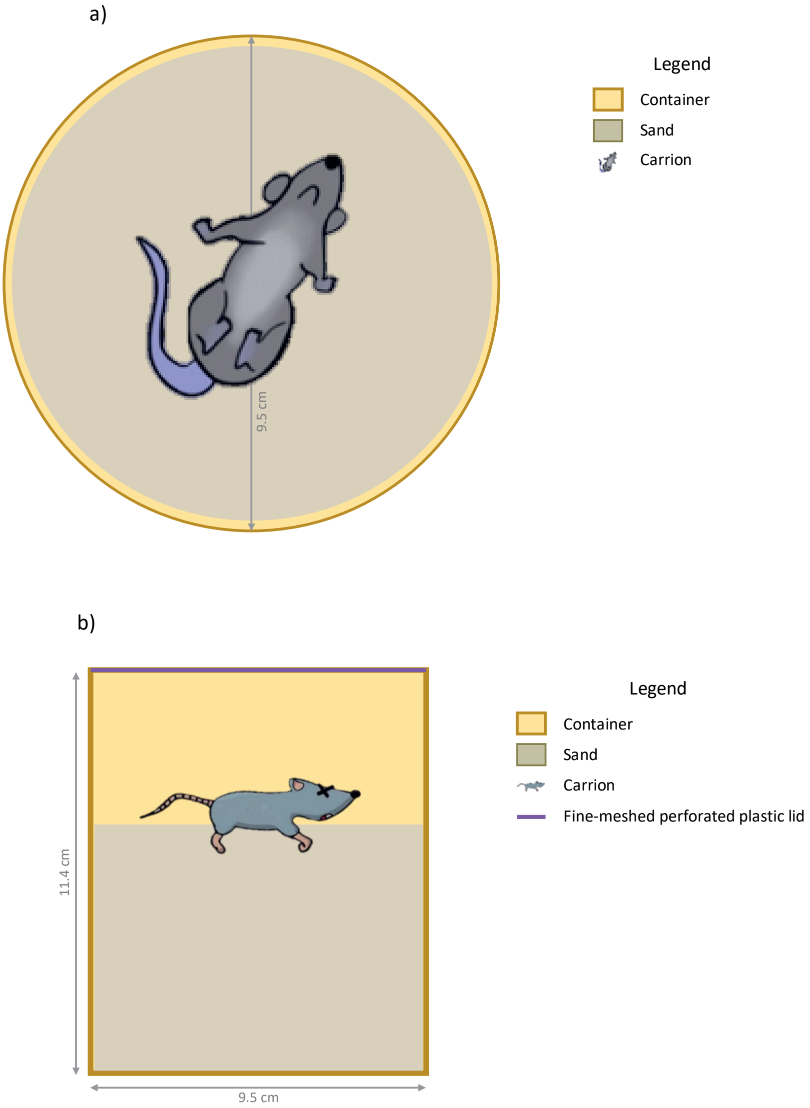
Generalized linear mixed-effects model (GLMM) (random factors)	Variance (σ2)	Std. dev.		Variance (σ2)	Std. dev.
Birds - depletion, first detection			- depletion, first scavenging		
- Area	0	0		0	0
- Carcass species	0	0		0	0
- Start month	111.5	10.56		101.5	10.07
Boar - depletion, first detection			- depletion, first scavenging		
- Area	353.6	18.8		<0.001	0.002
- Carcass species	0	0		<0.001	11.85
- Start month	257.4	16.04		<0.001	<0.001
Other mammals - depletion, first detection			- depletion, first scavenging		
- Area	153.9	12.4		104.1	10.202
- Carcass species	0	0		11.46	3.386
- Start month	183.4	13.54		87.62	9.361

Test statistics belonging to prediction 5: the carcass decomposition speed is not influenced by the proportion of carcass consumed by birds, but is accelerated when the proportions consumed by boars or other mammals increase.

Generalized linear mixed-effects model (GLMM) (fixed factors)	Coefficient (β)	SE	df	t	p	
Birds - depletion, first detection						
Intercept	6.902	13.459	16.93	0.513	0.615	
- Days first detection	40.897	15.241	22.418	2.683	0.013	*
- Temperature	2.06	1.265	13.316	1.629	0.127	
- Carcass initial state	-19.262	13.145	19.072	-1.465	0.159	
Birds - depletion, first scavenging						
Intercept	87.818	17.063	10.744	5.147	<0.001	
- Days first scavenging	-65.706	18.163	22.566	-3.618	0.001	*
- Temperature	0.147	1.242	21.002	0.118	0.907	
- Carcass initial state	-23.791	20.719	19.138	-1.148	0.265	
Boar - depletion, first detection						
Intercept	37.674	16.168	17.615	2.33	0.032	
- Days first detection	-3.509	13.876	43.614	-0.253	0.802	
- Temperature	0.973	1.09	33.62	0.893	0.378	
- Carcass initial state	0.039	13.726	41.816	0.003	0.998	

Generalized linear mixed-effects model (GLMM) (random factors)	Variance (σ2)	Std. dev.
Birds - depletion, first detection		
- *Area*	95.126	9.753
- *Carcass species*	<0.001	<0.001
- *Start month*	4.357	2.087
Boar - depletion, first detection		
- *Area*	<0.001	5.386
- *Carcass species*	<0.001	<0.001
- *Start month*	<0.001	0.002
Other mammals - depletion, first detection		
- *Area*	503.8	22.45
- *Carcass species*	<0.001	<0.001
- *Start month*	321.7	17.94

Appendix 7.1 Experimental design



Design of the experiment assessing the effect of microbes on nutrient fluxes related to carrion decomposition, showing the container top view (a) and front view (b).

Appendix 8.1 Photos of the experimental design

Aerial photographs from the locations where the experiment was executed: (a) Herikhuizen; and (b) e ekhuizen. The numbers indicate the parts of the locations where the different treatments were located: (1) allowing all scavengers; (2) excluding wild boar (Sus scrofa); (3) excluding all mammalian scavengers; and (4) excluding all vertebrate scavengers.

Photos of a fallow deer (*Dama dama*) carcass in each of the scavenger exclusion treatments: (a) allowing all scavengers; (b) excluding wild boar with a firm fence; (c) excluding all mammalian scavengers with an electricity fence; and (d) excluding all vertebrate scavengers using a cage.

A carcass (treatment 2) in the transportable scaffolding. This was used to lift the carcasses (to access the soil) and to put them back in exactly the same position as before.

Appendix 8.2 Test statistics and figures of the control samples

Test statistics belonging to the LMMs of the control soil samples.

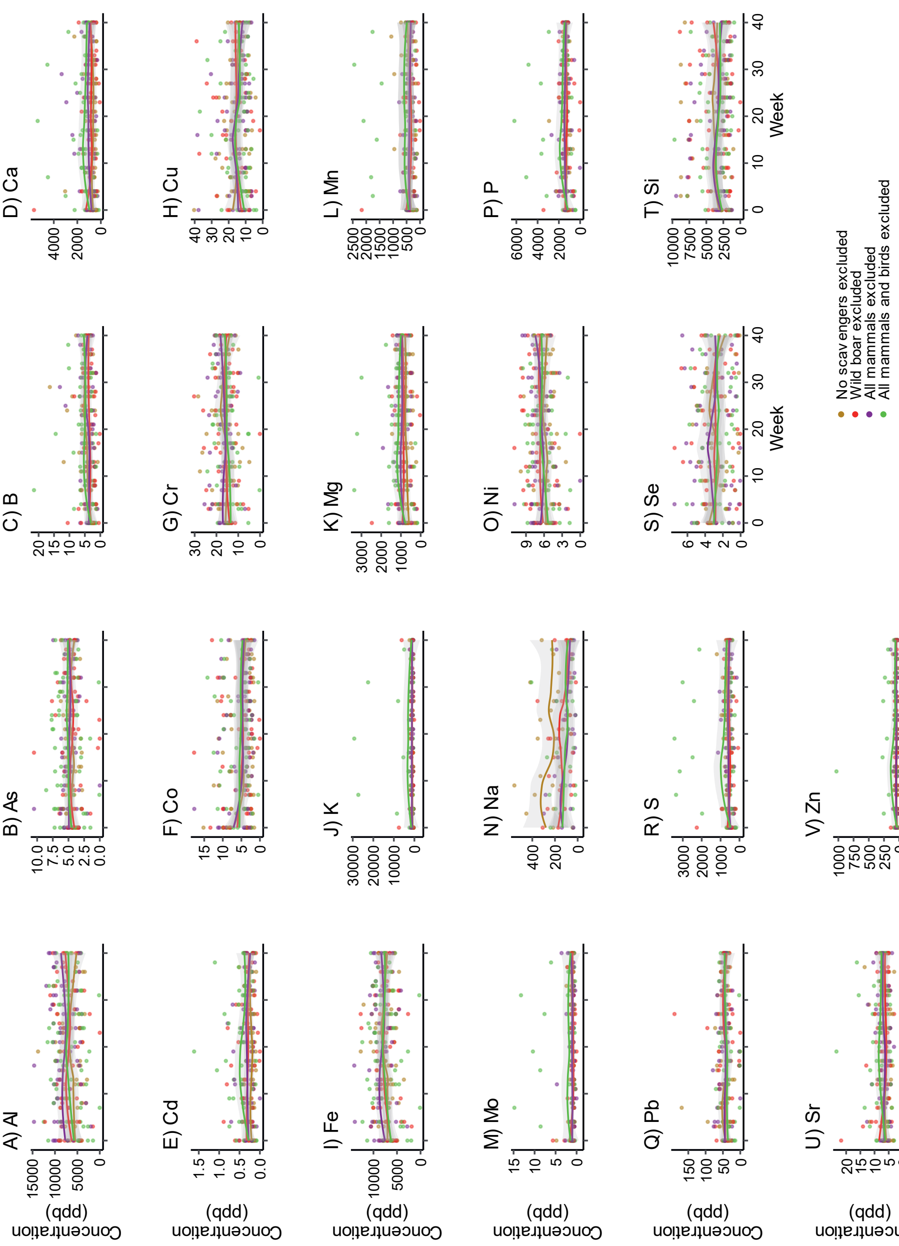
Element	Sum of Squares	Numerator Degrees of Freedom	Denominator Degrees of Freedom	F value	Adjusted p value
Al	3485740	3	243.104	0.327	0.979
As	2.056231	3	244.833	0.383	0.979
B	6.945086	3	244.383	0.518	0.979
Ca	903828.9	3	244.422	0.659	0.979
Cd	0.036743	3	242.738	0.432	0.979
Co	11.4317	3	243.974	0.935	0.979
Cr	16.53621	3	239.057	0.315	0.979
Cu	110.9044	3	236.481	1.005	0.979
Fe	5181358	3	241.988	0.515	0.979
K	1423367	3	244.174	0.064	0.979
Mg	108343.8	3	243.206	0.312	0.979
Mn	170708.5	3	245.703	0.647	0.979
Mo	0.816257	3	243.207	0.112	0.979
Na	6744.663	3	101.94	0.493	0.979
Ni	2.661034	3	244.002	0.356	0.979
P	85617.54	3	245.658	0.082	0.979
Pb	605.1686	3	244.172	0.731	0.979
S	88618.68	3	243.915	0.182	0.979
Se	2.538428	3	197.2	0.696	0.979
Si	4189220	3	244.099	0.999	0.979
Sr	37.58342	3	245.298	2.016	0.979
Zn	4355.319	3	239.713	0.313	0.979

Test statistics belonging to the LMMs of the control root samples.

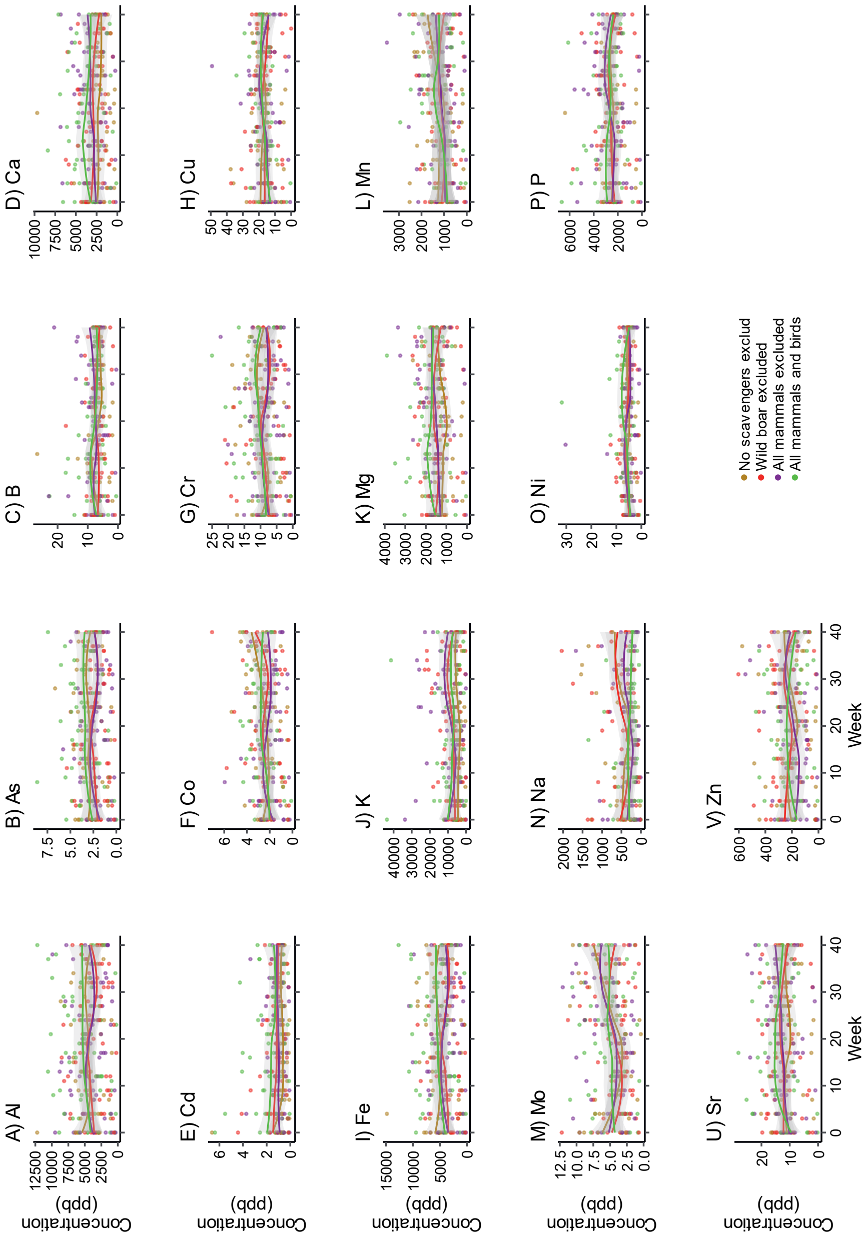
Element	Sum of Squares	Numerator Degrees of Freedom	Denominator Degrees of Freedom	F value	Adjusted p value
Al	14163854	3	245.709	1.012	0.547
As	3.136911	3	240.611	0.654	0.673
B	30.7333	3	240.78	0.99	0.547
Ca	10906357	3	244.946	1.954	0.547
Cd	2.540212	3	244.702	1.529	0.547
Co	5.757023	3	240.583	1.79	0.547
Cr	54.61888	3	243.395	1.09	0.547
Cu	202.0703	3	243.655	1.837	0.547
Fe	14739290	3	245.195	1.005	0.547
K	97925513	3	244.292	1.022	0.547
Mg	1100887	3	244.918	1.282	0.547
Mn	546403.1	3	244.445	0.575	0.695
Mo	11.4177	3	235.873	0.739	0.648
Na	312368	3	224.702	1.207	0.547
Ni	23.42884	3	243.213	0.874	0.589
P	3415026	3	244.569	1.254	0.547
Pb	317.4546	3	244.424	0.4	0.753
S	2523767	3	244.142	1.458	0.547
Se	6.540606	3	174.751	1.027	0.547
Si	14336349	3	243.213	0.435	0.753
Sr	99.80077	3	245.419	1.448	0.547
Zn	51654.77	3	245.241	1.323	0.547

Test statistics belonging to the LMMs of the control shoot samples.

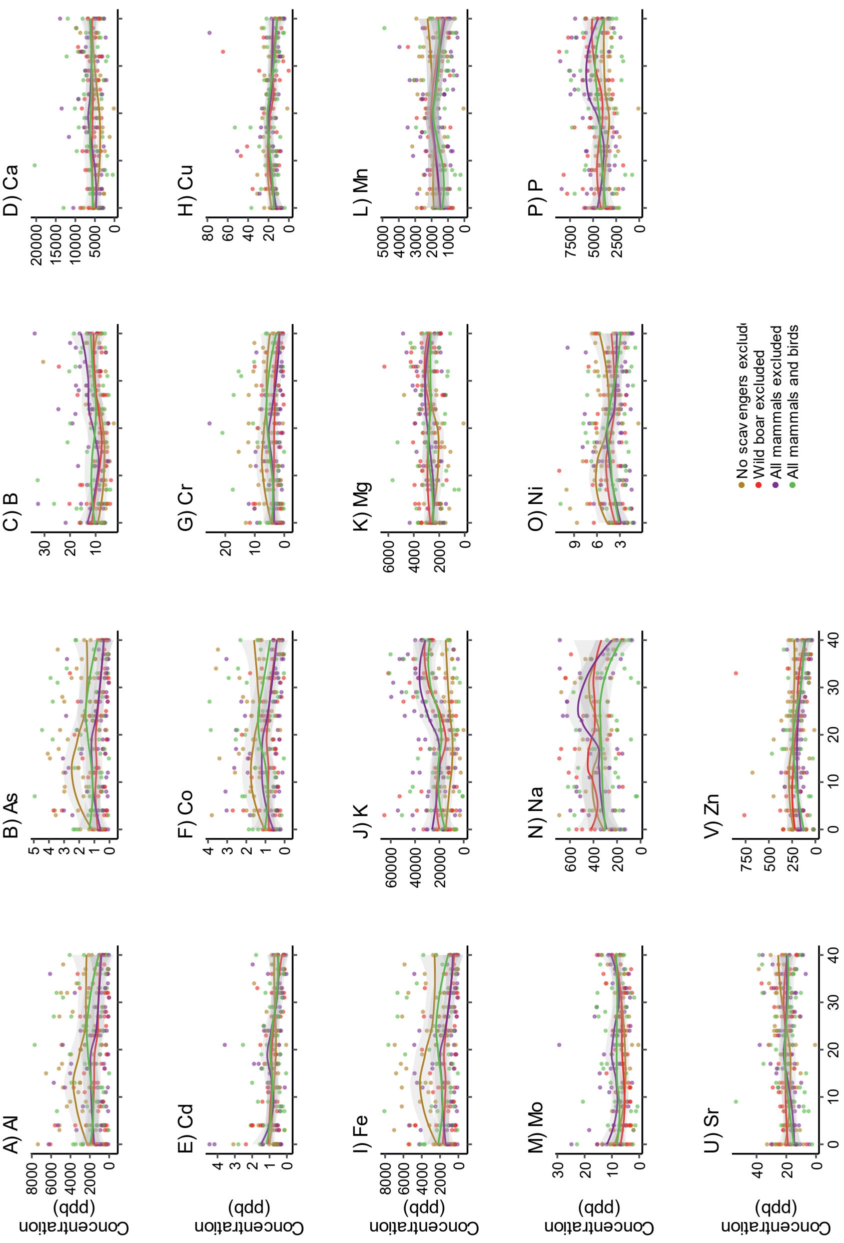
Element	Sum of Squares	Numerator Degrees of Freedom	Denominator Degrees of Freedom	F value	Adjusted p value
Al	2031125	3	236	0.28	0.965
As	0.472925	3	233	0.173	0.965
B	51.45427	3	217.064	0.799	0.965
Ca	7126969	3	231.014	0.51	0.965
Cd	1.243894	3	229.507	1.549	0.965
Co	1.207697	3	226.792	0.709	0.965
Cr	20.60693	3	229.508	0.499	0.965
Cu	79.73795	3	231.24	0.344	0.965
Fe	2328665	3	231.937	0.291	0.965
K	6.78E+08	3	231.4	1.585	0.965
Mg	464057.5	3	231.237	0.261	0.965
Mn	922685	3	230.778	0.807	0.965
Mo	50.2785	3	229.958	1.207	0.965
Na	67138.73	3	151.03	0.975	0.965
Ni	1.264184	3	233.263	0.13	0.965
P	4879887	3	231.264	0.861	0.965
Pb	199.5373	3	228.627	0.365	0.965
S	402333.7	3	232.011	0.092	0.965
Se	2.17702	3	182.483	0.534	0.965
Si	3.78E+08	3	234	0.654	0.965
Sr	97.55687	3	229.786	0.728	0.965
Zn	18337.59	3	230.189	0.598	0.965



Elemental concentrations of the control soil samples.



Elemental concentrations of the control root samples.



Elemental concentrations of the control shoot samples.

Appendix 8.3 Test statistics of the comparison of the initial elemental concentrations

Test statistics belonging to the Mann-Whitney U tests of the initial concentrations in the soil samples.

Element	W	Adjusted p value
B	468	0.691
Ca	462	0.691
Cu	462	0.691
K	432	0.877
Mg	458	0.691
Mn	431	0.877
Mo	511	0.691
Ni	459	0.691
P	461	0.691
S	459	0.691
Zn	461	0.691

Test statistics belonging to the Mann-Whitney U tests of the initial concentrations in the root samples.

Element	W	Adjusted p value
B	431	0.943
Ca	408	0.943
Cu	380	0.943
K	464	0.943
Mg	413	0.943
Mn	401	0.943
Mo	355	0.943
Ni	316	0.943
P	447	0.943
S	435	0.943
Zn	410	0.943

Test statistics belonging to the Mann-Whitney U tests of the initial concentrations in the shoot samples.

Element	W	Adjusted p value
B	302	0.733
Ca	360	0.945
Cu	511	0.118
K	404.5	0.777
Mg	369	0.945
Mn	414	0.733
Mo	382	0.942
Ni	398	0.785
P	414	0.733
S	435	0.733
Zn	438	0.733

Appendix 8.4 Test statistics of the samples on the carcass sites

Test statistics belonging to the interaction terms of treatment and sampling week of the LMMs of the soil samples beneath the carcasses.

Element	Sum of Squares	Numerator Degrees of Freedom	Denominator Degrees of Freedom	F value	Adjusted p value
B	0.577507	3	258.21	0.587	0.993
Ca	0.86183	3	259.473	0.42	0.993
Cu	21.81054	3	252.529	5.917	0.004
K	5.013046	3	260.122	0.04	0.993
Mg	0.042968	3	259.226	0.029	0.993
Mn	0.110249	3	259.673	0.032	0.993
Mo	0.239433	3	259.644	0.051	0.993
Ni	0.638478	3	258.516	1.357	0.94
P	1.230962	3	259.139	0.55	0.993
S	2.126002	3	259.683	0.537	0.993
Zn	482.2165	3	259.013	7.795	<0.001

Test statistics belonging to the Tukey post hoc tests of the statistically significant elements of the interaction terms of treatment and sampling week of the LMMs of the soil samples beneath the carcasses.

	Estimate	SE	df	t.ratio	p value
Element: Cu					
Treatment 1 x Treatment 2	-0.211	0.207	256	-1.02	0.738
Treatment 1 x Treatment 3	0.054	0.204	257	0.264	0.994
Treatment 1 x Treatment 4	-1.251	0.203	256	-6.171	<0.001
Treatment 2 x Treatment 3	0.265	0.188	253	1.409	0.495
Treatment 2 x Treatment 4	-1.04	0.188	252	-5.532	<0.001
Treatment 3 x Treatment 4	-1.305	0.184	252	-7.104	<0.001
Element: Zn					
Treatment 1 x Treatment 2	-0.27	0.833	264	-0.324	0.988
Treatment 1 x Treatment 3	-0.381	0.829	265	-0.459	0.968
Treatment 1 x Treatment 4	-6.025	0.829	264	-7.269	<0.001
Treatment 2 x Treatment 3	-0.111	0.753	259	-0.147	0.999
Treatment 2 x Treatment 4	-5.755	0.757	258	-7.599	<0.001
Treatment 3 x Treatment 4	-5.644	0.747	258	-7.555	<0.001

Test statistics belonging to the interaction terms of treatment and sampling week of the LMMs of the root samples on the edge of the carcasses.

Element	Sum of Squares	Numerator Degrees of Freedom	Denominator Degrees of Freedom	F value	Adjusted p value
B	54.14095	3	255.39	0.303	0.906
Ca	41.50442	3	255.192	1.191	0.575
Cu	248.2412	3	239.049	7.234	<0.001
K	2076.519	3	255.02	1.609	0.575
Mg	36.21557	3	255.029	0.948	0.657
Mn	3112.679	3	256.796	0.007	0.999
Mo	10.00443	3	251.365	0.69	0.769
Ni	14.53721	3	252.024	1.31	0.575
P	70.89681	3	255.04	2.015	0.575
S	144.1816	3	256.041	1.197	0.575
Zn	324.5851	3	253.847	0.347	0.906

Test statistics belonging to the Tukey post hoc tests of the statistically significant elements of the interaction terms of treatment and sampling week of the LMMs of the root samples on the edge of the carcasses.

	Estimate	SE	df	t.ratio	p value
Element: Cu					
Treatment 1 x Treatment 2	0.369	0.627	240	0.588	0.936
Treatment 1 x Treatment 3	0.33	0.63	241	0.523	0.953
Treatment 1 x Treatment 4	-2.751	0.652	240	-4.22	<0.001
Treatment 2 x Treatment 3	-0.039	0.573	239	-0.068	0.999
Treatment 2 x Treatment 4	-3.119	0.598	239	-5.212	<0.001
Treatment 3 x Treatment 4	-3.08	0.594	239	-5.185	<0.001

Test statistics belonging to the interaction terms of treatment and sampling week of the LMMs of the shoot samples on the edge of the carcasses.

Element	Sum of Squares	Numerator Degrees of Freedom	Denominator Degrees of Freedom	F value	Adjusted p value
B	1.347867	3	242.822	1.631	0.287
Ca	1.000824	3	245.073	1.717	0.287
Cu	14.08744	3	241.221	0.709	0.602
K	27.80677	3	245.036	2.418	0.184
Mg	1.406854	3	246.009	3.57	0.054
Mn	0.566442	3	245.842	1.051	0.453
Mo	1.77177	3	244.941	3.896	0.053
Ni	1.280269	3	243.979	1.207	0.423
P	5.027666	3	244.859	4.062	0.053
S	4.78E+00	3	245.271	1.714	0.287
Zn	6.093634	3	243.778	0.05	0.985

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AV
'24

About the author

Short biography

Elke Wenting was born in Nijmegen, the Netherlands, on 28th of May 1994. She grew up in Groesbeek, where animals always played an important role in her life. Always being surrounded by animals (e.g. horses, ducks, chickens, pigeons), she wanted to do "something with animals". In 2011, she started with the Bachelor's degree Animal Husbandry at HAS green academy in Den Bosch. During one of the BSc internships, she was involved in the "Circle of Life" project of ARK Rewilding Netherlands. This was her first experience in working on carcasses and scavengers. Since she wanted to learn more about ecological processes and wild-living animals, she followed the ecology specialisation of the Applied Biology program at the same institute. After obtaining her BSc degree in 2015, she started her Master's degree in Forest and Nature Conservation (ecology track) at Wageningen University in 2016. During this MSc program, she continued working on the scavenging process and she further developed her knowledge and interest in natural processes and nutrient cycles. She wrote a PhD grant proposal, which got funded by PE&RC. After obtaining her MSc degree in 2018, she started with her PhD research in the Wildlife Ecology and Conservation group (WEC). Besides that, she got involved in monitoring wolves in the Netherlands, that she coordinates for the southern Veluwe. As her PhD has now come to an end, she would like to continue working in the field of animal ecology and/or natural processes like nutrient cycles.

Korte biografie

Elke Wenting, geboren op 28 mei 1994 in Nijmegen, groeide op in Groesbeek, waar dieren altijd een belangrijke rol in haar leven speelden. Omringd door o.a. paarden, eenden, kippen en duiven, wilde ze later "iets met dieren" doen. In 2011 begon ze met de Hbo-opleiding Dier- en Veehouderij aan HAS green academy in Den Bosch. Ze kwam voor het eerst in aanraking met kadavers en aaseters tijdens een stage bij ARK Rewilding, waar ze werkte aan het project "Dood doet Leven". In het laatste jaar van haar Hbo-opleiding heeft ze de ecologiespecialisatie gevolgd van de opleiding Toegepaste Biologie omdat ze graag meer wilde leren over natuurlijke processen en in het wild levende dieren. Nadat ze in 2015 haar Bachelorsdiploma behaalde, startte ze in 2016 met de WO-master Bos- en Natuurbeheer (specialisatie ecologie) aan de Wageningen Universiteit. Tijdens deze master bleef ze zich ontwikkelen op het gebied van onderzoek naar kadavers en aaseters en groeide haar belangstelling voor natuurlijke processen en nutriëntenkringlopen. Ze schreef een onderzoeksvoorstel voor haar promotieonderzoek, dat werd gefinancierd door PE&RC. Nadat ze in 2018 haar MSc diploma behaalde, is ze in hetzelfde jaar begonnen met haar promotieonderzoek. In haar vrije tijd coördineert ze de wolvenmonitoring op de Zuid-Veluwe. Nu haar promotieonderzoek is afgerond, wil ze zich graag blijven bezighouden met dierecologie en/of natuurlijke processen zoals nutriëntenkringlopen.

Peer-reviewed journal publications

Wenting, E., Jansen, P. A., Pattipeilohy, L., van Lunteren, P., Siepel, H., & van Langevelde, F. (2024). Influence of tree cover on carcass detection and consumption by facultative vertebrate scavengers. Ecology and Evolution, 14, e10935. https://doi.org/10.1002/ece3.10935

Wenting, E., Siepel, H., & Jansen, P. A. (2023). Variability of the Ionome of Wild Boar (*Sus scrofa*) and Red Deer (*Cervus elaphus*) in a Dutch National Park, with Implications for Biomonitoring. Biological Trace Element Research, 1-29. https://doi.org/10.1007/s12011-023-03879-7

Wenting, E., Jansen, P. A., Laugeman, M. J., & van Langevelde, F. (2023). Leakage of Nutrients Into The Soil Due to Carrion Decomposition Can Enhance Plant Growth. Journal of Soil Science and Plant Nutrition, 1-6. https://doi.org/10.1007/s42729-023-01430-0

Wenting, E., Siepel, H., Christerus, M., & Jansen, P. A. (2023). Ionomic Variation Among Tissues in Fallow Deer (*Dama dama*) by Sex and Age. Biological Trace Element Research, 1-15. https://doi.org/10.1007/s12011-023-03724-x

Wenting, E., Rinzema, S. C., & van Langevelde, F. (2022). Functional differences in scavenger communities and the speed of carcass decomposition. Ecology and Evolution, 12, e8576. https://doi.org/10.1002/ece3.8576

Wenting, E., Siepel, H., & Jansen, P. A. (2020). Stoichiometric variation within and between a terrestrial herbivorous and a semi-aquatic carnivorous mammal. Journal of Trace Elements in Medicine and Biology, 62, 126622. https://doi.org/10.1016/j.jtemb.2020.126622

In preparation

Wenting, E., Patrick, P. A., Burggraeve, S., Delsman, D. F., Siepel, H., & van Langevelde, F. The influence of vertebrate scavengers on leakage of nutrients from carcasses. (submitted)

Wenting E.*, Eikelboom J. A. J.*, Siepel H., Broekhuis F., & van Langevelde, F. Influence of hunting strategies and large carnivore comeback on population dynamics of European facultative scavengers. (submitted)

Wenting, E., Siepel, H., Bakker, E. S., Strickland, M. S., Lynch, L., & Barton, P. S. Trace elemental cycles and the role of carcasses; a landscape view. (in preparation)

Wenting, E., Arnberg, M. P., Siepel, H., Delsman, D. F., van Langevelde, F., & Steyaert, S. M. J. G. Vertebrate scavenging mediates nutrient leakage from carrion into soil. (in preparation)

Situnayake, M., Pedersen, Å. Ø., Arnberg, M. P., Blaalid, R., Wetherbee, R., Kopp, K., Renčo, M., Wenting, E., Siepel, H., van Langevelde, F., Selva, N., Frank, S., Hjellupvik, T. G., Østnes, J. E., Skreå, H. F., Beumer, L., Mulley, M., van den Berg, J., Kooistra, L., & Steyaert, S. M. J. G. The landscape of death: vertebrate carrion contributes to the spatial heterogeneity of various ecological communities in an Artic tundra ecosystem. (in preparation)

Wenting, E., Verheesen, T. Y., Strijker, B. N., Jansen, P. A., & Siepel, H. Influence of vertebrate scavengers on soil mite communities below carcasses. (in preparation)

Other publications

Wenting, E., & Beekers, B. (2015). Mogelijke bijdrage van kadavers aan het broedsucces van Raven in Nationaal Park De Maasduinen. Natuurhistorisch Maandblad, 104, 81-85.

Wenting, E. (2024). Geen leven zonder dood. Vakblad Natuur Bos Landschap, 205, 14-17.

Supervised BSc and MSc students

Fleur van Beek – Can we relate ecological stoichiometry of mammals to tissue preference of avian and mammalian scavengers? (BSc)

Casper Bongers – The effects of environmental factors on the scavenging behaviour of vertebrate scavenging species (MSc)

Simon Burggraeve – All you can eat: how do scavengers influence nutrient fluxes? (MSc)

Melanie Christerus – Variation in elemental composition of tissues of Fallow deer (*Dama dama*) individuals of different sex and phases of life (MSc)

Devon Delsman – The legacy of cadavers on the nutrient cycle (MSc)

Jaap van Dorst – Analysis of variation in trace elemental value in tissues of four different animal species (BSc)

Felipe Griffioen – Carrion decomposition in Veluwezoom National Park – The effect on soil pH (BSc)

Stijn de Jong – Facultative scavengers can limit soil elemental influxes from cadavers (MSc)

Gijs Kloek – Circle of Life - identifying the beneficiaries! Assessing the possibilities for individual identification of red foxes (MSc)

Yvette Langenberg – The effect of tree cover on the diversity of facultative scavengers and decomposition rate (MSc)

Mathijs Laugeman – Effect of scavenger detection time and carrion condition on microbial and scavenger carrion consumption rates and larval development (MSc)

Jelle Lips – Circle of Life - a study on the impact of roe deer carcass provisioning on the vertebrate scavenger community in Dutch nature reserves (MSc)

Peter van Lunteren – Tree cover influences the partitioning of carrion resources among vertebrate scavengers (MSc)

Kimberley van der Meer – The scavenging behaviour of the Grey Wolf (*Canis lupus*): A case study at the Veluwe (BSc)

Jim Meuwissen – Nutrients in flow: from carrion to plant - An analysis of the effects manner of decomposition in carrion has on nutrient availability in soil and plants (MSc)

Willemijn Naaijer – Monitoring the black kite (*Milvus migrans*) (MSc)

Laura Oosting – The differences between scavenger community composition on carrion in forested and open terrain (BSc)

Luke Pattipeilohy – The effect of tree cover on carcass detection and depletion by vertebrate scavengers (MSc)

Salomé Rinzema – Diversity in scavenger communities and carcass decomposition (MSc)

Manouk Senhorst – The effects of carcass decomposition on accessible tissue types for scavengers (BSc)

Beau Strijker – The effect of carrion decomposition on the community structure of soil mites (BSc)

Akke-Wietske Terpstra – Differences in scavenging behaviour of vertebrates (BSc)

Tim Verheesen – Cascading effects of scavengers on soil mite communities below carcasses: an experiment (MSc)

Liona Voogt – Analyse van de elementsamenstelling van vegetatie rond kadavers - De invloed van elementsamentelling van vegetaties rond kadavers op het afsterven van omringende planten (BSc)

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review/project proposal (9 ECTS)

- Carcasses in the nutrient cycle: identifying knowledge gaps
- How do scavengers accelerate the terrestrial nutrient cycle

Post-graduate courses (3.5 ECTS)

- Professionele opleiding in wolvendossier; Zoogdiervereniging (2019)
- Canine systemic anatomy and physiology; London College of Osteopathy & Health Sciences, Canada (2021)

Invited review of journal manuscripts (1 ECTS)

- Ecological solutions and evidence: applied ecology of fear (2023)

Competence, skills and career-oriented activities (4.99 ECTS)

- Communication with the media and the general public; WGS (2018)
- Presenting with impact; WGS (2019)
- Supervising BSc & MSc thesis students; WGS (2020)
- Scientific integrity; WGS (2020)
- PhD Workshop carousel; WGS (2021)
- Global networks & international collaboration; WGS (2023)

PE&RC Annual meetings, seminars and PE&RC weekend/retreat (0.6 ECTS)

- PE&RC Last year retreat (2023)

Discussion groups/local seminars or scientific meetings (5 ECTS)

- Annual meeting ecologists Natuurmonumenten (2019-2024)
- Meetings project GPS-collared raven (2021-2024)
- Research day animal ecology & physiology (2022)
- NAEM (2022)
- Circle of Life symposium; ARK Rewilding Netherlands and WWF Belgium (2023)

International symposia, workshops and conferences (3.2 ECTS)

- NAEM; poster presentation (2019)
- NAEM; oral presentation (2024)

Societally relevant exposure (1.5 ECTS)

- Omroep Gelderland radio: wat is er nog over van de KadaverCam-ree (2019)
- Staatsbosbeheer magazine: er is leven na de dood (2020)
- Quest magazine: smullen van kadavers (2020)
- Vroege Vogels radio: unieke beelden van wolf en aaseters bij wolvenprooi (2020)
- Omroep Gelderland radio: slimme zwijnen verstoren onderzoek naar kadavers (2020)
- Vroege Vogels TV: Veluwezoom (2020)
- KIJK magazine: kadavers voor de wetenschap (2021)
- Trouw: de staat van ontbinding: nut en noodzaak van kadavers in het wild (2021)
- Omroep Gelderland Buitengewoon TV: dood doet leven (2022)
- Resource: dood doet leven (2022)
- Nieuwe Veluwe (2023)

Lecturing/supervision of practicals/tutorials (3.2 ECTS)

- Animal ecology lecture; RU (2019, 2020, 2022, 2023, 2024)
- Research master cluster proposal reviews (2021)
- Animal ecology practical; RU (2024)

BSc/MSc thesis supervision (6 ECTS)

- Ionome of mammals
- Scavenger community
- Carrion-related elemental fluxes
- Mite communities beneath carrion

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