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Annual and seasonal variations of essential minerals in free-ranging Scandinavian brown bears (*Ursus arctos*)

Madeleine Yngvell

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ANNUAL AND SEASONAL VARIATIONS OF ESSENTIAL MINERALS IN FREE-RANGING SCANDINAVIAN BROWN BEARS (*URSUS ARCTOS*)

EXERCISE THESIS

to obtain the title of
VETERINARY DOCTOR

STATE DIPLOMA

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the Paul Sabatier University of Toulouse*

by

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Source: picture taken by author.

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LIST OF ABBREVIATIONS AND SYMBOLS

•OH = Hydroxyl radical
AADC= Aromatic amino acid decarboxylase
ACE = Angiotensin converting enzyme
ACTH = Adreno-corticotropic hormone
ADH = Anti-diuretic hormone
ANOVA = One-way analysis of variance
ANP = Atrial natriuretic peptide
ASVCP = American Society of Veterinary Clinical Pathology
ATP = Adenosine triphosphate
Bpm = Beats per minute
Br = Bromine
BW = Body weight
Ca = Calcium
Ca²⁺ = Ionized calcium
Cl = Chloride
CNS = Central nervous system
Co = Cobalt
CO₂ = Carbon dioxide
Co²⁺ = Ionized cobalt
CPR = Cardiopulmonary resuscitation
CRH = Corticotropin-releasing hormone
CRT = Capillary refill time
Cu = Copper
Cu⁺ = Cuprous form of copper
Cu²⁺ = Cupric form of copper
DIOs = Iodothyronine deiodinases
DNA = Deoxyribonucleic acid
ECG = Electrocardiogram
EDEC = Estimated dietary energy content
EDTA = Ethylenediamine tetraacetic acid
ETT = Endotracheal tube
Fe = Iron
Fe²⁺ = Ferrous form of iron
Fe³⁺ = Ferric form of iron
FGF23 = Fibroblast growth factor 23
FO = Frequency of occurrence
FV = Fecal volume
g = Gram
GABA = γ-amino butyric acid
GPS = Global Positioning System
H₀ = Null hypothesis
H₁ = Alternative hypothesis
H₂O = Water
H₂O₂ = Hydrogen peroxide
HA = Hydroxyapatite
HClO = Hypochlorous acid

HE = Heparin
HIF = Hypoxia-inducible factors
HPAA = Hypothalamus-pituitary-adrenal axis
HR = Heart rate
I = Iodine
I⁻ = Iodide ion
I₂ = Inorganic iodine
ICP-MS = Inductively Coupled Plasma Mass Spectrometry
ICP-SFMS = Sector Field Inductively Coupled Plasma Mass Spectrometry
IM = Intramuscular
IO₃⁻ = Iodate
IP = Intraperitoneally
IV = Intravenous
K = Ketamine
K⁺ = Potassium
K₂-EDTA = Di-potassium Ethylenediaminetetraacetic Acid
K₃-EDTA = Tri-potassium Ethylenediaminetetraacetic Acid
Kg = Kilogram
Km = Kilometer
km² = Square kilometer
L = Liter
L-DOPA = Levodopa
m = Meter
MCOs = Multi-copper oxidases
Mg = Magnesium
mg = Milligram
min = Minute
mL = Milliliter
mm = Millimeter
Mn = Manganese
MnSOD = Manganese superoxide dismutase
MTZ = Medetomidine, tiletamine and zolazepam
MTZ+K = Medetomidine, tiletamine, zolazepam and ketamine
NA = Non available
Na = Sodium
Na⁺ = Sodium cation
NaCl = Sodium chloride
NINA = Norwegian Institute for Nature Research
NMDA = N-methyl-D-aspartic
O₂ = Oxygen
O²⁻ = Superoxide anion
P = Phosphorus
PaO₂ = Partial pressure of arterial oxygen
PEPCK = Enzyme phosphoenolpyruvate carboxykinase
PO = Per os
PO₄ = Phosphate
PTH = Parathyroid hormone
Q₁₀ = Rate coefficient for a 10°C change in body temperature
r = Ratio

RAAS = Renin-angiotensin-aldosterone system
Rb = Rubidium
RDDS = Remote drug delivery systems
RI = Reference interval
RNA = Ribonucleic acid
RNRs = Ribonucleotide reductases
ROS = Reactive oxygen species
RR = Respiratory rate
RT = Rectal temperature
S = Sulfur
SBBRP = Scandinavian Brown Bear Research Project
SC = Sub-cutaneously
Se = Selenium
SEPP1 = Selenoprotein 1
SF = Sector field
Si = Silicon
SNS = Sympathetic nervous system
SOD = Superoxide dismutase
SpO₂ = Hemoglobin oxygen saturation
T₃ = Triiodothyronine
T₄ = Thyroxine
T_A = Ambient temperature
T_b = Body temperature
TE = Trace element
TRH = Thyrotropin-releasing hormone
TrxR = Thioredoxin reductase isoenzyme
TSH = Thyroid-stimulating hormone
TZ = Tiletamine and zolazepam
UV = Ultraviolet
VHF = Very High Frequency
Zn = Zinc

1. ABSTRACT

Exploring blood chemistry values has the potential to improve our understanding of physiological changes in wildlife. However, it has been sparsely investigated in brown bears (*Ursus arctos*). The objectives of this study were to determine reference intervals for twelve blood chemistry variables and to evaluate differences between seasons and years. Thus, to establish reference values for biochemical variables in whole blood for free-ranging Scandinavian brown bears, 384 blood samples were collected from 164 different bears (93 females and 71 males) over a period of twelve years, from 2010 to 2022. The samples were collected during winter (February-March), spring (April-May) and summer (June), in the county of Dalarna in southcentral Sweden. The bears were chemically immobilized inside their den during winter, while being darted and immobilized from a helicopter during spring and summer. The drugs used for the chemical immobilization are a combination of tiletamine, zolazepam and medetomidine, with ketamine in addition in winter. To the best of our knowledge by visual observation, all bears appeared healthy and therefore eligible for capture. Essential minerals are one of the important nutritional components having a significant role in animal health, reproduction, immune system, metabolism, etc. They are crucial for optimum growth and proper muscle and nerve functions. The biochemical variables included in this study are the following twelve essential minerals: calcium, cobalt, copper, iodine, iron, magnesium, manganese, phosphorus, selenium, sodium, sulfur and zinc. Each of these twelve minerals were analyzed for each bear and collected from venous blood samples. Seasonal and annual variations of the mineral values were established for the brown bears. The seasonal differences found in the blood variables were mainly due to the physiological changes in bears' metabolism throughout the year. Indeed, the winter samples were collected while the bears were in hibernation, the spring samples after den emergence and the summer samples during their active phase. The annual differences found in the blood variables however probably reflect extrinsic factors such as variations in composition of their diet and different means in sample analysis between years (different operators, protocols, equipment, transportation and storage conditions etc.).

Key words: Scandinavian brown bear, *Ursus arctos*, whole blood, essential minerals, reference intervals, seasonal and annual variations

1. RÉSUMÉ EN FRANÇAIS

L'exploration des concentrations sanguines en minéraux essentiels permet d'améliorer notre compréhension des changements physiologiques rencontrés dans la faune sauvage. Cependant, il s'agit d'un sujet peu étudié chez l'ours brun (*Ursus arctos*). Les objectifs de cette étude sont d'établir des intervalles de référence en concentrations sanguines de douze minéraux essentiels et d'évaluer leurs variations entre saisons et années. Ainsi, pour établir ces valeurs de référence pour les ours bruns sauvages scandinaves, 384 échantillons sanguins ont été prélevés sur 164 ours bruns (93 femelles et 71 mâles) sur une période de douze ans, de 2010 à 2022. Les échantillons ont été prélevés en hiver (février-mars), au printemps (avril-mai) et en été (juin) dans le comté de Dalarna, dans le centre sud de la Suède. Les ours ont été immobilisés chimiquement à l'intérieur de leur tanière pendant l'hiver, tandis qu'ils ont été téléanesthésiés à partir d'un hélicoptère lors des captures au printemps et à l'été. Les molécules anesthésiques utilisées pour l'immobilisation chimique sont une combinaison de tilétamine, de zolazépam et de médétomidine, avec de la kétamine en plus en hiver. D'après leur observation à distance, tous les ours semblaient en bonne santé et donc éligibles à être capturés. Les minéraux essentiels sont des composants nutritionnels importants qui jouent un rôle significatif dans la santé animale, la reproduction, le système immunitaire, le métabolisme, etc. Ils sont essentiels à la croissance optimale des individus et au bon fonctionnement musculaire et nerveux. Les variables biochimiques incluses dans cette étude sont les douze minéraux essentiels suivants : calcium, cobalt, cuivre, iode, fer, magnésium, manganèse, phosphore, sélénium, sodium, soufre et zinc. Chez chaque ours, des prélèvements sanguins veineux ont permis d'analyser ces douze minéraux. Grâce à ce travail, des variations saisonnières et annuelles des concentrations en minéraux essentiels ont été établies. Les différences saisonnières dans les variables sanguines s'expliquent en partie par les changements physiologiques du métabolisme de l'ours brun tout au long de l'année. En effet, les échantillons d'hiver ont été prélevés alors que les ours étaient en hibernation, les échantillons de printemps après la sortie de la tanière et les échantillons d'été pendant leur phase active. Les différences annuelles constatées dans les variables sanguines reflètent probablement des facteurs extrinsèques tels que les variations dans la composition de leur régime alimentaire ainsi qu'une différence dans l'analyse des échantillons entre années (différents opérateurs, protocoles, équipement, conditions de transport et de stockage etc.).

Mots clés : Ours brun scandinave, *Ursus arctos*, concentrations sanguines, minéraux essentiels, intervalles de référence, variations saisonnières et annuelles.

Introduction

Les intervalles de référence des paramètres biochimiques sanguins couramment mesurés sont importants pour la surveillance de la santé au niveau individuel et à l'échelle d'une population. En effet, des paramètres sanguins spécifiques peuvent refléter certaines fonctions d'organes et donc être utilisés pour la surveillance de processus physiologiques et pathologiques d'organes, de l'équilibre électrolytique, des activités enzymatiques, etc. Un intervalle de référence (IR) "comprend les limites supérieures et inférieures d'un test de laboratoire basé sur un groupe de sujets apparemment en bonne santé" (définition en ligne de MedCalc ; Geffré et al. 2009). En général, les IR comprennent les valeurs dans lesquelles 95 % des individus apparemment en bonne santé se situeraient (Geffré et al. 2009 ; Horn, Pesce 2003). Ils représentent l'un des outils les plus largement utilisés dans la prise de décision médicale pour l'interprétation d'anomalies et de potentielles maladies. Les IR sont établis par analyse statistique à partir d'un large échantillon d'individus en bonne santé, afin d'être aussi significatifs que possible. Ils servent d'aide, notamment pour les professionnels de santé, dans l'évaluation et l'interprétation de résultats afin de déterminer si les valeurs obtenues se situent dans la fourchette attendue pour un paramètre donné. Ils contribuent ainsi au diagnostic et au suivi de certaines pathologies et à l'identification d'écarts par rapport à la normale. Afin d'obtenir des IR basés sur une population donnée, le sang doit être prélevé sur un grand nombre d'animaux (un minimum de 120 idéalement), avec un mélange de catégories d'âge et de sexes. L'échantillonnage d'un grand nombre d'individus constitue souvent un défi pour certaines espèces animales, et en particulier pour les animaux sauvages. Dans cette étude, des prélèvements sanguins ont été effectués sur une longue période en vue d'établir des IR pour certains paramètres biochimiques sanguins chez l'ours brun (*Ursus arctos*). Cela a permis de collecter un grand nombre d'échantillons (384 échantillons provenant de 164 ours différents, certains ayant été capturés plusieurs fois d'une année à l'autre et/ou même d'une saison à l'autre).

Des valeurs en dehors de l'IR de paramètres biochimiques sanguins reflètent souvent une pathologie, telle qu'une défaillance d'organe par exemple (Jones et al. 2018 ; Dimitrakakis et al. 2022 ; Park, Kim, Do 2021). Alors que les IR pour les variables biochimiques chez les animaux domestiques tels que les chats, chiens, vaches, chevaux, porcs, chèvres et moutons ont été largement étudiés dans le monde entier, il n'est pas aussi facile ni accessible d'établir de telles IR pour les animaux sauvages. L'une des principales difficultés rencontrées dans le cadre de la recherche sur les espèces sauvages et l'interprétation des valeurs obtenues à partir d'individus capturés est l'absence d'une "population contrôle/témoin", qui, dans le cas présent, serait constituée d'ours bruns sauvages n'ayant pas été chimiquement capturés. Relativement peu d'études, ont été menées sur les valeurs biochimiques de référence chez l'ours brun, et encore moins chez l'ours brun scandinave. Les rares données disponibles sur ce sujet proviennent souvent d'échantillons de petite taille, rarement prélevés sur une longue durée et sur des animaux en captivité. Des paramètres biochimiques sanguins ont déjà été évalués chez l'ours noir américain *Ursus americanus* (Matula, Lindzey, Rothenbacher 1980; Schroeder 1987; Hellgren, Rogers, Seal 1993), l'ours noir asiatique *Ursus thibetanus* en captivité (Yang et al. 2017), l'ours brun *Ursus arctos* (Halloran, Pearson 1972; Brannon 1985; Hissa et al. 1994; Huber et al. 1997; Græsli et al. 2014; Græsli et al. 2015) et l'ours polaire *Ursus maritimus* (Tryland et al. 2002; Fry et al. 2019). Certaines IR ont été évaluées chez des ours bruns sauvages scandinaves pour certaines de ces variables biochimiques, mais avec des paramètres différents. Par exemple, Græsli et al. (2014) ont évalué les IR biochimiques pour le calcium, fer, magnésium, sodium et phosphore inorganique chez des ours bruns sauvages scandinaves,

mais à partir de sérum. Tryland et al. (2002) ont évalué les IR du plasma pour le sodium, le magnésium, le calcium et le phosphore, mais chez des ours polaires vivant en liberté au Svalbard. Fuchs et al. (2021) et Fuchs et al. (2023) ont évalué les concentrations sanguines en plomb, arsenic, mercure et cadmium dans le sang total d'ours bruns sauvages scandinaves. Une autre étude récente a été menée sur les concentrations sanguines en plomb, arsenic, cadmium, mercure, sélénium et cuivre dans le sang total d'ours bruns sauvages scandinaves (Rikke Kristiansen Aarmo, 2021). À notre connaissance, il s'agit de la première étude examinant les valeurs dans le sang total afin d'établir des intervalles de référence pour les douze minéraux essentiels suivants chez les ours bruns sauvages scandinaves : calcium, cobalt, cuivre, iode, fer, magnésium, manganèse, phosphore, sélénium, sodium, soufre et zinc.

Les échantillons sanguins veineux ont été prélevés durant l'anesthésie pour le marquage, mise en place de colliers GPS et la collecte de plusieurs types de prélèvements (sang, graisse, muscle, peau, salive, fèces, poils, lait, etc.) pour des études de recherche faisant intervenir plusieurs équipes provenant de différents pays, selon les " Biomedical protocols for free-ranging Brown Bears, Wolves, Wolverines and Lynx " décrits par Arnemo et Evans (2017). L'anesthésie d'un animal sauvage nécessite beaucoup d'organisation, de logistique, de planification, de financement, d'autorisations des autorités légales, etc. (Kreeger TJ et al. 2023). L'utilisation d'animaux sauvages vivants dans le domaine de la recherche n'est accordée qu'après évaluation de sa nécessité et à condition que d'autres méthodes de collecte de données ne suffisent pas (e.g., le prélèvement d'ADN dans les excréments ou les poils). C'est ainsi que, chaque année, plusieurs équipes de différents pays menant divers projets de recherche se réunissent dans le centre de la Suède pour les captures d'ours d'hiver et d'été. De cette manière, plusieurs projets de recherche peuvent bénéficier de prélèvements d'échantillons ou de la collecte de données tout en anesthésiant les ours bruns le moins souvent possible afin de les perturber le moins possible. Le Scandinavian Brown Bear Research Project (SBBRP), créé en 1984, n'impliquait auparavant que des équipes de recherche scandinaves de Norvège et de Suède. Aujourd'hui, et depuis plusieurs décennies, les équipes impliquées proviennent de plusieurs pays d'Europe, afin de collecter des données sur cet incroyable spécimen qu'est l'ours brun scandinave. Parmi les projets en cours, citons la recherche biomédicale translationnelle sur l'atrophie musculaire (Gomez et al. 2021), l'endocrinologie, la cardiologie et les maladies cardiovasculaires (Frøbert et al. 2022 ; Frøbert et al. 2010 ; Frøbert et al. 2023 ; Thienel et al. 2023), l'hématologie et la biochimie (Græsli et al. 2014; 2015), les moteurs de l'hibernation et les changements physiologiques et physiques qui y sont associés (Alina Evans, Singh, Friebe, et al. 2016), le métabolisme lipidique pendant l'hibernation (Giroud et al. 2019 ; 2018 ; 2021), la reproduction (Alina Lynn Evans, 2016 ; Friebe et al. 2014 ; Lemièrre et al. 2022), l'écologie et la prédation (Sahlén et al. 2014 ; Thiel et al. 2022), la toxicologie (Fuchs et al. 2021 ; 2023), etc. Les ours bruns sont incroyables dans le sens où ils parviennent à rester dans un état hypométabolique inactif pendant cinq à sept mois, sans pâtir des conséquences que les humains subiraient à leur place. De surcroît, les ours bruns constituent un modèle naturel fascinant pour l'étude de leurs adaptations comportementales et physiologiques à de longues périodes d'inactivité, offrant des perspectives à la médecine humaine pour les personnes associées à un mode de vie sédentaire.

Bien qu'il ne soit pas possible de s'assurer que les valeurs des échantillons inclus dans cette étude reflètent réellement les valeurs physiologiques trouvées chez les ours bruns sauvages, elles pourraient fournir une première idée générale. Les échantillons sanguins ont été prélevés sur des ours bruns après immobilisation chimique, capturés par téléanesthésie

depuis un hélicoptère au printemps et en été. Des facteurs communs susceptibles d'interférer avec les variables biochimiques des échantillons prélevés incluent : le stress et l'activité physique accrue dus à la chasse, les médicaments anesthésiques (Arnemo JM, Caulkett N 2007), un jeûne prolongé, la déshydratation, le régime alimentaire, les maladies, les facteurs génétiques, le statut reproducteur (gestation, lactation, femelle en chaleur), les variations journalières et saisonnières, etc. (Irjala, Grönroos 1998). Tous ces facteurs doivent être pris en considération et doivent être évités au maximum, avec un protocole et des procédures d'échantillonnage standardisés. Alors que certains facteurs extrinsèques peuvent être plus ou moins faciles à éviter ou du moins à contrôler, les facteurs intrinsèques susceptibles d'interférer avec les variables sanguines sont difficiles à gérer. Les ours d'âge et de sexe différents présentent des différences de métabolisme, d'alimentation, de poids corporel, de comportement et de physiologie, qui peuvent se refléter dans les variables biochimiques.

L'hibernation constitue une période de la vie de l'ours d'une grande importance puisqu'il passe environ six mois à l'intérieur de la tanière (de novembre à avril). Les ours bruns sont soumis à une baisse de la température corporelle, une diminution du rythme cardiaque, du rythme respiratoire et de l'activité métabolique. Avant l'hibernation, ils accumulent des réserves corporelles (principalement en stockant de la graisse) en augmentant leur consommation de nourriture, ce qui se traduit par une prise de poids. Lorsque le printemps arrive et que les ours sortent de leur tanière, ils doivent à nouveau reconstituer leurs réserves corporelles après avoir perdu du poids pendant l'hiver, surtout si la mère était gestante et qu'elle a dû allaiter ses petits. Leur masse corporelle est très irrégulière tout au long de l'année, en fonction de la saison et de leur état physiologique associé. Les variations physiologiques selon les différentes saisons peuvent donc influencer les paramètres biochimiques sanguins, comme cela a été constaté pour certaines variables chez l'ours brun (Halloran, Pearson 1972 ; R. Hissa et al. 1994 ; Huber et al. 1997 ; Græsli et al. 2015).

Généralités sur les ours bruns scandinaves

L'ours brun scandinave appartient à l'espèce d'ours brun *Ursus arctos*, l'une des huit espèces d'ours de la famille des *Ursidae* (Dahle et al. 1998). Plus précisément, il appartient à la sous-espèce d'ours brun eurasiatique *Ursus arctos arctos*. Ces prédateurs se trouvent dans la péninsule scandinave, qui comprend la Norvège, la Suède, la Finlande et de petites parties de la Russie (Rovdata 2023). Leur corps mesure entre 130 et 250 cm de long, avec un cou épais, un profil facial bombé et une bosse nette au-dessus des épaules (Skandinaviska björnprojektet 2023). La tête est large, les oreilles petites et rondes, et la croupe est généralement plus basse que les épaules. Leur vision est beaucoup moins développée que l'odorat et l'ouïe, qui leur permet de ne voir qu'à de courtes distances (Marion 2018). Néanmoins ils possèdent un *tapetum lucidum*, une couche tissulaire à l'arrière de l'œil qui réfléchit la lumière, ce qui leur permet de voir dans l'obscurité (Marion 2018). Les femelles adultes pèsent entre 60 et 200 kg et les mâles entre 100 et 315 kg (Skandinaviska björnprojektet 2023 ; Rovdata 2023). Les ours bruns présentent un important dimorphisme de taille, qui peut s'expliquer en partie par la compétition à laquelle les mâles doivent faire face pendant la saison de reproduction. Cependant, en raison du chevauchement des tailles, il peut être difficile, voire impossible, de différencier une femelle célibataire d'un jeune mâle adulte. Les ours sont solitaires, sauf si la mère est suivie, auquel cas elle les nourrit et les protège pendant une période allant de 1,5 à 2,5 ans. Les empreintes d'ours sont larges, avec des empreintes marquées par cinq orteils, le plus grand étant à l'extérieur, avec de longues griffes. L'ours brun est un animal plantigrade,

signifiant qu'il marche sur la plante des pieds (site web Rovdata 2023). L'ours brun *Ursus arctos* est l'oursidé le plus répandu au monde. Autrefois, il couvrait toute l'Europe et la majeure partie de la Scandinavie. Aujourd'hui, son aire de répartition holarctique couvre des régions d'Asie, d'Europe et d'Amérique du Nord. Par le passé, elle couvrait également de petites parties de l'Afrique du Nord. Bien que la population mondiale d'ours bruns puisse être considérée comme relativement importante (plus de 200 000 individus (McLellan et al. 2017)), les populations locales actuelles sont fragmentées et diminuent globalement en raison de la chasse, des conflits humains et de la perte d'habitat. Il y a environ 2000 ans, la population originelle d'ours bruns en Europe était estimée entre 10 000 et plus de 100 000 individus (site internet European wilderness society 2023). En 2000, le nombre total d'ours bruns en Europe était d'environ 50 000 (dont ~14 000 en dehors de la Russie) sur une zone de plus de 2,5 millions de km² (800 000 km² en dehors de la Russie) (Zedrosser et al. 2000). Aujourd'hui, la population européenne d'ours bruns en dehors de la Russie est estimée à environ 17 000 individus (site internet European wilderness society 2023). Les populations d'ours en Europe sont très dispersées dans plusieurs pays avec des densités très faibles. La plus grande population européenne (en dehors de la Russie) se trouve probablement dans les Carpates, en Roumanie, avec une estimation de 6 000 individus (site internet European wilderness society 2023). En 2020, la Suède comptait environ 2 800 ours. La population norvégienne reste faible, avec un total de 150 ours bruns (65 femelles et 85 mâles) qui ont été enregistrés en Norvège en 2020 (Bjørn Henrik Stavdal Johansen, Nicklas Iversen 2021 ; Skandinaviska björnprojektet 2023).

La plupart des ours de Scandinavie se trouvent dans la forêt boréale, bien que certains se trouvent également dans les montagnes. Leurs déplacements au sein de la forêt et de l'habitat qu'ils utilisent reflètent probablement les endroits où les aliments constituant leur régime alimentaire sont disponibles (Skandinaviska björnprojektet 2023). Comme leur régime varie beaucoup en fonction de la saison, l'habitat qu'ils utilisent change également en fonction de la saison, lorsque les différents aliments sont disponibles dans des localisations différentes. En général, les mâles et les femelles préfèrent les terrains forestiers avec du relief aux terrains forestiers plus plats. Ils présentent l'avantage d'offrir davantage de ressources alimentaires et d'être moins fréquentés par l'homme. Le choix d'habitat de l'ours brun semble également être fortement influencé par les activités humaines. Ils évitent généralement les personnes et les zones urbaines. Les femelles ayant des petits d'un an choisiront des habitats avec un sous-bois moins dense avec une plus grande visibilité. Les ours sont généralement actifs tôt le matin et tard le soir. Ayant un mode de vie différent selon les saisons, la durée et le moment de leur période d'activité varient tout au long de l'année. Au début du printemps (juste après avoir quitté leur tanière), les ours ont tendance à être actifs pendant la journée et à se reposer plus longtemps. À l'automne, lorsque les ours sont en hyperphagie, c'est-à-dire qu'ils mangent continuellement afin de constituer des réserves de graisse pour l'hiver, ils ont tendance à être très actifs pendant la journée. Les ours bruns n'ont généralement pas de territoires qu'ils défendent, ils utilisent plutôt ce que l'on appelle un "domaine vital". Le domaine vital est la zone où l'ours vit et obtient les ressources nécessaires à sa survie et à sa reproduction, en d'autres termes, la zone dans laquelle il se nourrit et recherche des partenaires. Les domaines vitaux des ours se chevauchent généralement et la plupart des jeunes oursons établissent leur domaine vital en partie dans celui de leur mère (Skandinaviska björnprojektet 2023). Les femelles avec des oursons ont généralement des domaines vitaux de 100 à 150 km² alors que les femelles seules ont des domaines vitaux de 200 à 300 km². En règle générale, les mâles peuvent avoir des domaines vitaux compris entre 800 et 1000 km² (site web Rovdata 2023). La taille des domaines vitaux a tendance à augmenter lorsque la population d'ours est de faible

densité, et les domaines vitaux des mâles sont généralement plus grands que ceux des femelles, probablement parce qu'ils se déplacent sur de plus grandes distances que leurs homologues féminins à la recherche de partenaires. Les ours bruns femelles restent généralement près de la zone où ils sont nés et choisiront leur habitat dans leur domaine vital qui fournit des ressources alimentaires abondantes et minimise les perturbations causées par l'homme (Martin, Basille, Moorter, et al. 2010).

Objectifs de la thèse

Les objectifs de cette étude étaient d'établir des IR pour douze paramètres biochimiques chez des ours bruns sauvages scandinaves *Ursus arctos*, immobilisés chimiquement à l'aide d'une combinaison de médétomidine, de tilétamine et de zolazépam pendant le printemps et l'été, et d'une combinaison de médétomidine, de tilétamine, de zolazépam et de kétamine pendant l'hiver sur une période de douze ans, et d'évaluer les variations saisonnières et annuelles de ces variables biochimiques. Les douze éléments analysés dans cette étude sont le calcium, le cobalt, le cuivre, l'iode, le fer, le magnésium, le manganèse, le phosphore, le sélénium, le sodium, le soufre et le zinc. 384 échantillons prélevés à la veine jugulaire sur 164 ours différents ont été collectés lors des captures de printemps, d'été et d'hiver de 2010 à 2022, dans des tubes d'acide éthylènediamine tétraacétique (EDTA), d'oligo-éléments (TE) et d'héparine (HE).

Les minéraux constituent des composants essentiels des enzymes, des hormones et des cellules de l'organisme. Ce sont des éléments chimiques nécessaires sous forme de nutriments pour le bon fonctionnement de l'organisme et une vie saine. Ils jouent un rôle fondamental dans la digestion, les performances reproductives, le système immunitaire, le développement des os, des muscles, des dents, etc. (Rumosa Gwaze, Chimonyo, Dzama 2012). Les minéraux ont la capacité d'interagir avec les microbes, les virus et les macro-biomolécules par le biais de liaisons ioniques et/ou non covalentes et constituent donc de nouveaux intérêts pour la thérapie et la biotechnologie (Carter, Xu, Sadler 2021). Les minéraux jouent un rôle très important dans le maintien de l'homéostasie. L'homéostasie peut être définie comme un processus d'autorégulation par lequel les systèmes biologiques maintiennent leur stabilité tout en s'adaptant à des conditions externes fluctuantes. Il ne s'agit pas d'un processus statique et constant, mais plutôt d'un processus dynamique qui peut modifier les conditions internes en fonction des besoins pour survivre aux défis externes (Billman 2020). Elle explique comment les organismes peuvent maintenir des variables internes plus ou moins constantes, ce qui leur permet de s'adapter et de survivre lorsqu'ils sont confrontés à un environnement externe changeant (Billman 2020). Les mécanismes cellulaires automatiques, les contrôles neuronaux et endocriniens, ainsi que le comportement, contribuent tous à l'homéostasie. Elle est très développée chez les animaux terrestres à sang chaud, car ils doivent maintenir un certain équilibre des fluides, une température corporelle, un pH sanguin, des niveaux d'oxygène et un équilibre acido-basique dans des limites assez étroites des intervalles de référence. Le concept d'homéostasie permet d'appréhender les fonctions de l'organisme dans un état sain ou pathologique. En effet, sa perturbation entraîne des déséquilibres électrolytiques et des maladies. Le maintien de l'homéostasie nécessite une dépense d'énergie, les animaux doivent donc veiller à se nourrir suffisamment. Les minéraux se présentent sous la forme de macrominéraux ou de microminéraux. Les macrominéraux sont un groupe de minéraux dont les animaux ont le plus besoin dans leur alimentation pour assurer les nombreuses fonctions cellulaires dans de multiples organes. Il s'agit de minéraux dont l'organisme a besoin en quantités relativement importantes dans le cadre de

l'alimentation quotidienne (site web *Minéraux : fonctions et sources alimentaires* 2023). Les microminéraux sont souvent appelés oligo-éléments et se trouvent en plus petites quantités que les macrominéraux, mais remplissent néanmoins des fonctions d'importance physiologique (*Minerals : functions & food sources* 2023).

Les ours bruns scandinaves subissent des changements physiologiques tout au long de l'année, liés à leur régime alimentaire qui varie en fonction des saisons et d'autres facteurs plus ou moins connus. A l'automne, pendant la période d'hyperphagie, les ours se nourrissent d'aliments riches en sucre tels que les baies afin de stocker les graisses et d'augmenter leur masse corporelle en vue de l'hibernation (Hilderbrand et al. 1999 ; Stenset et al. 2016). Ils ne mangent pas, ne boivent pas, n'urinent pas et ne défèquent pas pendant une période de cinq à sept mois, au cours de laquelle ils dépendent uniquement de leurs réserves corporelles (principalement leurs réserves de graisse). Les aspects bien établis de la physiologie de l'hibernation des ours comprennent des diminutions de leur température corporelle (Hissa 1997), fréquence cardiaque, fréquence respiratoire, et du métabolisme (Folk, Minor 1972 ; Tøien et al. 2011 ; Tøien, Blake, Barnes 2015) pendant l'hibernation. Les femelles gestantes donnent naissance à des oursons à l'intérieur de la tanière et commencent à allaiter. Lorsque le printemps arrive et que les ours sortent de leur tanière, ils peuvent avoir perdu jusqu'à 43 % de leur masse corporelle et doivent reconstituer leurs réserves corporelles en prenant de la masse maigre et de la graisse. Cela correspond au début de leur phase active, où les ours recherchent activement des aliments riches en protéines, augmentant ainsi leurs déplacements au sein de leur habitat. En raison de ces changements, la physiologie varie considérablement tout au long de l'année, ainsi que certaines variables hématologiques et biochimiques illustrant bon nombre d'organes concernés. L'établissement d'intervalles de référence (IR) pour douze minéraux essentiels au printemps, été et hiver chez les ours bruns scandinaves pourrait permettre de mieux comprendre ces changements physiologiques, leur système de reproduction, l'hibernation et leur état de santé. Il existe toujours une concentration optimale en minéraux essentiels dont l'organisme a besoin. Une concentration supérieure ou inférieure à la plage optimale, se traduisant par un état de toxicité ou de carence, peut compromettre la santé de l'animal. La détermination des niveaux des paramètres sanguins tels que les minéraux essentiels est utile pour connaître les valeurs physiologiques auxquelles s'attendre et pour prédire les problèmes de santé susceptibles d'entraîner des mortalités (Kida et al. 2007). Les analyses sanguines sont capables de détecter des troubles métaboliques subcliniques et reflète donc l'état de santé des animaux (Caldeira et al. 2007). Ainsi, en plus de nous permettre d'acquérir plus de connaissances sur l'écologie et la biologie de l'ours brun, ces IR pourraient également s'avérer précieuses en cas de pathologies rencontrées chez les ours bruns en captivité. La comparaison des valeurs trouvées chez les ours bruns sauvages, qui devraient refléter l'état normal ou physiologique, avec celles trouvées chez les ours malades en captivité, pourrait conduire au développement de nouvelles thérapies cliniques et apporter des solutions médicales. Les résultats de cette étude fournissent des valeurs de référence qui aideront à comprendre la physiologie des ours bruns et à évaluer la santé de ces animaux en captivité.

Matériel et méthodes

Cette étude fait partie d'un projet de recherche de long terme sur la population d'ours bruns scandinaves dans les comtés de Dalarna et de Gävleborg, dans le centre-sud de la Suède (~61°N, 15°E). La taille de la zone d'étude est d'environ 13 000 km² et est principalement

couverte de forêts de conifères gérées de manière intensive dans un paysage vallonné. Les espèces dominantes sont le pin sylvestre (*Pinus sylvestris*) et l'épicéa commun (*Picea abies*) (Bischof et al. 2010 ; Stenset et al. 2016 ; Felton, Petersson, et al. 2020), mais des arbres à feuilles caduques comme le bouleau de montagne (*Betula pubescens*), le bouleau argenté (*Betula pendula*), le tremble européen (*Populus tremula*) et l'aulne glutineux (*Alnus incana*) sont couramment rencontrés (Zedrosser, Dahle, Swenson 2006). La strate terrestre est dominée par des arbustes nains, tels que la bruyère (*Calluna vulgaris*) et des baies, notamment des myrtilles, des airelles et des camarines, sur des sols principalement granitiques et des roches de gneiss (Elfström, Swenson, Ball 2009 ; Bischof et al. 2010).

La période de végétation dans le centre-sud de la Suède est de 170 à 190 jours, avec des précipitations annuelles moyennes de 600 à 1000 mm (dont 350 à 450 mm pendant la période de végétation). La couverture neigeuse dure généralement de fin octobre à fin avril/début mai et les températures journalières moyennes sont de -7°C en janvier et de +15°C en juin (Skogsstyrelsen rapport 2020, Forest Management In Sweden ; Zedrosser, Dahle, Swenson 2006). Le paysage vallonné est entrecoupé de lacs et de tourbières et de quelques champs agricoles à l'est. La zone d'étude est un paysage vallonné dont l'altitude varie de ≈150 m au-dessus du niveau de la mer à l'est à 850 m au-dessus du niveau de la mer à l'ouest. Les altitudes sont pour la plupart inférieures à la limite forestière, qui se situe autour de 850 m (Fuchs et al. 2021). La densité d'ours bruns estimée dans notre zone d'étude est d'environ 30 ours/1000 km² (Stenset et al. 2016 ; Bellemain et al. 2005).

Les ours bruns ont été immobilisés chimiquement à l'aide de pistolets à fléchettes (DANiNJECT®) avec une combinaison médicamenteuse de médétomidine, de tilétamine et de zolazépam (MTZ), à laquelle s'ajoute de la kétamine en hiver. Des prélèvements sanguins veineux ont été effectués pendant l'immobilisation à l'aide de tubes d'acide éthylènediamine tétraacétique (EDTA), d'héparine (HE) et d'oligo-éléments (TE) pour des études écologiques de marquage avec mise en place de colliers GPS au sein du SBBRP. Les ours bruns anesthésiés ont été placés en décubitus latéral avec les voies respiratoires dégagées. En résumé, 384 échantillons provenant de 322 captures ont été prélevés, ce qui signifie que pour certains ours, plus d'un échantillon a été prélevé. Au total, 384 échantillons de sang ont été prélevés au cours des années 2010, 2013 et 2017-2022, dont 131 dans des tubes EDTA, 232 dans des tubes TE et 21 dans des tubes HE.

Le sang a été prélevé dans la veine jugulaire des ours bruns à l'aide d'un système Vacutainer (BD Vacutainer®, BD Diagnostics, Preanalytical Systems, Franklin Lakes, NJ, USA). Des aiguilles de 18G ont été utilisées pour les adultes, tandis que des aiguilles de 20G ont été utilisées pour les oursons d'un an et les jeunes adultes. La prise de sang a été effectuée dans les 60 premières minutes de l'immobilisation. Le sang veineux utilisé pour l'analyse des minéraux essentiels a été prélevé dans des tubes contenant un anticoagulant. Les anticoagulants contenus dans les tubes étaient l'acide éthylènediamine tétraacétique (EDTA), l'héparine de sodium et l'héparine de lithium. Le sang a été prélevé dans des tubes contenant ces anticoagulants, appelés tubes K₂ et K₃-EDTA (principalement K₃-EDTA), tubes héparine et tubes oligo-éléments. Après le prélèvement du sang, les tubes sont retournés délicatement plusieurs fois (8-10 fois) afin que le sang se mélange à l'anticoagulant à l'intérieur du tube. Le sang a été congelé le jour même et conservé à -20°C jusqu'à son envoi au laboratoire. Tous les échantillons de sang ont été analysés dans le même laboratoire (ALS Scandinavia, Luleå, Suède). Les concentrations en minéraux essentiels ont été déterminées par spectrométrie de masse à plasma à couplage inductif (Rodushkin, Olofsson, Axelsson 2000).

Résultats

Statistiques descriptives

384 échantillons de sang ont été prélevés de la veine jugulaire de 164 ours bruns scandinaves dans le centre-sud de la Suède pour cette étude, dans les années 2010, 2013 et 2017 à 2022 lors des captures de printemps, d'été et d'hiver. Certains de ces 164 ours ont été capturés plusieurs fois au cours de cette période d'étude, 322 captures ont été réalisées au total (Illustrations n° 23 et 24). En résumé, 322 captures de 164 ours bruns sauvages scandinaves différents ont été effectuées au cours des années 2010, 2013 et 2017-2022, où 384 échantillons de sang ont été prélevés, dont 131 dans des tubes EDTA, 232 dans des tubes TE et 21 dans des tubes HE. Les 21 échantillons sanguins des tubes HE ont tous été prélevés au cours du printemps et de l'été 2021, tous provenant d'ours différents. Les 131 échantillons sanguins des tubes EDTA ont été prélevés au printemps et à l'été des années 2010, 2013, 2017, 2018 et 2019. Aucun ours n'a été échantillonné plus d'une fois avec les tubes EDTA au cours de chaque capture. Les 232 échantillons sanguins des tubes TE ont été prélevés au printemps, en été et en hiver au cours des années 2019, 2020, 2021 et 2022.

La population d'ours étudiée a d'abord été caractérisée en fonction de l'âge, du sexe, du poids corporel, de leur aire de répartition, et des saisons et années de prélèvement des échantillons sanguins (voir 6.1 Statistiques descriptives). Des tests de distribution normale et d'homogénéité des variances avec les variables saison, année et ours ont d'abord été effectués avec tous les échantillons regroupés (i.e., provenant des trois tubes d'échantillonnage EDTA, TE et HE). Des tests de distribution normale, d'homogénéité des variances et d'ANOVA non paramétrique ont ensuite été effectués pour comparer les valeurs moyennes et les variances entre les tubes, afin de voir si tous les échantillons pouvaient être analysés ensemble si les différences constatées n'étaient pas significatives. Des tests post-hoc incluant les tests de Wilcoxon et de Dunn ont été établis afin de déterminer quels groupes étaient responsables des différences significatives observées entre les groupes. Des boîtes à moustache et des diagrammes de dispersion ont été réalisés dans un premier temps avec l'ensemble des 384 échantillons afin d'évaluer les variations annuelles et saisonnières des concentrations en minéraux essentiels, et dans un second temps uniquement avec les 232 échantillons prélevés avec les tubes TE en raison des différences significatives dans les valeurs trouvées en fonction du type de tube.

Sur les 322 ours capturés, 227 étaient des femelles et 95 des mâles (Tableau 13). À l'exception de l'année 2010 où seulement quatre femelles ont été capturées par rapport à neuf mâles, toutes les années présentaient une majorité de femelles capturées. Le nombre d'ours capturés en 2010 et 2013 étant nettement inférieur à celui des années 2017-2022, les valeurs trouvées pour les proportions entre les sexes ne sont peut-être pas aussi représentatives. Nous constatons cependant que pour les années 2013 et 2017-2022, le pourcentage d'ours femelles capturés varie de 66,67 à 77,78 %. Au total, un pourcentage moyen de 70,50 % était des femelles et 29,50 % des mâles. Les femelles sont donc plus représentées dans cette étude et se doit d'être pris en compte dans l'interprétation des résultats. En 2010, 2013, 2017, 2018 et 2021, aucun échantillon n'a été prélevé en hiver, ni chez les mâles ni chez les femelles. En outre, les échantillons n'ont été prélevés uniquement lors des captures de printemps en 2010 et 2013. En 2017 et 2018, les mâles et les femelles ont été échantillonnés au printemps et en été. Des échantillons pour chaque saison et pour les deux sexes ont été prélevés en 2019,

2020 et 2022.

Parmi les 164 ours, 93 étaient des femelles et 71 des mâles. Plusieurs ours ont été recapturés au cours de plusieurs années, et certains même plusieurs fois au cours de la même année mais à des saisons différentes. Ainsi, un total de 322 captures a été réalisé au cours des années 2010, 2013 et 2017- 2022, dont 227 femelles et 95 mâles, avec des âges allant de 1 à 25 ans au moment de l'immobilisation (Tableau 15). Certains âges n'étaient pas disponibles (NA), soit parce que l'âge de l'ours était inconnu, soit parce que les données correspondantes étaient manquantes. Par exemple, une majorité d'ours âgés d'un an ont été capturés en 2010 ($n=5/13$, 38,46%), en 2017 ($n= 14/36$, 38,89%), en 2018 ($n=21/54$, 38,89%), et en 2020 ($n=15/67$, 22,39%). Une majorité d'ours de 3 ans a été capturée en 2013 ($n=3/9$, 33,33%), une majorité d'ours de 2 ans en 2019 ($n=11/51$, 21,57%), une majorité d'ours de 5 ans en 2021 ($n=11/56$, 19,64%), et si l'on exclut la majorité des ours d'âge inconnu en 2022 ($n=11/36$, 30,56%), les ours de 6 ans ont été principalement capturés en 2022 ($n=6/36$, 16,67%). Dans l'ensemble, les ours bruns âgés d'un an étaient les plus représentés dans cette étude ($n=68/322$, 21,12%).

Tous les ours échantillonnés semblaient en bonne santé d'après un examen clinique après avoir été immobilisés. Les prélèvements sanguins et d'autres tissus (muscles, peau, poils, fèces, etc.) n'ont été effectués qu'après avoir évalué la bonne santé des ours. Au moment de l'immobilisation et de l'échantillonnage, certaines femelles étaient des jeunes adultes ou des adultes célibataires, d'autres étaient allaitantes, d'autres encore avaient à leurs côtés des oursons de l'année ou des jeunes d'un an. Leur statut reproducteur a pu être déterminé par exemple lorsque leurs glandes mammaires étaient développées et des prélèvements de lait ont même pu être prélevés de leurs tétines sur certaines femelles. Afin de mieux comprendre la population étudiée, le poids corporel moyen des femelles, des mâles et des deux sexes en fonction de l'année de capture est présenté dans le Tableau 16.

Analyse des concentrations en minéraux essentiels dans les tubes EDTA, HE et TE

Des boîtes à moustache et des diagrammes de dispersion sont présentés dans les Figures 25 à 36 pour chaque minéral essentiel, affichant leurs valeurs et leurs variations annuelles. L'ensemble des 384 échantillons provenant des trois types de tubes (EDTA, TE et HE) sont présentés dans ces diagrammes de dispersion et les boîtes à moustache. Les boîtes à moustache affichant les concentrations en minéraux dans le sang à partir des 384 échantillons de sang pour chaque minéral essentiel révèlent des variations d'une année à l'autre. Tous les minéraux ne présentent pas la même tendance de variation. On observe par exemple une tendance pour plusieurs minéraux essentiels d'avoir des valeurs plus élevées en 2019 et 2022 par rapport aux autres années. À première vue, P, Zn, S et Se présentent des valeurs plus élevées en 2019 et 2022 que les autres années. D'autres minéraux, tels que Ca, Na, Mg et Fe, présentent des concentrations plus faibles en 2018, 2020 et 2021 par rapport aux autres années. En revanche, I, Co, Cu et Mn présentent une distribution relativement homogène des concentrations en fonction des années.

Étant donné que les concentrations en minéraux ne suivaient pas une distribution normale, un test ANOVA non paramétrique, le test de Scheirer-Ray-Hare, a été réalisé dans Rstudio pour étudier l'importance des différences avec les facteurs "année" et "ours". Toutes les p-value étaient nettement inférieures à 0,05, indiquant des différences significatives dans les concentrations d'une année à l'autre. Ce test ne permet toutefois pas d'identifier les groupes spécifiques qui diffèrent les uns des autres, mais nous indique qu'il existe des différences

entre les groupes dans leur ensemble. Les p-value $< 0,05$ indiquent qu'au moins un groupe est différent des autres. C'est pourquoi des tests post-hoc ont été effectués, en utilisant le test de Wilcoxon avec correction de Bonferroni pour les comparaisons multiples. Pour l'iode, le manganèse, le cobalt et le cuivre, le test de Wilcoxon n'a pas permis d'obtenir une p-value exacte. Les résultats du test de Wilcoxon pour ces quatre minéraux étaient basés sur des approximations. Une tentative d'effectuer un test post-hoc alternatif avec le test de Dunn a été réalisé mais les résultats ne reflétaient pas la tendance visuelle des concentrations en fonction des années. Une analyse plus poussée a été réalisée dans la partie « 6.3 Analyse des concentrations en minéraux dans les tubes TE ».

Des boîtes à moustache et des diagrammes de dispersion ont été réalisés pour chaque minéral essentiel avec l'ensemble des 384 échantillons, affichant les valeurs et leurs variations saisonnières au cours de chaque année. Par soucis de clarté, seuls les boîtes à moustache et les diagrammes de dispersion des tubes TE présentés dans les Figures 49 à 60 sont présentés dans cette étude.

Intervalles de référence selon les tubes EDTA, HE et TE

Comme indiqué dans l'introduction, pour obtenir des IR basés sur la population, le sang doit être prélevé sur un grand nombre d'animaux (un minimum de 120 idéalement), avec un mélange d'âges et de sexes. Dans cette étude, selon la définition ci-dessus, les IR ont été correctement obtenus pour les tubes TE (n=232) et les tubes EDTA (n=131), mais un nombre insuffisant d'échantillons concernant les tubes héparine (n=21) nous a permis de définir les intervalles obtenus comme des IR en tant que tels. Les intervalles obtenus avec les tubes d'héparine (Tableau 20) nous donnent une idée des valeurs pour chaque minéral essentiel, mais d'autres études à plus grande échelle sont nécessaires pour établir des IR fiables chez les ours bruns sauvages scandinaves.

Dans les Figures 25 à 36, des variations annuelles des douze minéraux essentiels ont été observées. Un des facteurs pouvant jouer un rôle dans ces variations est le type de tubes. Afin de pouvoir comparer les valeurs moyennes de chaque minéral en fonction des différents types de tubes (EDTA, HE et TE), des tests statistiques ont dû être réalisés. Un test de Shapiro-Wilk a été réalisé afin d'évaluer si les données suivaient une distribution normale et un test de Fligner-Killeen a été réalisé pour évaluer l'homogénéité des variances. Pour les deux tests, toutes les p-value étaient largement inférieures à 0,05, qui était le niveau alpha choisi. Les valeurs des minéraux essentiels ne suivaient pas une distribution normale et il y avait des différences significatives de variances entre les types de tubes. L'analyse de variance ANOVA à un facteur (ici le facteur « Tube ») suppose que les données suivent une distribution normale, ce qui n'était pas le cas des données utilisées dans cette étude. Par conséquent, le test statistique à effectuer était un test ANOVA non paramétrique, tel que le test de Kruskal-Wallis. Ce dernier a été réalisé pour évaluer s'il existait des différences significatives dans les concentrations sanguines des douze minéraux essentiels entre les trois types de tubes (EDTA, HE et TE). En d'autres termes, on a cherché à savoir si le choix du type de tube avait un effet significatif sur les concentrations de minéraux essentiels. Le test de Kruskal-Wallis a révélé une p-value bien inférieure à 0,05, ce qui indique des différences significatives dans les concentrations de minéraux entre les trois types de tubes. Afin de savoir plus précisément où se situent ces différences, une analyse post-hoc a été effectuée, en utilisant le test de Wilcoxon, qui ont comparé chaque paire de types de tubes. Cela a permis de voir quels minéraux essentiels avaient des concentrations significativement différentes d'un type de

tube à l'autre. Les résultats du test de Wilcoxon sont présentés dans le Tableau 22. Le sodium, le magnésium, le fer et l'iode sont les quatre minéraux essentiels dont les concentrations ne sont pas significativement différentes d'un tube à l'autre. En revanche, le phosphore et le manganèse présentent des concentrations significativement différentes entre les trois types de tubes (en orange dans le Tableau 22). Les différences significatives entre les tubes EDTA et TE concernent le phosphore, le zinc, le soufre, le manganèse et le sélénium. Les différences significatives entre les tubes EDTA et HE concernent le calcium, le phosphore, le cobalt, le cuivre, le manganèse et le sélénium. Les différences significatives entre les tubes TE et HE sont le calcium, le phosphore, le zinc, le cobalt, le cuivre et le manganèse. Nous ne pouvons donc pas regrouper toutes les valeurs moyennes (EDTA, HE et TE ensemble) dans l'analyse suivante puisqu'il existe des différences significatives dans les valeurs des valeurs moyennes entre les types de tubes. Afin de simplifier l'analyse, les variations saisonnières et annuelles des douze minéraux essentiels ont été analysées dans les sections suivantes avec les tubes TE uniquement.

Analyse des concentrations en minéraux essentiels dans les tubes TE

Après avoir réalisé un test de Shapiro-Wilk, nous avons pu affirmer avec un intervalle de confiance de 95 % que les valeurs des douze minéraux essentiels échantillonnés dans les tubes TE ne suivaient pas une distribution normale. Le test de Fligner-Killeen a ensuite révélé qu'il n'y avait pas de différences significatives dans les variances entre les groupes. Les concentrations minérales ne suivant pas une distribution normale, un test ANOVA non paramétrique, le test Scheirer-Ray-Hare, a été réalisé dans Rstudio pour étudier l'importance des différences avec les facteurs "année" et "ours". Toutes les p-value étaient largement inférieures à 0,05, ce qui indique des différences significatives pour toutes les concentrations en minéraux essentiels entre les années. L'une des boîtes à moustache les plus surprenantes était celui qui montrait les variations annuelles des concentrations en Na. Les années 2019 et 2022 présentaient des moyennes bien plus élevées que les années 2020 et 2021. De plus, entre les années 2021 et 2022, aucune valeur ne se chevauchait.

De même que pour les années, des boîtes à moustache et diagrammes de dispersion ont été effectués pour chaque minéral essentiel en fonction des saisons au sein des années 2019 à 2022. Les tests de Shapiro-Wilk et Fligner-Killeen ont retourné les mêmes résultats que pour l'analyse des variations annuelles, c'est-à-dire que les valeurs des douze minéraux essentiels échantillonnés dans les tubes TE ne suivaient pas une distribution normale et qu'il n'y avait pas de différences significatives dans les variances entre les groupes.

Avant de réaliser un test ANOVA pour tester les différences significatives des concentrations minérales en fonction des variables « saisons » et « ours », un test ANOVA non paramétrique, le test de Scheirer-Ray-Hare, a été réalisé afin d'évaluer les interactions potentielles entre les variables "année" et "saison". Toutes les p-value reflétant des différences significatives pour l'interaction "saison" x "année" étaient supérieures à 0,05, à l'exception de Zn qui avait une p-valeur de 0,46. Ainsi, à l'exception de Zn, aucune interaction entre les variables "année" et "saison" n'a été révélée. Sachant cela, un deuxième test de Scheirer-Ray-Hare a été effectué dans Rstudio pour étudier la significativité des différences avec les facteurs "saison" et "ours" (Tableau 23). Des différences significatives entre les groupes et donc un effet saison ont été observés pour le sodium, le fer, le zinc, l'iode, le soufre, le cobalt, le cuivre, le manganèse et le sélénium. Seuls le phosphore, le calcium et le magnésium ne présentaient pas de différence significative en fonction de la saison. De plus, il y avait des différences significatives entre les

groupes pour le manganèse et l'iode en fonction de la variable ours. Le fait que le Zn ait révélé une p-value significative avec le test de Scheirer-Ray-Hare et donc une interaction entre les variables saison et année nous empêche de tirer des conclusions quant à l'influence de la saison sur les concentrations de Zn. Le Fe, le S et le Cu ont montré des valeurs moyennes significativement différentes entre les trois saisons. Les autres minéraux essentiels ont également montré des différences entre les saisons, mais pas entre les trois saisons. Étant donné que Ca, P et Mg n'ont pas montré de p-value significatives dans le test de Scheirer-Ray-Hare du Tableau 23, les différences trouvées entre les saisons dans le test de Wilcoxon du Tableau 24 doivent être traitées avec précaution. Parmi les minéraux essentiels présentant des variations saisonnières significatives dans le Tableau 23, le Na, Fe, S, Co, Cu, Mn et Se avaient les valeurs moyennes les plus faibles au printemps. L'iode est le seul minéral essentiel dont les valeurs sont les plus élevées au printemps. Le Fe, S, Cu, Mn et Se avaient des valeurs les plus élevées pendant l'hibernation. Il est cependant délicat de comparer les valeurs saisonnières moyennes pour chaque minéral car la taille des échantillons par saison était inégale. La taille d'échantillonnage pendant l'hiver était bien plus faible que la taille d'échantillonnage pendant le printemps, qui était la saison avec le plus grand nombre d'échantillons. Pour le Ca et Mg, aucune différence significative entre les saisons n'a été révélée par le test de Scheirer-Ray-Hare, même s'il semble y avoir une différence saisonnière dans les valeurs lorsque l'on regarde les boîte à moustache (Figures 49 et 52). Au contraire, le Na ne semble pas avoir d'influence saisonnière sur les valeurs lorsque l'on examine les boîtes à moustache (Figure 51), mais la p-value a été révélée une valeur significative avec le test de Scheirer-Ray-Hare. Cela pourrait s'expliquer en partie par le fait que la taille des échantillons est inégale d'une saison à l'autre, avec beaucoup moins d'échantillons prélevés en hiver qu'au printemps.

Discussion

Les variations annuelles des concentrations des douze minéraux essentiels présentées dans les diagrammes en boîte des Figures 25 à 36 peuvent être liées à plusieurs facteurs. Elles pourraient s'expliquer par le fait que tous les échantillons n'ont pas été prélevés de la même manière d'une année à l'autre. Les variations annuelles des concentrations en minéraux pourraient tout d'abord être observées en raison de la différence de taille d'échantillons d'une année à l'autre, comme le montre le Tableau 11. La taille des échantillons varie de n=9 en 2013 à n=82 en 2019. L'échantillonnage n'est donc pas également représentatif de la population d'ours bruns d'une année à l'autre. Avec moins d'échantillons collectés en 2010 et 2013, la population n'est pas aussi représentative que dans les années 2019, 2020 et 2021 avec plus de 71 échantillons. De plus, les saisons peuvent avoir une influence sur les variations annuelles, car toutes les années n'ont pas eu une taille similaire d'échantillons pour chaque saison au sein de la même année. Comme le montre le Tableau 14, le nombre d'échantillons prélevés par saison varie d'une année à l'autre. Par exemple, les échantillons prélevés en hiver ne l'ont été qu'en 2019, 2020 et 2022. En 2010 et 2013, les échantillons n'ont été collectés qu'au printemps, avec une taille d'échantillons réduite par rapport aux autres années. Toutes les années n'ont pas fait l'objet d'un prélèvement d'échantillons pour chaque saison et dans la même quantité. En ce qui concerne la variable saison, les ours bruns subissent des changements physiologiques tout au long de l'année et comme le nombre d'échantillons prélevés pour chaque saison n'est pas le même pour toutes les années, certaines années n'ont pas reflété la variabilité des changements physiologiques, alors que d'autres années oui. Par exemple, en 2010 et 2013, avec aucun échantillon n'ayant été prélevé en hiver ou en été, seul l'état physiologique des ours au printemps a été représenté, tandis que les années 2019, 2020

et 2022 ont affiché des valeurs reflétant l'état physiologique des ours pendant les trois saisons (hiver, été et printemps). La déshydratation est un exemple de changements physiologiques observés entre les saisons. L'état de déshydratation des ours peut donc influencer les concentrations en minéraux, ce qui est particulièrement vrai en hiver (Welinder et al. 2016) et en cas de températures ambiantes élevées ou suite à des poursuites intenses. Ces biais sont réduits dans l'analyse des concentrations minérales sanguines provenant uniquement des tubes TE, affichées sur les boîtes à moustache des Figures 37-48. Les variations en concentrations des minéraux devraient être moins affectées par la taille des échantillons ($n = 63$ en 2019, $n = 71$ en 2020, $n = 57$ en 2021 et $n = 41$ en 2022) et le facteur saisonnier, car les échantillons ont été prélevés au cours des trois saisons (sauf en 2021 où seuls des échantillons ont été prélevés au printemps et en été).

Le sexe pourrait également influencer les variations annuelles affichées sur les boîtes à moustache des Figures 25-36 et 37-48. Comme le montrent les Tableaux 13 et 14, le pourcentage de mâles et de femelles échantillonnés diffère de manière plus ou moins importante chaque année. L'année 2010 est la seule année où une majorité de mâles a été échantillonnée, alors que les autres années ont été marquées par une majorité de femelles (plus de 66 %). Hormis 2010, les années présentent un ratio (r) femelles/mâles compris entre 2 et 3,5 ($r = 4/9 = 0,44$ en 2010 ; $r = 6/3 = 2$ en 2013 ; $r = 27/9 = 3$ en 2017 ; $r = 38/16 = 2,38$ en 2018 ; $r = 38/13 = 2,92$ en 2019 ; $r = 46/21 = 2,19$ en 2020 ; $r = 40/16 = 2,5$ en 2021 ; $r = 28/8 = 3,5$ en 2022). Si le ratio femelles/mâles avait été similaire d'une année à l'autre, le facteur sexe aurait potentiellement eu moins d'impact sur les différences de valeurs moyennes entre les années. Une étude menée en 2015 sur des ours bruns scandinaves sauvages a révélé des différences significatives dans les concentrations sériques en fonction du sexe des ours pour le Na, où les concentrations en Na sérique étaient significativement plus élevées chez les femelles que chez les mâles (Græsli et al. 2015). Bien que les niveaux de minéraux aient été évalués dans le plasma et non dans le sérum dans cette étude, cela soutient l'hypothèse selon laquelle le sexe pourrait avoir une influence sur les concentrations de minéraux dans le plasma chez les ours bruns sauvages. Il serait intéressant d'explorer d'autres études avec une analyse statistique de l'influence du sexe sur les concentrations minérales dans le sang total chez les ours bruns scandinaves (Annexe 8).

La différence d'âge des ours d'une année à l'autre pourrait également jouer un rôle dans les variations annuelles illustrées par les diagrammes en boîte des Figures 25-36 et 37-48. Comme le montre le Tableau 15, les catégories d'âge varient d'une année à l'autre. Une majorité d'ours d'un an a été capturée en 2010, 2017, 2018 et en 2020, une majorité d'ours de 3 ans a été capturée en 2013, une majorité d'ours de 2 ans en 2019, une majorité d'ours de 5 ans en 2021 et des ours de 6 ans ont été principalement capturés en 2022. L'âge est connu pour avoir une influence sur les concentrations sanguines en minéraux, en particulier pour les éléments toxiques (Fuchs et al. 2023), et pourrait donc avoir un impact sur les variations minérales annuelles dans cette étude puisque les catégories d'âge varient d'une année à l'autre. Les individus jeunes et âgés peuvent avoir des besoins en minéraux et des taux d'absorption différents. Une étude menée sur le cerf élaphe en 2013 a montré des différences significatives dans les concentrations minérales sériques entre les individus jeunes et adultes (Kučer et al. 2013). Par exemple, ils ont constaté une augmentation significative des niveaux sériques de Na avec l'âge. Dans notre étude, les niveaux de Na plasmatique dans les tubes TE ont montré les valeurs les plus élevées en 2022, lorsque la principale catégorie d'âge des ours capturés était de 6 ans, ce qui était plus élevé que les autres années (Tableau 15). Une étude menée en 2015 sur des ours bruns scandinaves sauvages a révélé des différences significatives dans les

concentrations sériques en fonction de l'âge des ours pour le Fe, le Mg et le P (Græsli et al. 2015). Il serait intéressant de mener d'autres études avec une analyse statistique de l'influence de l'âge sur les concentrations de minéraux dans le sang total chez les ours bruns scandinaves. Il serait même très intéressant d'étudier l'influence des facteurs âge et sexe sur les concentrations en minéraux dans le sang total des ours bruns scandinaves (Annexe 8). En outre, en relation avec les facteurs âge et sexe, le statut reproducteur peut influencer les variations annuelles des concentrations minérales. Les pourcentages de femelles célibataires ou allaitantes varient d'une année à l'autre et comme les femelles allaitantes peuvent avoir un métabolisme minéral modifié, cela pourrait jouer dans les variations annuelles et saisonnières.

Plus important encore, les minéraux essentiels chez les animaux sont apportés par leur régime alimentaire, de sorte que les variations en disponibilité alimentaire entre les années peuvent avoir un impact sur les concentrations minérales annuelles. En effet, l'analyse des minéraux sanguins est fréquemment utilisée chez les animaux comme moyen d'évaluer leur apport via la nutrition (Herdt, Rumbelha, Braselton 2000). Les variations de la qualité et de la quantité de nourriture peuvent influencer les concentrations en minéraux chez les ours bruns. Certaines années, les baies (l'un des aliments les plus importants dans le régime alimentaire de l'ours brun) peuvent être plus ou moins abondantes, ou les charognes plus ou moins nombreuses au printemps. Ces variations annuelles dans la disponibilité et de la composition alimentaire peuvent être influencées par des conditions environnementales telles que la température, les précipitations, la couverture neigeuse, etc. Des variations saisonnières et annuelles significatives ont été objectivées dans les composants alimentaires tels que les insectes, les vertébrés, les plantes et les baies (Stenset et al. 2016). Les variations constatées entre les années dans les concentrations sanguines de minéraux essentiels chez les ours bruns scandinaves peuvent ainsi être fortement liées aux variations de la disponibilité alimentaire. En effet, les concentrations en minéraux essentiels trouvés dans le sang total dépendent en partie des aliments contenant ces minéraux que les ours bruns ingèrent. Les variations saisonnières et annuelles peuvent coïncider avec les changements dans la disponibilité des ressources alimentaires, plus particulièrement la maturation des baies, la naissance des jeunes élans et la saisonnalité des plantes. Si certaines années sont "bonnes" pour les myrtilles, ce n'est pas nécessairement le cas pour les airelles ou les camarines, et vice versa. Les composantes en minéraux essentiels des différents aliments consommés par les ours étant différentes, les variations annuelles objectivées en minéraux essentiels dans cette étude peuvent donc être en partie liées aux variations annuelles de la disponibilité des baies. Dans cette étude, les variations saisonnières des concentrations minérales dans les tubes TE ont été trouvées pour Na, Fe, I, S, Co, Cu, Mn et Se. Aucune variation saisonnière n'a été statistiquement révélée pour Ca, P et Mg, et les résultats pour Zn n'étaient pas concluants puisqu'une interaction entre les facteurs année et saison a été objectivée. Ces variations saisonnières observées pour Na, Fe, I, S, Co, Cu, Mn et Se peuvent être fortement liées aux changements physiologiques du métabolisme d'une saison à l'autre chez les ours bruns scandinaves, en particulier les différences observées pendant l'hiver, lorsque les ours sont dans un état hypométabolique. Une étude menée sur des chèvres africaines en 2012 n'a pas révélé de différences significatives dans les niveaux de calcium et de phosphore sériques entre les saisons sèches et humides (Rumosa Gwaze, Chimonyo, Dzama 2012). Bien entendu, les concentrations minérales de notre étude ont été évaluées à partir du plasma d'espèces omnivores sauvages et non à partir de sérum d'herbivores domestiques. Même si les ours bruns subissent des changements physiologiques dans leur métabolisme entre les saisons et que certaines concentrations minérales dans le sang pourraient être attendues, le fait

qu'aucune différence significative dans les niveaux de calcium, de phosphore et de magnésium dans le sang n'ait été trouvée soutient l'hypothèse que ces minéraux sont régulés de manière homéostatique et maintenus dans des limites assez étroites (Rumosa Gwaze, Chimonyo, Dzama 2012). Une étude menée en 2015 sur des ours bruns sauvages scandinaves a révélé des différences significatives dans les concentrations sériques entre les saisons pour le chlorure, le fer, le magnésium, le phosphore et le sodium (Græsli et al. 2015). Un domaine de recherche intéressant serait d'approfondir l'influence d'autres facteurs que les composants alimentaires affectant les valeurs des minéraux essentiels chez les ours, et de voir comment ces variations affectent la biologie et le comportement de l'ours brun (comment ces variations affectent-elles le cycle d'hibernation, leur succès et leur cycle de reproduction, leur survie, etc.) Certaines études expérimentales ont déjà objectivé l'impact du changement climatique sur la disponibilité des ressources alimentaires. Le dégel hivernal survenant plus tard dans l'année entraîne des réductions importantes dans la production de baies, allant jusqu'à 52-95% chez les myrtilles et 11-75% chez les camarines (Bokhorst et al. 2011). Les baies représentent la principale source de nourriture pendant la période d'hyperphagie avant l'hibernation, mais avec cette réduction de la disponibilité des baies, on peut s'attendre à ce que les ours bruns scandinaves se tournent vers d'autres aliments pendant la période d'hyperphagie, comme c'est le cas dans le nord de l'Espagne (Rodríguez et al. 2007). Cela pourrait être l'une des explications des variations annuelles des intervalles de référence des minéraux essentiels constatées dans notre étude, les ours bruns changeant progressivement les ratios de leurs aliments pendant la période d'hyperphagie.

Un autre facteur susceptible d'affecter les résultats serait l'heure de la dernière prise alimentaire de l'animal. Le fait de savoir quand un animal a mangé pour la dernière fois peut s'avérer utile dans l'analyse des nutriments dans le sang. Après la consommation d'aliments, le corps commence le processus de digestion et la redistribution des nutriments dans l'organisme par le biais de la circulation sanguine. Il en résulte un pic dans la circulation des nutriments et des minéraux dans l'organisme lorsqu'ils pénètrent dans la circulation sanguine pour être redistribués aux tissus périphériques. La redistribution des nutriments dépend du nutriment en question, et certains sont digérés plus rapidement que d'autres. Par conséquent, la meilleure façon d'éviter les pics de concentrations de nutriments et les différences dans les valeurs restituées d'un animal à l'autre est de réaliser les prélèvements sanguins sur un animal à jeun. Les captures effectuées sur des animaux sauvages ne permettent pas de faire jeûner l'animal avant la prise de sang, de sorte que les ours échantillonnés dans cette étude avaient tous un statut différent en ce qui concerne le moment de leur dernier repas (sauf en hiver, où le jeûne commence à l'entrée de la tanière en octobre-novembre). Le fait d'avoir un statut différent de l'horaire du dernier repas de l'ours peut donc ajouter un biais dans l'analyse des minéraux essentiels.

Un autre facteur important susceptible d'avoir influencé les variations annuelles est le type de tubes utilisés pour les prélèvements sanguins, qui peut avoir entraîné une variabilité dans la quantification de certains minéraux et oligo-éléments dans le sang. Une mauvaise conception ou utilisation des dispositifs de prélèvement sanguin peut nuire à la précision des résultats des tests de laboratoire (Bowen, Remaley 2014). Le nombre d'échantillons prélevés dans les différents types de tubes (EDTA, HE et TE) n'a pas été le même pour toutes les années. Le Tableau 11 résume le nombre d'échantillons collectés chaque année en fonction du type de tubes. Par exemple, seuls des tubes EDTA ont été utilisés pour les prélèvements sanguins au cours des années 2010, 2013, 2017 et 2018. En 2020 et 2022, seuls des tubes TE ont été utilisés et les tubes HE n'ont été utilisés qu'en 2021. La plupart des échantillons de sang ont

été prélevés avec des tubes TE (n= 232), moins avec des tubes EDTA (n=131) et encore moins avec des tubes HE (n=21). Par conséquent, la taille de l'échantillon pour les tubes HE est plus de dix fois inférieure à celle de l'échantillon pour les tubes TE, leurs valeurs sont donc moins significatives. Les tests de Kruskal-Wallis et de Wilcoxon effectués ont montré qu'il existait des différences statistiquement significatives pour certaines concentrations minérales en fonction du type de tubes. P et Mn sont les deux minéraux qui présentent des différences significatives de concentrations sanguines entre les trois tubes. Certains minéraux ont montré des différences significatives entre deux types de tubes tandis que d'autres minéraux n'ont pas montré de différences significatives entre les trois tubes (i.e., Na, Mg, Fe et I). En outre, les tubes de sang K2 et K3-EDTA contiennent des anticoagulants qui chélatent le Ca (et d'autres minéraux tels que les ions Fe, Zn et Mg) (Staff 2013 ; Imafuku et al. 2002 ; Nkuna, Ijoma, Matambo 2022). Sachant cela, on aurait pu s'attendre à des valeurs plus faibles pour ces ions dans les tubes EDTA, mais cela n'a pas été le cas dans cette étude. Chez l'homme, il a été démontré que les éléments présents dans le sang/sérum à des concentrations supérieures à 10 ng/mL, c'est-à-dire Na, Ca, Mg, P, Fe, Br, Si, Zn, Cu, Rb, Se et I, la contribution de la contamination des dispositifs d'échantillonnage des tubes était négligeable, alors que pour la majorité des oligo-éléments présents dans l'organisme en plus faible quantité elle peut affecter de manière significative la détermination précise de leur concentration (Rodushkin, Odman 2001). Dans cette étude, les concentrations d'ions potentiellement affectés par les agents chélateurs dans les tubes EDTA (i.e., Ca, Mg, Fe et Zn) n'ont pas montré de valeurs significativement diminuées par rapport à celles trouvées dans les tubes HE et TE.

La méthode de transport, les conditions de stockage et la température des échantillons sanguins, le temps écoulé entre l'échantillonnage et le stockage, l'analyse en laboratoire (i.e., différentes personnes chargées de l'analyse d'une année à l'autre, changement d'équipement et/ou de méthode d'analyse, etc) doivent également être pris en compte. Avec des variations annuelles aussi importantes que celles présentées dans les Figures 25-36 et 37-48, le facteur "erreurs de manipulation/de laboratoire" pourrait avoir eu une forte influence. Par exemple, la Figure 39 qui présente les variations annuelles de Na, montre des différences significatives entre les années 2019 et 2022 et les années 2020 et 2021. Aucune valeur de Na ne se superpose dans les boîtes à moustache et les points de dispersion entre les années 2021 et 2022. Même si des études sur le cerf élaphe et l'ours brun scandinave montrent que l'âge et le sexe ont une influence sur les concentrations de Na chez les individus (Kučer et al. 2013; Græsli et al. 2015), il est peu probable que des différences aussi importantes soient uniquement dues à des facteurs liés à l'âge et au sexe. Par conséquent, l'un des principaux facteurs influençant les variations annuelles dans cette étude est supposé être dû à des erreurs de manipulation et de laboratoire dans l'analyse des minéraux. Il est possible que les protocoles, les analyseurs, les conditions de transport et de stockage aient été très différents d'une année à l'autre. Il serait intéressant de poursuivre des études en s'assurant que des méthodes strictement identiques sont utilisées d'une année à l'autre.

En résumé, les variations annuelles des douze minéraux essentiels peuvent être influencées par la taille de l'échantillonnage annuel, le sexe, l'âge et le statut reproducteur, la saison et les changements métaboliques physiologiques associés, les conditions environnementales ayant un impact sur la qualité et la quantité des composants alimentaires, le type de tube, le transport, le stockage et les méthodes d'analyse en laboratoire. Afin d'éliminer l'influence du choix du tube dans les variations de concentrations minérales observées entre les années, seules les concentrations collectées dans les tubes TE ont été analysées dans un deuxième temps dans la partie résultats. Les variations annuelles et saisonnières ont également été

objectivées mais avec des amplitudes de valeurs moins importantes que celles observées dans l'analyse avec les trois types de tubes. Les facteurs communs ayant le potentiel d'interférer avec les variables biochimiques des échantillons collectés comprennent : le stress et l'activité physique accrue due à la chasse, les médicaments anesthésiques (Arnemo JM, Caulkett N 2007), le jeun, la déshydratation, le régime alimentaire, les maladies, les facteurs génétiques, le statut reproducteur (femelles gestantes, allaitantes, en chaleur), les variations journalières et saisonnières, etc. (Irjala, Grönroos 1998). Tous ces facteurs doivent être pris en considération et doivent être évités au maximum, avec une cohérence dans les procédures d'échantillonnage et de protocole. Alors que certains facteurs extrinsèques peuvent être plus ou moins faciles à éviter ou du moins contrôlés d'une certaine manière, les facteurs intrinsèques susceptibles d'interférer avec les variables sanguines sont difficiles à gérer.

Les ours d'âge et sexe différents présentent des différences de métabolisme, d'alimentation, de poids corporel, de comportement et de physiologie, qui peuvent se refléter dans les variables biochimiques. C'était cependant l'objectif de cette étude, d'avoir une population avec un mélange d'âges, de statut reproducteur et de sexe afin d'obtenir des IR aussi représentatifs de la population d'ours bruns scandinaves que possible.

Des études complémentaires seraient intéressantes pour analyser les concentrations dans le sang total de ces douze minéraux essentiels chez d'autres espèces afin de comparer les résultats obtenus chez l'ours brun. Par exemple, il serait intéressant de choisir des espèces animales du même environnement que l'ours brun. Plus particulièrement, une espèce herbivore, comme l'élan *Alces alces* et une espèce carnivore, comme le loup *Canis lupus*. L'élan et le loup sont des espèces étudiées à l'Université des sciences appliquées de Norvège à Evenstad, et donc, comme pour les ours, des captures de ces espèces ont lieu afin de mener des études écologiques et la pose de colliers émetteurs. Les positions de certains individus ont été relevées, montrant une proximité avec l'aire d'habitat de l'ours brun (Annexe 9).

À plus grande échelle, il serait également intéressant de réaliser des études comparant les concentrations de minéraux essentiels trouvés chez les ours bruns sauvages scandinaves avec d'autres sous-espèces d'*Ursus arctos*. Les ours bruns ont des régimes alimentaires différents à travers le monde entier en fonction de leur situation géographique. La variation spatiale du régime alimentaire des ours bruns est le résultat des conditions environnementales (e.g., les facteurs climatiques), qui affectent la disponibilité locale de nourriture et les besoins nutritionnels et énergétiques des ours bruns (Bojarska, Selva 2012). Par conséquent, en fonction de la localisation des ours bruns, les proportions des composants alimentaires dans leur régime varient de largement herbivore à largement carnivore (Bojarska, Selva 2012). Par exemple, en comparant ces valeurs avec celles trouvées dans les populations d'ours en Europe (e.g., les populations pyrénéennes, cantabriques, carpatiques, caréliennes, dinariques et balkaniques (The IUCN Red List of Threatened Species 2018 ; IUCN/SSC 2023)), en Amérique du Nord (e.g., population canadienne, population d'Alaska, population du parc national de Yellowstone (*Bears of North America* [sans date])), au Japon (e.g., population de Hokkaido (Endo et al. 2021)) ou en Russie (Kudrenko et al. 2022) serait bénéfique pour évaluer l'impact du régime alimentaire sur les concentrations en minéraux essentiels. Étant donné que les différents composants alimentaires contiennent des concentrations différentes en minéraux essentiels, on peut s'attendre à ce que les valeurs et les intervalles de référence dans d'autres populations d'ours diffèrent des concentrations sanguines trouvées chez les ours bruns scandinaves.

Conclusion

Cette étude visait à établir des intervalles de référence pour douze minéraux essentiels dans le sang total d'ours bruns sauvages scandinaves (*Ursus arctos*), immobilisés chimiquement avec une combinaison de médétomidine, de tilétamine et de zolazépam au printemps et à l'été, et de la kétamine en plus en hiver. L'analyse a révélé des variations saisonnières et annuelles dans les concentrations en minéraux essentiels. Ces variations ont probablement été influencées par plusieurs variables, notamment le sexe, l'âge, le type de tube d'échantillonnage, les erreurs de manipulation et de laboratoire, le changement physiologique du métabolisme de la phase active à l'hibernation, et les conditions environnementales ayant un impact sur les composants du régime alimentaire de l'ours et leur disponibilité. Cette étude fournit des données sur les concentrations en minéraux essentiels dans le sang total, ce qui nous permet d'élargir nos connaissances sur leur physiologie et les impacts potentiels des changements environnementaux dans leur régime alimentaire. D'autres études à plus grande échelle et comparatives avec d'autres espèces sauvages et/ou sous-espèces d'ours bruns pourraient contribuer à approfondir nos connaissances sur la dynamique écologique au sens large.

2. INTRODUCTION

Reference intervals for commonly measured blood-based analytes are important for health monitoring on an individual or at population level. Indeed, specific blood analytes can reflect certain organ functions and therefore be used to monitor physiological and pathological processes in organs, electrolytic balance, enzyme activity etc.

A reference interval (RI) “includes upper and lower limits of a lab test based on a group of apparently healthy subjects” (MedCalc online definition; Geffré et al. 2009). Usually, RIs include the values in which 95% of apparently healthy individuals would fall in (Geffré et al. 2009; Horn, Pesce 2003). They represent the most widely used medical decision-making tool for interpretation of abnormalities and potential diseases. RIs are established through statistical analysis from a large sample of healthy individuals, to be as significant as possible. They serve as a guideline, notably for healthcare professionals, to assess and interpret test results, and determine whether they fall within the expected range for a given parameter. Thus, they help in the diagnosis and monitoring of certain medical conditions, and in the identification of deviations from the norm. Extensive guidelines on formulating such RIs have been established by the American Society of Veterinary Clinical Pathology (ASVCP) (Friedrichs et al. 2012). In order to obtain population-based RIs, blood should be sampled from a large number of animals (a minimum of 120 ideally), with a mixture of ages and genders. Sampling a large number of individuals often poses a challenge in different animal species, and especially in wildlife species. Establishing RIs for blood analytes in brown bears over a long period of time, which is the case in this study, allows us to collect a large number of samples (384 samples from 164 different bears, some being captured several times from one year to the next and/or even from one season to the next within the same year).

Values outside of the RI (higher or lower) of blood analytes often reflect a pathology, such as an organ malfunction or failure for example (Jones et al. 2018; Dimitrakakis et al. 2022; Park, Kim, Do 2021). While RIs for biochemical variables in domestic animals such as cats and dogs, cows, horses, pigs, goats and sheep have been widely studied all across the world, one can imagine that establishing such RIs for wildlife species isn't so easy or accessible. One of the biggest challenges when doing research on wildlife and processing the obtained values from captured individuals is not having a “control population”, which in this case are the free-ranging bears that have not been captured. Few studies, listed below, have been carried out regarding biochemical reference values in brown bears (*Ursus arctos*), even less so in Scandinavian brown bears. The data available on such a topic often comes from small sample sizes, not always over a long period of time and from animals in captivity. Biochemistry data has previously been evaluated in the American black bear *Ursus americanus* (Matula, Lindzey, Rothenbacher 1980; Schroeder 1987; Hellgren, Rogers, Seal 1993), captive Asiatic black bears *Ursus thibetanus* (Yang et al. 2017), brown bears *Ursus arctos* (Halloran, Pearson 1972; Brannon 1985; Hissa et al. 1994; Huber et al. 1997; Græsli et al. 2014; Græsli et al. 2015) and polar bears *Ursus maritimus* (Tryland et al. 2002; Fry et al. 2019). Some RIs have been evaluated in free-ranging Scandinavian brown bears for some of these biochemical variables but with different parameters. For example, Græsli et al. (2014) evaluated biochemical reference intervals for calcium, iron, magnesium, sodium and inorganic phosphorus in free-ranging Scandinavian brown bears but from serum. Tryland et al. (2002) evaluated reference intervals from plasma for sodium, magnesium, calcium and phosphorus but in free-ranging

polar bears in Svalbard. Fuchs et al. (2021) and Fuchs et al. (2023) evaluated concentrations of lead, arsenic, mercury, and cadmium in whole blood of free-ranging Scandinavian brown bears. Another recent study was conducted on lead but also arsenic, cadmium, mercury, selenium and copper concentrations in whole blood in free-ranging Scandinavian brown bears (Rikke Kristiansen Aarmo, 2021). To our knowledge, this is the first study reviewing values in whole blood and establishing reference intervals for the following twelve essential minerals in free-ranging Scandinavian brown bears: calcium, cobalt, copper, iodine, iron, magnesium, manganese, phosphorus, selenium, sodium, sulfur and zinc.

The venous blood samples were collected during anesthesia for marking, radio collaring and several types of sample collecting (blood, fat, muscle, skin, saliva, feces, hair, milk, etc.) for research studies involving several teams from different countries, according to the “Biomedical protocols for free-ranging Brown Bears, Wolves, Wolverines and Lynx” described by Arnemo and Evans (2017). Anesthetizing a wild animal requires a lot of organization, logistics, planning, funding, authorizations from legal authorities, etc. (Kreeger TJ et al. 2023). The use of live wild animals in research is granted only after assessing its necessity and provided that other methods of data collection wouldn’t suffice (e.g., DNA sampling via feces or hair). And so, every year, several teams from different countries with various research projects gather in south-central Sweden for the winter and summer captures. That way, multiple research projects get to collect their samples or data while anesthetizing the brown bears as few times as possible so as to disturb the bears as little as possible. The Scandinavian Brown Bear Research Project (SBBRP), created back in 1984, used to involve only Scandinavian research teams from Norway and Sweden. Nowadays and for the last couple of decades, the teams involved come from several different countries in Europe, to collect data from the incredible specimen which is the Scandinavian brown bear. Some examples of the ongoing projects include translational biomedicine research on muscle disuse and atrophy (Gomez et al. 2021), endocrinology, cardiology and cardiovascular diseases (Frøbert et al. 2022; Frøbert et al. 2010; Frøbert et al. 2023; Thienel et al. 2023), hematology and biochemistry (Græsli et al. 2014; 2015), the drivers of hibernation and the associated physiological and physical changes (Alina Evans, Singh, Friebe, et al. 2016), the lipidic metabolism during hibernation (Giroud et al. 2019; 2018; 2021), reproduction (Alina Lynn Evans, 2016; Friebe et al. 2014; Lemièrre et al. 2022), ecology and predation (Sahlén et al. 2014; Thiel et al. 2022), toxicology (Fuchs et al. 2021; 2023), etc. Brown bears are incredible in that they manage to lie in an inactive hypometabolic state during five to seven months, without suffering the consequences humans would undergo if they would do it. Among other reasons, brown bears serve as a fascinating natural model for studying their behavioral and physiological adaptations to long periods of inactivity, offering insights to human medicine for people associated with a sedentary lifestyle.

Although it isn’t possible to ascertain that the values from the samples included in this study truly reflect the physiological values found in free-ranging brown bears, they may give a preliminary general idea. The blood samples were collected from brown bears after chemical immobilization, darted from a helicopter during spring and summer. Common factors having the potential to interfere with the biochemical variables from the collected samples include: stress and increased physical activity due to the chase, the anesthetic drugs (Arnemo JM, Caulkett N 2007), starvation, dehydration, diet, diseases, genetic factors, reproductive status (pregnant, lactating, female in heat), daily and seasonal variations, etc. (Irjala, Grönroos 1998). All these factors need to be taken into consideration and should be avoided to a maximum, with a consistency in the sampling and protocol procedures. While some extrinsic factors may

be more or less easy to avoid or at least control in some ways, intrinsic factors potentially interfering with the blood variables are challenging to manage. Bears of different ages and gender show differences in metabolism, diet, body weight, behavior and physiology, which may be reflected in the biochemical variables.

Hibernation constitutes a period in the bear's life of huge importance since it spends approximately six months inside the den (from November to April). Brown bears experience a drop in body temperature, decreased heart rate, respiratory rate and metabolic rate. Prior to hibernation, they build up body reserves (mostly by storing fat) by increasing their food consumption resulting in weight gain. When spring comes and the bears emerge from their den, they must rebuild their body reserves again from all the weight loss they've undergone during winter, especially if the mother was pregnant and ends up nursing cubs. Their body mass is very inconsistent throughout the year, depending on the season and their physiological state associated. The physiological variations according to the different seasons may therefore influence the biochemical blood analytes, as it has been recorded for some variables in brown bears (Halloran, Pearson 1972; R. Hissa et al. 1994; Huber et al. 1997; Græsli et al. 2015).

The objectives of this study were to establish RIs for twelve biochemical variables in whole blood in free-ranging Scandinavian brown bears *Ursus arctos*, chemically immobilized using a combination of medetomidine, tiletamine and zolazepam during spring and summer, and a combination of medetomidine, tiletamine, zolazepam and ketamine during winter over a twelve-year period of time, and to assess the seasonal and annual variations in the biochemical variables.

3. BACKGROUND

3.1 The Scandinavian Brown Bear

3.1.1 Characteristics

3.1.1.1 – Physical characteristics and distribution

The Scandinavian brown bear belongs to the brown bear species *Ursus arctos*, which is one of the eight species of bears in the *Ursidae* family (Dahle et al. 1998). More specifically, it belongs to the Eurasian brown bear subspecies *Ursus arctos arctos*.

Table 1: Taxonomy of the Scandinavian brown bear. Source: Table made by author based on the Brown bear fact sheet website <https://ielc.libguides.com/sdzg/factsheets/brownbear/taxonomy>

Class	Order	Family	Genus	Species	Subspecies
Mammalia	Carnivora	Ursidae	Ursus	Ursus arctos	Ursus arctos arctos

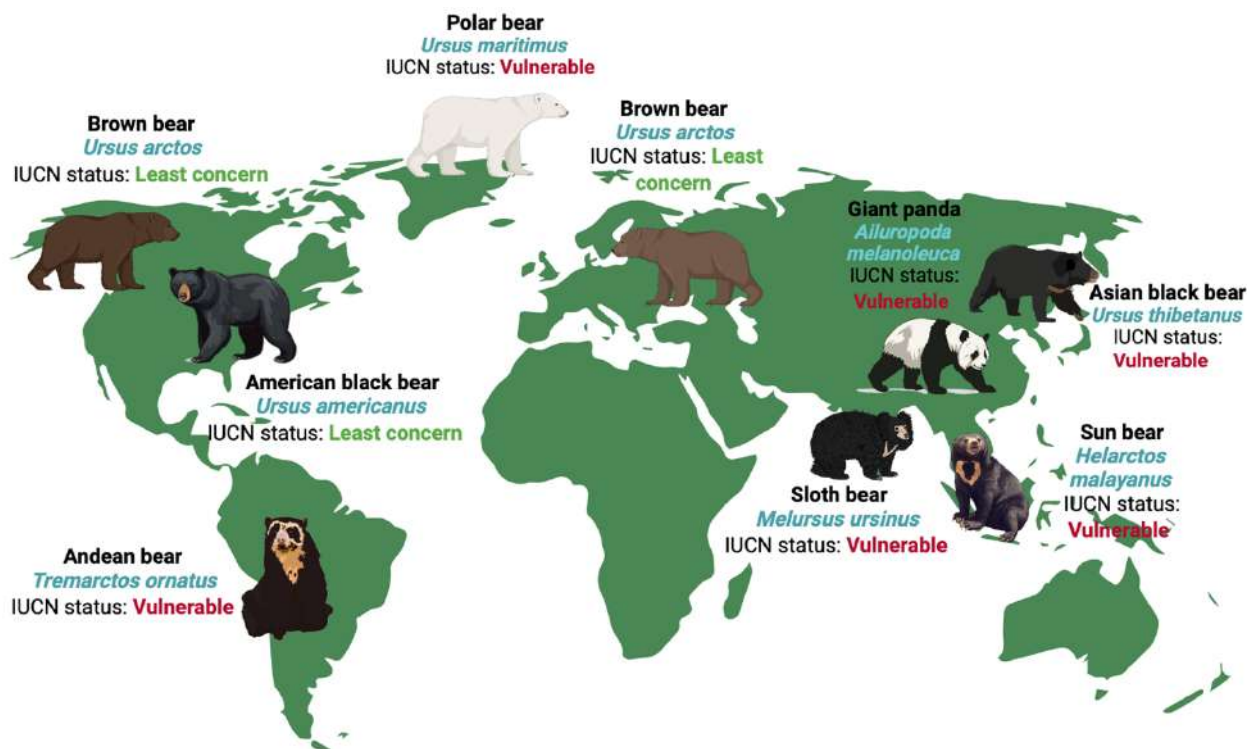


Figure 1: Worldwide distribution of the eight species of bears. Source: BioRender, by author.

These apex predators are found in the Scandinavian Peninsula which includes Norway, Sweden, Finland and small parts of Russia (Rovdata website 2023). Their body length is 130-250 cm long, with a thick neck, a dished face profile, and a clear hump over the shoulders (Skandinaviska björnprojektet 2023). The head is broad with small, round ears. Their rump is usually lower than their shoulders. Their sight is much less developed than their sense of smell or their sense of hearing (Marion 2018). Their sight only allows them to see from short

distances, but they possess a *tapetum lucidum*, a layer of tissue at the back of the eye that reflects light causing the eyes to glow when light strikes them at night, allowing them to see in the dark (Marion 2018). Unlike their name suggests, brown bears don't always have brown fur. Their color can vary from light grey-yellow to almost black, although most bears are the typical brown. The bear cubs can be recognized with a distinct bright collar around their neck that fades away with age. Adult females weigh between 60-200 kg and males between 100-315 kg (Skandinaviska björnprojektet 2023; Rovdata website 2023). The largest free-ranging brown bear recorded in Sweden weighed 343 kg (Skandinaviska björnprojektet 2023). Brown bears show large size dimorphism, which can partly be explained by the competition males have to face during breeding season. However, with the overlap in size, it can be difficult if not impossible to differentiate a single female with a young adult male. They are solitary, unless the mother has cubs in which case she will nurse and protect them for a period of time of 1.5 up to 2.5 years. Bear tracks are large with marks made by five toes, with the largest toe on the outside, and long claws. As a general rule, the claws of the front paw leave clearer traces on the ground than the back paw, although it isn't always easily perceptible depending on the terrain (Rovdata website 2023). The brown bear is a plantigrade animal, meaning it walks on the soles of its feet. The front paw is slightly shorter, although wider, than the back paw (Rovdata website 2023).

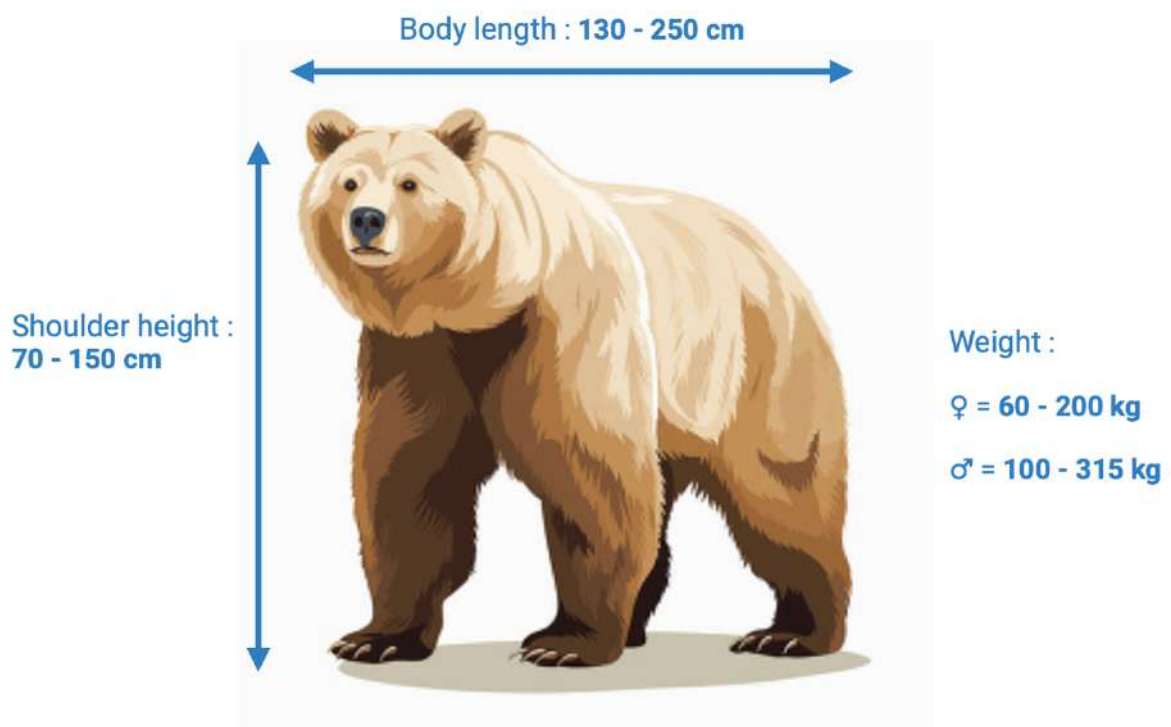


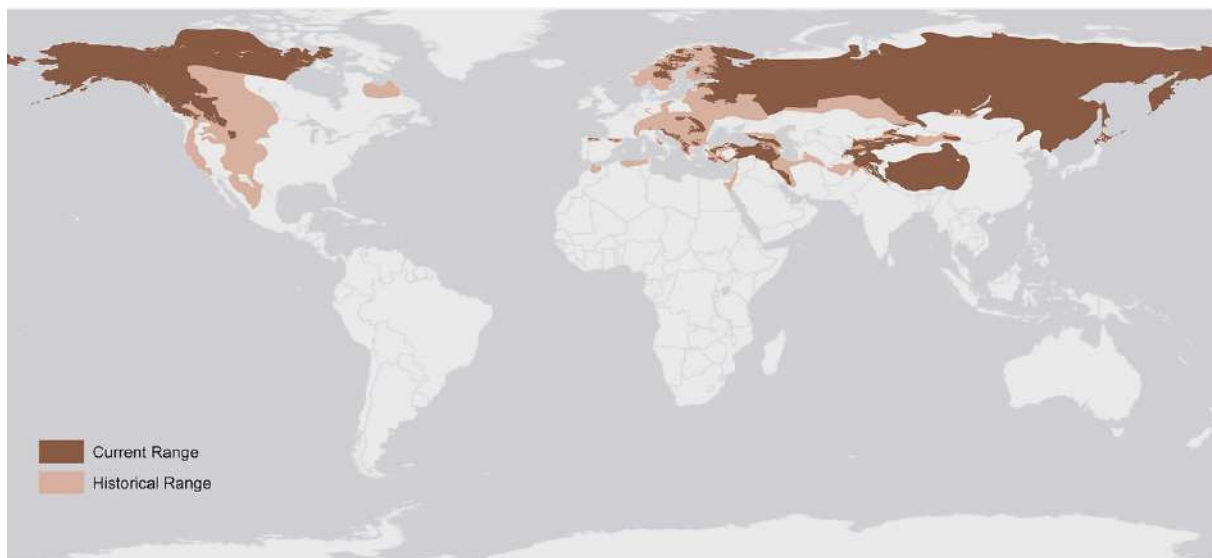
Figure 2: Physical dimensions of a Scandinavian brown bear. Source: BioRender, by author.



Illustration No. 1: Tracks and paws of Scandinavian brown bears. Upper right: back track in the snow of a subadult brown bear; lower left: front paw of a subadult brown bear; lower right: back paw of a subadult brown bear - spring captures 2023. Source: Photographs taken by author; drawing of the bear tracks: <https://rovdatab.no/Brunbjørn/Sporogsporetegn.aspx>

3.1.1.2 – History

The brown bear *Ursus arctos* is the most widely distributed ursid in the world. It once ranged across all of Europe and most of Scandinavia. Nowadays, its Holarctic range covers areas in Asia, Europe and North America. It also used to cover small parts in North Africa in the past.



*Figure 3: Historical (light brown) and current (dark brown) worldwide ranges of the brown bear *Ursus arctos*. Source: Scandinaviska Björnprojektet website <https://www.brownbearproject.com/history>*

Although the worldwide brown bear population can be considered fairly large (exceeding 200,000 individuals (McLellan et al. 2017)), localised populations are fragmented and globally decreasing because of hunting, human conflict and habitat loss. In total, 44 subpopulations of brown bears have been identified (McLellan et al. 2008; Calvignac et al. 2009, Proctor et al., 2012). In Europe, the original brown bear population around 2,000 years ago was believed to range from 10,000 to over 100,000 individuals (European wilderness society website 2023). In the year 2000, the total number of brown bears in Europe was about 50,000 bears (with ~14,000 outside of Russia) within an area of more than 2.5 million km² (800,000 km² outside of Russia) (Zedrosser et al. 2000). Today, the current European brown bear population outside of Russia is estimated to be around 17,000 individuals (European wilderness society website 2023). Bear populations in Europe are very scattered across several countries with very low densities. The largest European single population (outside of Russia) is likely to be in the Carpathian Mountain parts of Romania, with an estimate of 6,000 individuals (European wilderness society website 2023).

In Scandinavia, the bear population began to steadily decline just after the 17th century. By the 18th century, the brown bear was fully absent from the southern parts of Sweden. The Scandinavian Brown Bear Research Project (SBBRP) estimates there were around 4,700-4,800 bears in Scandinavia by the middle of the 19th century, with about 3,100 individuals in Norway and 1,650 in Sweden (Kindberg et al. 2011; Skandinaviska björnprojektet 2023).

Politics weighed heavily on the existence of large carnivores at that time. The governments of both Sweden and Norway sought to eradicate the bear, and both countries achieved their means by paying relatively large bounties. In 1845, a new law was passed with the aim of wiping out the large carnivores. Hunters received good income from national and local bounties as well as the sales they made from the meat and skins. Between 1856 and 1893, bounties were paid for a total of 5,164 bears in Norway and 2,605 in Sweden. These killings led to a rapid population decline in both countries. The decline was faster in Sweden since the population was smaller at that time. Thus, bears quickly disappeared from the Swedish and

Norwegian landscapes and were forced to retreat to the most inaccessible mountain areas in the north. The Swedish Hunters' Association and the Swedish Academy came up with plans at the turn of the century 20th century to save Sweden's bear population from extinction. It was around that time that Sweden and Norway parted ways in terms of bear management goals. In the 1920s, Sweden protected the bear, whereas hunting was still permitted in Norway (Kindberg et al. 2011; Skandinaviska björnprojektet 2023). Sweden abolished bounties in 1893 and enforced several measures that stopped the population decline by around 1930. There were only ~130 surviving bears left in four remaining subpopulations in Sweden at that time. Norway on the other hand abolished bounties in 1930, but local bounties were still allowed until 1972. The last 'native Norwegian' female bear was shot in 1956 in Southern Norway's Vassfaret valley, after which the brown bear was extirpated from the country (Skandinaviska björnprojektet 2023). It was only by 1973 that the bear became protected in Norway (Kindberg et al. 2011; Skandinaviska björnprojektet 2023). By that time, there was barely a single bear left, and it wasn't until 2001 that the first bear cubs were recorded in Southern Norway, which spread from Sweden. This is why most of the brown bears in Norway are found along the Swedish border.

In 1942, the Swedish bear population was estimated at 294 bears. It was then decided that their population was large enough to allow hunting again, and so license hunting was reintroduced in Sweden in 1943. From 1943 to 1994, the bear population increased ~1.5% per year. In 1994, the SBBRP estimated the Scandinavian population was about 650-700 individuals in Sweden and 22-35 in Norway (Kindberg et al. 2011; Skandinaviska björnprojektet 2023). Since then, the brown bear population has increased and stabilized. As of 2018, there were about 2,600 bears in Sweden. The Norwegian population remains small, a total of 150 brown bears (65 females and 85 males) were recorded in Norway in 2020 (Bjørn Henrik Stավdal Johansen, Nicklas Iversen 2021; Skandinaviska björnprojektet 2023).

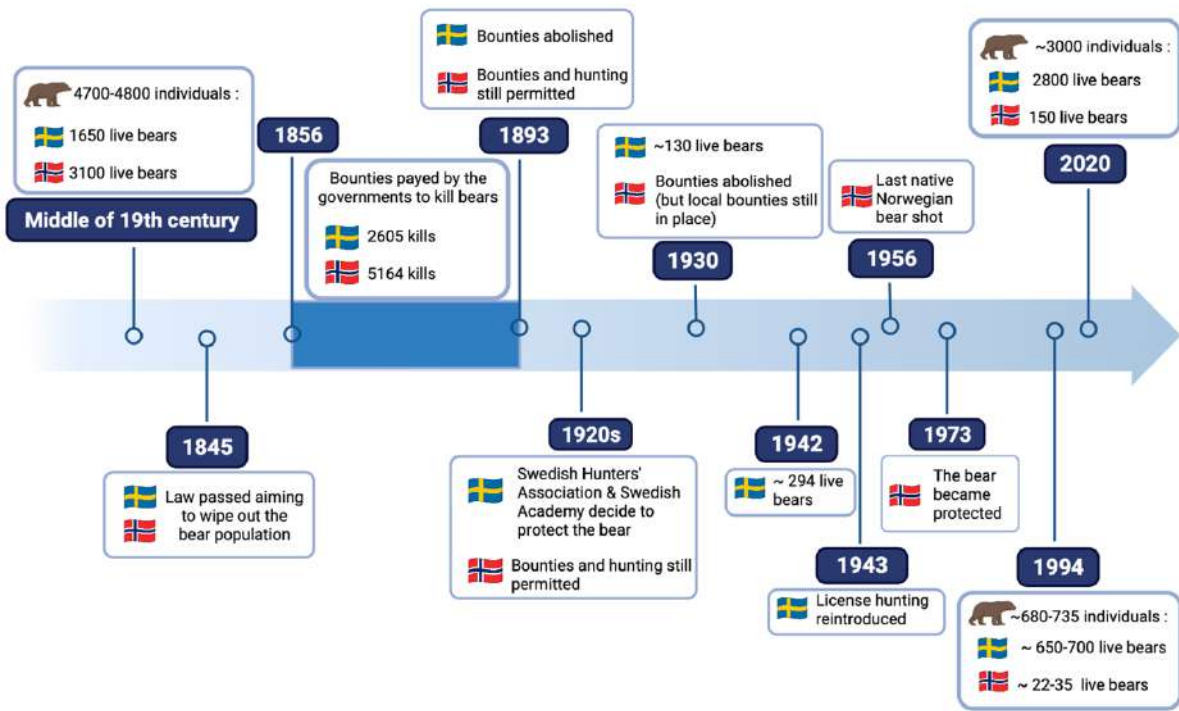


Figure 4: Timeline of the history of the Scandinavian brown bear in Sweden and Norway from 1845 to 2020. Source: BioRender, by author.

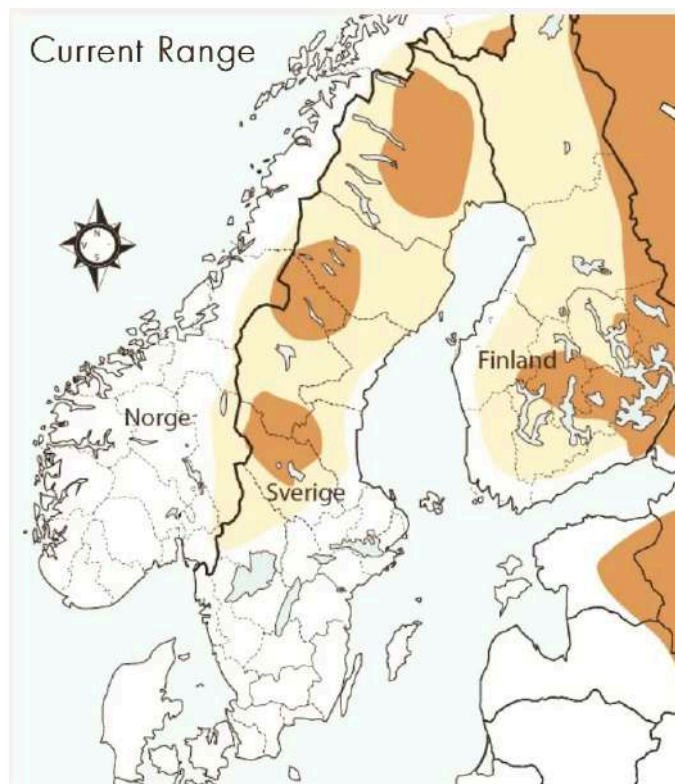


Figure 5: Current range of the Scandinavian brown bear. The dark brown color shows the highest bear densities. Source: Scandinaviska Björnprojektet website <https://www.brownbearproject.com/history>

3.1.1.3 – Habitat use and movement

Most bears in Scandinavia are found in the boreal forest, although some can be found in the mountains as well. Their movement patterns within the forest and which habitat they utilize likely reflect where their important food items are available (Skandinaviska björnprojektet 2023). Since their diet varies so much depending on the season, the habitats they use change with the season when different foods are available in different places. In general, both males and females prefer rugged forest terrain over flatter forest terrain and marshland. Forests in rugged terrain seem to have two main advantages: on one hand, they may offer more food resources and on the other hand, they may not be visited so often by people and therefore be safer for the bears. The brown bear's movement patterns and habitat choices also appear to be heavily influenced by humans and human activity. They will generally avoid both people and urban areas.

Bears spend a good amount of time resting, and in general, they choose resting sites that are relatively hidden with relatively dense vegetation and little visibility. However, the bear's reproductive status influences the resting site selection. For example, females with yearlings will choose habitats with a less dense understory and higher visibility. These sites tend to be in dense forests and have a higher proportion of tall pine trees that cubs may climb if threats would appear such as adult males.

Bears are commonly active early in the morning and late at night. Having different lifestyles depending on the season, the length and timing of their active period varies throughout the year. During early spring (right after they have left their den), bears tend to be active during the day and rest for longer periods of time. During fall when bears are in hyperphagia, or in other words continuously eating to build up fat reserves for winter, they tend to be highly active during the day.

Brown bears usually don't have territories that they defend, rather, they use what is called a "home range". A home range is the area where the bear lives and obtains the resources required for survival and reproduction, in other words, the area in which it forages and searches for mates and other resources. Bear home ranges typically overlap, and most young cubs establish their home ranges partly within their mothers' home ranges (Skandinaviska björnprojektet 2023). The home range size of bears in Scandinavia varies greatly. Females with cubs usually have home ranges between 100 and 150 km² whereas single females have home ranges from 200 up to 300 km². As a general rule, males can have home ranges between 800 and 1,000 km² (Rovdata website 2023). Home range sizes tend to increase when the bear population is of low density, and male home ranges are generally larger than the females', likely because they move across larger distances in search of mates than their female counterparts. Female brown bears usually stay close to the area where they were born and will select their habitat within their home range that provides abundant food resources and minimizes human-caused disturbance (Martin, Basille, Moorter, et al. 2010).

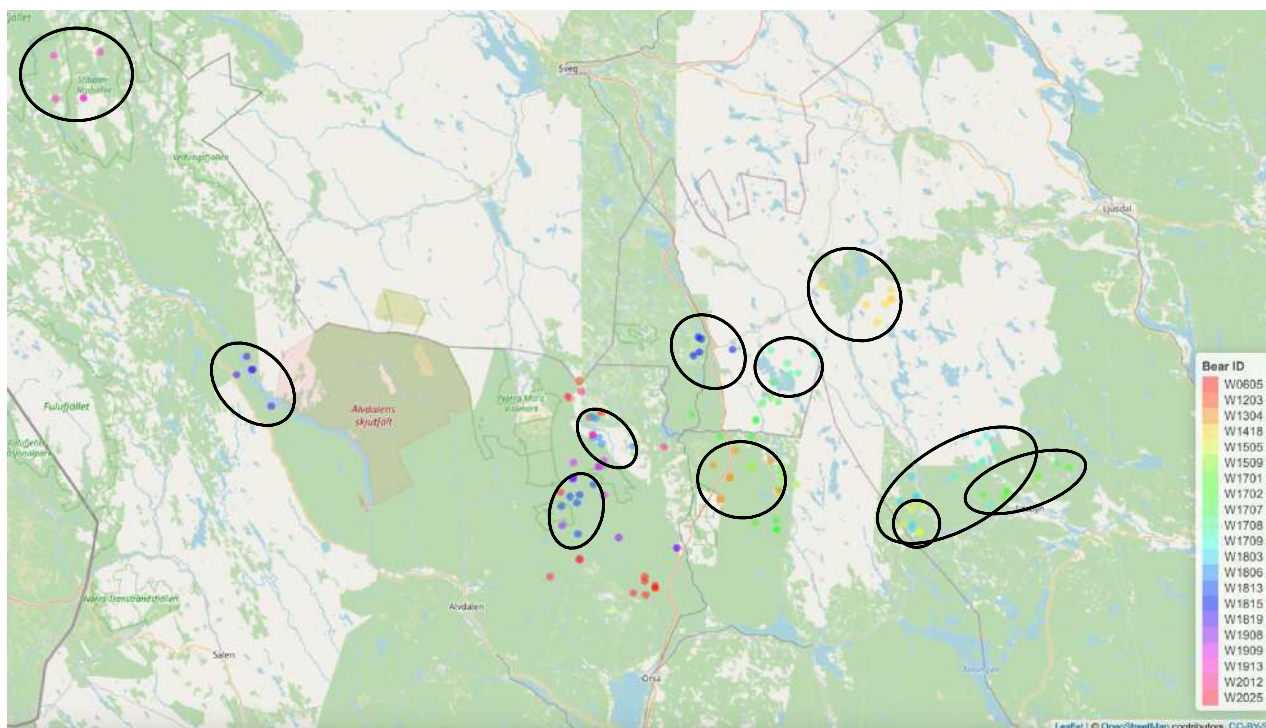


Figure 6: Distribution of bears sampled more than 4 times in the years 2010, 2013, and 2017-2022. The legend on the bottom right shows that each individual bear has its own color. The black ovals encircle the locations of the same captured bear. Source: Rstudio, by author.

21 Scandinavian brown bears were sampled more than 4 times over the period of time from 2010 to 2022. The map in Figure 6 shows that these bears that got sampled more than 4 times were captured and sampled in the same area from one season to the next or from one year to the next. The black ovals help us not only to visualize the areas where the same bear got recaptured, and by extension part of its habitat range, but also that bear home ranges overlap.

3.1.2 Hibernation

Many animal species can drastically slow down their metabolism to enter low-energy states. This condition, characterized by reduced metabolic rate, body temperature (T_b) and complete inactivity, is known as torpor. Within mammals, torpor is most commonly observed in small endotherms, usually weighing under 10 kg. It is a strategy applied in members of at least seven mammalian orders: Monotremata, Marsupialia, Insectivora, Chiroptera, Rodentia, Carnivora and Primates (Lyman et al. 1982). Hibernation, also known as successive torpor bouts (which consist of three phases: entry, stable period and arousal), is a powerful energy-saving strategy. It is associated with various physiological adaptations, such as a decrease in the heart rate (HR), respiratory rate (RR), T_b and metabolic rate. But these physiological variations don't happen overnight. Physical activity, HR, and T_b start dropping several weeks before the brown bears enter the den (Alina Evans, Singh, Friebe, et al. 2016).

Hibernation constitutes a long period of fasting strategy for brown bears for survival in harsh environmental conditions. In the past, it was mainly viewed as an adaptation to the cold. Research has been ongoing on multiple hibernators from different habitats, showing that hibernation is a phenomenon that can be observed from the arctic to the tropics. It appears

that lack of food, which takes place for part of the year in dry tropical areas as well as in cold temperate and arctic areas, is the main reason for hibernation to occur (Geiser 2013). Thus, it's the association of low temperatures with low food abundance that drives some species to hibernate (Geiser, 2013; Ruf & Geiser, 2015).

The term hibernate comes from latin "hibernare", which translates into "to pass the winter". But the term hibernation used nowadays isn't exclusively restricted to the winter season. The most typical hibernation season for lots of hibernators is from fall to spring, but it can take place during other periods of time (Geiser 2020): some hibernate from autumn to summer (e.g., the little brown bat *Myotis lucifugus*, the jumping mouse *Zapus princeps*), some from summer to spring (e.g., the woodchuck *Marmota monax*, the Anatolian ground squirrel *Spermophilus xanthopymnus*, the echidna *Tachyglossus aculeatus*), some from autumn to spring (e.g., the European hedgehog *Eracineus europaus*, the big brown bat *Eptesicus fuscus*, the European hamster *Cricetus cricetus*, the alpine marmot *Marmota marmota*, the yellow-pine chipmunk *Tamias amoenus*, the brown bear *Ursus arctos*), some from autumn to winter (e.g., the African hedgehog *Atelerix frontalis*, the armadillo *Zaedyus pichiy*), some from winter to spring (e.g., the pygmy slow loris *Nycticebus pygmaeus*, the black bear *Ursus americanus*), and some can even hibernate throughout the whole year in specific conditions (e.g., the eastern pygmy possum *Cercartetus nanus*, the edible dormouse *Glis glis*).

Over the past few years, more research has focused on the mechanism of hibernation. Such studies have been undertaken on small hibernators, but also larger mammals such as the brown bear *Ursus arctos*, to try to understand the main drivers of hibernation: does it involve climatic factors (e.g., ambient temperature (T_A), photoperiod, snow depth), physiological variations, an endogenous circannual clock? While small mammals have the ability to rely on an extreme drop in T_b , brown bears face different energetic challenges due to their low surface-to-volume ratios, such as slower cooling rates and the inability to rely on important drops in T_b for metabolic rate reduction (Geiser 2004). Indeed, the mechanisms of metabolic rate reduction are thought to differ between large and small hibernators (Geiser 2004). Large hibernators, such as brown bears, are expected to rely to a greater extent on active metabolic suppression to reduce their metabolism, due to their larger body size, compared to small hibernators, which benefit more from the Q_{10} effect in torpor. The " Q_{10} rule" states that the metabolic rate of a body changes exponentially with the variation in temperature. The correlation between metabolic rate and temperature is based on the " Q_{10} rule" that explains the variation in metabolic rate by a specific factor for every 10°C change in T_b (e.g., $Q_{10}=3$ results in metabolic rate being cut to 1/3 for every 10°C temperature drop). Using active metabolic suppression, brown bears are able to reach metabolic rates (with T_b above 30°C) as low as small hibernators (with $T_b < 5^\circ\text{C}$) in deep torpor (Tøien et al. 2011). Bears are different from other hibernators in that their T_b drops less drastically (37-38°C in summer to 30-33°C in winter) than in small hibernators, which can drop to near hibernaculum temperatures (Revsbech et al. 2012), and in some cases even drop below freezing. One of the most dramatic drops in T_b is documented in the Arctic ground squirrel, going down as low as -2,9°C (Barnes 1989). While small hibernators show spontaneous, periodical arousals to normothermic levels of T_b , this is not the case for hibernating bears. An explanation as to why that is would be because the bear's T_b does not decrease below 30°C, a level that may reflect a threshold below which neural deficits, such as loss of neuronal structure (Barnes 1989), begin to occur that require regular returns to high T_b (Tøien, Blake, Barnes 2015). Another interesting fact in hibernating mammals is the reduced circulating leukocytes, although the cause and mechanism are yet unknown. This has been reported in hibernating captive Asiatic black

bears, displaying a 23% drop compared to non-hibernating captive bears (Asano et al. 2007). A study on white blood cell counts in free-ranging Scandinavian brown bears both during summer and winter over a two-year time displayed that bears had fewer circulating innate immune cells during hibernation, reflecting potentially a suppressed innate immune system (Sahdo et al. 2013).

During hibernation, brown bears fuel their energy needs by mobilizing lipid sources, which come from all the fat they store during the fall. The bear's body mass increases drastically during fall (up to 50%) within a short period of time due to hyperphagia and fat deposition. These lipid sources are of low unsaturation, keeping unsaturated fatty acids in their membranes for the organs to function properly (e.g., heart, brain) (Giroud et al. 2019). Building up large fat reserves is crucial if they want to last through the winter, since they lose weight at a rate of ~ 300-400 g per day. Furthermore, bears that leave the den periodically during winter due to disturbances lose more weight than bears that stay in the den, suggesting that leaving the den results in a greater loss of energy.

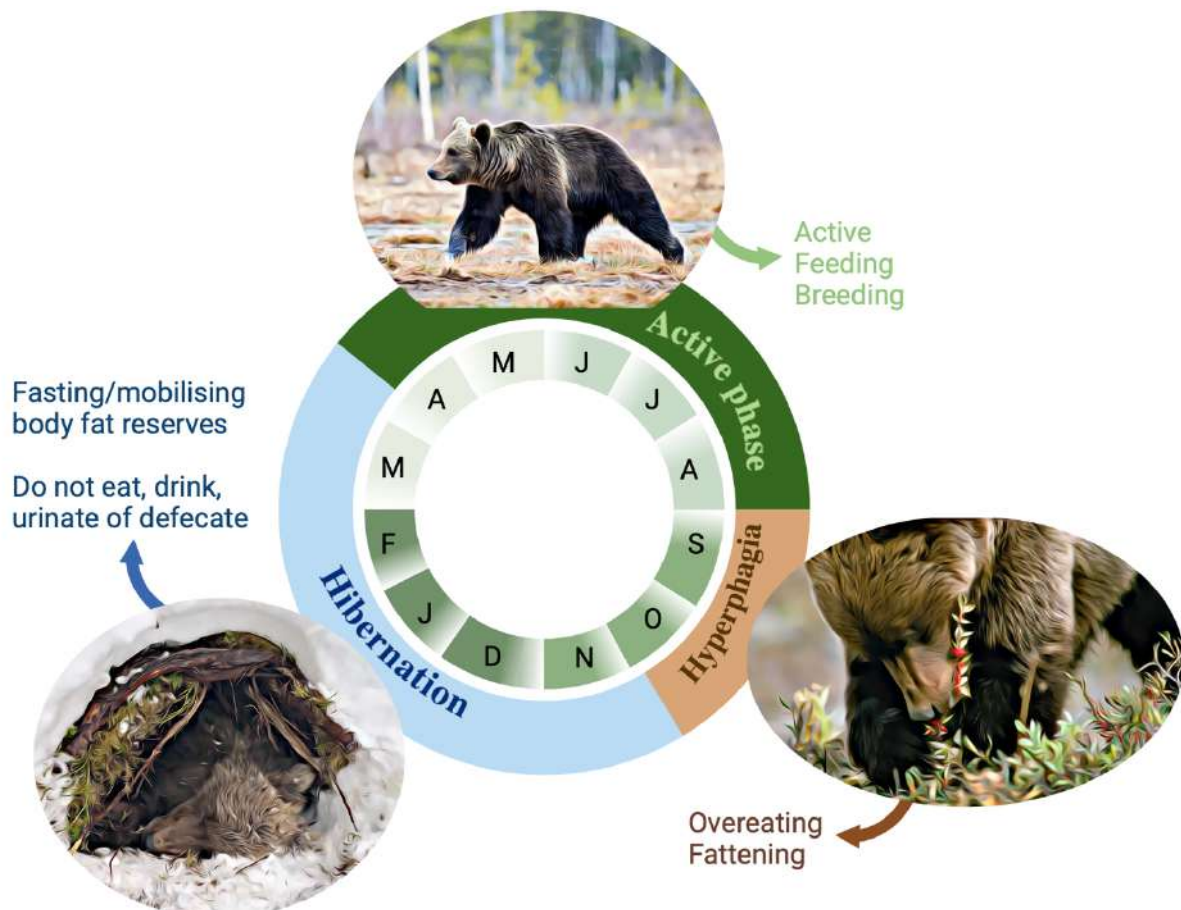


Figure 7: Annual cycle of Scandinavian brown bears. Source: BioRender, by author.

Spending several months in a hypometabolic state requires adaptations in the brown bear's physiological parameters. The rates of O₂ consumption in hibernating bears are downregulated by 75% (Tøien et al. 2011). Despite the decreased ventilation and HR, brown bears don't experience deleterious effects such as hypoxia, muscle mass atrophy, vascular

thrombosis, atherosclerosis (Frøbert et al. 2010) or heart failure (Nelson, Robbins 2010) during or after hibernation thanks to cell preservation strategies for circulatory and innate immune systems, muscle and bone. During winter, Scandinavian brown bears equipped with abdominal bio loggers hibernate from November to April at T_b of $33,6 \pm 0,8$ °C (min = $30,6$ °C, max = $35,3$ °C) (Alina L. Evans et al. 2016). During summer (June to August), T_b in brown bears is normally $37,6 \pm 0,3$ °C (Tøien et al. 2011; Alina L. Evans et al. 2016). Their HR during winter averages 15 ± 3 beats per minute (bpm) (min = 10, max = 28) (Alina L. Evans et al. 2016). In summer, the average is 65 ± 20 bpm (Blanchet et al. 2019) with the lowest HR during end of April (~50 bpm) and highest around mid-July (~75 bpm). Brown bears' respiratory rate drops from 10-12 breaths per minute during summer to 5–7 breaths per minute during winter (Laske, Garshelis, Iazzo 2011; Evans et al. 2012).

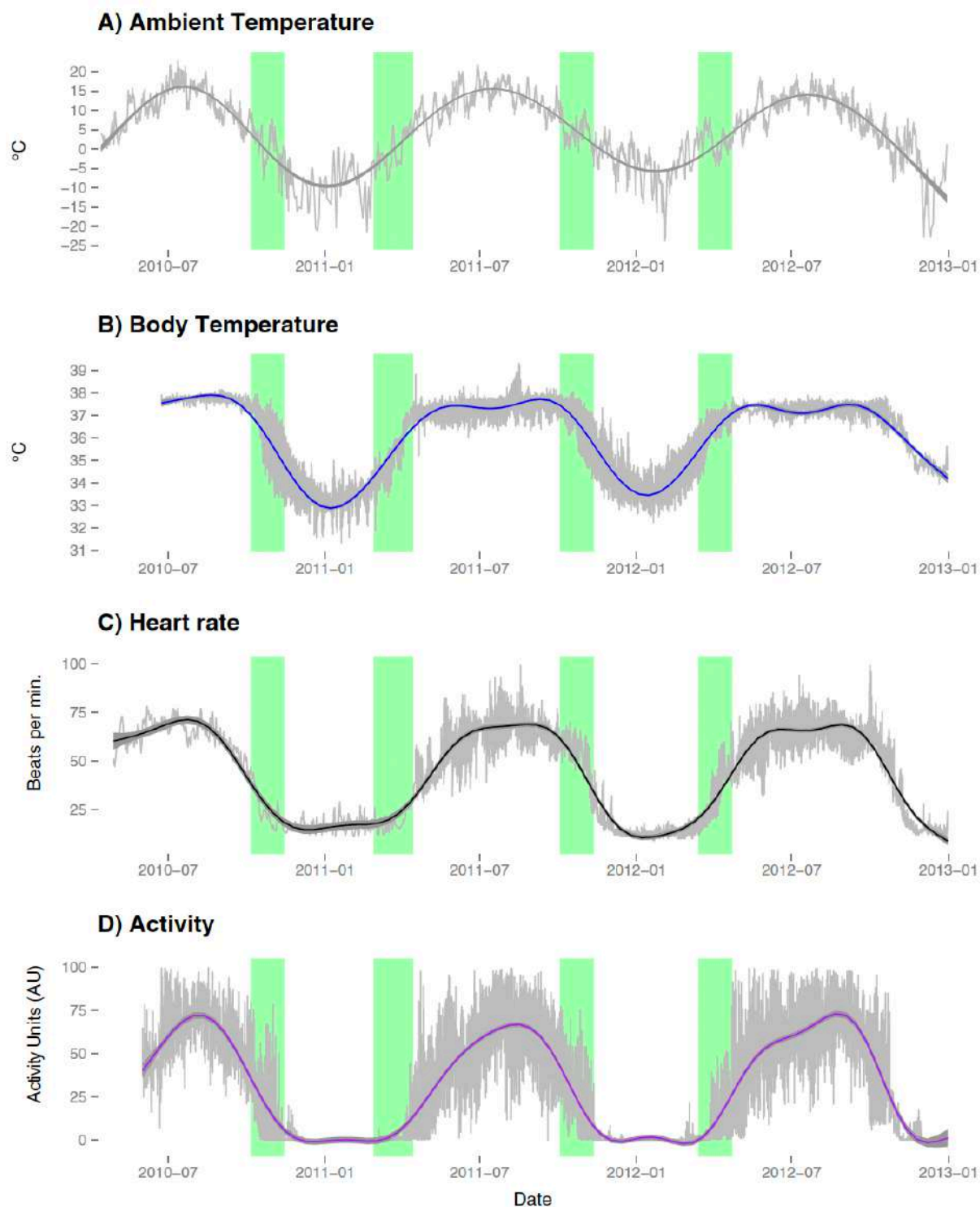


Figure 8: Average of the daily mean values for ambient temperature (a) bear body temperature (b), heart rate (c) and activity level in accelerometry units (d) for 14 individual free-ranging brown bears in central Sweden collected over three years. The X-axis indicates the time of year. Green vertical bars indicate the den entry and exit periods. The width of the green bars denotes the range of den entry and exit dates across all individuals. Source: Alina Evans, Singh, Friebe, et al. 2016.

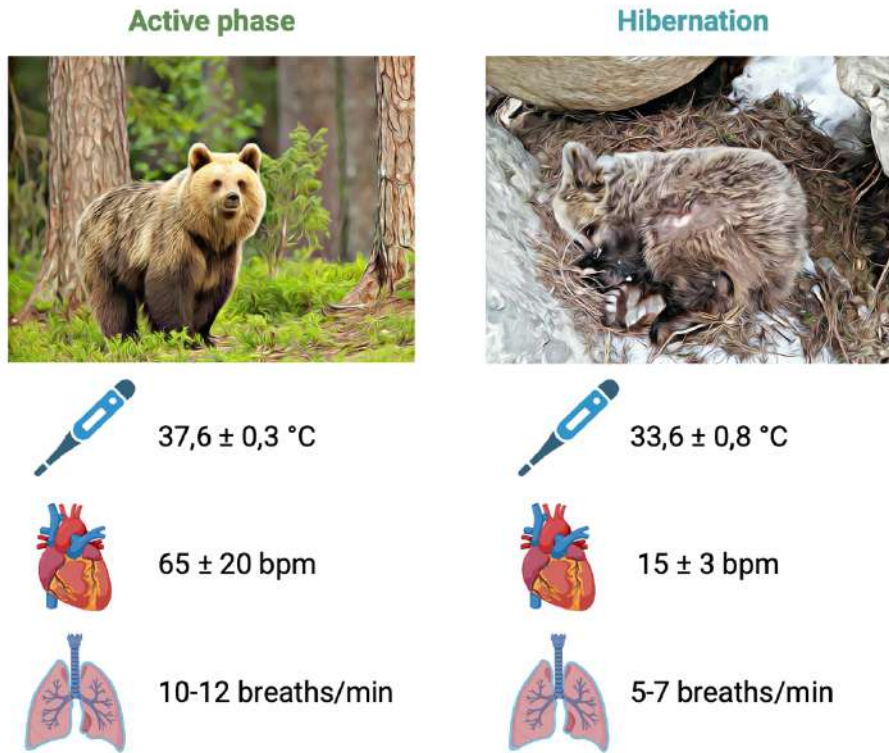


Figure 9: Body temperature, heart rate and respiratory rate in Scandinavian brown bears during summer vs hibernation. Source: BioRender, by author.

Den entry in Scandinavian brown bears is mainly driven by environmental factors, such as T_A and snow depth, while den exit is mainly driven by physiological cues. They enter their den when snow arrives and the T_A reaches 0°C (Alina Evans, Singh, Friebe, et al. 2016).

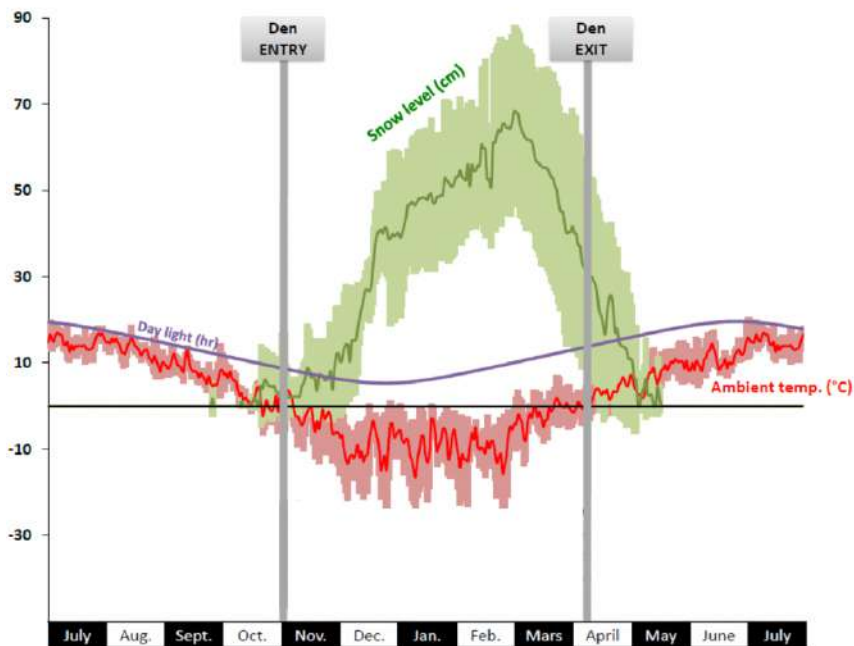


Figure 10: Den entry and exit dates in Scandinavian brown bears based on data collected from April 2010 to June 2012. Source: Alina Evans, Singh, Friebe, et al. 2016.

Surprisingly, the brown bear's T_b starts to increase gradually as early as two months before the den exit, with T_A being the driver of such an increase. While den emergence begins with an increase in T_A , T_b constitutes the first physiological parameter to change during arousal from hibernation, followed by the HR (one month later), the heart rate variability (20 days before den exit), and activity (10 days before den exit) (Alina Evans, Singh, Friebe, et al. 2016). Activity resurgence contributes to the increase in T_b and the return to euthermia, allowing brown bears to exit their den when T_b almost reaches euthermia (36.7 ± 0.15 °C; Alina Evans, Singh, Friebe, et al. 2016).

The dens chosen by the brown bears can be ant hills, rock crevices or caves, under boulders, nests (made out of piled plants, branches and moss), uprooted trees, soil, etc. The most common type of dens remains excavated abandoned anthills in Scandinavia's southern bear population. Brown bears increase the den's insulation properties by lining the bottom and the entrance with vegetation (e.g., grasses, tree branches and twigs, moss, dwarf shrubs, berry foliage). However, in rare cases, open dens can be used, mainly by large males. Dens are often centrally located in the bear's home range.



Illustration No. 2: Left and middle: the den of a three-year-old brown bear; right: the den, under a rock, of a two-year-old brown bear - pictures taken from the 2023 winter captures. Source: Photographs by author.

The factors influencing den-site selection in brown bears include physiographic features (steep slopes and high elevation providing good drainage properties, stability, security and minimal disturbance), good thermal insulation (sites with good snow loads that persist for a long time and avoiding moist areas, to reduce energy expenditure), near abundant spring and/or fall food resources (to reduce energy expenditure pre- and/or post-hibernation) (Pigeon et al. 2014) and close to previous year's den-site (reducing the energy cost of exploring new denning areas; Sorum et al. 2019). Indeed, disturbances such as den collapses or flooding would be energetically costly due to involuntary arousal (Tietje, Ruff 1980) and therefore impact the hibernation cycle, obliging the bears to draw more from their body reserves, thus impacting their reproductive success (Linnell et al. 2000), and survival rates of both adults and offspring (Boyles, Brack 2009; Sorum et al. 2019). Leaving the den periodically can also affect their physical condition when they finally emerge in the spring. For example, 60% of pregnant females that changed dens during winter emerged in the spring without cubs, whereas only

6% of those that stayed in their original den emerged without cubs. Therefore, the bears need to choose the best den site to reduce these risk factors. Brown bears are thought to show a high degree of fidelity regarding denning areas (Linnell et al. 2000, Ciarniello et al. 2005, Manchi, Jon E. Swenson 2005). It would be more advantageous for them to return to a well-known area rather than exploring new denning areas (Miller 1990; Manchi, Swenson 2005), to avoid potential fights with unknown congeners, risk lower food availability during spring or fall and lower quality den sites. Disturbances during the hyperphagia period (e.g., bear and moose hunting) and the hibernation period may have more or less important consequences on behavior, survival rate on both adults and juveniles, reproductive rate and success, habitat use (e.g., den abandonment), body condition, and foraging opportunities (Ordiz et al. 2008; Welch et al. 1997; Elowe, Dodge 1989; Linnell et al. 2000; Swenson et al. 1997). Usually, bears will choose their dens located at least two kilometers from the nearest infrastructure with regular human activity (buildings, open roads).

Studies on the impact of captures on hibernating free-ranging Scandinavian brown bears showed an increase in their HR and T_b for up to four weeks post-capture (Alina L. Evans et al. 2016). However, there was no detectable influence of capture on the overall depth and length of hibernation. It is important to keep in mind that capturing brown bears during hibernation causes such disturbances, which makes it important to proceed with these annual captures only after assessing carefully their necessity. Indeed, doing these captures during winter and summertime, over a long period of time, allows us to understand and manage the Scandinavian brown bear population, its behavior, ecology (reproduction, diet, environment, interactions etc.), and also pursue leads in medicinal research, with brown bears acting as translational models for human health and disease. There have already been some groundbreaking discoveries, and yet there is so much more to learn from how their bodies adapt to such long periods of stillness. Upon rewarming during early spring, dramatic metabolic changes occur: organs and tissues are rapidly re-perfused, while metabolic and oxidative processes rise to a peak. Hibernating brown bears undergo more or less half a year of inactivity and yet these large hibernators are resilient to muscle disuse atrophy (Luu et al. 2020), nor do they suffer from change in bone structure, oxidative damage or other significant deleterious effects. Since they do not urinate or defecate during hibernation, several physiological adaptations have to be put in place. In order to avoid poisoning and organ failure provoked by their waste products (e.g., nitrogenous substances such as urea and creatinine), recycling pathways fall into place among other sorts of adaptations (e.g., the bladder becomes permeable allowing the water and nitrogenous substances of the urine to re-enter the blood stream (Brown et al. 1971; Nelson et al. 1975)). Moreover, brown bears do not suffer from severe sarcopenia, osteoporosis, nor vascular thromboembolism. Trying to elucidate the mystery of how they manage to avoid such pathologies with their unique physiology may provide insight into the treatment of people suffering from similar disorders. While a sedentary lifestyle in humans increases the risk of developing serious health problems such as metabolic syndromes, osteoporosis, diabetes, heart failure and other cardiovascular diseases, and muscle mass atrophy, brown bears adapted in a way to avoid such diseases.

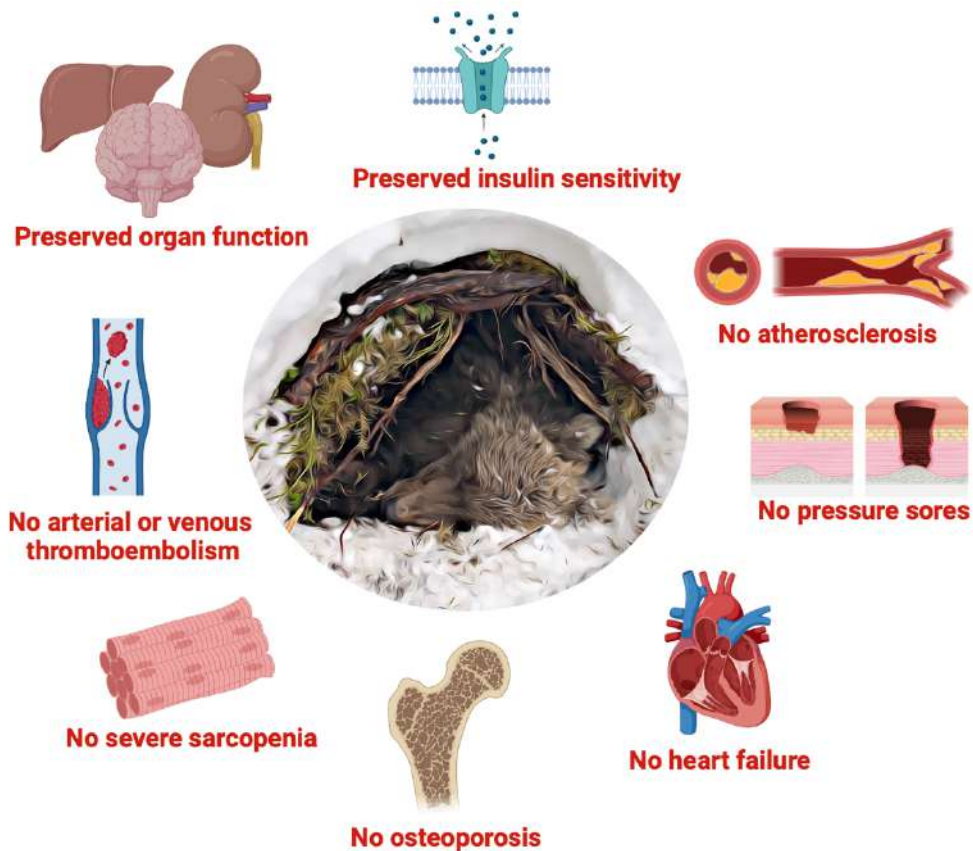


Figure 11: The unique physiology of hibernating Scandinavian brown bears allowing them to overcome diseases seen in humans related to physical inactivity. Source: BioRender, by author.

Hibernation represents a big part in the life of brown bears, since they spend five to seven months a year in their dens, during which they do not drink, eat, urinate or defecate. However, females may give birth and nurse their offspring in the den. While the mother continues hibernating, the cubs don't hibernate alongside but show normal physical and physiological activities. The time spent hibernating may vary from one year to the next, as it is partly dependent on T_A . Generally, Scandinavian brown bears hibernate from the end of October until mid-April, but it can even span until May (Sahlén et al. 2014; Friebe, Swenson, Sandegren 2001). The variability in den entry and exit dates could be explained by the T_A variation from one year to the other. As climate change projections predict warmer temperatures during winter (Jacob et al. 2014), the time brown bears spend hibernating is expected to get shorter. With higher T_A , and by extension delayed first snowfalls, den entries would occur later in the year, thus affecting the timing of different life events of the bear. The consequences of climate disruption, such as mismatches between thermal and photoperiod cues (Bradshaw, Holzapfel 2010), would impose several physiological adaptations, leading to shortened hibernation period and potentially prolonging den-entry periods. Human activities are still high during brown bears' den entry time (e.g., bear hunting occurring in Sweden from August 21st to October 15th or when the quota has been filled (Bischof et al. 2010), moose hunting), which is when the risk period for human injuries caused by bears is the highest (Sahlén et al. 2014). Of course, the timing of den entry isn't only influenced by T_A , but also food availability, sex, reproductive status, age and body size (Manchi, Swenson 2005; Friebe, Swenson, Sandegren 2001). For example, pregnant females often have earlier den entries compared to nonpregnant ones (Friebe, Swenson, Sandegren 2001) and other bears.

3.1.3 Reproduction

Brown bears are polygamous, meaning that males mate with multiple females and females with multiple males (Steyaert et al. 2011). Female brown bears are in estrous from mid-May through early July (Skandinaviska björnprojektet 2023). During the mating season, both males and females can travel long distances within their home range to find mates, although males travel farther than females. The most successful breeders are males who are large, old, and have the highest genetic variation, making them fierce competitors to other younger or smaller bears (Skandinaviska björnprojektet 2023). This is at least the case in the central and southern parts of Sweden and Norway, but not in northern Sweden. Up north it is reported that the young males have greater breeding success, possibly because illegal hunting removes the larger males from the competition. However, within the category made up of young males, the larger ones in that area tend to be the most successful breeders.

There is a strong possibility that females mate with multiple males to protect their cubs from infanticide (Skandinaviska björnprojektet 2023). Infanticide is when one animal kills another individual of the same species while they are young and still dependent on their mother. This behavior is “sexually selected” if it is based on competition for sexual partners, which is the case for Scandinavian brown bears. It occurs when a male’s reproductive success has a chance of increasing when cubs are no longer present. For the male, the conditions of his reproductive output benefiting from killing the cubs are as such: he must not be the cub’s father, the mother is coming into heat earlier than she otherwise would have, and he can be the father of her next litter (Skandinaviska björnprojektet 2023). Thus, in order to boost their reproductive success, males may select to kill unknown cubs. On the other hand, the female’s reproductive success always suffers from infanticide. Females can only produce so many litters during their life span, not to mention their production and care are energetically costly. They employ several strategies to protect their cubs from males. This includes mating with multiple males to “hide” the paternity, moving little within their home range to avoid detection, physically defending their offspring, and staying in areas with escape routes for the cubs such as dense forests with a high proportion of tall pine trees for them to climb up to, or using areas where males are unlikely to roam. Brown bears have the ability to have shared paternity resulting from multiple mating. This occurs in 14% of litters with >2 cubs and 28% of litters with >3 cubs (Skandinaviska björnprojektet 2023). And so, since males do not kill their own offspring, the mother can “hide” the paternity better by mating with multiple males.

As with most other animal species, mortality in bears is greatest during the first year of life. From their birth to 1.5 years old, the mortality rate is about 40-50%, meaning about half of all bear cubs die before they are able to leave their mother’s side (Skandinaviska björnprojektet 2023). About 80% of that mortality is likely due to infanticide by male bears when the mother is in estrous. Apart from sexually selected infanticide, bears are known to kill other bears (at least young subadults) although it is relatively uncommon. Only a few documented cases of adult bears killing other adult bears have been documented, all of which were adult females with yearlings. The most probable hypothesis as to why such events occur is the result of the mother vigorously defending her cubs against a male bear. Legal bear hunting in Scandinavia takes place from late August to mid-October, or when the quota has been filled. In fact, most of the bear kills occur during the first three days of hunting season (Stenset et al. 2016). Most of the adult mortality is thus caused by humans. Within the SBBRP, humans were the number one cause of death for their radio-collared bears, with approximately 80% caused by some form of human activity (e.g., legal hunting, poaching, road accidents). Even with legal hunting

put in place, it is very likely that poaching represents an important source of mortality for Scandinavia's bear population.

Brown bears spread very slowly. This can partly be explained by the fact that females do not move very far across their habitat range (at least less than males), they have a small number of cubs (usually two) and they keep their cubs for a long time (1.5 to 2.5 years). Furthermore, Scandinavian females have their first litter when they are between four and five years old, with slight differences in age depending on the geographical location: between five and seven years old in the northern populations, and between four and six years old in the southern populations. In south-central Sweden, the mean age of primiparity of female brown bears is 4.7 ± 0.7 years old (Zedrosser et al. 2009). Female productivity begins to decrease after they reach 20 years of age, and they stop reproducing altogether after 28. Bears can live for up to 30 years in the wild, although it is rare for the Scandinavian individuals to live that long, being a hunted population.

In Scandinavia, female brown bears commonly give birth to between one and four cubs, two being the most common number. They hibernate with their mother the following fall and stay by her side at least for the first year. Most cubs leave their mother during the following estrous season when they are 1.5 years old (one or two months after the family has emerged from the den). In northern Sweden, about half of all litters stay with their mother for an extra year until they are 2.5 years old. This is also observed in brown bears in North America (Skandinaviska björnprojektet 2023). Even in southern Sweden, the trend is that more litters are staying longer until they reach 2.5 years old. The reasons for this changing pattern are still unclear, although it may have to do with the increased density of bears in the area, or because family groups are protected by hunting regulations.

Female brown bears exhibit delayed embryo implantation. The breeding occurs between mid-May and early July, but the fertilized egg implantation inside the uterine wall takes place several months later in order to synchronize the birth within the hibernation period. This is called embryonic diapause, which is a widespread strategy among plants, insects, fish, birds and mammals (Mead 1993; Thom, Johnson, MacDonald 2004). It is a condition of temporary suspension of development of the embryo, occurring due to suppression of cell proliferation at the blastocyst stage (Lopes, Desmarais, Murphy 2005; Wimsatt 1963; Foresman, Daniel 1983). The unattached blastocyst remains inside the uterine lumen for four to five months, where it undergoes reduced metabolic activity and has a very low mitotic index. The start, maintenance and escape from diapause are regulated by cascades of environmental, hypophyseal, ovarian and uterine mechanisms that vary among species (Lopes, Desmarais, Murphy 2005). Studies show that progesterone is partly implicated in the mechanism of diapause, with concentrations maintained low during delayed implantation and rising significantly at the time of implantation (Sato et al. 2000; Lemière et al. 2022). During the embryonic diapause, the corpus luteum formed after ovulation remain quiescent and produce low but sufficient levels of progesterone to keep the blastocysts viable in the uterine lumen (Mead 1993; Lemière et al. 2022). Embryonic diapause allows the female bears to re-enter the phase of estrous within the same breeding season, enabling the cubs to have different fathers (Spady, Lindburg, Durrant 2007). Reactivation of the corpus luteum leads to the end of the embryonic diapause, and is associated with a significant rise in progesterone concentrations at the time of implantation (Sato et al. 2000). To our knowledge, bears are the only mammalian species with delayed implantation, gestation, parturition and lactation occurring during hibernation (Friebe et al. 2014). The female must not only mobilize energy from her fat

stores for her own survival, but for the survival and growth of her offspring as well. Females with young may lose up to 43% of body mass during denning (Garshelis, 2009). They give birth between late December and early February inside their den. They are even able to vary the date of birth and the growth rate of their cubs in response to their fat stores (Friebe et al. 2014). Thus, female brown bears give birth and nurse their newborn cubs for the first three to five months of their life while fasting in hibernation. Females with good body condition (i.e., large amounts of body reserves) give birth earlier than females with poorer body condition and also lactate longer with milk of higher quality and quantity (Friebe et al. 2014). The milk of a female brown bear is energetically rich, made up of 22-24% fat (Jenness, Erickson, Craighead 1972). The composition of the milk varies depending on the brown bear subspecies, which could be partly explained by the different diets they have, along with other environmental and physiological factors. Furthermore, the nutritional composition of milk changes depending on how long the mother has been lactating. The milk composition during hibernation is different than during summer and at the lactation peak. The newborn cubs need a lot of energy with milk rich in carbohydrates at the beginning to ensure good growth during the hibernation period. Thus, the carbohydrate composition in the milk is relatively high in the den compared to after denning whereas the milk is two times higher in protein content after denning than during. The water values and fat content aren't constant throughout the lactation cycle either (Riley Woodford 2007). The highest fat content in the milk of wild grizzly bears *Ursus arctos horribilis* averages 22.3 % (n= 8, [18.3 – 26.5]), in the Kodiak bear *Ursus arctos middendorffi* 14.4 % (n=1), in the Hokkaido brown bear *Ursus arctos yesoensis* 23.2% (n=2, [19.6-26.8]) (Jenness, Erickson, Craighead 1972) and in the polar bear *Ursus maritimus* 31 % (Baker, Harington, Symes 2011; Jenness, Erickson, Craighead 1972). As a comparison, cow milk or even human maternal milk is only made up of 3.5 to 4% fat (Marion 2018). Newborn blind cubs only weigh between 250 and 600 grams (g) at birth (SBBRP site; Jean-Pierre Digard 2002; Hissa 1997), so they must rapidly gain weight in order to face the outside world a few months later. From birth until den emergence (i.e., three to five months later), cubs only consume about 9% of their annual milk intake. While being inside the den and when the activity of the mother bear is low after parturition, the cubs will crawl to the nipples closest to the pelvis to nurse. After hibernation, when the bear family emerges from the den, they will usually nurse from the top four nipples. The position adopted by a lactating female is quite similar to the one in humans: the mother is sitting comfortably, or laying down on her back, while the cubs are choosing the best of the six teats to suckle. When exiting the den, the bear cubs generally weigh five to six kilograms (kg) (Marion 2018). Even though the milk composition and quantity produced varies, females continue lactating while they still have their cubs, whether they are cubs of the year, yearlings or two-year-olds (Fuchs et al. 2021). Cubs of the year going into hibernation with their mother the next fall can weigh up to 40 kg (Rovdata website 2023). Studies on grizzly bear cubs show a mean milk intake and growth during hibernation of 353 ± 54 and 98 ± 22 g/day respectively (Farley, Robbins 1995). Scandinavian brown bears being generally smaller in weight and body size than grizzly bears, the milk intake and growth during hibernation could be expected to be lower. The peak lactation occurs in June and July.

There is still much to be learned about the reproductive biology of ursids such as factors triggering implantation and birth, hormone and estrous cycling, reproductive cycles, etc. Most of the knowledge today comes from studies carried out on captive individuals. But thanks to biologists recording T_b , HR, activity, etc., more and more studies are able to see the day and learn more about the reproductive cycle of the free-ranging Scandinavian brown bear population. As described previously, hibernation is possible through physical and physiological

variations, which is all the more the case for pregnant hibernating brown bears. A study on reproduction factors in free-ranging Scandinavian brown bears in 2014 compared the mean daily T_b and activity levels of pregnant and nonpregnant females during preimplantation, gestation, and lactation. They found that T_b and activity are higher during the gestation period than during the rest of hibernation, including the preimplantation and lactation periods, and drop at parturition (Friebe et al. 2014; Hissa 1997; Tøien et al. 2011). Indeed, the development of the embryo requires eutheria. The T_b of pregnant females during gestation is around 37-38°C, around 34°C during preimplantation and around 34.5°C during lactation (Friebe et al. 2014; Lemière et al. 2022). These T_b are significantly higher than that of nonpregnant females during the corresponding periods (T_b of nonpregnant females around 33°C). The T_b in pregnant females increases in late November/early December and remains high until parturition in January/February. The trend in T_b of pregnant females decreases slowly as the gestation advances (Friebe et al. 2014). They also tested whether age, litter size, primiparity, environmental conditions, and the start of hibernation had an influence on the timing of parturition. The mean date of implantation was December 1st ± 12 days, the mean date of parturition was January 26th ± 12 days, and the mean duration of the gestation period was 56 ± 2 days (Friebe et al. 2014). A more recent study conducted in 2022 on 24 free-ranging Scandinavian brown bears between 2011 and 2018 established the mean dates of corpus luteum reactivation, implantation and parturition in two study areas in Sweden (Lemière et al. 2022). They also studied the influence of primiparity, age, and bilberry abundance on the parturition date. They found that in young adults, low berry abundance the previous year was associated with a later parturition (predicted delay of 21 days) whereas adult females were not affected by the previous year's berry abundance. In the southern study area, the mean date of corpus luteum reactivation was November 19th ± 11.40 days in young adults and November 8th ± 3.96 days in adult females, the mean date of implantation was November 30th ± 10.44 days in young adults and November 20th ± 2.68 days in adult females, and the mean date of parturition was January 20th ± 8.50 days in young adults and January 11th ± 3.05 days in adult females (Lemière et al. 2022). In the northern study area, the mean date of corpus luteum reactivation was November 23rd ± 4.15 days in adult females, the mean date of implantation was December 5th ± 5.81 days and the mean date of parturition was January 22nd ± 5.86 days. The mean gestation period found in females was 51 ± 1.10 days (Lemière et al. 2022). Other studies of the reproductive organs of captive and free-ranging brown bears and American black bears showed similar results, indicating that implantation occurs in late November/early December, and parturition in late January/early February (Wimsatt 1963; Tsubota, Kanagawa 1993; Kordek WS, Lindzey JS 1980; Hensel, Troyer, Erickson 1969). Pregnant females tend to stay longer inside their den than nonpregnant ones or other bears. On average, pregnant females spent 196 days in the den, about one month longer than females that entered the den with cubs (161 days) or adult solitary bears (168 days) (Friebe, Swenson, Sandegren 2001).



Figure 12: Annual reproductive cycle of the Scandinavian brown bear. Source: BioRender, by author based on the data found in Friebe et al. 2014 and Marion 2018.

There seems to be a strong correlation between the female's body condition and her reproductive success. In other words, nutrition plays a key role in litter sizes. The females that manage to store large amounts of fat reserves and that are well-nourished have larger litter sizes and shorter litter intervals (Rogers 1987; Stringham 1990; Welch et al. 1997; McLellan 2011). Studies show that a minimum amount of 19% body fat content in brown bears prior to hibernation is necessary for reproduction (Beecham 1980; Elowe, Dodge 1989; Atkinson, Ramsay 1995; López-Alfaro et al. 2013). Furthermore, depending on the number of cubs and the length of lactation, the body fat content necessary to support reproduction can even vary from 19 to 33% (López-Alfaro et al. 2013).

3.1.4 Feeding habits

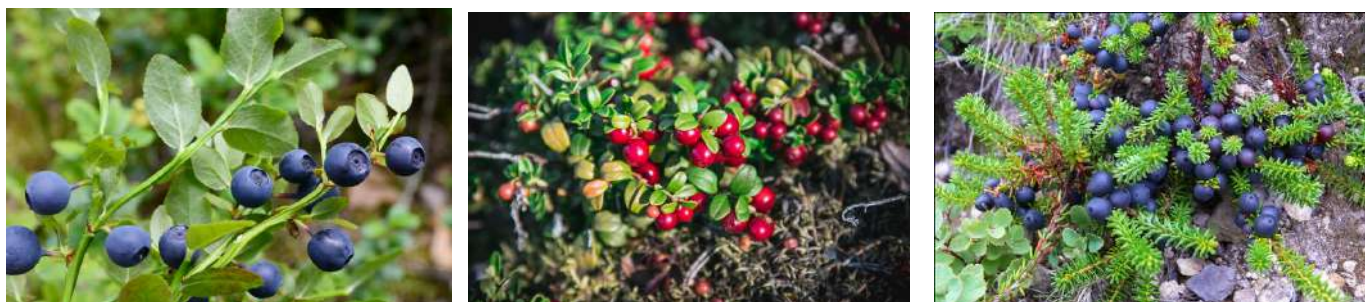
Brown bears are classified as carnivores but should more accurately be described as omnivores, and more specifically opportunistic omnivores. Their diet varies throughout the year depending on the available food during each season (Alina L. Evans et al. 2016). Also, the diet of brown bears can vary dramatically across their broad range that spans much of the northern hemisphere. Nutritional differences in the habitats occupied by brown bears often lead to variations in body and litter size, population densities, and mineral concentrations (Zedrosser et al. 2011a; Hilderbrand et al. 1999; Mowat et al. 2005; Efford, Borchers, Mowat 2013). For instance, Grizzly brown bears are much bigger in weight and size than the

Scandinavian ones. One explanation for such dietary variations is that brown bears are opportunistic, or flexible omnivores (Robbins, Schwartz, Felicetti 2009), and food composition and availability are different from one country (or areas within the same country) to another. Therefore the diet of brown bears can range from largely herbivorous to largely carnivorous, depending on the geographical location but also the season (Hilderbrand et al. 1999; Edwards et al. 2011). For example, bears in Yellowstone National Park in the United States of America tend to have a high proportion of ungulate meat in their diet, and bears in Alaska consume fish such as salmon to build and maintain their large body mass. On the other hand, bears further south in Europe exploit the hard and soft mast that dominate the landscape. In Scandinavia, they get most of their energy intake from berries, ants, and moose calves, but they also eat a wide variety of other foods including herbs, grasses, and fungi. They have a varied diet throughout the year but overall, plant foods dominate their diet. Berries are one of their most important food sources, which are especially important in autumn for building up fat reserves in preparation for hibernation. Food abundance can influence survival, body mass, reproductive success, habitat use and movements in brown bears (Stenset et al. 2016). For example, poor food abundance during fall has been found to affect negatively the bears' survival and reproductive success, and to increase their range of movement, therefore increasing their energy expenditure in search of food (Ian McT. Jonkel 1971; Noyce, Garshelis 2011; Powell et al. 1996).

During spring, or den emergence, bears need food that can rebuild the body's muscle mass they lost over the hibernation period, rather than renew its fat reserves for the coming winter. Therefore, protein-rich foods tend to dominate the diet during spring and early summer. During early spring, Scandinavian brown bears in south-central Sweden mostly feed on ants, which represent an important food source for them. Ant species selected by bears (the most common ones being *Formica* spp. and *Camponotus herculeanus*) tend to be relatively high in energy content, low in formic acid, and to behave quite passively when their colonies are ravaged (Ian McT. Jonkel 1971; Noyce, Garshelis 2011). Altogether, ants make up an estimated 20% of the total annual energy intake of the Scandinavian brown bear. Furthermore, myrmecophagy doesn't seem to show interseasonal variation, however, the frequency of occurrence of ant genera varies significantly by season (Stenset et al. 2016). Newborn ungulate calves are also a very important part of the bear's diet in spring and early summer, which contribute to the protein-rich diet brown bears need. If isolated from their mother, moose calves represent easy preys. They constitute the most consumed ungulate species by bears in Scandinavia, which are an important part of their diet from mid-May through the end of June. In the southern study area, it is estimated that each individual bear older than two years old takes about 6.5 moose calves/year (Skandinaviska björnprojektet 2023). Of these calves, the majority (93%) are taken during the first four weeks after birth. In total, moose make up about 14-30% of a bear's annual energy intake. In reindeer herding areas, reindeer calves also represent important contributors to the bear's diet and a continual source of conflict for reindeer herders, but this concerns mostly the bear's northern distribution (Skandinaviska björnprojektet 2023). Bears only occasionally prey on adult ungulates, such as moose and reindeer, but are generally not very effective predators of these adult species. However, bears are incredibly efficient scavengers. For example, after den emergence when the ice and snow have melted, they will eat the carrions that died before or during winter that resurface from the snow. They can also steal kills from wolves, in the areas where the two predators overlap. Theft of preys killed by wolves often occurs during spring when they are preying on larger-bodied prey (e.g., last year's calves and adults). While the importance of fat or energy reserves at the beginning of hibernation has been long recognized,

the importance of consuming high-protein foods after hibernation may be equally important (López-Alfaro et al. 2013).

Scandinavia's abundant berry crop has matured by late summer, and by and large, bears in Scandinavia switch during that time period to the sugar-rich berries they feed on for the remainder of the season. The majority of the fat reserves come from carbohydrates from berries during hyperphagia in autumn (Welch et al. 1997). Bears must build up fat reserves for their winter hibernation period, and berries contain high-calorie carbohydrates that are generally easily converted into fat. Bears in Scandinavia prefer (in order of preference) blueberries, crowberries, and lingonberries. Brown bears select berry species that maximize their energy intake, in addition to growing in a clustered manner thus allowing bears to take larger bites, to reduce search time and movements and therefore energy expenditure, maximizing the efficiency of foraging (Welch et al. 1997). The carbohydrate content in bilberries is higher than in crowberries and lingonberries (31.1%, 14.4 and 15.4% respectively) (Eriksson, Ehrlén 1991). An adult bear can eat up to a third of its own weight in berries in one day, which corresponds to about 20,000 calories daily. Altogether, berries make up about 45% of a bear's annual energy intake in Sweden. The most important berry species consumed by the bears are bilberries *Vaccinium myrtillus*, crowberries *Empetrum hermaphoditum* and lingonberries *Vaccinium vitis-idaea* (Illustration No. 3; Stenset et al. 2016). Bilberries, also known as European wild blueberries, is a low-growing shrub native to northern Europe and contain a variety of phenolic compounds including flavonols, tannins, ellagitannins and phenolic acids, with anthocyanins (300-700mg/ 100g fresh fruit) making up the largest part of its phytochemical mix (Chu et al. 2011; Upton 2001; Seeram 2008). These phenolic compounds are redox-active antioxidants and iron chelators, interesting lines of investigation in human health and disease prevention (Benzie 2003; Zafra-Stone et al. 2007). Indeed, edible berries have demonstrated a wide range of biomedical functions, including cardiovascular disorders, advancing age-induced oxidative stress, inflammatory responses, degenerative diseases etc. Anthocyanins are also known to improve neuronal and cognitive brain functions and to protect genomic DNA integrity (Zafra-Stone et al. 2007). Besides the antioxidant virtues of anthocyanins contained in bilberries in relation to health benefits, other properties are involved such as cell-signaling pathways, gene expression, DNA repair, cell adhesion, antineoplastic and antimicrobial effects (Packer, Cadenas 2007; Zafra-Stone et al. 2007; Seeram 2008; Kowalczyk et al. 2003; Benzie, wachtel Galor 2010). The incredible fact that brown bears don't suffer from muscle atrophy, osteoporosis, organ or brain damage, atherosclerosis or other cardiovascular diseases during or after emerging from six months of hibernation could partly be linked to the chemical compounds in their diet.



*Illustration No. 3: Pictures of the most common eaten berries by Scandinavian brown bears. From left to right: Bilberries (*Vaccinium myrtillus*), lingonberries (*Vaccinium vitis-idaea*) and crowberries (*Empetrum hermaphroditum*). Source: Google images.*

These berry species brown bears feed on can show rather important annual variations in berry yields in the Scandinavian boreal forests (Selås 2006; Stenset et al. 2016).

*Table 2: Annual contribution of berry species (bilberries *Vaccinium myrtillus*, lingonberries *Vaccinium vitis-idaea* and crowberries *Empetrum hermaphroditum*) to the autumn diet of brown bears, during 1994-1996 and 2000-2001 in south-central Sweden. They are presented as frequency of occurrence (FO) and fecal volume (FV). Source: Stenset et al. 2016.*

Dietary item	1994 (n = 44)		1995 (n = 58)		1996 (n = 30)		2000 (n = 109)		2001 (n = 59)	
	FO	FV	FO	FV	FO	FV	FO	FV	FO	FV
<i>Vaccinium myrtillus</i>	80	43	85	60	83	61	88	20	95	36
<i>Vaccinium vitis-idaea</i>	41	11	29	4	67	16	21	1	81	11
<i>Empetrum hermaphroditum</i>	82	26	81	23	30	2	94	46	85	19
Berries	98	82	98	90	100	79	99	67	97	66

Furthermore, human-induced habitat changes such as forest management can influence annual and spatial food availability for the bears, especially berries and ants, which make up a large amount of their diet (Kardell 1979; Frank et al. 2015). Another factor impacting the brown bears' diet is climate change which is bound to have severe effects in a more or less distant future on the availability of berries throughout the Swedish and Norwegian landscapes. This poses a serious concern since berries represent the main food intake during late summer and fall for fat acquisition prior to hibernation. Indeed, bilberries for example need moderate shade and humid ground conditions to grow. Scat collection from brown bears during these captures over a long period of time allows us to analyze the dietary components and their variations throughout the years. In addition, Scandinavia represents one of the most impacted areas by climate change with great variability of annual trends in temperature (Walther et al. 2002). It is also predicted that the higher latitudes are to expect later winter thaws, which have been reported to cause major reductions in berry production (especially bilberries) and plant cover (Bokhorst et al. 2008; 2011). While berries represent almost half of the annual energy intake for brown bears in south-central Sweden, the situation is different in Norway. A study conducted in Nord-Trøndelag showed that only 6-17% of local bear annual energy intake consisted of berries, but 65-87% was from meat, mainly sheep *Ovis aries* (Skandinaviska björnprojektet 2023). This shows just how important the availability of the various food items is for a bear's food choices. Free-ranging sheep grazing in large pastures are relatively easy for bears to kill, especially with no dogs guarding or enclosures for them to go in at night. Animal fat (e.g., sheep fat) can be converted into fat reserves more easily than

berries. In that part of Norway with relatively large numbers of free-ranging sheep, bears will prefer feeding on sheep, and especially the fattest parts of the sheep, to berries. On the other hand, there are virtually no free-ranging sheep within the bear's habitat range in Sweden, although bears will scavenge on hunter-killed ungulate remains during the fall in both countries (Skandinaviska björnprojektet 2023).

The Scandinavian brown bear's diet has been studied in different areas in Scandinavia for the past decades (Stenset et al. 2016), through various methods including visual observation (directly, through foraging signs or camera traps), scat sample analysis, or even stomach content analysis of deceased individuals. Bear scats vary according to what the bear has eaten. What is more, bears don't have very good digestion, so whole berries, hair, ants, plant residues and even bits of bone can be seen in bear droppings. Stenset et al. (2016) set out to retrace the seasonal and annual variations in the diet of Scandinavian brown bears in southcentral Sweden.

Table 3: Mean seasonal diet of brown bears in south-central Sweden, based on combined data of fecal analyses from 1994-1996 and 2000-2001. Data are presented as frequency of occurrence (FO), percent fecal volume (FV) and estimated dietary energy content (EDEC). Contributors below 1% are indicated by t (trace) for clarity. Empty cells indicate that the item was not recorded in that season and dashes (-) indicate that these categories were not recorded in all years in that season. Source: Stenset et al. 2016.

Food item	Spring (n = 141)			Summer (n = 93)			Autumn (n = 305)		
	FO	FV	EDEC	FO	FV	EDEC	FO	FV	EDEC
Berries	44.7	16.3	8.7	21.7	2.7	1.8	98.3	76.8	68
<i>Vaccinium myrtillus</i>	4.0	1.0	0.5	7.8	1.8	1.3	86.2	44.1	39.6
<i>Vaccinium vitis-idaea</i>	33.8	10.7	6.0	t	t	t	47.9	8.5	7.6
<i>Empetrum hermaphroditum</i>	22.3	5.3	2.3	6.8	t	t	74.3	23.1	20.9
<i>Vaccinium uliginosum</i>							3.3	t	t
<i>Sorbus aucuparia</i>				t	t	t	2	t	t
<i>Rubus idaeus</i>							1.6	t	t
<i>Oxycoccus quadripetalus</i>	t	t	t	t	t	t			
Insects	61.7	11.7	20.5	78.3	16.7	28.2	53.4	4.9	14.2
Hymenoptera	t	t	t	t	t	t	10.3	t	t
Coleoptera	t	t	t	t	t	t	1.5	t	t
Ants	60.3	11.7	20.3	78.3	16.7	28.2	32.5	4.1	11.1
<i>Formica</i> v.	56.0	10.7	16.8	55.7	5.0	8.3	27	1.6	4.3
<i>Camponotus herculeanus</i>	9.5	1.5	2.8	48.0	10.0	18.2	13.9	1.7	4
<i>Myrmica</i> spp.	3.7	t	t	14.8	t	t	6.6	t	t
<i>Cerviformica</i> spp.	3.5	t	t	t	t	t	12.7	t	1.1
Insect fragments*	—	—	—	—	—	—	52	t	t
Egg/larvae*	—	—	—	—	—	—	60.3	1.7	3.9
Fungi				3.7	t	t	17.6	1.8	0.8
Cryptogams	33.3	t	t	15.3	1.0	t	23.1	1.2	0.5
Graminoids	66.3	21.7	3.0	65.7	25.0	3.7	39.2	2.7	0.9
Forbs	8.2	2.2	t	54.0	20.3	3.7	21.9	2.5	1.2
Horsetails	3.2	1.2	t	6.8	3.5	t	1.1	t	t
Shrub material*	—	—	—	—	—	—	98.2	7.2	1.6
Vertebrates	40.5	22.2	60.8	44.3	15.0	62.3	13.7	2.5	13.8
Other	73.7	16.7	—	77.3	16.0	—	56	5.6	—

*These items were not identified during 1994–1996, when the spring and summer samples were collected and analyzed.

Table 4: Frequency of occurrence (FO) of food item categories in Scandinavian brown bear fecal samples during spring, summer and autumn seasons, during the years 1994-1996 and 2000-2001 in South-central Sweden. "tr" ("trace") indicates contributions below 1%. Source: Stenset et al. 2016.

Season	Category	1994	1995	1996	2000	2001
Spring	berries	66	14	54		
	insects	76	74	35		
	fungi	0	0	0		
	cryptograms	59	21	20		
	graminoids	83	66	50		
	forbs	24	tr	0		
	horsetails	tr	9	0		
	vertebrates	35	26	29		
Summer	berries	26	17	22		
	insects	80	77	78		
	fungi	0	0	11		
	cryptograms	26	20	0		
	graminoids	91	73	33		
	forbs	91	27	44		
	horsetails	tr	3	17		
	vertebrates	40	43	50		
Autumn	berries	98	98	100	99	97
	insects	39	26	30	79	93
	fungi	0	7	3	15	63
	cryptograms	tr	tr	tr	72	42
	graminoids	41	tr	23	47	85
	forbs	16	7	tr	13	73
	horsetails	5	0	tr	0	0
	vertebrates	9	14	27	13	3

As we can see from the tables, the bear's diet composition shows great variation among seasons (Tables 3 and 4) and years (Table 4). And so, in spring, the major food items found in the scat samples were ants, plant material (graminoids, cryptograms, forbs), vertebrates and berries consumed from the previous autumn prior to hibernation. The vertebrates are essentially adult moose since it is before the calving season. The insect category is almost entirely made up of ants whether it's in spring, summer or fall. Within these major food items, the vertebrates are the dietary component that contributes to the majority of the bear's energy intake during spring, with an estimated dietary energy content (EDEC) of 60.8% (Table 3). This chart also shows that the food items contributing the most to the bear's energy intake don't necessarily make up for the majority of the fecal volume (FV) nor the frequency of occurrence (FO). For example, insects were the food item found most often in terms of frequency (FO) but only contributed to a third of the vertebrates' EDEC and made up approximately 10% of the FV. In contrast, vertebrates were found less frequently in the scat samples (FO = 40.5) but made up a bit more than 20% of the FV and constituted the major dietary item in terms of EDEC, suggesting that some food items are better digested than others. In summer, the majority of the EDEC is brought from vertebrates (62.3%) and ants (28.2%) even though plant materials (graminoids and forbs) make up for almost half of the total fecal volume, suggesting poor digestibility of plant materials. The Scandinavian brown bear's autumn diet shows the most extensive diet of all the seasons, as we can see in the FO column. Berries make up for the most important food item during fall, with an FO of 98.3, an FV of 76.8 and an EDEC of 68%. Insects and vertebrates are also important dietary components during autumn.

As for the annual fluctuations in dietary items, significant variations were found for the insect and vertebrate categories in terms of FO, and in the fungi and plant categories in terms of FV. Berries as a group did not show significant variations, however, the proportion of berry species showed significant differences among years in terms of FO and FV (e.g., for bilberries *Vaccinium myrtillus*, FV= 43 in 1994, FV=60 in 1995, FV=20 in 2000, FV=36 in 2001). Thus, the most consumed berry species show good and poor years, based on the FO and FV of the bear scats. Although Scandinavian brown bears show a clear preference for bilberries if the availability supports it, they will switch the major berry species ingested depending on the overall berry abundance. FO and FV of combined berries in the bears' diet seem to be constant across years, suggesting that bears compensate for changes in the abundance of one species by switching to other species (Stenset et al. 2016). For example, as seen in Table 2, in terms of FO and FV, 1996 was one of the best years for bilberries and lingonberries but a very poor year for crowberries, whereas 2000 was a poor year for bilberries and lingonberries but the best year for crowberries. Of course, this 2016 study is based on data collected from over 20 years ago, so the FO, FV and EDEC values in the various dietary items nowadays would most definitely differ with the increasing variations due to climate change and human-induced activities. But it still tells us how Scandinavian brown bears are adaptable to food availability variations, given their opportunistic feeding lifestyle.

3.2 The Scandinavian Brown Bear Research Project

The Scandinavian Brown Bear Research Project (SBBRP) studies free-ranging brown bears in their natural surroundings in Scandinavia and is one of the world's longest running research projects. It started in 1984 in Sweden, and since then the project has marked and followed over 2421 individual bears from 1984 to 2022 (Arnemo, unpublished data). Three years after its inception, the project became a collaboration between Sweden and Norway. A wide range of research topics are covered such as biology, physiology, anatomy, genetics, behavior, human-bear interactions and conflicts, population ecology, health, research ethics (e.g., short, mid and long-term effects of captures, immobilization, use of tracking devices). The aim of this multidisciplinary project is to provide wildlife management authorities in Norway and Sweden with knowledge that can form a solid scientific basis for the current and future management of brown bears. Since the beginning of the project, they have been communicating and cooperating with administrative authorities, hunting associations and its members, Sami people and locals. The SBBRP is funded by the Swedish Environment Agency (SEPA or Naturvårdsverket) and the Norwegian Environment Agency (NEA or Miljødirektoratet).

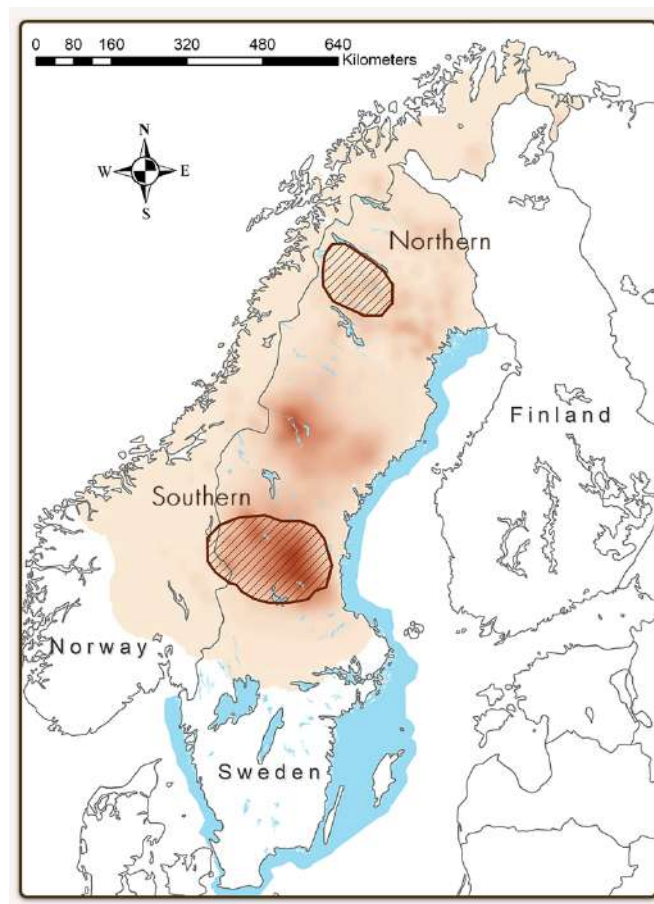


Figure 13: Study areas of the SBBRP. Source: Scandinaviska Björnprojektet website <https://www.brownbearproject.com/history>

The SBBRP has conducted most of its field-based work in two different areas in Scandinavia (Figure 13). The Southern study area (~61°-62°) includes an area of approximately 20,000 km² in the counties of Dalarna, Gävleborg and Jämtland in Sweden and Hedmark in Norway. The altitude ranges from 200 to 1,000 meters (m) above sea level. The Northern study area (~66°-67°) was located in Norrbotten county in Sweden. For practical reasons and because the south-central region is considered to be the core of the brown bear range in Scandinavia with higher bear densities, the Northern study area has been inactive since 2013. Both study areas are made up of hilly landscapes, with intensively managed boreal forests. The dominating tree species are Scots pine (*Pinus silvestris*) and Norway spruce (*Picea abies*), interspersed with water bodies such as lakes and bogs.

The project provides long-term, continuous data on bear ecology that becomes more and more valuable with each passing year. Long-term projects based on data collected over a long period of time allow us to get more information, making the data more significant and decreasing the potential biases (e.g., if a set of data is collected only once or for a short period of time, biases such as warmer or colder temperatures, one gender category being more represented than the other, poor variety of age categories, temperatures affecting the availability or composition in their usual diet etc. may affect the values and not represent the studied population's real physiological values). They also provide insight on annual variations and since the same bears can get recaptured several times, offer follow-ups whether it is for basic physiology, reproduction, behavior, ecology or other fields of work that are studied. For instance, capturing the same bear over a certain amount of years enables to study mid- and

long-term effects of captures and immobilization, hence providing adjustments and/or improvements. Over the last several decades, the project has made important scientific contributions to our understanding of an extensive range of subjects including brown bear biology and ecology, animal welfare, and human health. The SBBRP and its increasing number of collaborators (including the Norwegian Institute for Nature Research (NINA)) have published over 350 scientific peer-reviewed articles, contributed more than 150 management-related reports, and produced a wide array of content (articles, broadcasts, posters, etc.) aimed for the general public. During the winter captures in 2023 for example, a team of reporters came along with the research team to film several steps of the captures in order to create a broadcast intended for the general public explaining what the project entails. They provide reliable information, contributing to reducing people's fear of large carnivores and supply leads on how to better coexist (see Annex 1 for links). The project has educated multiple new generations of science professionals by mentoring numerous post-doctoral researchers, PhD students, master's students, bachelor's students, and volunteers from all around the world. The long-term goal of the SBBRP is to continue to monitor the Scandinavian brown bear population and facilitate local management decisions, contribute to the understanding of brown bear biology and ecology in the context of our changing world (e.g., climate change, increasing human-induced activities), and provide nature-based solutions based on brown bear physiology to the field of human medicine.

The SBBRP monitors brown bears in Scandinavia using GPS/VHF-collaring, den surveys, scat sampling, habitat surveys, berry monitoring, infanticide cases and human-bear interactions. Radio-marked bears are the basis for management tools and population models to monitor the status but also the development of the brown bear population. These close long-term studies enable us to document fluctuations in demography, life history, individual brown bear densities along with changes in climate and environment, management, and land use. The SBBRP also receives relevant and updated data for population models and can thus develop and quality-assure monitoring and inventory systems, such as population size estimation. Between the winter, spring and summer captures, the SBBRP captures more or less 80 brown bears every year (Arnemo, unpublished data).

3.3 Immobilization

3.3.1 Drug administration sites

Immobilization drugs are most of the time administered intramuscularly (IM) in wildlife species. For bears in general, the preferred drug administration sites one should aim for are the lower regions of the hindlimbs, where the muscle mass is large and deep (Figure 14; Illustration No. 4) (Kreeger TJ et al. 2023; Zedrosser et al. 2011b). The dart-gun should be aimed towards the rear of the hindquarters, to avoid the femur which could end up in several complications (see 3.3.8 Potential complications). Another possible location is on their shoulders (Figure 14; Illustration No. 5) (Kreeger TJ et al. 2023). Absorption from large fat deposit sites is slow, unpredictable and not always fully known. Such areas of fat deposits should therefore be avoided and implicates a knowledge of the animal's anatomy and ecophysiology to know which regions to aim for and the ones to avoid. Brown bears have large amounts of fat deposits around the rump and should as such be avoided. This is especially true during the summer captures when the bears have started regaining body mass (lean mass and fat deposit) compared to their state in spring when a major part of their fat stores have

been consumed during hibernation. This is also why higher doses and longer dart needles (5 millimeters (mm) longer) are used for summer captures compared to the spring ones.

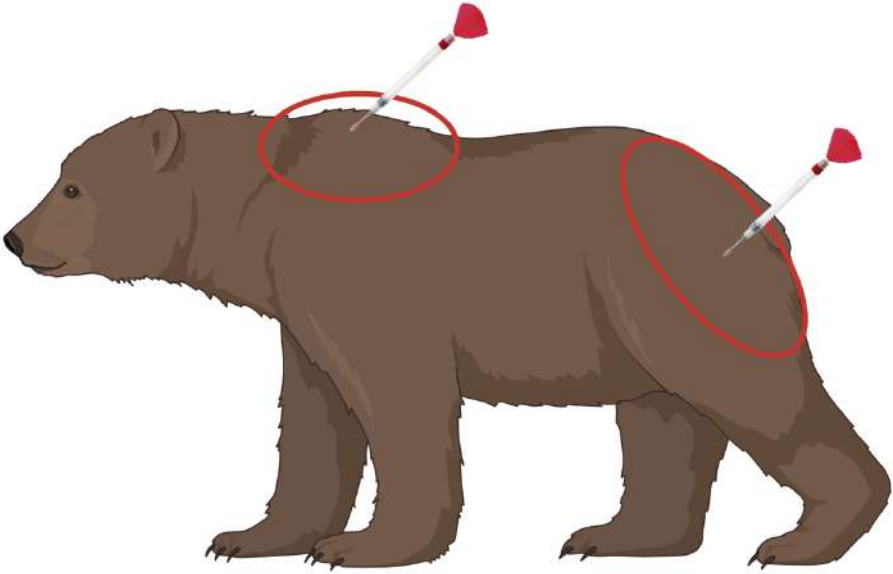


Figure 14: Drug administration sites to aim for in Scandinavian brown bears. Source: BioRender, by author.



Illustration No. 4: Anesthetic dart located in the left hindlimb of a brown bear - spring captures 2023. Source: Photograph by author.



Illustration No. 5: Anesthetic dart located in the left shoulder of a two-year-old brown bear - winter captures 2023. Source: Photograph by author.

3.3.2 Immobilization procedures and material

Most wildlife immobilizations are done remotely, meaning that there is no direct contact between the darter and the animal. Darts containing anesthetic drugs are the usual means of delivering the drugs to wildlife animals, which is the case for the Scandinavian brown bears used in this study and all of the research projects involved with the SBBRP. These darts are propelled by systems described as remote drug delivery systems (RDDS). RDDS are defined as

“mechanical devices capable of administering a single dose to an unrestrained animal, usually by means of a ballistic projectile” (Kreeger TJ et al. 2023). Modern dart propelling systems originated in the 1950’s, which is when the first projectile dart capable of delivering a liquid drug was reported (Crockford et al. 1957). That first projectile dart became the predecessor of the darts still used today for wildlife immobilization. Since then, many RDDS have been developed but only a few have proven reliable enough. The RDDS used during fieldwork for the brown bear captures are CO₂-powered tranquilizing rifles (DANiNJECT®, Børkop, Denmark). The darts inserted in the rifles are lightweight darts with barbed needles which have two side-ports (DANiNJECT®), to securely retain the dart in the animal. The size of the darts and needles are decided according to which categories the bears fall into: yearlings, subadults (two- and three-years-old) and adults. Yearlings and subadults are darted with 1.5 mL darts, but yearlings are shot with 1.5x25 mm needles whereas subadult are targeted with 2.0x30 mm needles. Adult bears are darted with 3 mL darts with 2.0x40 mm needles. During summer, the adults are darted with longer needles so that they can penetrate through the adipose tissue into the muscles and avoid slower and unpredictable drug delivery inside their fat deposits. Advantages of RDDS compared to other equipment used for capturing wildlife include targeting specific animals (as opposed to baiting or trapping), the drug administration is made based on body mass (visual observation by the experienced biologists allows them to estimate relatively accurately the weight of the animal to capture) and a wide range of volumes can be delivered. For the Scandinavian brown bears, 1.5 or 3 mL darts are used, but darts able to contain larger volumes exist. However, the target animal needs to be approached quite closely (ideally approximately 10-15m separating the bear from the helicopter, if not less), and they require fairly intense practice and experience by the user. In addition, all darts that use explosive charges to inject drugs into the targeted animal cause more or less severe hemorrhage and hematoma (Cattet et al. 2010), and misplaced darts can hit and break bones or even kill the animal.



Illustration No. 6: DANiNJECT® rifles used for the brown bear captures. Source: <https://daninject.com.au/products/model-im/>

Capturing free-ranging brown bears with such devices require a lot of planning and rigorous packing, so as to be prepared for every possible outcome and setbacks. The guns should be tested before, to ensure the pressure settings are up to standard. All the equipment should be packed systematically, with spare parts and drugs in order to be prepared for every possible outcome. When a lot of people are involved in projects like this, it can be easy to forget necessary equipment which can result in big problems and even put the animal’s health and

safety at risk. Communication, planning and list checks are paramount for good success rate in the research project. During all chemical immobilization and capture operations, a veterinarian must be part of the team in order to provide guidance and chemical immobilization procedures and techniques.

3.3.3 Spring and summer captures

As a result of its forestry management, Sweden has a lot of clear cuttings which facilitates darting brown bears from a helicopter, which is not the case in the rest of Europe. For example, in Croatia and Slovenia, Aldrich-type foot snares at feeding sites are used to capture brown bears, with different designs of trap sites: “American cubby”, “European cubby”, “trail” set, or “passage” type (Annex 2; Pereira et al. 2022; Huber 1996; Kaczensky et al. 2002).

Most of the bear captures occur during spring. The use of the helicopter enables to capture more bears than in winter, in fact, 98% of the brown bear captures have been made from a helicopter (Arnemo, unpublished data). The snow cover is usually still in place, and ambient temperatures are not as high as in summer, so there is less chance for the bear to overheat during the chase. Summer captures are more difficult than spring captures due to the lack of snow cover, the presence of open water, high ambient temperatures and increased dose requirements correlated with the seasonal changes in the bear’s physiology and body fat (Arnemo, Evans, Fahlman 2012).

During both spring (April-May) and summer (June) captures, the immobilization of the bears is done by helicopter. The shot is made by an experimented shooter outside of the helicopter, attached with a safety harness. For safety reasons, the dart gun should remain unloaded until the decision to pursue the animal is made. Helicopter pursuit along the rear one-third of the running bear is a suitable sight for the shooter to aim for the hindlimb muscles or back muscles near the shoulders. From the moment the bear has been located from the helicopter, the intense chase generally lasts no longer than one minute. Sometimes it takes a bit longer especially if there are forests around for the bear to run through, making it impossible to get close enough for an accurate shot. The helicopter pilot then needs to lure the bear back into the clear cut, and possibly hinder the bear from getting too close to water bodies or steep slopes by getting in his way, forcing the bear to change direction. Bears that haven’t been chased or captured by helicopter before are usually relatively naïve and won’t run away immediately when hearing the sound of the helicopter. Darting these bears is generally quicker compared to the ones that have previously been captured if the snow conditions and the landscape permit it (open spaces, clear-cuts, good snow cover). To keep the stress level and physiological side effects such as hyperthermia and lactic acidemia to a minimum, the intensive chasing period causing intense running should be kept under three minutes. In addition, the maximum amount of time permitted for the total pursuit (time from initial observation to successful darting, covering the alternating periods of intensive and extensive pursuit) is set at 30 minutes, which is the cut-off generally set for all wildlife species chased by helicopter, after which time the chase stops whether the shooter was about to get a clear shot or not. The cut-off may be shorter, depending on environmental factors such as the weather, chasing area, and after assessment of the bear’s physical or physiological condition (Arnemo, Evans, Fahlman 2012).

Most of the time when the dart strikes the animal intramuscularly at the right location of the body, the bear shows definite signs of being drugged within five to ten minutes. Even if the bear seems immobilized lying down on the ground, there can always be surprises with the bear waking up and running away or towards the person trying to approach it. Before making physical contact, a good method to know if the bear is properly anesthetized is throwing snow balls or sticks at it. If it doesn't trigger any response, the radio-collaring and sampling process can begin. The bear is either taken into the helicopter and brought to a meeting point where the ground team is waiting, or staying at the location where it was immobilized. When carrying a yearling out of the helicopter, it is important to know how to carry it properly so it can breathe correctly, without causing tissue damage and with its mouth and respiratory tract facing the ground so that in case of vomiting, it doesn't reach the lungs (Illustration No. 7).



Illustration No. 7: How to properly carry a bear cub. Source: Photograph by Alexandra Thiel.

For bigger bears too heavy to carry, a sledge is usually used as a mean of transportation from the helicopter to the meeting point (Illustration No. 8).



Illustration No. 8: Transportation mean for subadult and adult bears to-and-fro the helicopter/meeting point. Source: Photograph by author.

After making sure the bear is completely down, the first thing to do is to cover its eyes with an eye cover and provide oxygen to the animal. The eye cover attenuates visual stimuli and protects the bear's eyes from ultraviolet (UV) radiations that can seriously damage the eye (e.g., cataract, photokeratitis, pinguecula, photo conjunctivitis), especially with the snow reflecting the rays (Alebrahim et al. 2022). Protective eye lubricant (Viscotears®) is put in the eyes to prevent dry eyes, infection and corneal ulcers. Oxygen deprivation causes tissue damage, in particular brain damage. Oxygen supplementation is therefore administered intranasally from portable oxygen cylinders (which store gaseous oxygen under high pressure) or portable battery-driven oxygen concentrators (which extract nitrogen from the air and produce oxygen on site; Fahlman et al. 2012) at different flow rates depending on the bear's weight (0.5-3 L/min). Generally, two nasal lines are inserted inside the nostrils up to the medial canthus of the eye (Illustration No. 9).



Illustration No. 9: Two two-year-old brown bears blind-folded and supplemented with oxygen provided by oxygen cylinders – winter captures 2023. Source: Photographs by author.

In relation to the body mass of the bears, an overview of the recommendations on optimal oxygen flow rates for brown bears anesthetized with medetomidine, tiletamine and zolazepam (MTZ) are shown in Table 5.

Table 5: Optimal oxygen flow rate recommendations for Scandinavian brown bears anesthetized with MTZ during spring and summer. Source: Table made by author based on the oxygen supplementation recommendations by Arnemo, Evans 2017.

Weight of the bear (kg)	≤ 25	25 - 50	51 - 200	> 200
Optimal oxygen flow rate (L/min)	0.5	1	2	3

In addition, when oxygen (O₂) supplementation is discontinued, hypoxemia quickly recurs, attaining values not significantly different from pre-O₂ values, and should thus continuously be provided throughout the anesthesia (Figure 15; Fahlman et al. 2014).

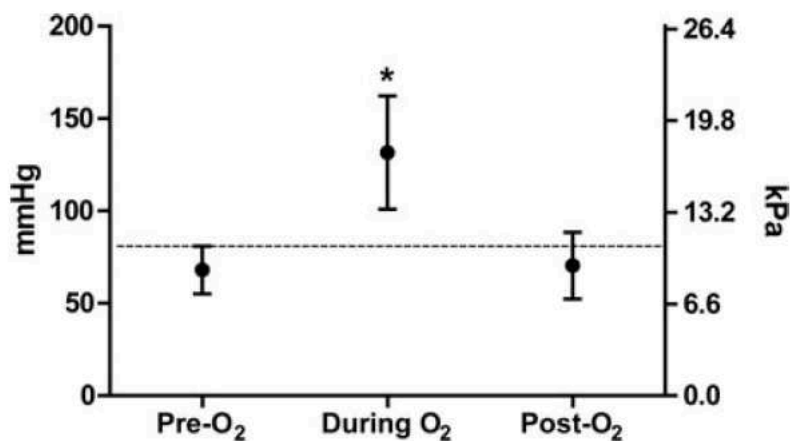


Figure 15: Partial pressure of arterial blood (PaO₂) before (pre-O₂), during, and after (post-O₂) intranasal oxygen therapy administered at 0.5-3 L/min from a portable oxygen cylinder to 11 anesthetized brown bears (*Ursus arctos*) with MTZ. “*” indicates significant differences ($p < 0.05$) from the pre- and post-O₂ samples. The dotted line represents the mean target PaO₂ of 11.1 kPa (individual range 10.1-11.6 kPa). Source: Fahlman et al. 2014.

While bears captured during winter were all equipped with global positioning system (GPS) collars and very high frequency (VHF) transmitters, entailing they had already been captured before, some bears during spring and summer got captured for the first time. In such cases, their age needed to be determined by extracting a premolar tooth and later on measuring its cementum layer (Illustration No. 10). Approximately five minutes before tooth extraction, local anesthetics were injected over the maxillary foramen, in addition to an anti-inflammatory IM (carprofen or meloxicam). The extracted tooth was then preserved in a two-mL cryogenic vial containing 96% alcohol (Arnemo, Evans 2017). On newly captured bears, a microchip was also inserted sub-cutaneously (SC) at the base of the nose, as well as tattooing inside the upper lip of the mouth. Ear tags were once used on brown bears as a means of marking, but stopped being used because they caused too many ear infections.



Illustration No. 10: Anne Randi Græsli and Alexandra Thiel collecting a premolar tooth from a first-time-captured anesthetized brown bear in order to assess its age. Source: Photograph by author.

During spring, summer and winter captures, the radio collars are fitted according to the body size, age and sex of the bear, and the weight of the collar shouldn't exceed 2% of the bear's body mass (Illustration No. 11). If the bear was already wearing a collar from a previous capture, its neck should thoroughly be checked for hair loss and potential skin irritation (Arnemo, Evans 2017). The radio collars are equipped with a transmitter (VHF) and GPS unit. The collars should be fitted so that it can be put in place and pulled off over the head, without sitting too tight or too loose. A way of checking the proper fitting is to be able to pass a flat hand between the collar and the neck. Collars equipped with drop-off mechanisms or break-away zones are put on growing bears and bears whose age are uncertain (Arnemo, Evans 2017). It is sometimes difficult to put on collars fitting really large males, with necks as or bigger than their heads.



*Illustration No. 11: Boris Fuchs replacing the radio collar on a two-year-old brown bear.
Source: Photograph by author.*

It is during the spring and summer captures the bear cubs get captured, which is not the case during the winter. They can be cubs of the year, yearlings or subadults still by their mother's side (Illustration No. 12).



Illustration No. 12: Cubs of the year with their mother, after the marking and sampling procedures during the spring captures 2023. Source: Photographs by author.

3.3.4 Winter captures

The basic rules of approaching a free-ranging brown bear vary depending on the season of the captures. During winter, the approach should be as quiet as possible, especially when the team is composed of a lot of people. Most of the time the den will be covered in snow, creating a layer of insulation (in terms of heat, noise, etc). One should exercise extra caution when the layer of snow on the ground is thin, as the noise generated will be greater and the den might not be covered entirely by snow. This could result in the bear waking up and running away from the den. In addition to disturbing the hibernation cycle and causing den abandonment, it could be dangerous not only for the bear itself (waking up and running while being in hypometabolic state) but also for the group of researchers around it. Even in a hypometabolic state, brown bears are still very sensitive to human disturbance. In one study, bears exposed to human activity within 200 m abandoned their dens, moving an average of 5.1 km and up to 30 km before selecting a new den (Swenson et al. 1997). In another recent study, den abandonment rates of bears in Sweden averaged 22%, with most abandonments occurring during early denning (before the 15th of December) (Sahlén et al. 2014). These studies on den abandonment in Scandinavian brown bears were documented based on non-research human disturbance, so we can imagine that the research activity on bears increases the percentage of den abandonments. A recent study on den abandonment caused by captures of Scandinavian brown bears showed a 92% rate (12 out of 13 captures) of den abandonment (Alina L. Evans et al. 2016). Furthermore, bears that abandon their den during early spring will usually not try to find a new one, instead they will wake up prematurely and not spend time looking for a new one until next fall. Therefore, knowing the general denning areas may help to put in place restrictions to human activity to avoid disturbances as much as possible, and by extension lower the risk of conflict and bear-related injuries. Arousal from hibernation is known to be energetically expensive for small hibernators. For example, in arctic ground squirrels (*Urocitellus parryii*), arousal episodes are the most energetically costly component of hibernation, accounting for the majority of energetic costs during the hibernation period (Karpovich et al. 2009). That is why all the precautions must be taken to make sure the probability of waking up the bear unnecessarily is as low as possible, so as not to disturb the hibernation cycle from the same year as the capture, but maybe also the hibernation cycles for the years to come.

During winter captures, only single two- or three-year-old subadults are targeted. Their weight ranges from 25 to 50 kg and in some rare cases more than 50 kg (one three-year-old female captured during the 2023 winter captures weighed up to 74 kg, but that was exceptional). Their weight is systematically determined during the captures (Illustration No. 13). Capturing adults older than three years old or females with cubs would be too dangerous both for the bears and the research team, in case of an accidental escape from the den.



Illustration No. 13: Weighing of a brown bear – spring captures 2023. Source: Photograph by research team.

Oxygen supplementation is administered intranasally from portable oxygen cylinders (or rarely from oxygen concentrators, but their efficiency is limited below -10°C) at different flow rates depending on the bear’s weight. Since only single two- or three-year-old subadults are targeted during the winter captures, the adequate oxygen flow rates are between 0.5-1L/min. In relation to the body mass of the bears, an overview of the recommendations on optimal oxygen flow rates for brown bears anesthetized with MTZ + ketamine (K) is shown in Table 6.

Table 6: Optimal oxygen flow rate recommendations for Scandinavian subadult brown bears anesthetized with MTZ+K during winter. Source: Table made by author based on the oxygen supplementation recommendations by Arnemo, Evans 2017.

Weight of the bear (kg)	≤ 55	> 55
Optimal oxygen flow rate (L/min)	0.5	1

It is important to keep in mind that everything takes more time in cold weather conditions so planning and good organization are all the more important (time of approach on snow shoes or cross-country skis with all the equipment, finding the bear’s den with the VHF devices, doing surgery in extreme cold conditions with only a thin layer of surgical gloves etc.). Each person involved should know precisely its task before the start so that the marking and sampling procedures go as quickly and efficiently as possible. When the dens are relatively close to the forest roads, the research team reaches the den on foot using snow shoes or cross-country skis using VHF location devices.



*Illustration No. 14: Equipment check of the VHF devices before heading out to track the bears.
Source: Photograph by Erlend Moe, Lars Bjarne Mythen.*

If the den is located too far from the roads, snowmobiles are required, but the last hundred meters are made on foot. Once the den has been located, the snow covering the den's entrance needs to be removed with shovels. This needs to be done rather quickly and as quietly as possible so the chances of the bear waking up are kept to a minimum. Metal grids are then put in front of the den entrance, and an experienced shooter darts the bear (Illustration No. 15). The dart gets generally fired from 0.3-3.5 meters distance (Græsli et al. 2015). The darts contain a volume of 3 mL of drug combination, with a barbed needle measuring 2.0x30 mm. After waiting 10 to 15 minutes for the anesthetic drugs to act, the immobilized bear is brought outside of the den (Illustration No. 16), where all the radio collaring and sampling procedures described above in "3.3.3 Spring and summer captures" are put in place.



Illustration No. 15: Left: Metal grids put up in front of the den after shoveling the snow by the den's entrance. Right: Anesthetizing the bear inside the den with a dart-gun – winter captures 2023. Source: Photographs by author.



Illustration No. 16: A three-year-old female brown bear being carried out of the den – winter captures 2023. Source: Photograph by Erlend Moe, Lars Bjarne Mythen.

3.3.5 Anesthetics

As previously said, the anesthetic drugs are administered intramuscularly during winter as well as spring and summer captures. Intravenous (IV) injections are usually reserved for antagonists during emergencies. If an anesthetic drug is to be administered intravenously, it must be done with extreme caution and with the adapted monitoring material, as well as the proper antagonists ready nearby. IV injections often have a rapid onset of action, and for some drugs, more than others, may cause respiratory depression or in worse cases respiratory arrest (Karpovich et al. 2009).

The brown bears are immobilized with a combination of medetomidine, tiletamine and zolazepam (MTZ), with ketamine (K) in addition during winter. The bears during winter are in a hypometabolic state so they don't need a dose as high as in spring or summer. The lower doses administered in winter not only reduce the costs related to the drugs but also generate fewer side effects. Ketamine constitutes the preferred additional drug if needed, both in summer and winter. During an evaluation of the physiologic effects of immobilizing captive and free-ranging brown bears with the drug combination of MTZ, marked lactic acid and hyperthermia were documented in free-ranging bears and hypoxemia was demonstrated in both captive and free-ranging bears (Græsli et al. 2015). O₂ is systematically given during the captures. Indeed, the benefits of O₂ supplementation in Scandinavian brown bears have been proven to diminish many complications such as respiratory depression and hypoxemia (Fahlman et al. 2012; 2014).

3.3.5.1 Medetomidine and atipamezole

Medetomidine is a very potent, selective and specific agonist for alpha-2 adrenoceptors in the central and peripheral nervous systems (Veterinary Anaesthesia - 11th Edition, 2014; Virtanen 1989). Alpha-2 adrenoceptor agonists are a group of sedative-analgesic drugs that act on pre- and postsynaptic alpha-2 adrenergic receptors in the central and peripheral nervous systems to inhibit the release of norepinephrine. Medetomidine, or 4-(1-(2,3-dimethylphenyl) ethyl)-1H-imidazole, is a racemic mixture of two optical enantiomers, dexmedetomidine and levomedetomidine, the former being the pharmacologically active component (Gaynor 2009; Kreeger TJ et al. 2023). Even if the relative potency (mg:mg) between dexmedetomidine and medetomidine is 2:1, dexmedetomidine in wildlife doesn't appear to offer significant advantages over medetomidine (Bouts et al. 2010; 2011; Fandos Esteruelas et al. 2017). Medetomidine is a non-irritant solution, that can be administered IM, IV, SC or even via the transmucous membrane. This anesthetic compound is lipophilic, which facilitates its rapid absorption after IM administration, with peak plasma concentrations reached after ~30 minutes (Veterinary Anaesthesia - 11th Edition, 2014). It is metabolized by the liver with a half-life of 0.5-1.5 hours in dogs, cats and horses (Bettschart-Wolfensberger et al. 1999; Grimsrud et al. 2014; Salonen 1989). Medetomidine is often used in combination with injectable anesthetic agents such as tiletamine-zolazepam, ketamine and propofol for general anesthesia, although opioids and benzodiazepines can also be included in such protocols (Veterinary Anaesthesia - 11th Edition, 2014). Furthermore, in case of emergency endotracheal intubation, medetomidine provides good laryngeal relaxation (Textbook of Rabbit Medicine - 3rd Edition, 2022). Medetomidine induces sedation and analgesia with some significant cardiovascular side effects such as bradycardia, arterial hypertension followed by hypotension, and reduced cardiac output in a dose-dependent way. Indeed, a

biphasic blood pressure response occurs after administration of medetomidine, with an initial hypertension due to increased vascular resistance, followed by hypotension which is secondary to decreased release of norepinephrine (Doherty, Valverde 2007). The binding of alpha-2 adrenoceptor agonists on alpha-2 adrenoceptors stimulates negative feedback preventing further release of norepinephrine (= noradrenaline) from the presynaptic nerve endings. This leads to decreased arousal and an inhibition of the afferent pain pathway (Posner, Burns 2009). It is also a respiratory depressant. Other actions of clinical significance include intestinal relaxation with decreased motility, uterine stimulation, hyperglycemia through reduced renin and insulin secretions, and platelet aggregation. Because it causes peripheral vasoconstriction, the mucous membranes can take a slight mauve appearance that could be mistaken with cyanosis. Its vasoconstriction properties may also render blood sampling or IV fluid therapy more difficult, which is why blood sampling should be performed as quickly as possible after having stabilized the anesthetized bear with O₂.

Atipamezole, or 4-(2-ethyl-2,3-dihydro-1H-inden-2-yl)-1H-imidazole hydrochloride, is a specific alpha-2 adrenoceptor antagonist that rapidly reverses all sedative, analgesic and cardiovascular effects associated with medetomidine (and other alpha-2 adrenoceptor agonists; Virtanen 1989). The recommended posology is a dose of five times that of the medetomidine dose (Arnemo, Evans, Fahlman 2012; Vainio, Vähä-Vahe, Palmu 1989). The marketed solutions containing atipamezole used during the bear captures (Antisedan®) are five times the concentration of the marketed medetomidine so that equal volumes of agonist and antagonist may be used. Furthermore, an overdose of atipamezole doesn't appear to cause major harm in most animal species (Veterinary Anaesthesia - 11th Edition, 2014).

3.3.5.2 Tiletamine and ketamine

Tiletamine is a dissociative agent similar to ketamine, but at least twice as potent and has a longer-lasting effect (Veterinary Anaesthesia - 11th Edition, 2014). Tiletamine, or 2-(ethylamino)-2-(2-thienyl)-cyclohexanone hydrochloride, is classified as a N-methyl-D-aspartic (NMDA) receptor antagonist. Dissociative anesthesia generally implies dissociation from the surroundings, with superficial sleep mediated by the interruption of neuronal transmission between the conscious and unconscious parts of the brain. The dissociative anesthetics act on NMDA, opioid, monoaminergic and muscarinic receptors (Dugassa, Fromsa Merga 2018). They bind themselves to the phencyclidine-binding site, which prevents glutamate, an excitatory neurotransmitter, from binding. The effects resulting from the inability of glutamate to bind with the NMDA receptors are depression of the thalamocortical, limbic, and reticular activating systems (Dugassa, Fromsa Merga 2018). The marketed solution used for the brown bear captures is made of a combination between tiletamine and zolazepam which is a benzodiazepine agent (Zoletil®, 250 mg of tiletamine – 250 mg of zolazepam). It is a 1:1 dry powder preparation that needs to be reconstituted with sterile water or another drug. Adding 4.4 mL of sterile water to the vial results in a concentration of approximately 100 mg/mL (Kreeger TJ et al. 2023). Its lyophilized (freeze-dried) form allows it to have a long shelf life. The combination of tiletamine and zolazepam is quite popular for use in wild animals since it can be turned into highly concentrated solutions, suitable for dart guns.

Ketamine is a racemic mixture composed of equal parts of two optical enantiomers, S-ketamine and R-ketamine. Ketamine is administered IM during the bear captures, in the darts in winter and as a supplemental drug if needed in spring, summer and winter. In case of an IV injection, it should be given very slowly, because a rapid injection can cause apnea. Tiletamine,

as ketamine, can be administered by a wide number of routes: IM, IV, SC, per os (PO) or intraperitoneally (IP). Its plasma half-life in cats is two to four hours (Veterinary Anaesthesia - 11th Edition, 2014). The central nervous system (CNS) effects induced by tiletamine are highly species-specific. Tiletamine and ketamine present multiple advantages. They are effective on many species, in particular wildlife animals, their high therapeutic indexes make them safe drugs to use, provide peripheral analgesia (good somatic but poor visceral analgesia), have minimal respiratory effects, and provide good cardiovascular support. Dissociative drugs such as tiletamine and ketamine are known to have rough inductions and recoveries and poor muscle relaxation without tranquilizers. This is why an alpha-2 agonist or opioid is usually administered together. Other side effects include spontaneous involuntary muscle movements, convulsions (particularly with high doses or prolonged administration), pronounced salivation (possibility of controlling it with atropine) and lacrimation, irritation upon injection due to the low pH of the solution and production of a variety of hematologic, serum, chemical, and endocrine alterations (Kreeger TJ et al. 2023; Dugassa, Fromsa Merga 2018). The use of dissociative drugs implies retained reflexes like corneal, palpebral, laryngeal, pharyngeal, swallowing reflexes and jaw tone (Dugassa, Fromsa Merga 2018; Hampton et al. 2019). These reflexes should therefore not be used to assess the depth of anesthesia. Dissociative agents are metabolized in the liver by N-demethylation via cytochrome P-450 enzymes, conjugated to water-soluble glucuronide derivatives and excreted in urine (Kreeger TJ et al. 2023). During cyclohexane anesthesia, the eyes usually remain open, and should therefore be protected from drying out and UV light, as said previously (see "3.3.3 Spring and summer captures").

3.3.5.3 Zolazepam

Zolazepam is a benzodiazepine agonist, which is the same group that diazepam, midazolam and clonazepam belong to. Drugs of this group provide good muscle relaxation, antianxiety actions, anticonvulsant effects (particularly effective in decreasing cyclohexane-induced convulsions) and anterograde amnesia (Roth et al. 1984; Veterinary Anaesthesia - 11th Edition, 2014; Curran 1986). They operate their main sedative effects through a depression of the limbic system and their muscle relaxation ability through inhibition of the interneuronal neurons at spinal levels. They potentiate the inhibitory effects of γ -amino butyric acid (GABA) neurotransmitters. They do not activate the GABA_A receptors within the CNS directly but enhance the GABA effects by increasing the frequency of the opening of the chloride channels, therefore enhancing the potentiation of the neuronal inhibition that is mediated by GABA neurotransmitters (Kreeger TJ et al. 2023; Olkkola, Ahonen 2008). Benzodiazepines can be administered IM, IV, SC and PO (Kreeger TJ et al. 2023). Zolazepam's plasma half-life goes up to five hours in cats (Veterinary Anaesthesia - 11th Edition, 2014). The elimination of benzodiazepines is achieved through hepatic oxidation and glucuronide conjugation with excretion in urine and feces. It is impossible to induce anesthesia on animals using solely benzodiazepines, and should henceforth be combined with other central nervous depressant drugs to achieve it. Furthermore, they cause minimal respiratory and cardiovascular depression, although they may add to the depressant effects of the other anesthetic drugs used (Veterinary Anaesthesia - 11th Edition, 2014; Kreeger TJ et al. 2023).

Using a combination of tiletamine and zolazepam provides a good safety margin, as well as few respiratory, cardiovascular and thermoregulatory side effects. The main disadvantage of this combination is the lack of a reversal agent, rendering the recovery times long. A good way to reduce the recovery time due to this combination is to add an alpha-2 adrenoceptor agonist

to the mixture, in this case medetomidine, so that the doses of tiletamine-zolazepam may be reduced, therefore reducing their side effects which entails decreasing the recovery time. Another factor contributing to the reduction of the recovery time is the use of atipamezole, a reversal agent for medetomidine.

3.3.5.4 Current anesthetic protocol for immobilizing Scandinavian brown bears

The current anesthetic protocol (Table 7) in April-May is based on more than 38 years of fieldwork experience, bibliographic research, and captures of 2421 free-ranging Scandinavian brown bears from 1984 to 2022 (Arnemo, Evans 2017).

Table 7: Anesthetic standard drug doses of MTZ used when darting Scandinavian brown bears from a helicopter in April-May. Source: Table made by author based on the brown bear capture protocol by Arnemo, Evans, Fahlman 2012; Arnemo, Evans 2017.

Age and size categories	Yearlings	Small bears (two and three years old)	Adult females & small males	Adult males	Large adult males
Weight (kg)	15-45	45-70	70-120	120-200	>200
MTZ dose IM (mg)	1.66 M + 83.3 TZ	2.5 M + 125 TZ	5 M + 250 TZ	10 M + 500 TZ	15 M + 750 TZ
Supplemental drug	2 mg/kg of ketamine IM, every ~20 minutes				
Darts	1.5 mL	1.5 mL	3 mL		
Barbed needles	1.5x25mm	2.0x30mm	2.0x40mm		
Reversal drug = Atipamezole (mg)	6.25	12.5	25	50	75

For bears captured in summer (June), or in rare cases in fall, the spring doses were increased by 25-50%. Longer needles were also used correlated with the higher amount of fat deposits, in order to avoid delayed absorption from subcutaneous fat.

For hibernating bears captured during wintertime (February-March) in 2022 and 2023, the spring doses of medetomidine were reduced, correlated with their hypometabolic state and reduced drug elimination. For instance, subadult bears weighing 45-65 kg received a combination of 1 mg medetomidine, 125 mg of tiletamine-zolazepam and 100 mg ketamine, which provided sufficient anesthesia. For smaller subadults weighing 20-30 kg, a reduced dose can be used. For two-year-old bears 1.5 mL darts were used, and 3 mL darts for three-year-old bears. 2.0x30 mm barbed needles were used for both age groups (Arnemo, Evans, Fahlman 2012; Arnemo, Evans 2017).

The MTZ ratio is kept constant so that doses can be split or combined. The immobilization procedure generally requires the injection of supplemental drugs, which is a dose of 2 mg/kg of ketamine IM, every 20 minutes. Advantages of the use of MTZ in brown bears include rapid

induction, the ability to reach surgical anesthesia, the ability to be partly reversible with atipamezole, and that the doses are very safe to use. High doses of MTZ have been tested on captive brown bears and show very high therapeutic indexes. In the event of accidental mishandling, massive overdoses can be tolerated, up to ten times the standard dose. Tiletamine-zolazepam (TZ) was formerly the drug combination of choice for the immobilization of bear species. This combination has a wide margin of safety and has no major cardiopulmonary or thermoregulatory side effects in bears. However, its main disadvantage is its extended recovery times. While medetomidine causes cardiopulmonary and thermoregulatory side effects (it can cause hypotension and bradycardia, disrupts thermoregulatory capabilities, and may even cause vomiting in canids and felids), it can be antagonized by atipamezole (Antisedan®). Thus, combining medetomidine with TZ enables the effective dose of TZ to be reduced by as much as 75%, with a possibility to be partly reversible, leading to shortening the recovery times (Arnemo, Evans, Fahlman 2012).

3.3.5.5 Post-operative analgesia

For the post-operative analgesia, 0.4 mg/kg meloxicam was administered SC as soon as possible after the bear's immobilization and before surgical procedures were initiated during the spring and summer captures. Due to decreased metabolic rate and drug excretion, the meloxicam dose injected in bears during winter was reduced to 0.1 mg/kg (Arnemo, Evans, Fahlman 2012).

3.3.6 Immobilization signs

The immobilization signs the helicopter crew can start looking for after darting the brown bear are as following: slight behavioral changes (e.g., pace slowing down after an intensive chase or motion stopping altogether), abnormal behavior, aimless walking, agitated walking and/or running, licking, head posture lowering, straddle-legged stance, unsteady stance or walk, several attempts of rising up again after falling down, and finally lateral recumbency (Kreeger TJ et al. 2023). Urination and defecation can also occur. Then, after making sure the brown bears is properly immobilized from afar (by throwing snowballs or sticks, then poking the bear gently with a stick for example), signs of immobilization one can look for up close are: loss of jaw tone (no resistance to having their jaw opened), loss of pedal reflex (when pinching a toe, the limb doesn't withdraw), loss of palpebral reflex (when touching the medial canthus of the eye, the bear doesn't blink), loss of swallowing reflex (when pulling the tongue and releasing, the bear doesn't swallow). The ideal desired stage of anesthesia during the captures based on Guedel's classification is the Stage III, Plane II of anesthesia (Annex 3). This stage, also known as "Surgical Anesthesia", is characterized by regular depth and rate of respiration, loss of reflexes mentioned above, normal pulse and blood pressure, normal pupils with fixed eyes (Kreeger TJ et al. 2023). The aim is to ensure unconsciousness, amnesia and analgesia throughout the immobilization process.

3.3.7 Monitoring

The immobilized brown bears should be monitored and clinically examined throughout the anesthesia by experienced professionals (veterinarians, experienced biologists and technicians). If side effects such as respiratory depression, hypoxemia, cardiac depression/arrest, or thermoregulatory dysfunction occur, the persons in charge of

monitoring should rapidly recognize the signs and administer the adequate antidote or perform the correct technical gestures (e.g., endotracheal intubation, cardiopulmonary resuscitation (CPR)).

Equipment enabling good monitoring of the brown bear includes a stethoscope, a thermometer (with plastic covers), eye ointment (Ocrygel®), blindfolds, earplugs, a pulse oximeter, oxygen supplies (portable O₂ cylinders or battery-driven O₂ concentrators), an ambu-bag (or ventilation bag), different sizes of endotracheal tubes, a laryngoscope, electrical clippers (essential for IV access), fluids (several bags of Ringer-acetate), surgical kits (in case wound treatment is needed), a waterproof insulated cover (Wolverine Bag®, or Jervenduk®), iSTAT device (portable handheld blood analyzer) and electrocardiogram (ECG) monitor for research purposes, material for potential euthanasia (firearm, drugs).

By and large, the respiration (RR, amplitude) and T_b are the two main parameters to monitor carefully during the captures. Especially during winter, it is important to isolate the bear from the snow-covered ground with a Wolverine Bag® (Jervenduk®) to avoid T_b from dropping too low, but also for measurements requiring the animal to be isolated from the ground (whatever the season) such as bioelectrical impedance to assess body fat percentage. During summer or even spring on the other hand, the chances of the bear overheating due to the chase, stress and T_A increase. Monitoring the T_b with regular measurements of the rectal temperature (RT) allows to act quickly. “Normal” T_b during the active phase (April/May-October) in brown bears is 37-37.5°C, and during winter 33.6 ± 0.8 °C (Arnemo, Evans 2017; Alina L. Evans et al. 2016; Tøien et al. 2011). Hyperthermia is defined as a RT > 2°C above normal temperature (Kreeger TJ et al. 2023). The causes include metabolic heat generated by physical activity (e.g., helicopter pursuit), heat absorption from environment (i.e., warm T_A, direct exposure to sun, confinement in a poorly ventilated space), infections (bacterial, viral), and drug-induced alteration of thermoregulatory centers. The signs of hyperthermia are an elevated RT (> 2°C above normal RT), very warm extremities to the touch (i.e., ears, feet), rapid HR and irregular pulse, rapid breathing, and in the worst case scenario coma or death (Kreeger TJ et al. 2023). Hyperthermia during the spring and summer captures (T_b > 39.0°C) can be reduced by cooling the bear down with putting snowballs, water or humid moss on the axilla, groin, belly, or even the tongue. In order to reduce the risk of hyperthermia in the first place, the bear is brought to a location offering shade when possible, otherwise sheets or covers can be installed hanging over the bear in order to provide shade. Immersing the whole body of the animal in water probably remains the most rapid means of decreasing its T_b (Kreeger TJ et al. 2023). This is relatively easy for small animal (e.g., wolverines, lynx), provided that a pond, lake, or other type of water source is near. However, this is almost never possible for brown bears who are much heavier. If the T_b doesn't go down within 15 minutes, IV fluid therapy should be put in place (10-15 mL/kg) using cool (20°C) Ringer-acetate (Arnemo, Evans 2017). IV fluid therapy is also very useful during winter, since blood samples are collected on already-dehydrated bears. Usually 1L of Ringer-acetate is administered to the bear, with the fluid bag being kept close to the chest of the person helping administering it (Illustration No. 17).



Illustration No. 17: Alexandra Thiel and Jon Arnemo administering intravenous fluids for rehydration of a two-year-old brown bear – winter captures 2023. Source: Photograph by author.

On the other hand, hypothermic bears ($T_b < 36^\circ\text{C}$) should be protected from cold surfaces and wind. Hypothermia is defined as a $RT > 2^\circ\text{C}$ below normal temperature (Kreeger TJ et al. 2023). The causes of hypothermia include drugs (generating a decreased metabolism and/or endogenous heat production, and alterations of thermoregulatory centers), cold T_A , loss of insulation (e.g., wet fur, malnourished individual hence decreased fat deposits, prolonged recumbency in one position), and inadequate circulation (e.g., shock) (Kreeger TJ et al. 2023). The signs of hypothermia are a decreased RT ($> 2^\circ\text{C}$ below normal RT), shivering, decreased HR, cold extremities, and a decreased blood pressure (Kreeger TJ et al. 2023). Avoiding hypothermia can be achieved by placing the bear on an insulated, waterproof cover (Jervenduk®), hot water bottles can be placed on the groin and/or axilla, and covering the animal to retain heat (Kreeger TJ et al. 2023). If prolonged hypothermia occurs, body warm (38°C) Ringer-acetate should be administered to the bear (Arnemo, Evans 2017).

In order to assess correct blood oxygenation, the gums in the mouth of the bear should be checked. The color of the mucous mouth membrane provides good information on the matter, as well as the capillary refill time (CRT). A well oxygenated bear should have pink gums and a $\text{CRT} < 2$ seconds. When anesthetizing brown bears, the aim is to have partial pressure of arterial oxygen (PaO_2) between 80 and 100 mmHg. Hypoxemia, which is the term defining low levels of O_2 in the blood, is a common side effect when conducting arterial blood gas analysis, both in captive and free-ranging brown bears immobilized with different doses of MTZ (Arnemo, Evans 2017). The levels of hypoxemia are defined according to the degrees of severity: the

hypoxemia is mild when PaO₂ is between 60-80 mmHg, marked when it is between 40-60 mmHg and severe when PaO₂ falls below 40 mmHg. With over 39 years of experience with brown bear captures as well as other research studies on the matter, optimal O₂ flow rates have been determined (Fahlman et al. 2014) and should be monitored with extreme care. If the bear doesn't seem to breathe correctly, before using drugs or intubation, one should check for kinked nasal lines, lines blocked with mucus or blood, lines falling out of the nares when moving or manipulating the bear, lines coming off the O₂ concentrator or cylinder. The cardiorespiratory function is monitored via a pulse oximeter, with the sensor placed on the bear's tongue. The hemoglobin oxygen saturation (SpO₂) should be above 95%. Often when the pulse oximeter shows a SpO₂ below 95%, the sensor is just misplaced and needs to be correctly replaced. The clip of the oximeter is generally attached on the tongue, or eventually another thin non-pigmented tissue. If the sensor is correctly placed however and the SpO₂ shows a decreasing trend, it means the bear is suffering from hypoxemia, thus needing supplementation with intranasal O₂. It is not uncommon however for the SpO₂ to fall below 95% in bears anesthetized with alpha-2 adrenoceptor agonists, so the trend of the SpO₂ is more important than its absolute value. Other signs of respiratory depression in anesthetized brown bears are noisy breathing, few and/or shallow breaths, and cyanosis expressed by blue/grey gums. In case of apnea (= absence of breathing), intubation with an endotracheal tube and assisted ventilation with the ambu-bag should be put in place. In addition, doxapram at 5-10 mg/kg IV can be given if the bear doesn't resume breathing correctly on its own (Arnemo, Evans 2017). These measures are of course put in place after ensuring the basics which are ensuring free airways and correct positioning of the animal (potential pressure on the diaphragm), and stop administering further immobilization drugs. If all these efforts still don't result in the animal breathing, acupuncture can be tried, by inserting a needle into the upper lip between and below the nares, though its effectiveness has shown mixed success in stimulating respiration in immobilized animals.

3.3.8 Potential complications

Even with an experienced helicopter pilot, a well-trained and experienced research team on animal handling, veterinary anesthesia, safety, medical training and a good knowledge of the physiology, anatomy and metabolism of Scandinavian brown bears, complications during the immobilization process may occur. These include wounds (Illustration No. 18) and darts striking the bones (e.g., the femur if aiming the hindlimb, the scapula or the vertebral column if aiming the shoulders), which is painful for the animal, may cause fractures, bone marrow infections caused by bacteria transported along the needle, and may not inject the anesthetic drugs properly due to blockage of the dart needle. Extracting the dart from a bone almost always necessitates the use of pliers, and even then, may be impossible to extricate. Lethality due to darts striking bones is rare but can happen.



Illustration No. 18: Front paw injury, probably during the chase from the helicopter. Source: Photograph by author.

Even with years of studies and experience with anesthetic drugs, one must keep in mind they can never be considered 100% safe or devoid of toxicity, and there is always a risk even when administered in healthy individuals. Every anesthetic drug group has its advantages and disadvantages, which should be known to know how to act if they should arise. The risk of severe side effects, injuries or death can never be completely eliminated, depending on various factors, especially with wildlife species in harsh weather conditions. These are a few examples of the reason why it is so important to chemically immobilize wildlife only if it is necessary to capture live animals, to accomplish well thought-out research projects or management goals. Jon M. Arnemo wrote *“One should ascertain if the capture is really necessary and whether the risk of killing the animal justifies the planned gains”* in the Handbook of Wildlife Chemical Immobilization, 5th edition, 2018.

Respiratory depression (hypoventilation, increased CO₂ levels in the blood) or arrest is the most common medical emergency encountered during the immobilization of brown bears. Most physiological studies involving the analysis of blood gas show that the drugs and their doses used during anesthesia in free-ranging and captive wild animals demonstrate hypoxemia (Fahlman et al. 2012; Mich et al. 2008; Read 2003). Such events have been documented in free-ranging and captive brown bears that had been immobilized with MTZ, whether the hypoxemia was mild or severe (Fahlman et al. 2012). O₂ supplementation should therefore always be put in place as soon as the ground team receives the anesthetized bear until the reversal agent is given at the end of the procedure. Other complications include cardiac depression or arrest, thermoregulatory dysfunction (hypothermia or hyperthermia), hyper- or hypotension, aspiration of vomitus/saliva, drug overdose in individuals with poor body condition etc. The drugs used in the anesthetic protocol disrupt the thermoregulatory capabilities of the brown bear, in addition to the physical exertion of the chase prior to immobilization resulting in elevated body temperatures. Either hypo- or hyperthermia can kill

a bear. Monitoring the RT from start to finish is paramount. Additionally, high ambient temperatures (especially in June during the summer captures) make the captures more difficult and increase the risk of morbidity or mortality, with the body temperatures rising high during the chase. Warm weather conditions often require changing the original plans, like doing the chase very early in the morning and very late at night, making sure the marking and sampling take place in a location with access to shade, etc.

Some situations can get a bit stressful especially during spring captures when there are families of three or four bears simultaneously immobilized, during which silly accidents can occur such as accidental auto-injections of the drugs in the handler. The emergency kits should therefore contain drugs to counter act the effects in such cases.

The darts are equipped with a location detection microchip, which permits to recover the missed or lost darts during the chase, using a Recco® device. When the darter misses the animal during the pursuit, the people present inside the helicopter should drop an object as quickly as possible (e.g., a sweater) to locate more easily the spot where the dart ended up. They then go back looking for the lost dart, using the Recco® device which is a hand-held scanner emitting louder sounds the closer you get to the dart. It is less difficult to locate the missed dart during wintertime because the tracks can be used to help during the search (Illustration No. 19). It is important to try to recover all the missed darts for several reasons: from an ecological point of view so as not to leave plastic or other waste in nature, for safety reasons so that people don't accidentally step on the dart injecting themselves with the drugs or even deliberately misuse the drugs, for safety reasons for other animals accidentally stepping on the dart.



Illustration No. 19: Use of the Recco® device for the recovery of lost or missed darts. Source: Jon M. Arnemo.

Darting wild animals with dart-guns inevitably generates more or less severe hemorrhage and hematoma (Cattet et al. 2010), and misplaced darts can hit and break bones, penetrate through the thorax resulting in pneumothorax, cause major tissue damage or even kill the animal. The animals should always be darted with the lowest pressure setting that still ensures good ballistic trajectory of the dart and proper injection of the drugs, particularly for the smaller bears (cubs of the year, yearlings, small subadults).

In some cases, the shooter manages to aim for the animal but the dart falls on the ground right after, making it impossible to know whether the animal got part of the drugs injected, all of it, or none of it, or sometimes the dart remains on the animal but it doesn't seem to go down after a while. It is recommended to allow 10 to 15 minutes maximum to pass before re-administering a second dose. If the animal shows no drug effect after 10-15 minutes, the shooter darts the animal a second time with a full dose, which is the safest thing to do when using safe drugs such as MTZ with high therapeutic indexes (Arnemo, Evans 2017; Kreeger TJ et al. 2023).

Other factors increasing the risk of morbidity and mortality are the presence of open water and bare grounds. Drowning constitutes the first cause of mortality during bear captures (Table 8).

Table 8: Scandinavian brown bear mortalities related to captures (+ 30 days post-capture) immobilized with MTZ, from 1992 to 2023. Source: Jon M. Arnemo.

Year	Sex	Age (years)	Cause of death
1993	M	2	Stress
1997	M	3	Stress
1999	F	1	Stress
2006	M	1	Dart trauma; penetration of heart
2006	F	11	Drowning
2007	M	4	Drowning
2008	M	4	Unknown; 3 days post capture
2009	M	2	Unknown; 10 days post capture
2012	F	9	Dart trauma; pneumothorax
2013	M	3	Killed by another bear 20 days post capture
2015	F	2	Drowning
2016	M	Adult	Unknown; 3 days post capture
2016	F	Adult	Unknown; 2 days post capture
2016	M	Adult	Drowning
2017	M	2	Killed by another bear 27 days post capture
2021	M	1	Drowning
2021	F	2	Unknown; 3 days post capture
2022	F	3	Unknown (likely drowning); < 24 hours post capture
2023	F	5	Unknown; likely 2 weeks post capture

Drowning can occur if the bear was darted in an area containing bogs or rivers or other types of water bodies that the helicopter crew didn't see from the helicopter, or if the bear was able to run far enough to find a source of water after overheating from the chase (Illustration No. 20). If the situation seems to be safe for the handlers, the bear should as quickly as possible be pulled out of the water, or at least have its head safely moved away from it. "Stress" includes the stress from the chase, from the drugs (e.g., respiratory depression, circulatory problems) and hyperthermia.



Illustration No. 20: Scandinavian brown bears falling into bogs as the anesthetic drugs kick in.
Source: Jon M. Arnemo.

The ideal darting area would be in a wide clear-cut with open terrain, no dense forest or roads around, no powerlines, on a flat landscape covered with snow, with no water bodies around. Of course, such ideal scenarios rarely occur.

As mentioned above, the drugs used may create respiratory depression. In that case, the best response is to intubate the bear and provide assisted oxygenation, and as a second step use doxapram from the emergency kit. The endotracheal tube (ETT) along with the ambu-bag could be considered as one of the most important emergency tools. Doxapram is a pyrrolidinone derivative with central nervous system-stimulant (analeptic) effects. It generates a direct stimulation of the medullary respiratory center and the activation of the carotid and aortic chemoreceptors, thus acting on both central and peripheral chemoreceptors, leading to increased depth and rate of respiration (Shah, Di Fiore, Martin 2019; Kandasamy, Carlo 2017). One should exercise caution when using doxapram, since its onset of action causes an increase in the metabolic rate, therefore increasing the chances of the animal waking up.

While mortality directly due to the anesthetic drugs is rare, death due to the global process of chemical immobilization may occur: fractures or serious wounds happening during the chase, dart trauma, drowning, falling off cliffs, crossing the road and causing accidents when vehicles hit them, suffocating (if the nostrils are facing the ground, bent neck, an object in front of the nostrils), during the recovery with long-term effects from stress such as capture myopathy, unusual long recovery, attacks from other bears while still being sedated. Broadly speaking,

most of animal mortality in relation to captures is due to human error which can be avoided with good planning, organization, organized protocols, frequent trainings, patience etc. Some examples of human errors include lack of training or experience, lack of monitoring, forgotten equipment or drugs, dart misplacement, and prolonged chasing.

The SBBRP considers that a mortality rate $\leq 2\%$ is possible and achievable, including the 30 days post-capture mortality (“Arnemo’s 2%-rule”). This is especially true if a project has been going on for a long period of time, and the protocol gets perfected with years of experience, with the usage of improved drug delivery methods. Table 9 shows a decrease in mortality rate after 1992, which corresponds to the year the team changed the anesthetic drugs.

Table 9: Mortality rate in free-ranging Scandinavian brown bears immobilized with MTZ during captures from 1984 to 2022. Source: Arnemo and Evans, unpublished data.

Year	Number of captured bears	Mortality rate
1984-1991	175	4% (7/175)
1992-2022	2246	0.8% (18/2246)

3.3.9 Reversal of immobilization

After all the marking and sampling procedures are finished, a reversal drug is administered IM. Medetomidine is an alpha-2-adrenoceptor agonist and one of the anesthetic drugs used for the immobilization. It can be antagonized by atipamezole, which is the reversal agent used for Scandinavian brown bears at a dose of 5 mg of atipamezole per mg of the total medetomidine dose. Because TZ has such a long elimination time, atipamezole should not be injected before 50 to 60 minutes after darting, so as not to harm the bear with a rough recovery including incoordination, excitation and convulsions. However, in case of an emergency requiring the bear to be woken up, atipamezole can be given at any time, in which case the IM administration of midazolam at 0.1-0.2 mg/kg is recommended to help the bear calm down (Arnemo, Evans 2017).

Wildlife animals that have been anesthetized should be monitored during their recovery, to make sure everything goes smoothly and potentially step in if mishaps should occur, but also to write down the positive and negative outcomes, in order to adapt the anesthetic protocol for the following captures. Being near wild animals recovering from anesthesia is dangerous for many reasons, partly because of their unpredictable responses due to the drugs. Watching the brown bears recover from the anesthesia from a distance was carried out at the start of the SBBRP. As years went by and the SBBRP gained more knowledge about the bear’s physiology and the anesthetic protocol got improved, brown bears seemed to show smooth recoveries, no longer requiring supervising during their recovery. With the current anesthetic protocol used for immobilizing brown bears, it is known that their recovery goes well. Thus, at the end of the procedure, while most of the research team packs up all the equipment and head back to the helicopter and/ or cars, a few people stay behind to place the bears in a good recovery location, safely positioned and inject the reversal drug. A good recovery location can be described as a quiet place, far away from bogs, lakes and roads, in the shade during spring and summer, close to the edge of the forest. Potential dangers and side effects during and

immediately after the recovery include hypothermia (in case of procedures that last a long time), hyperthermia (due to high T_A , drugs, direct exposure to the sun and due to the intense chasing prior to capture), lack of fear, traffic, poaching, open water (even after making sure no water bodies were seen within a certain radius) and intraspecific strife (males attacking other males or young, males trying to mount immobilized females still in estrous etc.) (Arnemo, Evans 2017). All GPS and radio-instrumented bears are closely monitored the day after the capture.

3.4 Essential minerals studied

Minerals constitute essential components of body enzymes, hormones and cells. They are chemical elements essentially required in the form of nutrients for the proper functioning of the body and healthy life (online definition, "Many definition of minerals" 2023). They play a fundamental role in digestion, reproductive performance, immune system, development of bones, muscles, teeth, etc. (Rumosa Gwaze, Chimonyo, Dzama 2012). Minerals have the ability to interact with microbes, viruses and macro-biomolecules through ionic bonds and/or non-covalent bonds and therefore constitute new interests in therapy and biotechnology (Carter, Xu, Sadler 2021). Minerals play a very important role in the maintenance of homeostasis. Homeostasis can be defined as a *"self-regulating process by which biological systems maintain stability while adjusting to fluctuating external conditions"* (Billman 2020). It is not static and constant, but rather a dynamic process that can change internal conditions as required to survive external challenges (Billman 2020). It explains how organisms can maintain more or less constant internal variables, allowing them to adapt and survive when faced with a changing external environment (Billman 2020). Automatic cellular mechanisms, neuronal endocrine controls, as well as behavior, all contribute to homeostasis. It is highly developed in warm-blooded animals living on land, because they must maintain a certain fluid balance, T_b , blood pH, O_2 levels, and acid-base balance within rather narrow limits of reference intervals. The concept of homeostasis permits us to comprehend the functions of the body in a healthy or pathological state. Indeed, its disruption leads to electrolyte imbalances and disease. Maintaining homeostasis requires the expenditure of energy, so animals must make sure to obtain sufficient nutrition. Minerals can be found in the form of macrominerals or microminerals. Macrominerals are a group of minerals the most needed by animals in their diet to fulfil a plethora of cellular functions in multiple organs. They constitute minerals that the body needs through daily diet in relatively large amounts (Minerals: functions & food sources 2023). Microminerals are often referred to as trace minerals and are found in smaller quantities compared to macrominerals, but still fulfil functions of physiological importance (Minerals: functions & food sources 2023).

3.4.1 Calcium

Calcium (Ca) is classified as an alkaline earth metal in chemistry and has an atomic number of 20 in the periodic table of elements (Annex 6; PubChem 2023). The mineral found in the greatest abundance in mammals is Ca (David et al. 2023). It is also the fifth most abundant element of Earth's crust after oxygen, silicon, aluminium and iron (Calcium - Element information, properties and uses | Periodic Table 2023). This macromineral is essential for skeletal growth and healthy bones and teeth. It also plays a key role in regulating blood pressure levels. About 99% of Ca in animal bodies is located in the skeleton (Annex 4; The roles of calcium and phosphorus 2014), but the remaining 1% present in blood still plays a crucial

role in numerous functions of the body. The majority of Ca in bones and teeth is found in the form of hydroxyapatite (HA) crystals, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, which are phosphocalcic compounds making up approximately 65% of the weight of bones (Cooper, Guan 2016). This compound is often used in bone implants (orthopedic and dental). The animal's bone is a heterogeneous composite material consisting of a mineral phase (mainly composed of HA), an organic phase (~90% type I collagen) and water (Boskey 2013). The relative amount of each of these constituents in bones varies with sex (Gregson et al. 2013), age (Boskey, Coleman 2010), anatomic sites (Donnelly et al. 2012), and health status (Boskey, Mendelsohn 2005). Bones are dynamic tissues that are remodelled throughout life, through bone cells called osteoclasts and osteoblasts, responsible for bone resorption and bone formation respectively. The only source of Ca is from the diet (Weaver, Peacock 2011).

Ca is present in the serum in the proportions of 50% in the ionized state (Ca^{2+}), 10% under the form of complexed Ca (mainly to citrate and PO_4 ions) and 40% of protein-bound Ca (of which 80% is bound to albumin and 20% to globulin) (Favus, Bushinsky, Lemann 2006). In a nutshell, Ca exists in 3 forms in serum: protein-bound, ionized (Ca^{2+}) and complexed (or chelated). Calcitriol (1,25-dihydroxyvitamin D3), calcitonin and the parathyroid hormone (PTH) play an important role in maintaining a steady level of Ca in animals' bloodstreams. These 3 hormones regulate Ca transport in three major transport systems: the gut, kidneys and bone (Yu, Sharma 2023). A decrease in serum Ca concentrations induces the release of the PTH from the parathyroid glands, which will launch the cascade responsible for increasing the Ca concentration in the blood stream involving the kidneys, bones, and small intestine. PTH acts on the kidneys, increasing Ca reabsorption in the ascending loops of Henle, the distal convoluted tubule and the collecting duct (Yu, Sharma 2023). In response to the action of PTH on kidneys, they also stimulate the secretion of the active form of vitamin D, calcitriol, thus stimulating Ca absorption through the small intestine. PTH also acts on the bones, by stimulating bone cells called osteoclasts, which are involved in bone reabsorption and the release of free Ca. On the other hand, an increase in serum Ca concentrations induces the release of calcitonin (a peptide hormone) from the thyroid gland, which will have an impact on decreasing Ca absorption from the small intestine, Ca reabsorption from the kidneys, and Ca resorption from the bones by stimulating the osteoblasts (Yu, Sharma 2023). This complex system allows for rapid control and adjustments of blood Ca concentrations, though at the expense of the skeleton (Weaver, Peacock 2011).

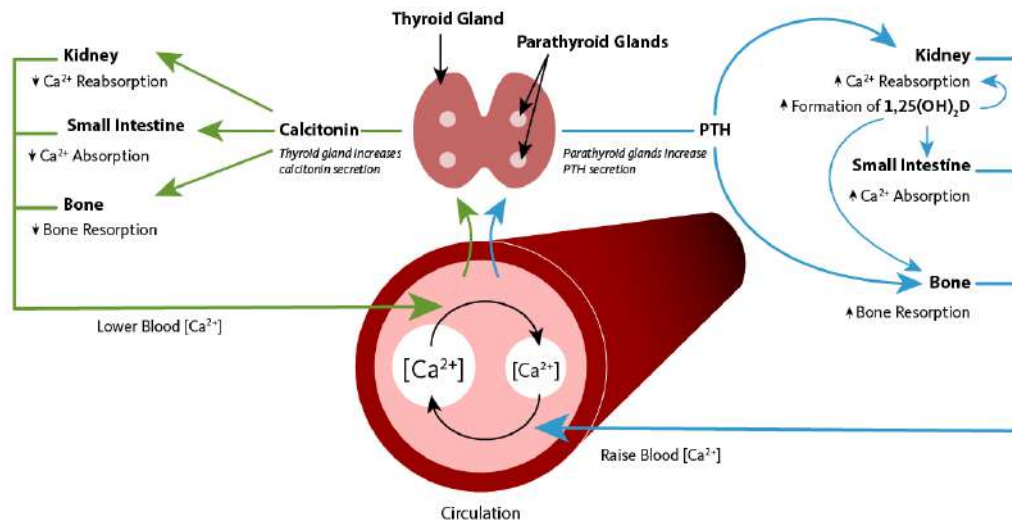


Figure 16: Calcium homeostasis. Source: Oregon state University website <https://lpi.oregonstate.edu/mic/minerals/calcium>

Ca plays a fundamental role in transferring information between cells and the transmission of nerve impulses. For instance, it plays a major role in muscle contraction (smooth, skeletal and cardiac muscles) and blood coagulation, vasoconstriction and vasodilation, nerve impulse transmission, enzyme activation, metabolic reactions, protein synthesis, maintenance of acid-base and osmotic balance (Danks 2014).

Mild hypercalcemia is usually asymptomatic (Yu, Sharma 2023). Elevations in Ca concentrations can lead to nonspecific symptoms such as nausea, vomiting, confusion, abdominal pain, constipation, depression, weakness, myalgias, arthralgias, polyuria, polydipsia, nocturia etc. The physical exam can display hypertension, bradycardia, hyperreflexia and tongue fasciculations. On the other hand, hypocalcemia can lead to syncope, congestive heart failure, numbness, muscle spasms, tetany, bronchospasm, laryngospasms and dysphagia, depression, fatigue, seizures etc. Chronic hypocalcemia can lead to coarse hair, brittle nails, psoriasis, dry skin, pruritus, poor dentition, and cataracts. Physical exams can show neural hyperexcitability, psychological disturbances, and cardiac arrhythmias (Yu, Sharma 2023). Chronically low Ca intakes in growing individuals interfere negatively with reaching optimal peak bone mass, contributing later on to accelerated bone loss and osteoporosis.

Multiple nutrients and minerals interact with Ca and are essential for Ca concentration maintenance in the body. For instance, vitamin D is required for optimal Ca absorption, by the means of its active form, calcitriol. Dietary Ca plays a major role in Ca loss (Weaver, Peacock 2011). Probably because of the competition between sodium and Ca reabsorption, as well as the effect of sodium on PTH secretion, high-sodium intake results in an increased loss of Ca in the urines. High-protein intake enhances intestinal absorption and urinary excretion of Ca (Ross et al. 2011). Phosphorus, which can be found in protein-rich food, tends to increase the excretion of Ca in urines.

3.4.2 Cobalt

Cobalt (Co) is classified as a transition metal in chemistry and has an atomic number of 27 in the periodic table of elements (Annex 6; PubChem 2023). Co compounds have been used as blue coloring agents in glass and ceramics for thousands of years. Co traces have been found in Egyptian pottery dating back to 2600 BC, in Greek vases and pottery from the early Christian era, in Chinese pottery from the Tang (600-900 AD) and Ming (1350-1650 AD) dynasties era, etc. Leonardo Da Vinci even used Co as a blue pigment in oil paints. Co metal was isolated by the Swedish scientist G. Brandt in 1735 and in 1790 T.O Bergman established Co as an element (Donaldson, 1986). Come the twentieth century, Co was used for industrial purposes. It was first added in 1923 to produce hard metals (Anon., 1989). Co is classified as a compound that is known to “possibly” be carcinogenic to humans, meaning there is sufficient evidence that they cause cancer in animals but not enough evidence that they cause cancer in humans (Jensen, Tüchsen 1990). Therefore, Co levels in whole blood, plasma, serum, and urine are used as a biological indicator of exposure to Co (Ferioli *et al.*, 1987). Pulmonary effects (interstitial fibrosis and obstructive lung disease, asthma) are one of the major problems in relation to Co, particularly in the hard-metal industry, where Co-containing dust is generated.

On a biological level, this micromineral is a component of vitamin B12 (also called cobalamin) involved in the formation of red blood cells and nerve cell functions. Due to its erythropoietic effect, Co has been used for the treatment of anemia (Berk *et. al* 1949). Co is also involved in folate and fatty acid metabolism. At the cellular level, cobalamin acts as a cofactor of two enzymatic reactions involving methionine synthase and methyl-malonyl-co A mutase. Methionine synthase helps to convert homocysteine to methionine, which is an amino acid that converts into S-adenosylmethionine and participates in several methylation processes of cells. These methylation reactions are necessary for the synthesis of many molecules such as phospholipids, neurotransmitters, and the regulation of gene expression (Al Amin, Gupta 2023). In serum, Co binds to albumin, and the concentrations of free ionized Co^{2+} are estimated around 5-12% of total Co concentration. In human red blood cells, the membrane transport pathway for Co^{2+} uptake appears to be shared with Ca^{2+} , but with an irreversible uptake since Co^{2+} is effectively bound in the cytosol and is not itself released by the Ca-pump (Simonsen, Harbak, Bennekou 2012). Co is ingested as cobalamin via meat products and in an inorganic form via plant products (AFSSA 2010).

3.4.3 Copper

Like Co, copper (Cu) has been used as a blue coloring agent in glass and ceramics for thousands of years. Because minerals such a malachite ($\text{Cu}_2(\text{CO}_3)(\text{OH})_2$) are cheap and abundant, metallic Cu has been used in many industrial applications (e.g., coins, ornamental jewelry). Cu is classified as a transition metal in chemistry and has an atomic number of 29 in the periodic table of elements (Annex 6; PubChem 2023). As a member of the transition metal series, Cu and six other metals in the series (chromium, iron, cobalt, manganese, nickel and zinc) constitute the bulk of essential metals in biological systems (National Research Council (US) Committee on Copper in Drinking Water 2000).

On the biological level, the micromineral copper (Cu) is a cofactor for several enzymes, involved in oxidation-reduction reactions in various metabolic pathways. These enzymes regulate redox processes in tissues with a high metabolic rate, such as the liver. Almost two

thirds of the body's Cu is located in the skeleton and muscle (A.C. Ross et al. 2012; Institute of Medicine (US) Panel on Micronutrients 2001). In biological systems, Cu shifts between the cuprous (Cu^+) and cupric (Cu^{2+}) forms. Cu is mainly eliminated from the body through bile and a small amount via urines (Office of Dietary Supplements - Copper 2023). Cu homeostasis is maintained by its absorption from the intestine and excretion through bile and in a smaller amount the urine (Institute of Medicine (US) Panel on Micronutrients 2001). Cu also plays a key role in the good functioning of blood cells, angiogenesis, formation of bones and nerves, neurohormone homeostasis, regulation of gene expression, brain development, immune system functioning and skin pigmentation (Office of Dietary Supplements - Copper 2023). Copper-dependent enzymes, or cuproenzymes (e.g., cytochrome c oxidase, superoxide dismutase, ceruloplasmin, lysyl oxidase, dopamine- β -hydroxylase), participate in energy production (ATP), neuropeptide activation, iron metabolism, tissue formation, and neurotransmitter synthesis (Copper 2014; Office of Dietary Supplements - Copper 2023; A.C. Ross et al. 2012; Erdman, MacDonald, Zeisel 2012; Institute of Medicine (US) Panel on Micronutrients 2001). Cuproenzymes are essential for several physiological processes within the brain and nervous system, including neurotransmitter synthesis and the formation and maintenance of myelin. For example, dopamine- β -hydroxylase catalyzes the conversion of dopamine to the neurotransmitter norepinephrine (Copper 2014; O'Dell, Sunde 1997). Multi-copper oxidases (MCOs) are Cu-dependent ferroxidases, involved in iron (Fe) metabolism and homeostasis. Cu and Fe metabolism are intimately related. For instance, Cu depletion results in an Fe deficiency-like anemia, and Fe accumulates in the liver of Cu-deficient animals (Arredondo, Nunez 2005; Copper 2014). Anemia resulting from Cu deficiency may be linked to low ceruloplasmin activity, impairing the release of Fe from stores in the liver and reducing Fe delivery to the erythroid marrow, hence resulting in an Fe-restricted erythropoiesis (A.C. Ross et al. 2012). Indeed, Cu depletion reduces the activity of ceruloplasmin in humans, leading to hepatic Fe overload, and increasing the risk for oxidative damage and liver cirrhosis (Thackeray et al. 2011; Prohaska 2016). Thus, the anemia resulting from defective Fe mobilization due to decreased ceruloplasmin activity is unresponsive to Fe therapy but corrected by Cu supplementation (Harris, Klomp, Gitlin 1998). MCOs oxidize ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) which enables binding to transferrin (i.e., the main Fe carrier) in the blood, allowing Fe transport to sites of utilization (e.g., the bone marrow). One abundant MCO is ceruloplasmin, and carries more than 95% of the total Cu in healthy human plasma (Hellman, Gitlin 2002). This cuproenzyme is also involved in the acute-phase reaction of inflammation and in scavenging free radicals to protect cells against oxidative damage. Defense against oxidative damage, which the Scandinavian brown bear must deal with more significantly during hibernation, depends mainly on the Cu-containing superoxide dismutase (SOD) (Allen, Klevay 1994). SOD functions as an antioxidant by catalyzing the conversion of reactive oxygen species, such as the superoxide anion (O_2^-) and the hydroxyl radical ($\bullet\text{OH}$), to hydrogen peroxide (H_2O_2), which is subsequently reduced to water by other oxidant systems.

The effects of Cu deficiency, which is rare in humans, include anemia, hypopigmentation, connective tissue disorders, cardiovascular disease, osteoporosis and other bone defects (e.g., demineralization), fatty liver disease and abnormal lipid metabolism (e.g., hypercholesterolemia), ataxia, and an increased risk of infection due to neutropenia (Erdman, MacDonald, Zeisel 2012; A.C. Ross et al. 2012; Prohaska 2014). Cu depletion may occur due to intestinal defects, genetic conditions (e.g., Menkes disease) or high supplemental zinc intake (Copper 2014). It is however difficult to assess Cu status in humans because no definitive biomarker exists for detecting Cu deficiency. Animal studies show that Cu deficiency is associated with cardiac abnormalities and leads to changes in blood lipid levels, which

represents a risk factor for atherosclerotic cardiovascular disease (A.C. Ross et al. 2012). Another micromineral impacting Cu levels in the blood is zinc (Zn). Studies show that excess intake of supplemental Zn may result in Cu depletion. The exact mechanism is currently unknown but may involve the sequestration of Cu within intestinal mucosal cells thus limiting its bioavailability (Fischer, Giroux, L'Abbé 1981).

On the other hand, exposure to excessive Cu levels can result in liver damage and gastrointestinal symptoms (e.g., abdominal pain, cramps, vomiting, diarrhea, nausea; National Research Council (US) Committee on Copper in Drinking Water 2000; Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes and its Panel on Folate, Other B Vitamins, and Choline 1998). Cu toxicity is rare in healthy individuals who don't have any hereditary Cu homeostasis defect (such as Wilson disease), but has been reported in people who consume water containing high levels of Cu as a result of stagnant water in Cu-containing pipes or Cu alloys in water distribution systems that allow Cu to infiltrate the water (National Research Council (US) Committee on Copper in Drinking Water 2000; Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes and its Panel on Folate, Other B Vitamins, and Choline 1998). Environmental Cu contamination increases with human-induced activities such as mining activities, use of pesticides containing Cu, near industrial settings or waste disposals (Achour et al. 2009; Ahmed et al. 2018).

3.4.4 Iodine

Iodine (I) is classified as a halogen in chemistry and has an atomic number of 53 in the periodic table of elements (Annex 6; PubChem 2023). I is found in the environment as well as in biological systems as a micromineral. Most of the Earth's iodine is found in oceans in the form of iodide ion (I^-) and the content of iodine in soil varies depending on the region, which in turn affects the I content of crops. The older an exposed soil surface, the more likely the I has been washed away by erosion (Iodine 2014).

I is a micromineral making up the thyroid hormones thyroxine (T_4) and triiodothyronine (T_3), the former being the most abundant circulating thyroid hormone. I finds its way into the body through dietary intake and is quickly and almost entirely absorbed in the stomach and duodenum (Institute of Medicine (US) Panel on Micronutrients 2001; Zimmermann, Jooste, Pandav 2008). Iodine can be ingested in several chemical forms including sodium and potassium salts, inorganic iodine (I_2), iodate (IO_3^-) and iodide (I^-). Its excretion is mainly through urine (Office of Dietary Supplements - Iodine 2023). Iodine plays an important role in the normal growth, neurological development and metabolism of animals (Iodine 2014). In order to meet the body's demand for thyroid hormones, the thyroid gland traps I circulating in the bloodstream to incorporate it into the large glycoprotein thyroglobulin. Thereafter, thyroglobulin can be hydrolyzed by lysosomal enzymes, generating thyroid hormones that are stored and released into the circulation when needed. In the brain and the liver in particular, T_4 can be converted into T_3 by selenium-containing enzymes called iodothyronine deiodinases (DIOs), capable of catalyzing the deiodination (i.e., removal of iodine) from iodothyronines (Iodine 2014). More specifically, DIO1 and DIO2 are the enzymes responsible for the deiodination of thyroxine (T_4), generating the biologically active triiodothyronine (T_3). T_3 can bind to thyroid receptors in the nuclei of cells and regulate gene expression. As a result, thyroid hormones are able to regulate various physiological processes including growth,

development, metabolism and reproductive function (A.C. Ross et al. 2012). They represent a major physiological regulator in brain development in mammals.

The regulation of thyroid function is a complex process, involving the pituitary gland and the hypothalamus. The hypothalamus can secrete thyrotropin-releasing hormone (TRH), acting thereafter on the pituitary gland and generating the secretion of thyroid-stimulating hormone (TSH), which in turn stimulates I trapping, thyroid hormone synthesis and the release of T₄ and T₃ by the thyroid gland. When adequate circulating serum T₄ and T₃ concentrations are reached, a negative feedback system acting both on the hypothalamus and the pituitary gland allows the decrease in TRH and TSH production (Iodine 2014). If the blood T₄ concentrations were to decrease, TSH secretion would increase from the pituitary gland due to a low negative feedback, resulting in I trapping and increased production and release of both T₄ and T₃ by the thyroid gland into circulation.

Hypothalamic-pituitary-thyroid-axis

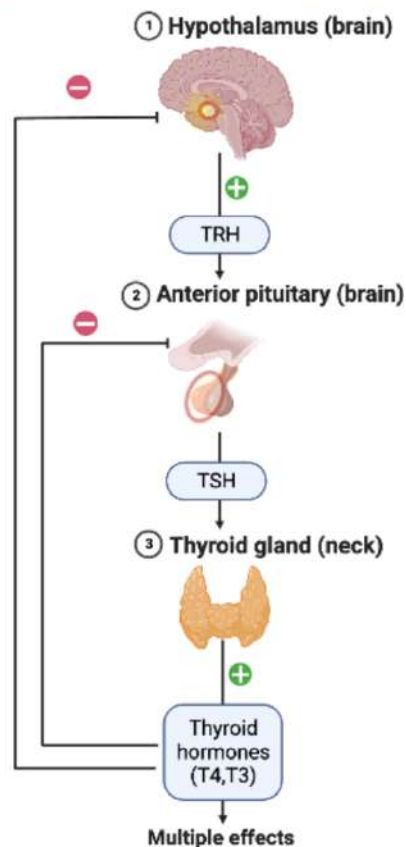


Figure 17: Hypothalamic-pituitary-thyroid-axis. "+" indicates increase secretion; "-" indicates the negative feedback. Source: <https://www.yourhormones.info/glands/thyroid-gland/>

Despite significant progress over the past years, Fe and I deficiencies remain major public health issues, affecting $\geq 30\%$ of the global population (Zimmermann 2006). More than 120 countries worldwide have introduced programs of salt fortification with I in order to correct I deficiency in the concerned populations (Iodine 2014). Daily consumption of salt fortified with I has been proven to be an effective strategy for the prevention of I deficiency disorders (Kapil 2007). Impaired thyroid function and thyroid hormone synthesis may disrupt the ability to thermoregulate correctly on exposure to cold, in particular in individuals exacerbating iron deficiency (Iron 2014). The thyroid gland of a healthy adult concentrates 70-80% of the total

body I content (Iodine 2014). Other organs capable of concentrating I include salivary glands, breast, choroid plexus, and gastric mucosa (Institute of Medicine (US) Panel on Micronutrients 2001). Insufficient I intake impairs the production of thyroid hormones, potentially leading to hypothyroidism, or other serious health problems such as thyroid gland enlargement (i.e., goiter) or cretinism (i.e., severe physical and mental retardation) (Iodine 2014). Moreover, I deficiency is a leading cause of preventable brain damage worldwide (Kapil 2007; Iodine 2014). In adults, hypothyroidism causes lethargy, hyporeflexia and poor motor coordination (Jaggy, Oliver 1994; Joffe, Sokolov 1994). Studies show that animals with polyneuropathy associated with primary hypothyroidism may present a wide range of clinical signs, including neurologic signs that can range from peripheral vestibular signs, lower motor neuronal deficits, and laryngeal paralysis, to megaesophagus (Jaggy, Oliver 1994). The most common clinical signs in canines are signs of a decreased metabolic rate (e.g., unexplained weight gain without polyphagia, obesity, lethargy, mental dullness, cold intolerance, lack of coordination) and dermatologic manifestations (loss or thinning of the fur, excess shedding or scaling, recurring ear and skin infections, thickening of skin, dark pigmentation of the skin) (Jaggy, Oliver 1994). Iodine deficiency results in inadequate production of T_4 and in response to its poor blood levels, the pituitary gland increases its output of TSH. But since the production of T_4 is impaired, its blood levels stay low, leading to persistently elevated TSH levels that could generate hypertrophy of the thyroid gland (i.e., goiter) (Vargatu 2016). It happens mainly because of poor dietary intake, which occurs when the soil is poor in I, causing a low concentration in food products (Kapil 2007). Iodine deficiency-induced hypothyroidism has multiple deleterious effects in all stages of development but is most damaging on the brain (Iodine 2014). With the aim of correcting such deficiencies, too much of a rapid increase in I intake can be deleterious and act in a harmful way in the opposite way, i.e. leading to iodine-induced hyperthyroidism. Maternal I deficiency during pregnancy has consequences on both mother and child, potentially leading to both maternal and fetal hypothyroidism, miscarriage, preterm birth, and neurological impairments in offspring (Iodine 2014). Because of their special needs during their life stages, pregnant women, lactating mothers and young infants are the most vulnerable to iodine deficiency (Iodine 2014). In addition, during pregnancy and lactation, the fetus and infant are completely reliant on the mother's intake of I for thyroid hormone synthesis. The most important biological role played by T_4 is in the early fetal stage, ensuring growth, differentiation and maturation of different organs (Kapil 2007). It is during fetal and early postnatal stages that the early development of the brain occurs in humans (Bernal, Nunez 1995; Bernal 2000). Insufficient intake of I lead to insufficient production of the thyroid hormones, which adversely affect the muscle, heart, liver, kidneys and the developing brain causing retarded maturation, intellectual deficits, and neurological impairments (Kapil 2007; Bernal 2000). Thyroid hormone deficiency during these critical transition periods may lead to irreversible brain damage, the consequences depending on the severity and duration of the deficiency and its time of onset (Escobar, Obregon, Rey 2004; Anderson, Schoonover, Jones 2003). In contrast, hyperthyroidism can cause hyperreflexia, irritability and anxiety among other symptoms.

I has a connection to other minerals and vitamins, including selenium, Fe and vitamin A. Concurrent deficiencies in selenium (Se), Fe or vitamin A may aggravate the effects of I deficiency (Iodine 2014). As mentioned above, Se-containing iodothyronine deiodinases (DIOs) are enzymes (or selenoenzymes) required for the conversion of T_4 to the biologically active thyroid hormone T_3 . Studies on mice reveal that DIO1 activity may also be involved in regulating I homeostasis (Schneider et al. 2006). Furthermore, glutathione peroxidases are selenoenzymes that protect the thyroid gland from hydrogen peroxide-induced damage

during thyroid hormone synthesis (Schomburg 2011). Fe deficiency anemia can impair thyroid metabolism (which will be described more in detail in the section below “3.4.5 Iron”) in the following ways: (1) by altering the TSH response of the pituitary gland; (2) by reducing the activity of heme-containing thyroid peroxidase that catalyzes the iodination of thyroglobulin for the production of thyroid hormones; and (3) in the liver by limiting the conversion of T_4 to T_3 , increasing T_3 turnover and decreasing T_3 binding to nuclear receptors. Correcting Fe deficiency anemia has been shown to improve the efficacy of I supplementation in humans to reduce thyroid disorders (Zimmermann 2006; Hess 2010; Iron 2014). In some countries in north and west Africa, vitamin A deficiency and I deficiency-induced goiter may coexist with up to 50% of children (Iodine 2014). Vitamin A status, like other nutritional factors such as Se and Fe, seems to influence the response to I supplementation programs in I-deficient populations (Zimmermann et al. 2004). Studies in animal models on vitamin A deficiency revealed interference with the pituitary-thyroid axis by (1) increasing the synthesis and secretion of TSH by the pituitary gland, (2) participating in the hypertrophy of the thyroid gland, (3) reducing I uptake by the thyroid gland and impairing the synthesis and iodination of thyroglobulin, and (4) increasing circulating concentrations of thyroid hormones (Zimmermann 2007).

3.4.5 Iron

Iron (Fe) is the fourth most abundant element of Earth’s crust after oxygen, silicon and aluminium, and one of the most studied micronutrients in nutrition science (Erdman, MacDonald, Zeisel 2012; Marriott et al. 2020). Fe is classified as a transition metal in chemistry and has an atomic number of 26 in the periodic table of elements (Annex 6; PubChem 2023).

Fe is a micromineral involved in blood oxygenation, as it constitutes blood cells, in charge of transporting O_2 and CO_2 . Fe is found in biological systems under two oxidation states, ferrous (Fe^{2+}) and ferric (Fe^{3+}) forms. It is a component in several enzymes and proteins involved in essential biological functions such as energy production, cell growth and replication, DNA synthesis and O_2 transport. For instance, hemoglobin, myoglobin, cytochromes and peroxidases require Fe-containing heme as a prosthetic group for their biological activities (Iron 2014). Heme is an Fe-containing compound found in several biologically important molecules (Annex 5). Heme Fe is provided by hemoglobin and myoglobin found in animal food sources and is much better absorbed than nonheme Fe found in plants and animal food sources. Some Fe-dependent proteins are heme-containing proteins, also called hemoproteins. A few examples of Fe-dependent proteins include hemoglobin, myoglobin and neuroglobin which are involved in O_2 transport and storage; cytochrome c oxidase which is involved in electron transfer, cytochrome P450 oxidase, myeloperoxidase, peroxidases, catalase, cyclooxygenase which are involved in oxidase activity; succinate dehydrogenase, NADH dehydrogenase, isocitrate dehydrogenase, ferredoxin-1 which are involved in energy production; DNA polymerases, DNA helicases which are involved in DNA replication and repair; tyrosine, tryptophan, phenylalanine and lysine hydroxylases which are enzymes requiring Fe as a cofactor for catalytic activities; ferritin, transferrin, haptoglobin, hemopexin and lactoferrin which are proteins responsible for Fe transport and storage (Iron 2014).

A total of 75% of total body Fe is functional and 25% is stored within cells as ferritin or hemosiderin. Hemoglobin is the primary protein found in red blood cells and represents about two thirds of the body’s Fe (Winter, Bazydlo, Harris 2014). Blood cells contain 3.5 mg of Fe per

gram of hemoglobin in humans (Iron 2014; Saljoughian 2007). Hemoglobin plays a vital role in transporting O₂ from the lungs to the tissues of the body. In addition to this, myoglobin operates in the transport and short-term storage of O₂ in muscle cells, providing an adequate amount of O₂ to meet the demands of working muscles (Erdman, MacDonald, Zeisel 2012). Inadequate oxygenation of body tissues (i.e., hypoxia) observed for example during a stroke, ischemia, or inflammatory disorders generates several compensatory physiological responses. These include erythropoiesis, angiogenesis and increased production of enzymes implicated in anaerobic metabolism (Iron 2014). Under hypoxic conditions, hypoxia-inducible factors (HIF) lead to increased synthesis of proteins involved in compensatory responses to hypoxia. Fe-dependent enzymes of the dioxygenase family, prolyl hydroxylases and asparaginyl hydroxylase are implicated in HIF regulation (Iron 2014). Another major role that Fe is involved in is DNA replication and repair. Ribonucleotide reductases (RNRs) are Fe-dependent enzymes that catalyze the synthesis of deoxyribonucleotides required for DNA replication and repair in response to DNA damage. Other enzymes crucial to these processes are DNA polymerases and DNA helicases, which are composed of Fe-S cluster proteins. The latter constitute small inorganic protein cofactors serving a variety of functions in biological systems.

Regarding energy metabolism, cytochromes (heme-containing enzymes) play a major role in mitochondrial electron transport required for cellular energy production (Iron 2014). During the synthesis of adenosine triphosphate (ATP), which is the primary energy storage compound in cells, cytochromes serve as electron carriers. For example, cytochrome P450 is involved in the detoxification and metabolism of drugs and pollutants, as well as in the metabolism of several biological molecules (e.g., steroids, prostaglandins, fatty acids, vitamin A, D and K) (Erdman, MacDonald, Zeisel 2012). Fe is implicated in antioxidant functions via catalases and peroxidases, which are heme-containing enzymes that protect cells against the accumulation of hydrogen peroxide (H₂O₂) by catalyzing a reaction converting H₂O₂ to H₂O and O₂. This micromineral is an important player in the immune system as well. Some white blood cells manage to kill bacteria by engulfing them and exposing them to reactive oxygen species (ROS). Hypochlorous acid (HClO) is an example of ROS, synthesized by neutrophils and produced by heme-containing enzyme myeloperoxidase catalysis (Erdman, MacDonald, Zeisel 2012). In the thyroid gland, heme-containing thyroid peroxidase catalyzes the iodination of thyroglobulin for the production of thyroid hormones. We therefore understand that Fe deficiency impacts thyroid metabolism (Iron 2014). Fe is also required for several additional vital functions including reproduction, growth, and healing.

Fe is an essential mineral but can potentially be toxic to the body because free Fe inside the cells can lead to the creation of free radicals, thereafter causing oxidative stress and cellular damage (Iron 2014). Fe homeostasis is therefore crucial and requires rigorous regulation. Inadequate dietary Fe intake, acute and chronic inflammatory conditions and obesity are known to be associated with alterations in Fe homeostasis (Pusatcioglu et al. 2012). Hepcidin, a Fe regulatory hormone, plays a key role in the regulation of Fe metabolism. This liver-derived peptide that is primarily synthesized and secreted by liver cells is described as the key modulator of cellular Fe export (Anderson et al. 2007). Circulating hepcidin reduces Fe export into the plasma by binding to the iron-efflux protein ferroportin-1 on the surface of enterocytes, macrophages and other cell types causing its internalization and even degradation (Anderson et al. 2007). Ferroportin-1 regulates the release into plasma of Fe from cells such as enterocytes, hepatocytes and Fe-recycling macrophages (Fleming 2008). When Fe concentration in blood is low and in situations of Fe-deficiency anemia, hepcidin expression is minimal, to maximize Fe absorption from the diet and Fe mobilization from body stores. On

the other hand, in excess Fe levels situations, hepcidin inhibits dietary Fe absorption, boosts cellular Fe sequestration and reduces Fe bioavailability. Heparin is up-regulated in conditions of endoplasmic reticulum stress and inflammation, and down-regulated in situations of hypoxia and anemia (Pusatcioglu et al. 2012). Indeed, most infectious agents require Fe to grow and spread, just like the infected host in order to build up an effective immune response. Sufficient Fe is crucial for the differentiation and proliferation of T lymphocytes and the formation of ROS required for killing pathogens (Bhaskaram 2001). Should infection or inflammation occur, up-regulation of hepcidin is being set in motion leading to a decrease in serum Fe concentrations and an increase in serum ferritin (Fe storage protein) concentrations.

As stated previously (in “3.4.3 Copper”), the metabolism of Fe and Cu are intimately related. But Cu isn’t the only mineral that has a connection to Fe: zinc (Zn), calcium (Ca) and iodine (I) are also closely linked. For instance, Zn is essential to maintain adequate erythropoiesis. When Zn deficiency coexists with Fe deficiency, Fe-deficiency anemia may appear (Kelkitli et al. 2016). Indeed, Zn is the co-factor of several enzymes and plays a role in Fe metabolism. Ca has been known to impair Fe absorption in patients taking Fe and Ca supplements or ingesting Fe- and Ca-rich food. Studies show that Ca decreases Fe absorption from both nonheme (i.e., most Fe supplements and food sources other than red meat, poultry and seafood) and heme sources (Hurrell, Egli 2010). It is therefore recommended for individuals taking Fe as well as Ca supplements to take them two hours apart (Iron 2014). Iodine (I) is also intertwined with Fe in the sense that Fe-deficiency anemia can impair thyroid metabolism in the following ways: (1) by altering the TSH response of the pituitary gland; (2) by reducing the activity of thyroid peroxidase that catalyzes the iodination of thyroglobulin for the production of thyroid hormones; and (3) in the liver by limiting the conversion of T_4 to T_3 , increasing T_3 turnover and decreasing T_3 binding to nuclear receptors. Therefore, correcting Fe-deficiency anemia has been shown to improve the efficiency of I supplementation in humans to attenuate thyroid disorders (Zimmermann 2006; Hess 2010).

Fe deficiency is the most common nutritional deficiency worldwide and is associated with serious health risks (Iron 2014). Amongst other disorders, it leads to microcytic anemia due to impairments in hemoglobin synthesis and red blood cell formation (Iron 2014). Fe deficiency in children has been associated with poor cognitive development and abnormal behavior patterns (Iron 2014). In humans, Fe deficiency is a common comorbidity in patients with heart failure, with a prevalence reaching up to 59% (Alnuwaysir et al. 2021). Most of the symptoms or Fe deficiency are a result of the associated anemia, such as fatigue, accelerated HR, palpitations, and rapid breathing during exercise. Indeed, the reduced hemoglobin content of red blood cells results in decreased O_2 delivery to active tissues of the body, as well as decreased myoglobin levels in muscle cells therefore limiting the amount of O_2 that can be delivered to mitochondria for oxidative metabolism (Iron 2014). Furthermore, impaired thyroid function and thyroid hormone synthesis may disrupt the ability to thermoregulate properly on exposure to cold in Fe-deficient individuals. If Fe deficiency should occur during early childhood, impairment in psychomotor development and short- and long-term behavioral and cognitive alterations have been reported (Jáuregui-Lobera 2014). In addition, pica (i.e., a behavioral disturbance characterized by the action of ingesting non-nutritive substances, which is repeated for a period of at least a month and is developmentally inappropriate) may be a symptom and even a cause of Fe deficiency (Ganesan, Vasauskas 2023; Thomas, Falko, Zuckerman 1976).

On the other hand, excessive Fe levels in the body causing toxic Fe deposition in vital organs can occur in individuals affected by hereditary hemochromatosis, which is a genetic disorder that causes the body to absorb too much Fe from the diet (Iron 2014). These Fe depositions in organs have been associated with numerous chronic conditions, such as liver cancer and type II diabetes mellitus (Iron 2014). Organs suffering from Fe excess include the heart, liver, skin, pancreas, bones, joints, and pituitary gland (Winter, Bazydlo, Harris 2014). Tight regulation of Fe is needed because excessive Fe levels are highly toxic and humans can only excrete small amounts through the skin, sweat, sloughing of enterocytes, menstrual and blood loss (Pusatcioglu et al. 2012). Since the human body lacks efficient functions in excreting excessive Fe, it can be stored in the form of ferritin and hemosiderin to minimize the toxicity of Fe (Saito et al. 2012). Fe recycling systems are well-developed, with up to 35 mg being recycled daily, of which about 20 mg or Fe can be recovered daily from heme recycling originating from senescent red blood cells engulfed by macrophages in the spleen. The Fe recovered from this heme recycling is either deposited to the ferritin of spleen macrophages or exported to ferroportin-1 to transferrin (which happens to be the main Fe carrier in the blood) that delivers Fe to other tissues (Iron 2014; Erdman, MacDonald, Zeisel 2012).

3.4.6 Magnesium

Magnesium (Mg) is classified as an alkaline earth metal in chemistry and has an atomic number of 12 in the periodic table of elements (Annex 6; PubChem 2023). Magnesium is a macromineral closely related to calcium and phosphorus and is mostly found in bone structures (Cherian 2019; Farag et al. 2023). About 50 to 60% of all the Mg in the body is found in bones, the remainder is found inside cells and in soft tissues (primarily muscle) (Simonsen, Harbak, Bennekou 2012) (A.C. Ross et al. 2012). It contributes to the structure of bones and plays a key role in DNA and RNA synthesis. Less than 1% of total Mg is found in blood serum (Magnesium 2014). Mg is a cofactor for hundreds of enzymes inside the body. It acts on the regulation of diverse biochemical reactions in the body and is involved in more than 300 metabolic pathways, including nucleic acid and protein synthesis, Fe transport, cell signaling, muscle and nerve function, blood pressure regulation, inflammation management, and structural functions. Mg is also required for energy production, oxidative phosphorylation and glycolysis (Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes 1997; Coates et al. 2015; A. Catharine Ross et al. 2012). In the intracellular fluid, Mg is a cation involved in the metabolism of carbohydrates and proteins (Cherian 2019). Mg is linked with other macrominerals in that it plays an important part in the active transport of calcium and potassium ions across cell membranes, a process necessary for the proper functioning of nerve impulse conduction, muscle contraction and normal heart rhythm (A. Catharine Ross et al. 2012). Dietary Mg is mostly absorbed in the ileum (Cherian 2019).

Mg deficiency can impair PTH secretion by the parathyroid glands, and lessen the responsiveness of osteoclasts to PTH, therefore disrupting vitamin D and Ca homeostasis. Some individuals are more sensitive to Mg deficiency, in particular those with gastrointestinal or renal disorders, and older people (Magnesium 2014). Insufficient dietary intake and/or low serum concentrations in Mg have been reported to have increased risk of developing cardiovascular disease, osteoporosis, metabolic disorders (e.g., hypotension, insulin resistance resulting in type II diabetes mellitus, lack of energy), and muscle cramps. Early signs of Mg deficiency include loss of appetite, nausea, vomiting and weakness. Severe Mg

deficiency can lead to hypocalcemia and hypokalemia because of the disruption of mineral homeostasis (Coates et al. 2015). Grass tetany is the most common Mg deficiency in grazing animals, which can occur in the spring season when rapidly growing grass has a slow plant intake of Mg from the soil (Cherian 2019). It is characterized by uncoordinated gait (staggering), convulsions, extreme sensitivity to noise and touch, coma, and even death (Cherian 2019; Filley 2018). Mg homeostasis is largely controlled by the kidneys, and when Mg concentrations in the bloodstream are too low, urinary excretion is reduced (Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes 1997).

3.4.7 Manganese

Manganese (Mn) is classified as a transition metal in chemistry and has an atomic number of 25 in the periodic table of elements (Annex 6; PubChem 2023). It is the 12th most abundant element and fifth most abundant metal in Earth's crust (Professional Paper 2017; Williams et al. 2012).

Mn is a micromineral partly involved in the functioning of enzymes related to the Krebs cycle. It is also involved in the formation of bone and amino acids, lipid (e.g., cholesterol) and carbohydrate metabolism via manganese-activated enzymes (Micronutrients 2001; Manganese 2014; Institute of Medicine (US) Panel on Micronutrients 2001). Manganese metalloenzymes include arginase, glutamine synthetase, phosphoenolpyruvate decarboxylase, and manganese superoxide dismutase (MnSOD). Glycosyltransferases and xylosyltransferases which are important in proteoglycan synthesis and thus bone formation, are sensitive to Mn concentrations in animals (Institute of Medicine (US) Panel on Micronutrients 2001). The Mn-containing enzyme pyruvate carboxylase and the Mn-activated enzyme phosphoenolpyruvate carboxykinase (PEPCK) are two actors critical in gluconeogenesis (i.e., the production of glucose from non-carbohydrate precursors) (Manganese 2014). Another example of an important Mn-containing enzyme is the arginase, required by the liver for the urea cycle, where the ammonia generated is detoxified during amino acid metabolism (O'Dell, Sunde 1997). In the brain, the Mn-activated enzyme glutamine synthetase converts the amino acid glutamate to glutamine. Glutamate is an excitotoxic neurotransmitter and a precursor to an inhibitory neurotransmitter, γ -amino butyric acid (GABA) (Albrecht et al. 2007; Avila, Puntel, Aschner 2013). MnSOD is the principal antioxidant enzyme in the mitochondria. Mitochondria are very vulnerable to oxidative stress since they consume over 90% of the oxygen used by cells (Manganese 2014). The superoxide radical ($O_2^{\cdot -}$) is one of the reactive oxygen species produced during ATP synthesis in the mitochondria. MnSOD catalyzes the conversion of $O_2^{\cdot -}$ to H_2O_2 which can subsequently be converted to H_2O and O_2 by other antioxidant enzymes (O'Dell, Sunde 1997).

Mn intakes in humans come from dietary intake via ingestion, or through inhalation. Only a small amount of dietary Mn is absorbed by the body. It is then very rapidly excreted into the gut through bile and thereafter the feces (Britton, Cotzias 1966). Mn is taken from the blood by the liver and transported to extrahepatic tissues by transferrin, which is identified as the major Mn-binding protein in plasma (Davidsson et al. 1989) and possibly α_2 -macroglobulin (Rabin et al. 1993) and albumin (Davis, Wolf, Greger 1992). Mn is a nutritionally essential mineral but can potentially be toxic in high levels in the body, which needs to tightly regulate Mn homeostasis. There is a potential risk for Mn toxicity when bile excretion is low (e.g.,

difficulties in maintaining Mn homeostasis in neonates, cholestatic liver disease). Furthermore, Mn exposure may start before birth, coming from maternal exposure through inhalation and/or ingestion of food items from environmental pollution (Milatovic et al. 2011). Mn toxicity is mostly associated with a neurological disorder called manganism. The symptoms include irritability, aggressiveness, hallucinations, tremors, motor difficulties, and facial muscle spasm. In humans, children exposed to high levels of Mn in drinking water for example may develop several detrimental effects, particularly behavioral problems and in their ability to learn and remember (Milatovic et al. 2011).

Mn deficiency results in abnormal skeletal development in a large amount of animal species. Studies conducted in humans show decreased plasma Mn concentrations in osteoporotic women. The formation of healthy bone and cartilage requires proteoglycans that are synthesized by glycosyltransferases, which are enzymes using Mn as a preferred cofactor (Erdman, MacDonald, Zeisel 2012; Ziegler, Filer 1996). Wound cicatrization is a complex process that requires an increased production of collagen. Mn plays a key role for the activation of prolylase, an enzyme that provides the amino acid proline, for collagen formation in human skin cells (Muszyńska, Pałka, Gorodkiewicz 2000). Prolylase deficiency is a known genetic disorder, resulting in abnormal wound healing, among other problems, characterized by abnormal Mn metabolism (Ziegler, Filer 1996). Glycosaminoglycan synthesis, which requires Mn-activated glycosyltransferases, may also have an important role in wound healing. Other signs of Mn deficiency expressed by animals include impaired growth, reproductive function, glucose tolerance and alterations in carbohydrate and lipid metabolism (Institute of Medicine (US) Panel on Micronutrients 2001).

Mn interacts with other elements in the body such as Fe, Ca and Mg. For starters, Fe and Mn share common absorption and transport proteins including the divalent metal transport 1, the lactoferrin receptor, transferrin and ferroportin (Ye et al. 2017). Therefore Mn absorption from dietary intake decreases as the meal's Fe content increases (Ziegler, Filer 1996). Studies conducted on Mn absorption, retention, balance and status and Fe status show a significant negative association between Mn intestinal absorption and plasma ferritin concentrations. Intestinal absorption of Mn is increased during Fe deficiency and decreased when Fe stores are elevated (i.e., high serum ferritin concentrations; Finley 1999). Fe deficiency also increases the risk of Mn accumulation in the brain (Aschner, Dorman 2006). Fe status impacts not only Mn intestinal absorption but also its biological half-life. Furthermore, daily Fe supplementation has been associated with decreased blood Mn concentrations and decreased MnSOD activity in leukocytes (Davis, Greger 1992). In contrast, low ferritin concentrations are associated with increased Mn absorption (Finley 1999). In humans, men have been known to absorb less Mn than women, which may be related to the fact that men usually have higher Fe stores than women (Finley, Johnson, Johnson 1995). Other elements such as Ca, Fe, P and Mg have been found to affect Mn retention (Freeland-Graves, Lin 1991; Greger 1998; Lutz, Schroff, Scharrer 1993). Mg supplementation has been shown to slightly decrease Mn bioavailability in healthy adults, either by increasing its excretion or by decreasing its intestinal absorption (Manganese 2014).

3.4.8 Phosphorus

Phosphorus (P) is classified as a nonmetal in chemistry and has an atomic number of 15 in the periodic table of elements (Annex 6; PubChem 2023). Similarly to Ca and Mg, the majority of

phosphorus (about 85%) is contained in bones and teeth (The roles of calcium and phosphorus 2014; Office of Dietary Supplements - Phosphorus). The remaining 15% is distributed throughout soft tissues, with only 0.1% in the extracellular fluids (Favus, Bushinsky, Lemann 2006). As the elemental phosphorus is only present as part of organic and inorganic compounds, and not in a “free” form in biological systems, it will be referred to as phosphate and abbreviated PO_4 . PO_4 is also a structural component in DNA and RNA, responsible for the storage and transmission of genetic information (Erdman, MacDonald, Zeisel 2012). Its presence in cell membranes as phospholipids (e.g., phosphatidylcholine) plays a major role, as well as its provision of energy at a cellular level in the form of ATP. Many sugars and proteins are phosphorylated in the body, as well as enzymes, hormones and cell-signaling molecules which depend on phosphorylation for their activation (Office of Dietary Supplements - Phosphorus 2023). In addition, PO_4 plays an important role in the regulation of the activation of enzymes, gene transcription, maintenance of normal pH in extracellular fluid, and intracellular energy storage and signaling. Furthermore, the phosphorus-containing molecule 2,3-diphosphoglycerate binds itself to hemoglobin in red blood cells, participating in the regulation of oxygen delivery to the tissues of the body (A.C. Ross et al. 2012). PO_4 is mainly absorbed passively in the small intestine, although some absorption is done by active transport (Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes 1997). Dietary phosphorus deficiency is quite uncommon, with symptoms including loss of appetite, muscle weakness, bone fragility, numbness in the extremities and rickets in children (characterized by soft and deformed bones in growing children, as a result of impaired incorporation of Ca and PO_4 into the skeleton) (Office of Dietary Supplements - Phosphorus 2023).

PO_4 and Ca are interrelated in the sense that both their metabolism is regulated by calcitriol and PTH for example. Like Ca, the kidneys, bones and intestines regulate PO_4 homeostasis (Office of Dietary Supplements - Phosphorus 2023). The serum PO_4 concentration is thus determined by the balance between intestinal absorption of PO_4 from the diet, storage in the skeleton, and excretion through urine. The reabsorption of PO_4 from urine in the kidneys takes place in the renal proximal tubules, through sodium-phosphate cotransporters, which play a key role in serum PO_4 homeostasis. Renal PO_4 reabsorption is controlled by the PTH and fibroblast growth factor 23 (FGF23) (Calvo, Sherman, Uribarri 2019). PTH acts on Ca and PO_4 but with an opposite outcome. When a drop in serum Ca levels is detected, PTH is secreted by the parathyroid glands, resulting in a decrease in the urinary Ca excretion but an increase in urinary PO_4 excretion. Other actions participating in restoring serum Ca concentrations is the release of bone mineral (both Ca and PO_4) and the stimulation of intestinal absorption of both Ca and PO_4 through the medium of circulating calcitriol. Thus, even though Ca and PO_4 have homeostasis-regulation hormones in common, the PO_4 balance is maintained neutral through phosphaturic effects in the event of hypocalcemia. FGF23 plays a central role in PO_4 homeostasis at several levels. This hormone is secreted by bone-forming cells (osteoblasts) in response to an increase in PO_4 intake. Through a negative feedback loop, FGF23 inhibits the production and stimulates the degradation of calcitriol, and increases urinary PO_4 excretion (independently of PTH and calcitriol). In addition, as previously stated, the main structural compound in bones and teeth is hydroxyapatite (HA) crystals, which are phosphocalcic compounds (Trautvetter et al. 2018). Several other hormones also appear to contribute to PO_4 homeostasis, such as estrogen, adrenaline (Office of Dietary Supplements - Phosphorus 2023), insulin, and hormones of the pituitary axis. When the kidney function declines resulting in chronic kidney disease for example, PO_4 excretion is impaired, leading to hyperphosphatemia (Calvo, Sherman, Uribarri 2019).

3.4.9 Selenium

Selenium (Se) is classified as a nonmetal in chemistry and has an atomic number of 34 in the periodic table of elements (Annex 6; PubChem 2023). Se is a micromineral, essential for various functions in the body in small amounts, but like most essential minerals, it can be toxic at high levels, and therefore requires careful homeostasis. Se intake varies according to geographic location. The levels and chemical forms of Se in plant-based food vary according to soil composition, Se content in the soil, agricultural practices, plant species and pollution. The major forms of Se in the diet are highly bioavailable. Most Se in animal tissues is present as selenomethionine or selenocysteine. The latter is the form of Se that accounts for the biological activity of the element (Compounds 2000). The liver represents a major storage site for Se (Selenium 2014) and the excretory metabolites appear primarily in the urine. The known biological functions of Se include reproductive success (i.e., spermatogenesis, male fertility), defense against oxidative stress, regulation of thyroid hormones, and regulation of the redox status of vitamin C and other molecules (Compounds 2000). Se acts in synergy with the antioxidant vitamins, vitamin C and vitamin E by regenerating them from their oxidized forms and promoting maximal antioxidant protection (Murer et al. 2014; May et al. 1997; Li et al. 2001). One of the most essential roles of Se to biological systems, and specifically to the cellular redox balance, resides from its presence as selenocysteine in the catalytic site of selenoproteins. Other minerals that are critical components of antioxidant enzymes include Cu (as superoxide dismutase), Zn (as superoxide dismutase) and Fe (as catalase). Most animals require Se for the appropriate functioning of several Se-dependent enzymes, called selenoproteins. Se exerts various biological functions mainly as part of the amino acid selenocysteine which is found in at least 25 selenocysteine-containing proteins. In humans, 25 genes code for selenocysteine-containing proteins (Mariotti et al. 2012). In animals, 14 selenoproteins have been characterized to date (Hofstee, Cuffe, Perkins 2020; Compounds 2000). Some well characterized selenoproteins, of which some are oxidant defense enzymes, include glutathione peroxidases, thioredoxin reductases, iodothyronine deiodinases and methionine sulfoxide reductase B1 (Selenium 2014). During protein synthesis, the amino acid selenocysteine is incorporated into elongation proteins at specific locations in the amino acid sequence in order to form functional selenoproteins. For example, Se is a component of the enzymes glutathione peroxidases, which functions are to protect tissues and membranes against oxidative stress. More specifically, Se-dependent glutathione peroxidases designated as GPx1-4 and GPx6 defend against oxidative stress (Flohé 1988; Compounds 2000). These GPx isoenzymes reduce potentially damaging ROS such as H₂O₂ and lipid hydroperoxides, to products like water and alcohols. More specifically, GPx4 plays a role in spermatogenesis and male fertility, along another plasma protein called selenoprotein P (SEPP1), which is produced by the liver and secreted in the plasma (Compounds 2000). GPx4 reduces phospholipid hydroperoxides, thus protecting immature spermatozoa cells against oxidative stress in the testicles, where it is also involved in sperm motility. Studies conducted on SEPP1 show its crucial role in supplying Se to the testes by conducting experiments on animal models lacking the SEPP1 gene, resulting in infertility due to poor Se tissue bioavailability, defective GPx4 synthesis and impaired sperm maturation (Boitani, Puglisi 2008). SEPP1 constitutes the major form of Se transport to peripheral tissues and appears to be especially crucial for Se homeostasis in the brain and testes (Hill et al. 2012). Furthermore, SEPP1 is involved in the regulation of glucose metabolism and insulin sensitivity (Misu et al. 2010). In mammals, three selenocysteine-containing thioredoxin reductase (TrxR) isoenzymes have been identified in

the thioredoxin system (cytosolic TrxR1, mitochondrial TrxR3, and testes-specific thioredoxin glutathione reductase TGR). TrxRs catalyze the reduction of a wide range of substrates in different cellular compartments and serve as electron donors for the regeneration of small antioxidants, possibly recycling ascorbic acid (Vitamin C), α -lipoic acid, α -tocopherol (vitamin E) and coenzyme Q₁₀ from their oxidized forms (Arnér 2009). TrxR1 is regarded as one of the major antioxidant enzymes and redox regulators in mammalian cells (Selenium 2014). Three Se-dependent iodothyronine deiodinases regulate thyroid hormone metabolism (Berry, Larsen 1992). The thyroid gland releases very small amounts of biologically active thyroid hormone T₃ and larger amounts of the inactive form T₄. Most of the T₃ hormones in the circulation and inside cells is generated by a reaction involving the removal of one iodine atom from T₄, in a reaction catalyzed by Se-dependent iodothyronine deiodinases enzymes (DIO1 and DIO2) (Annex 7; Selenium 2014). Some studies suggest that high serum Se concentrations may have adverse effects on glycemic control. Individuals with high Se status and/or individuals at risk for type II diabetes mellitus should avoid taking Se supplements (Laclaustra et al. 2009).

Se deficiency impairs thyroid hormone metabolism by inhibiting the synthesis and activity of the iodothyronine deiodinases, which convert T₄ to the metabolically active form T₃ (Arthur, Nicol, Beckett 1992; Berry, Larsen 1992). However, even when severe, isolated Se deficiency does not usually result in obvious clinical illness (Selenium 2014). Too low concentrations of Se in an animal's diet can result in white muscle disease, which is a nutritional myopathy, found in various animal species (e.g., horses, sheep, goats, cattle; Delesalle et al. 2017). Scandinavia, in particular south-central Sweden, has a Se-poor soil (Alexander, Alehagen, Aaseth 2020; Alloway 2013). On the other hand, excess of Se intake can occur in animals grazing or feeding on plants or forage with high Se contents.

Over the years, there has been lots of research on the effect of Se supplementation on the incidence of cancer in animals. In fact, more than two thirds of over 100 published papers in 20 different animal models of spontaneous, viral and chemically induced cancers reveal that Se supplementation significantly reduces tumor incidence (Combs, Gray 1998).

3.4.10 Sodium

Sodium (Na) is classified as an alkali metal in chemistry and has an atomic number of 11 in the periodic table of elements (Annex 6; PubChem 2023). 40% of total body Na is found in bones and 60% in the fluid inside and outside of cells (Erdman, MacDonald, Zeisel 2012). Na when combined with chloride (NaCl), regulates the amount of water at the cellular level and thus involved in almost all organic processes. Na and chloride (Cl) are the principal electrolytes (i.e., minerals that the body requires in relatively large amounts) of the extracellular fluid compartment in biological systems. Extracellular compartment includes blood plasma, interstitial fluid (i.e., fluid between cells) and transcellular fluid (e.g., joint fluid, cerebrospinal fluid). While potassium (K⁺) is the principal cation inside of cells, Na⁺ is the principal cation in extracellular fluid, with concentrations 10 times lower inside than outside cells (Sodium (Chloride) 2014). Hence, K⁺ diffuses easily out of the cells whereas Na⁺ diffuses easily into the cells. Na is therefore a determining factor for the extracellular fluid osmolality, and a key element in many processes of secretion in the body (Bigiani 2020). The concentration differences between K⁺ and Na⁺ across cell membranes create an electrochemical gradient, known as the membrane potential. A cell's membrane potential is maintained by ion pumps

located in the cell membrane, especially Na^+/K^+ ATPase pumps. As their name suggests, these pumps use ATP to pump Na^+ out of the cell in exchange for K^+ (Figure 18). Magnesium (Mg^{2+}) and phosphate are also implicated in the functioning of these pumps. Large proportions of energy are dedicated to maintaining Na^+/K^+ concentration gradients, emphasizing on its importance in sustaining life. In addition, tight control of cell membrane potential is critical for nerve impulse transmission, muscle contraction and cardiac function (Clausen 2013; Larsen, Stoica, MacAulay 2016; Shattock et al. 2015).

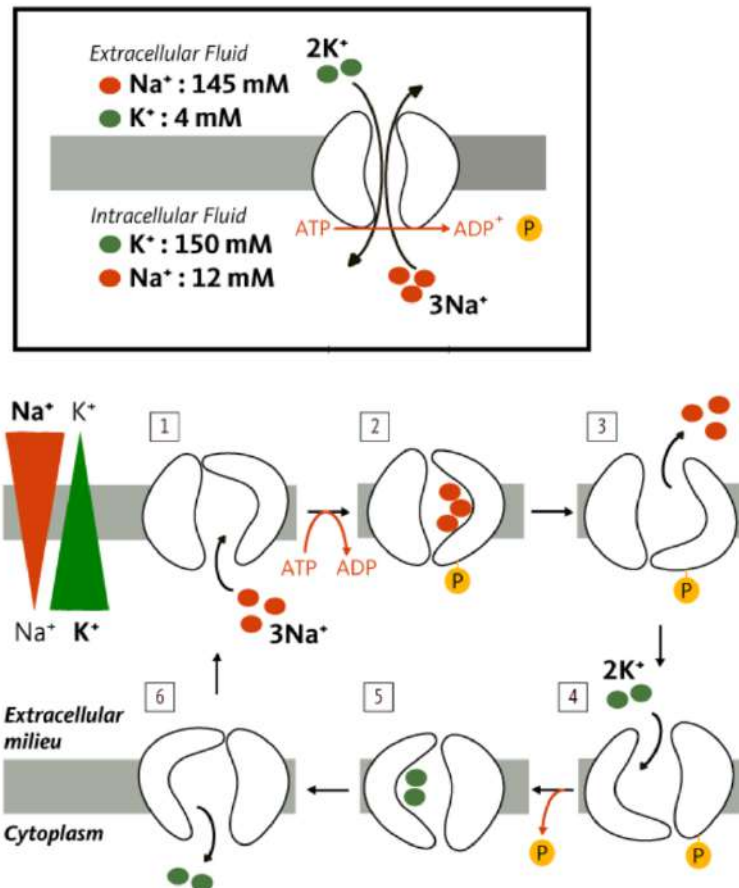


Figure 18: Simplified model of the Na^+/K^+ ATPase pump and the membrane potential. Source: Oregon state University website <https://lpi.oregonstate.edu/mic/minerals/sodium>

Na^+ is involved in a plethora of physiological processes, including the generation of electrical impulses in neurons and muscle cells and therefore nerve impulse transmission, muscle contraction and heart contraction, regulation of blood pressure and blood volume. Na homeostasis is therefore primordial, involving multiple mechanisms, including urinary excretion and its tight regulation to ensure that the amount lost via renal excretion compensates for the amount of Na consumed (Sodium (Chloride) 2014).

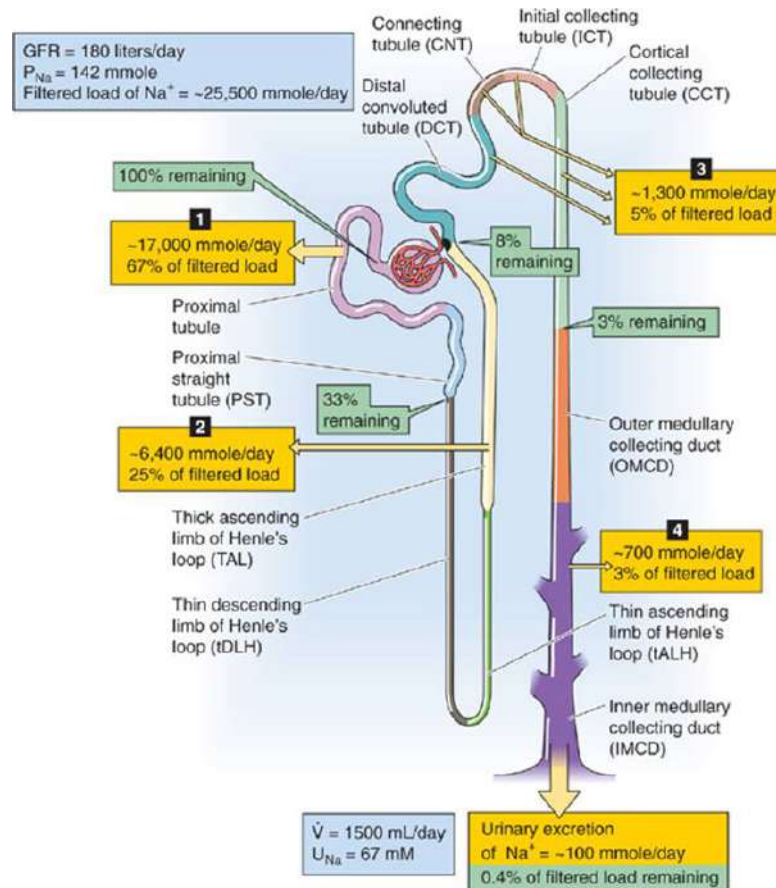


Figure 19: Segmental distribution of sodium reabsorption along the nephron. Source: <https://doctorlib.info/physiology/medical-physiology-molecular/36.html>

The proximal tubule of the nephron absorbs about two thirds of filtered Na^+ , without dissociating salt and water absorption. The thick ascending limb absorbs 25% of filtered Na^+ , but no water. Losses of Na through the kidneys, gastrointestinal tract and sweating have to be balanced by the ingestion of foods containing this mineral (Bigiani 2020). Usually we consume Na in the form of NaCl, i.e. the common table salt. Little Na occurs naturally in food, instead it is added to maintain or increase food shelf life. The lowest salt intakes are associated with diets of unprocessed foods, especially fruits and vegetables. In human adults, the National Academy of Medicine established an adequate intake for Na of 1.5 g/day, equivalent to 3.8 g/day of NaCl. Absorption of Na in the small intestine plays a key role in the absorption of other elements, such as Cl, amino acids, glucose and water (Sodium (Chloride) 2014). Disturbances in Na concentrations in the extracellular fluid are associated with disorders of water balance. Because Na is the primary determinant of extracellular fluid volume, including blood volume, various physiological mechanisms in charge of regulating blood volume and blood pressure work by adjusting the body's Na concentrations. Pressure receptors in the circulatory system (i.e., baroreceptors) sense changes in blood pressure, thereafter sending excitatory or inhibitory signals to the nervous system and/or endocrine glands to affect the regulation of Na by the kidneys (Sodium (Chloride) 2014). Generally, Na retention also results in water retention and Na loss results in water loss (Bailey, Sands, Franch 2012). Mechanisms contributing to a large multifactorial homeostatic control system governing blood volume and blood pressure through regulation of Na balance include the renin-angiotensin-aldosterone system (RAAS), anti-diuretic hormone (ADH) and the dopaminergic system (Sodium (Chloride) 2014).

In cases of serious blood loss, dehydration, or other significant processes inducing a significant decrease in blood volume or pressure, the kidneys release renin into the circulation. Renin is an enzyme that splits a large protein called angiotensinogen into a small peptide called angiotensin I (Sodium (Chloride) 2014), which is itself split into a smaller peptide, angiotensin II by the angiotensin converting enzyme (ACE). ACE is present on the inner surface of blood vessels and in the lungs, liver and kidneys. Angiotensin II stimulates the constriction of small arteries, resulting thereafter in an increase in blood pressure. It is also a stimulator of aldosterone synthesis by the adrenal glands. Aldosterone, a steroid hormone, acts on the kidneys to increase the reabsorption of Na and the excretion of K. This leads to increased Na retention and water retention, resulting in increased blood volume and blood pressure (A.C. Ross et al. 2012).

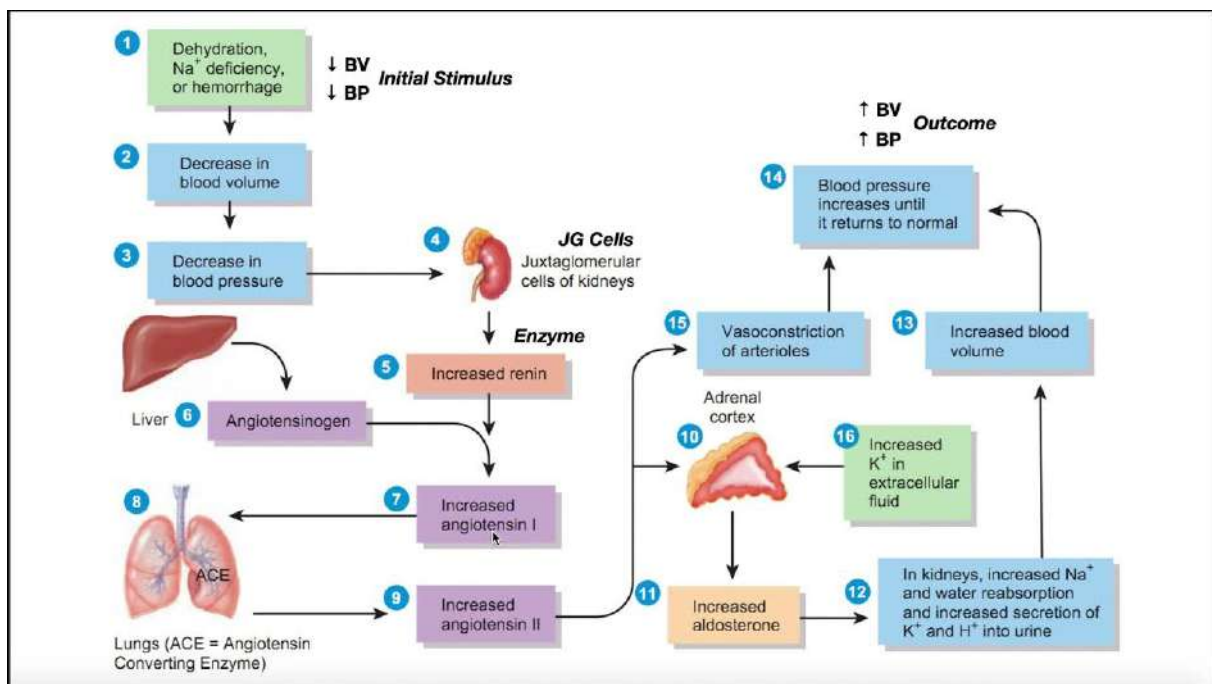


Figure 20: Simplified model of Renin-angiotensin-aldosterone system. Source: https://www.youtube.com/watch?app=desktop&v=_S59qSuosgE

A significant decrease in blood volume or pressure also stimulates the secretion of an anti-diuretic hormone (ADH, also known as arginine vasopressin) by the posterior pituitary gland (Sodium (Chloride) 2014). Alongside the RAAS, ADH stimulates epithelial Na channels in apical cell membranes along kidney nephron distal tubules in order to increase Na and water reabsorption. With its antidiuretic and antinatriuretic properties, it allows the kidneys to increase body water retention predominantly by increasing the cell surface expression of aquaporin water channels in the collecting duct as well as increasing the osmotic driving forces for water reabsorption (Kortenoeven et al. 2015).

The dopaminergic system plays an important role in Na homeostasis. In addition to its role as an essential neurotransmitter, dopamine serves important physiologic functions in organs such as the kidney. The main source of renal dopamine comes from the decarboxylation of levodopa, also known as L-DOPA or L-3,4-dihydroxyphenylalanine from plasma (Daubner, Le, Wang 2010). L-DOPA is taken up by the renal tubules from circulation (i.e., glomerular

filtration) and is converted into dopamine by aromatic amino acid decarboxylase (AADC) (Zhang et al. 2011). AADC activity is higher in the proximal tubules of the nephron, which is where the conversion from L-DOPA to dopamine mainly occurs. Dopamine then binds to the various dopamine receptors (Goldberg 1977) distributed along the proximal tubules and the thick ascending limbs of the loop of Henle to regulate Na transport. Dopamine promotes Na excretion (i.e., natriuresis) by inhibiting the Na⁺/H⁺ exchanger and Na⁺/phosphate co-transporter in apical cell membranes, and the Na⁺/bicarbonate co-transporter and Na⁺/K⁺ ATPase in basolateral cell membranes (Sodium (Chloride) 2014). The inhibitory effect of dopamine on Na⁺/K⁺ ATPase is enhanced by the natriuretic hormone, atrial natriuretic peptide (ANP), which is secreted by heart muscle cells into circulation (Rayner, Ramesar 2015).

Hyponatremia (i.e., abnormally low Na concentrations in blood) is a common disturbance in the electrolyte balance among older adults and individuals with hypertension, kidney disease and heart disease (Sodium (Chloride) 2014; Mohan et al. 2013). Hyponatremia may result from increased fluid retention (i.e., dilutional hyponatremia, due to inappropriate ADH secretion associated with CNS disorders or use of certain drugs, or excessive water intake for example) or increased Na loss (e.g., severe or prolonged vomiting or diarrhea, excessive and persistent sweating, the use of some diuretics (e.g., furosemide), some forms of kidney disease, etc.). Inadequate Na intakes rarely result in hyponatremia because the kidneys increase the excretion of water in order to maintain serum osmolality (i.e., electrolyte-water balance). Symptoms of hyponatremia include headache, nausea, vomiting, muscle cramps, fatigue, disorientation, and fainting (Sodium (Chloride) 2014). Severe acute hyponatremia may lead to brain edema, associated with neurological consequences and other serious repercussions such as seizures, coma, brain damage, and even death if not diagnosed and treated rapidly (Giuliani, Peri 2015). Cardiovascular morbidity and mortality have been associated with mild chronic hyponatremia with long-term adverse health effects such as bone loss and fractures, gait instability and falls, attention deficits etc. Na has been identified as a nutrient of public health concern for overconsumption (Sodium (Chloride) 2014). Excessive intakes of Na lead to an increase in extracellular fluid volume, as water is drawn out of the cells to maintain normal Na concentrations. As long as the body's needs in water can be met, normally functioning kidneys can excrete the excess of Na and maintain homeostasis (Erdman, MacDonald, Zeisel 2012). The ingestion of large quantities of salt may lead to nausea, vomiting, diarrhea (leading to disturbances in electrolyte balance), and abdominal cramps (Drug-Nutrient Interactions 2023). Hypernatremia is much less common than hyponatremia and is rarely caused by an excessive Na intake, but rather from excess water loss (e.g., burns, respiratory infections, renal loss due to kidney malfunction, osmotic diarrhea, hypothalamic disorders) or reduced water intake, generally accompanied by an impaired thirst mechanism (Reynolds, Padfield, Seckl 2006; A.C. Ross et al. 2012). Symptoms of hypernatremia associated with dehydration from excessive water loss include dizziness, fainting, low blood pressure, and decreased urine production. Severe hypernatremia may lead to altered mental status, lethargy, irritability, convulsions and coma fainting (Sodium (Chloride) 2014). Moreover, excess dietary Na intake represents a major contributor to hypertension, which is a leading preventable risk factor for cardiovascular disease. Other deleterious consequences for Na overconsumption include gastric cancer, osteoporosis and kidney stones (Sodium (Chloride) 2014). Chronic hypertension damages the heart, blood vessels and kidneys, increasing the risk of heart disease, stroke and hypertensive kidney disease. Moreover, studies in animals and humans report an association between high-salt intake and pathological alterations in the structure and function of large elastic arteries, independently of changes in blood pressure (Safar et al. 2000). Endothelial dysfunction is considered to be an early step in the

development of atherosclerosis. Indeed, the resulting loss of normal nitric oxide- mediated endothelium-dependent vasodilation results in widespread vasoconstriction and coagulation abnormalities (Ras et al. 2013).

3.4.11 Sulfur

Sulfur (S) is classified as a nonmetal in chemistry and has an atomic number of 16 in the periodic table of elements (Annex 6; PubChem 2023). Sulfur is a macromineral of great importance in the formation of amino acids and some vitamins, and required for the growth of all living organisms. It is present in cells and extracellular compartments as part of amino acids cysteine and methionine (Bohrer, Takahashi 2016). S serves as a structural component of skin, hair, feather, wool, cartilage and connective tissue and is required by the body mainly as a component of S-containing organic compounds (Cherian 2019). These include the hormone insulin (in the form of disulfide bonds (Chang et al. 2004)), the anticoagulant heparin, and chondroitin sulfate (Cherian 2019). S is found in enzymes such as glutathione peroxidase which plays an important role in cell protection from oxidative stress (Miękus et al. 2020). It is also commonly found in mammals in the form of Fe-S clusters in metalloproteins and in bridging ligands (e.g., in cytochromes; Francioso et al. 2020). The S compounds found in food are amino acids or vitamins including methionine, cysteine, homocysteine, cystine, taurine, lipoic acid, glucosinolates, and thiamin and biotin which are two B vitamins, involved in lipid and carbohydrate metabolism and the regulation of acidity in the body (Cherian 2019; Sulfur 2020; Francioso et al. 2020). S also plays a role in energy metabolism as a component of the coenzyme A (Cherian 2019; Meriläinen et al. 2008). This micromineral is needed for multiple chemical reactions involved in the metabolism of drugs, xenobiotics and steroids (e.g., sulfation is needed in the detoxification of pharmacological agents by the liver; Pang et al. 1995).

Dietary inorganic S is very poorly absorbed. S deficiency in the diet leads to disturbances in the formation of proteins, can cause weight gain, reduced feather and wool growth (Cherian 2019). Because of its very poor and slow absorption, S toxicity is very rare (Cherian 2019).

3.4.12 Zinc

Zinc (Zn) is classified as a transition metal in chemistry and has an atomic number of 30 in the periodic table of elements (Annex 6; PubChem 2023). Zn is a micromineral acting as a cofactor for several enzymes and is a structural component of a wide variety of proteins, neuropeptides, hormone receptors and polynucleotides (Baum, Shor-Posner, Campa 2000). It is found in the structure of metalloproteins and more than 300 types of enzymes (Kelkitli et al. 2016). Among the best-known Zn-dependent hormones or enzymes are the copper zinc superoxide dismutase (SOD) which plays a key role in the antioxidant defense system, and thymulin, a nonapeptide hormone produced by thymic epithelial cells, which is essential for the formation of T-lymphocytes (Baum, Shor-Posner, Campa 2000). Zn plays a role in the metabolism of lipids, carbohydrates, nucleic acids and proteins, as well as in cell membrane stabilization, protein synthesis, normal tissue growth, wound healing, immune function, neurotransmission, vision, reproduction, and intestinal ion transport (Önal et al. 2011; Terrin et al. 2015; Institute of Medicine (US) Panel on Micronutrients 2001; Marriott et al. 2020; A. Catharine Ross et al. 2012). Numerous reactions of cellular metabolism are Zn-dependent. The functions of Zn can be divided into three categories: catalytic, structural, and regulatory (A.

Catharine Ross et al. 2012). Regarding its catalytic role, over 50 different enzymes depend on Zn for their ability to catalyze vital chemical reactions (Vallee, Falchuk 1993). Zn-dependent enzymes can be found in all six classes of enzymes (i.e., oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases) (King 2011; Cornish-Bowden 2014). Zn can have a direct catalytic role or a structural role during enzymatic reactions. For instance, Zn plays a key role in the folding of some proteins (Zinc 2014). A finger-like structure called “zinc finger motif” stabilizes the structure of several proteins (e.g., the superfamily of nuclear receptors that bind and respond to steroids, estrogens, thyroid hormones, vitamin A and D, allowing them to bind to DNA and act as transcription factors to regulate gene expression (Mangelsdorf et al. 1995)). The removal of Zn from Zn-containing proteins results in protein misfolding and thereafter loss of function. Metallothioneins are metal-binding cysteine-rich proteins composed of a Zn-binding motif with a high affinity for this micromineral, as well as a Cu-binding site, and are involved in the regulation of metal ion homeostasis, cellular defense against oxidative stress and detoxification of heavy metals (Atrián-Blasco et al. 2017; Hijova 2004). Studies conducted over the last two decades have reported the role of Cu, Zn and Fe and oxidative stress in several neurodegenerative diseases like Alzheimer’s, Parkinson’s, and prion diseases. Metallothioneins seem to have a protective role in such neurodegenerative processes (Hijova 2004). Beside its regulatory role in gene expression, Zn also plays a role in cell signaling, hormone release and nerve impulse transmission (Zinc 2014). Studies have linked dysfunctional Zn signaling with several diseases including cancer, obesity, cardiovascular disease and type II diabetes (Norouzi et al. 2018). Zn interacts with nutrients such as Cu, Fe, Ca, folate and vitamin A. For instance, a high intake in Zn over a long period of time interferes with Cu bioavailability, by inducing the intestinal synthesis of metallothioneins, which trap Cu within intestinal cells and therefore prevent systemic absorption of Cu (i.e., Wilson’s disease) (Zinc 2014). The reciprocal isn’t true, meaning that high Cu intake doesn’t affect Zn absorption (Erdman, MacDonald, Zeisel 2012). High intakes of dietary Ca also impair Zn absorption in animals (Erdman, MacDonald, Zeisel 2012). Vitamin A and Zn interact in multiple ways. Zn status influences several aspects of vitamin A metabolism (i.e., transport, absorption, utilization (Christian, West 1998)). Zn is a component of retinol-binding protein, which is a protein necessary for transporting vitamin A in the blood. Zn is also required for the enzyme that converts vitamin A (= retinol) to retinal. This latter form of vitamin A is essential for rhodopsin synthesis, which is a protein in the eye that absorbs light and is involved in dark adaptation. Thus, Zn deficiency has been associated with the elevation of hepatic vitamin A and its decreased release from the liver into the bloodstream, which might contribute to symptoms of night blindness associated with Zn deficiency (Christian, West 1998; Boron et al. 1988). Adequate Zn intake is also essential in maintaining the integrity of the immune system, in particular for normal development and function of cells that mediate both innate and adaptive immune responses (Maares, Haase 2016; Baum, Shor-Posner, Campa 2000). It is essential for T-cell division, maturation and differentiation, lymphocyte response to mitogens, programmed cell death of lymphoid and myeloid origins, gene transcription, and bio membrane function (Baum, Shor-Posner, Campa 2000). A close relationship exists between Zn and insulin action. In pancreatic β -cells, Zn is involved in insulin synthesis and storage in secretory vesicles. When blood glucose concentrations increase, Zn is released alongside insulin. Zn stimulates glucose metabolism and uptake by insulin-sensitive tissues by triggering the intracellular insulin signaling pathway (Norouzi et al. 2018).

In animals and humans, Zn deficiency results in a rapid and marked atrophy of the thymus, as well as impaired cell-mediated cutaneous sensitivity, lymphopenia, and reduced antibody responses (Baum, Shor-Posner, Campa 2000). Beside resulting in altered host defense,

increased risk of inflammation, increased susceptibility to a variety of infectious agents and even death can be associated with Zn deficiency (Maares, Haase 2016; Baum, Shor-Posner, Campa 2000; Fischer Walker, Black 2004). Indeed, pathogens also require Zn to multiply and invade, which underlies the importance of having a well-established antimicrobial defense mechanism that can sequester free Zn away from microbes (Subramanian Vignesh, Deepe 2016). An adequate Zn status is particularly important for brown bears during hibernation when they have fewer circulating innate immune cells, reflecting potentially a suppressed innate immune system (Sahdo et al. 2013).

3.5 Potential impacts of immobilization on blood variables

During the captures and immobilization process, several factors can potentially affect the blood variables. These include the immobilization drugs (medetomidine, tiletamine, zolazepam, ketamine), the duration of anesthesia, the chasing prior to darting inducing stress, etc. There are many different definitions of “stress”, and it is difficult to find a definition with a general consensus among people. Here we will define stress as “*the internal physiological and biochemical changes that alter homeostasis when an animal is subjected to physical and psychological perturbations*” (Kreeger TJ et al. 2023). This definition doesn’t refer as stress entirely as a pejorative thing, it also includes *eustress* (i.e., “good stress”) which is a natural part of an animal’s daily life and can benefit it by increasing productivity and motivation that could be important for its survival. If the stress response threatens the animal’s well-being leading thereafter potentially to disease or pathologies, the animal experiences *distress*, i.e., “bad stress”(Arnemo JM, Caulkett N 2007). Stress includes physical stress (e.g., fighting, running, restraint) and psychological stress (fear, panic, pain, unease), and animal captures almost always involve both. The actual or perceived threat to a biological being is referred as a “stressor”, and the response to this stressor is referred as the “stress response”. Stressors can be physical (e.g., trauma, surgery, intense heat or cold), chemical (e.g., anesthetics drugs, reduced oxygen supply, acid-base imbalance), physiological (e.g., hemorrhage, shock, pain, infection) or emotional (e.g., anxiety, fear) (Arnemo JM, Caulkett N 2007). The relationship between stressors and disease is impacted by the nature, number, magnitude and persistence of the stressors, as well as the organism’s biological vulnerability (i.e., intrinsic factors such as genetics or constitutional factors) and learned pattern of coping (Schneiderman, Ironson, Siegel 2005). When a stressor is recognized and the individual is exposed to stress, neural and hormonal responses are activated and two main responses occur in the CNS: the hypothalamus activates the sympathetic nervous system (SNS) and the hypothalamus-pituitary-adrenal axis (HPAA) (Arnemo JM, Caulkett N 2007). The main neural response to acute stress is the intense and rapid activation of the SNS, also known as the “fight or flight response”. The adrenal medulla releases a massive amount of catecholamines: epinephrine (also known as adrenaline) and norepinephrine (also known as noradrenaline). The huge outpouring of these catecholamines inside the body (the amount depends on the type and intensity of the stressors, but can go up to 300 times the normal concentration of circulating catecholamines; Arnemo JM, Caulkett N 2007) induces multiple physiological effects that enhances the animal’s ability to cope with situations of emergencies. Such physiological variations include increased HR and arterial blood pressure, vasodilatation leading to an increased blood flow to skeletal and cardiac muscles, increased cardiac output, decreased motility and contraction of sphincters in the gastrointestinal tract, increased cellular metabolism, inhibition of insulin secretion and stimulation of glucagon secretion increasing glycemia, ocular effects (dilatation of the pupil, adjustment of the eyes for distant vision),

increased alertness and mental activity, etc. All these adaptations allow the animal to perform more strenuous exercise than usual.

The major hormonal response during acute but also chronic stress is the activation of the hypothalamic-pituitary-adrenal axis (HPAA) (Arnemo JM, Caulkett N 2007). Neural input to the hypothalamus in response to a stressor leads to the secretion of the corticotropin-releasing hormone (CRH) which in turn stimulates the release of adreno-corticotrophic hormone (ACTH). The latter circulates to the adrenal cortex and stimulates the release of glucocorticoid hormones (cortisol and corticosterone). Canids secrete more or less equal amounts of cortisol and glucocorticoids whereas felids, ungulates and primates secrete mainly cortisol (Arnemo JM, Caulkett N 2007). Indeed, plasma cortisol concentrations are used as an indicator to stress in animals, and is even considered to be the main stress biomarker in animal species despite its variability and short life-span (Mitchell, Hattingh, Ganhao 1988; Crookshank et al. 1979). Morton et al. conducted a study in 1995 on 712 individual animals of 18 different wildlife species, to determine plasma cortisol concentrations after chemical or physical restraint, trauma or tranquilization. They noticed a rise in the cortisol levels after capture in all species except one (Morton et al. 1995; Græsli et al. 2015). Other biomarkers that could be used as reliable stress indicators include biomarkers of the energetic metabolism (e.g., lactate, glucose) and biomarkers of the inflammatory system (e.g., haptoglobin, serum amyloid A (particularly in ruminants) and C-reactive protein) (Ataallahi, Nejad, Park 2022; García-Torres et al. 2021; Ogi, Gazzano 2023). Besides increasing the release of glucocorticoid hormones, ACTH may have a direct key role in resisting stress. ACTH constitutes one of several hormones facilitating learning and behavior. Therefore an increase in ACTH during stress may teach the body on how to cope better with similar stressors in the future by enabling the learning of appropriate behavioral responses (Arnemo JM, Caulkett N 2007).

Other hormones than hypothalamic CRH stimulate the secretion of ACTH and glucocorticoids, such as vasopressin (released from the pool stored in the posterior pituitary gland) and catecholamines. The predominant metabolic effect of glucocorticoids secreted during a stressful event is to mobilize fuel. Because of the catabolism of protein stores in muscles, the amino acid plasma concentrations increase. Triacylglycerol catabolism in adipose tissue also increases, leading to increased glycerol and fatty acids. Increased gluconeogenesis from amino acids, glycerol and lactate in addition to the inhibition of glucose uptake and oxidation by many cells contribute to the increase in blood glucose concentrations. The catabolism of labile body protein stores leading to an increased release in amino acids provides a potential source of amino acids for tissue repair if injuries were to happen. This way, an increased pool of amino acids, glucose and fatty acids is available for use as needed by the animal undergoing stress stimuli (Arnemo JM, Caulkett N 2007). A long period of stress or prolonged activation of the HPAA involving high amounts of glucocorticoids inside the body may inhibit specific immune responses due to the anti-inflammatory properties of glucocorticoids and their ability to induce a shift in composition of white blood cells.

During winter, some bears get darted inside their dens without waking up, but sometimes some wake up or even manage to flee for a short distance, inducing a higher level of stress on the bears waking up compared to the ones who stayed asleep. During the spring and summer captures, the helicopter pursuit results in physical and psychological stress on the brown bears, especially if the chase goes on for a while. Ideally, in order to evaluate the magnitude of the impact of drugs and stress on the blood variables of brown bears, samples should be taken on bears that have not received immobilization drugs and are not in a state of stress,

and compare those blood samples (which would be the control group) with the ones sampled from bears which have been submitted to a certain level of stress and anesthetic drugs. Of course, with wild animals such as free-ranging brown bears, this scenario is not possible since immobilization with anesthetic drugs are indispensable and bears are not used to humans handling them whatsoever. It is important to keep in mind that stress can be expressed for a short period of time (i.e., acute stress), involving the activation of the SNS and adrenalin secretion but also for a much longer duration (i.e., chronic stress that can last for hours, days, years). Generally, acute stress doesn't result in any significant pathology or disorder, whereas chronic stress is known to cause pathologies measured by immunosuppression, infertility, state of deterioration etc. (Arnemo JM, Caulkett N 2007; Fischer, Romero 2019; Karaer, Čebulj-Kadunc, Snoj 2023; Romano et al. 2010).

The intense chasing during helicopter pursuit not only causes stress reactions in bears but also affects muscle enzymes. Although immobilizing brown bears by helicopter pursuit induces stress on several levels (i.e., physical, chemical, psychological and emotional), a study conducted in 2003 comparing different methods of brown bear captures has shown that this chemical immobilization method had less adverse effects regarding muscle enzyme alterations than a physical restraint method using a leg-hold snare prior to chemical immobilization (Cattet et al. 2003). For instance, serum concentrations of alanine aminotransferase, aspartate aminotransferase, and creatine kinase were higher in physically restrained grizzly bears, suggesting higher muscle injury than in bears immobilized by remote injection from a helicopter. In addition, serum concentrations of Na and Cl were higher in physically restrained bears, attributed to reduced body water volume because of water deprivation and increased water loss while trying to escape from the leg-hold snare (Cattet et al. 2003). In any case, the reduction of time pursuit and anesthesia help in minimizing the negative physiological effects of brown bears.

4. OBJECTIVES OF THE THESIS

The goals of this thesis were firstly to summarize some features of the Scandinavian brown bear's lifestyle through its life history, reproduction cycle, hibernation physiology and diet in order to get an overview on the studied species. Then in a second part, twelve essential minerals were studied in order to establish reference intervals in whole blood, as well as their seasonal and annual variations. This required bibliographic research and the analysis of data already collected during fieldwork. The twelve elements included in this study were calcium, cobalt, copper, iodine, iron, magnesium, manganese, phosphorus, selenium, sodium, sulfur and zinc. 384 samples taken from the jugular vein of 164 individual bears were collected during spring, summer and winter captures from 2010 to 2022, in ethylenediamine tetraacetic acid (EDTA), trace element (TE) and heparin (HE) tubes.

Scandinavian brown bears undergo physiological changes throughout the year, linked with their diet that varies with each changing season and other known or unknown factors (e.g., environmental factors, intrinsic factors). During fall, or hyperphagia period, the bears feed on sugar-rich food items such as berries in order to store fat and increase their body mass in preparation for hibernation (Hilderbrand et al. 1999; Stenset et al. 2016). They do not eat, drink, urinate or defecate for a period of five to seven months, in which time they solely rely on their body reserves (mostly their fat stores). Well-established aspects of bear hibernation physiology include decreases in Tb (Hissa 1997), HR, RR, and metabolism (Folk, Minor 1972; Tøien et al. 2011; Tøien, Blake, Barnes 2015) during hibernation. Pregnant females give birth to cubs inside the den and begin lactation. When spring comes and the bears emerge from their den, they may have lost up to 43% of their body mass and must rebuild their body reserves by gaining lean mass as well as fat. This corresponds to the start of their active phase, where the bears actively seek protein-rich food items thus increasing their movements within their habitat range. Due to these changes, physiology varies dramatically throughout the year, with hematological and biochemical variables illustrating many of the organ systems involved. Establishing reference intervals (RIs) for twelve essential minerals during spring, summer and winter in Scandinavian brown bears may provide insights on understanding better these physiological changes, their reproductive system, hibernation, and health in general. There is always an optimal concentration of essential minerals required by the organism. A concentration above or below the optimal range, translating into a toxicity or deficiency state, may compromise the animal's health. Determination of levels of blood parameters such as essential minerals is useful in knowing the physiological values to expect and in predicting health problems that might result in mortalities (Kida et al. 2007). Blood profiling is capable of detecting subclinical metabolic disorders and therefore reflects animals' health status (Caldeira et al. 2007). And so, besides allowing us to gain more knowledge on the brown bear's ecology and biology, these RIs could also be valuable in case of pathologies found in captive brown bears. Comparing the values found in free-ranging brown bears, that should reflect the normal or physiological state, with the ones found in sick captive bears, could lead to the development of novel clinical therapies and may bring medical solutions in view of healing them. The results of this study provide reference values that will aid in understanding the physiology of brown bears and in assessing the health of these animals in captive environments.

5. MATERIALS AND METHODS

5.1 Study Area and Animals

This study is part of a long-term research project on the Scandinavian brown bear population in Dalarna and Gävleborg counties, in south-central Sweden (~61°N, 15°E; Figure 21).

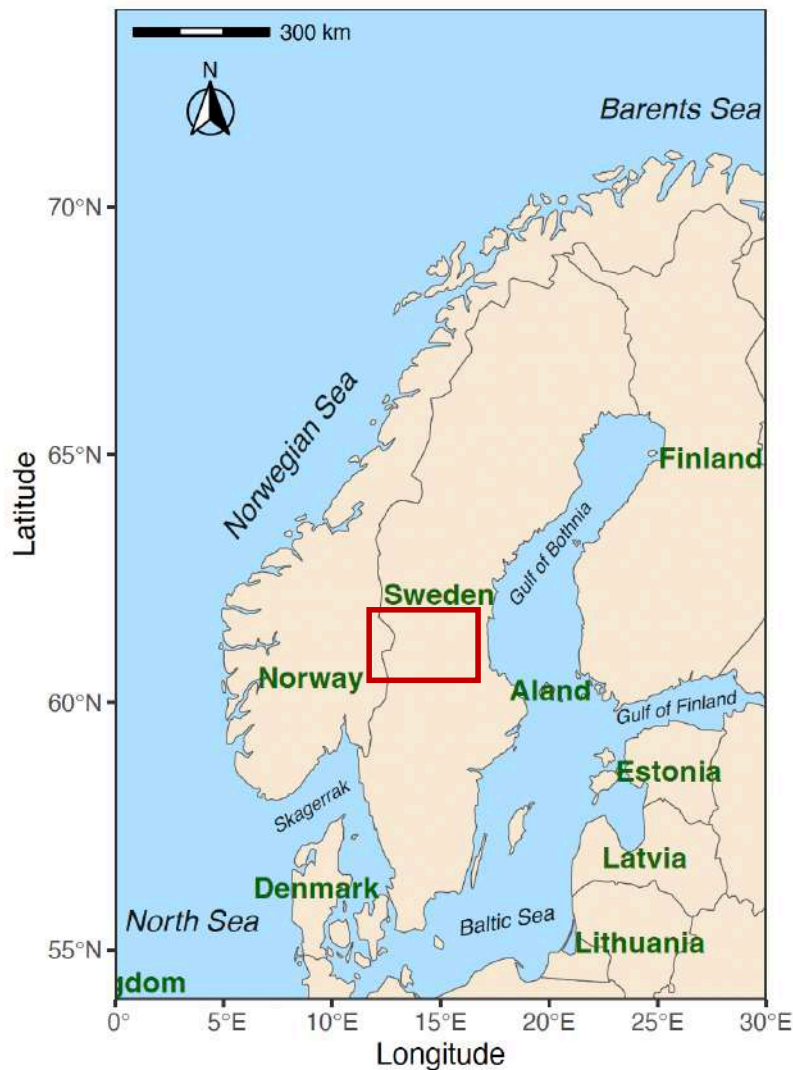


Figure 21: Map of the study area delimited by the red rectangle. Source: Rstudio, by author.

The size of the study area approximates 13,000 km² and is predominantly covered with intensively managed coniferous forest in a rolling landscape. The dominating tree species are Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*) (Bischof et al. 2010; Stenset et al. 2016; Felton, Petersson, et al. 2020), but deciduous trees like mountain birch (*Betula pubescens*), silver birch (*Betula pendula*), European aspen (*Populus tremula*) and grey alder (*Alnus incana*) are commonly found (Zedrosser, Dahle, Swenson 2006). The shrub layer is mainly made up of common juniper *Juniperus communis*, willows *Salix* spp., and rowan *Sorbus aucuparia*, and the ground layer is dominated by dwarf shrubs, such as heather *Calluna vulgaris* and berries including bilberries, lingonberries and crowberries, on primarily granite and gneiss bedrock (Elfström, Swenson, Ball 2009; Bischof et al. 2010). Even-aged forest management with tree retention is the dominating management system in Swedish forest

today (Roberge et al. 2020; Felton, Löfroth, et al. 2020). This system is characterized by the maintenance of a more or less even age structure of trees within individual forest stands, with one single tree cohort (not considering the trees retained for environmental conservation purposes), established through regeneration after clearcutting. Forest stands are typically planted and commercially thinned a couple of times before being felled through clearcutting, at an age of 80 to 100 years. It is then followed by scarification and replanting of seedlings (Martin, Basille, Van Moorter, et al. 2010; Swenson et al. 1999; Roberge et al. 2020).

The vegetation period in south-central Sweden is 170-190 days, with 600-1,000 mm average annual precipitation (of which 350-450 mm during the vegetation period). Snow cover usually lasts from end of October to late April/early May and the mean daily temperatures are -7°C in January and $+15^{\circ}\text{C}$ in June (Roberge et al. 2020; Zedrosser, Dahle, Swenson 2006). The hilly landscape is interspersed with lakes and bogs and a few agricultural fields in the east. The study area is a rolling landscape with elevations ranging from ~ 150 m above sea level in the east to 850m above sea level in the west. The altitudes are mostly below timberline which is around 850m (Fuchs et al. 2021). Most of the human settlements are concentrated in the north and south. Few high-traffic roads, used for forestry and private properties are distributed throughout the study area at a density of 0.14 km/ km². Isolated houses (mainly cabins) and both paved and gravel roads with low traffic volume are distributed throughout the study area (0.3 cabins/ km² and 0.7 km low-traffic roads/ km²) (Martin et al., 2010).

The brown bear density estimate in our study area is around 30 bears/1,000 km² (Stenset et al. 2016; Bellemain et al. 2005). The core area where Scandinavian brown bear density is the highest is represented in red on the maps in Figure 22, which is the area corresponding to our study area in south-central Sweden (red rectangle).

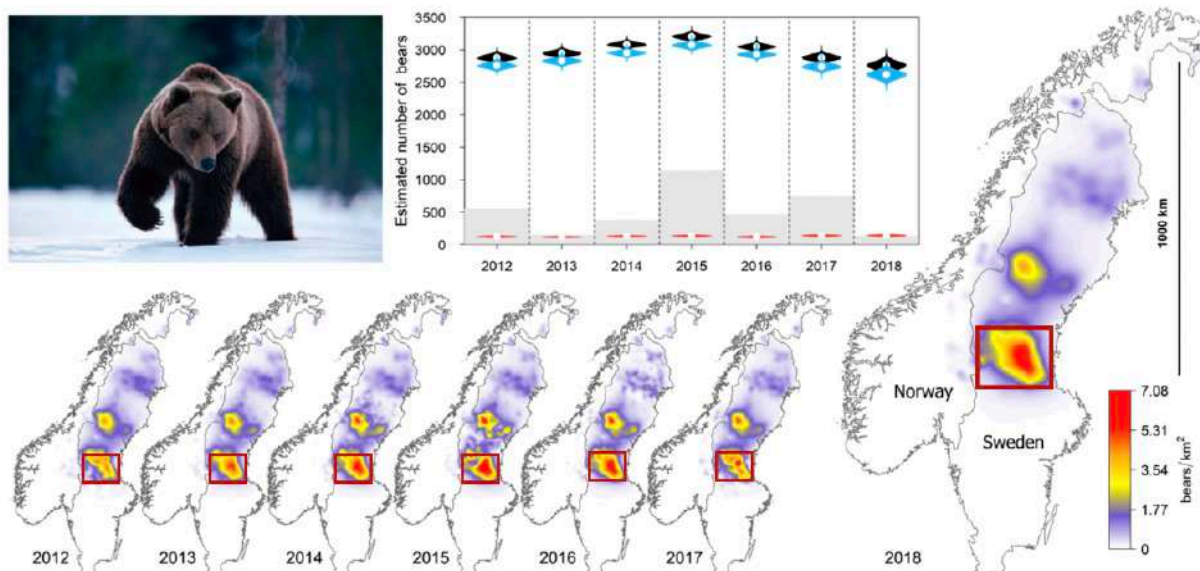


Figure 22: Annual density maps for Scandinavian brown bears (*Ursus arctos*) in Norway and Sweden with corresponding population estimates from 2012 to 2018. The red rectangles delimit our study area. On the chart, the black violins represent the total bear population estimate; the blue violins the population estimate in Sweden; the red violins the population estimate in Norway. Source: Bischof et al. 2020.

5.2 Sampling procedures

The brown bears were chemically immobilized using dart-guns (DANiJECT®) with a drug-combination of medetomidine, tiletamine and zolazepam (MTZ), with ketamine in addition in winter. Venous blood samples using ethylenediamine tetraacetic acid (EDTA), heparin (HE) and trace element (TE) tubes were collected during the immobilization for ecological studies involving radio collaring and marking in the SBBRP. The anesthetized brown bears were placed in lateral recumbency, with their airways cleared, and their head facing upwards if the terrain was hilly, with the mouth and nose pointed down. In summary, 384 samples from 322 capture events were collected, which means that in some bears more than one sample were taken. In total, 384 blood samples were collected in the years 2010, 2013 and 2017-2022, of which 131 were sampled in EDTA tubes, 232 in TE tubes and 21 in HE tubes.

Blood was sampled from the brown bears' jugular vein, using a vacutainer system (BD Vacutainer®, BD Diagnostics, Preanalytical Systems, Franklin Lakes, NJ, USA). 18G needles were used for adults whereas 20G needles were used for yearlings and subadults. Blood sampling was conducted within the first 60 minutes of the immobilization. The venous blood used for analysis of the essential minerals was collected in tubes containing an anticoagulant, preventing the blood from clotting. Anticoagulants in the tubes included ethylenediamine tetraacetic acid (EDTA), sodium heparin and lithium heparin. The blood was collected in tubes containing these anticoagulants, called K₂ and K₃-EDTA (but mainly K₃-EDTA) tubes, heparin tubes, and trace element tubes. Their characteristics are described in Table 10. After sampling the blood, the tubes were gently inverted several times (8-10 times) in order for the blood to mix with the anticoagulant inside the tube. If blood was collected and clotted, the sample was no longer usable and blood was redrawn. The blood was frozen the same day and kept at -20°C until shipment to the laboratory. All blood samples were analyzed at the same laboratory (ALS Scandinavia, Luleå, Sweden). Concentrations of elements were determined by double focusing sector field Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Rodushkin, Olofsson, Axelsson 2000).

Table 10: Characteristics of the blood sampling tubes: EDTA, TE and HE. Source: Table made by author based on tube characteristics on the Greiner Bio-one website <https://shop.gbo.com/en/row/products/preanalytics/venous-blood-collection/vacurette-tube/trace-elements/trace-elements-nh-sodium-heparin/>

Tube	Laboratory	Whole name	Volume	Anticoagulant	Cap color
EDTA	Greiner Bio-One International AG, Kremsmünster, Austria	Vacurette® Di- and Tri-potassium Ethylenediaminetetraacetic Acid (K ₂ and K ₃ -EDTA) Tube	4 mL	K ₂ and K ₃ -EDTA contain two (K ₂) or three (K ₃) chelated potassium ions. EDTA (sprayed-dried on the walls of the tube for K ₂ -EDTA or as liquid for K ₃ -EDTA) inhibits clotting by chelation of Ca ²⁺ and Mg ²⁺ (and even iron and zinc)	Lavender, black ring
TE		Vacurette® NH Trace Elements Sodium Heparin Tube	6 mL	Sodium heparin, sprayed-dried on the walls of the tube. Activates antithrombins, blocking the coagulation cascade by preventing formation of fibrin.	Royal blue, black ring
HE		Vacurette® LH Lithium Heparin Tube	4 mL	Lithium heparin, sprayed-dried on the walls of the tube. Activates antithrombins, blocking the coagulation cascade by preventing formation of fibrin.	Green, black ring

The anticoagulant effect of EDTA is achieved by its chelating activity resulting in the loss of free Ca ion in the plasma which is essential for blood coagulation. EDTA is also known for chelating other metals, i.e., Fe, Mg and Zn (Staff 2013; Imafuku et al. 2002; Nkuna, Ijoma, Matambo 2022).



Illustration No. 21: Jon M. Arnemo and Anne Randi Græsli, two wildlife veterinarians, sampling blood from the jugular vein and muscle tissues – winter captures 2023. Source: Photograph by author.

Some bears got recaptured several times over the years and/or from one season to another during the same year but not systematically, and sometimes several blood tubes were collected from the same bear during the capture, which is why there are more blood samples than bears. An overview of the number of samples from each type of blood tubes can be found in Table 11.

Table 11: Total number of samples taken each year according to the different types of tubes. Source: Rstudio, by author.

Year	2010	2013	2017	2018	2019	2020	2021	2022	Total
Total number of samples	13	9	36	54	82	71	78	41	384
n EDTA tubes	13	9	36	54	19	0	0	0	131
n TE tubes	0	0	0	0	63	71	57	41	232
n HE tubes	0	0	0	0	0	0	21	0	21

EDTA tubes were used for blood sampling during the years 2010, 2013, 2017, 2018 and 2019. In addition, in 2010, 2013, 2017 and 2018 only EDTA tubes were used. TE tubes were used for blood sampling during the years 2019, 2020, 2021 and 2022. In 2019, both EDTA and TE tubes were used. HE tubes were only used in the year 2021. In 2020 and 2022, only TE tubes were used. In 2021, TE and HE tubes were used.

Out of the 164 different bears captured, 89 bears were sampled more than once: the number of samples per bear ranges from 1 to 8. Most of the bears (45.73%) got sampled only once, and some got sampled twice (18.29%) or three times (17.68%), but taking more than 3 samples was not very frequent (Table 12).

Table 12: Number of blood samples taken per bear during the captures in 2010, 2013, and 2017-2022. Source: Rstudio, by author.

Number of blood samples per bear	Number of bears	Percentage (%)
1	75	45.73
2	30	18.29
3	29	17.68
4	9	5.49
5	8	4.88
6	7	4.27
7	4	2.44
8	2	1.22

In this study, the aim was to analyze the concentrations of twelve essential minerals in whole blood in Scandinavian free-ranging brown bears and to see their seasonal and annual variations. Some bears were captured several times (some once each year but during multiple years, some several times each year (e.g., summer and spring within the same year), and some several times a year during multiple years). Knowing that, tests were run to see if the same bear got sampled more than once during each capture. All the bears that got blood sampled in HE tubes were different bears, all occurring during spring and summer in the year 2021. All bears that got blood sampled in EDTA tubes were only sampled once at each capture. Regarding the TE tubes, there was only one bear that got blood sampled twice during spring 2021 using TE tubes. They occurred however on a different date (April 19th 2021 and May 28th 2021) so during two different captures, but within the same season.

5.3 Sample preparation and laboratory analysis

The biochemical profile included calcium, cobalt, copper, iodine, iron, magnesium, manganese, phosphorus, selenium, sodium, sulfur and zinc concentrations from whole blood.

All measurements of element concentrations were done using a double-focusing sector field (SF) inductively coupled plasma mass spectrometry (ICP-MS) ELEMENT XR (Thermo Scientific, Bremen, Germany) instrument (Rodushkin et al. 2005). ICP-MS is an analytical technique that can be used to measure elements at trace levels in biological fluids (Wilschefski, Baxter 2019). One of the biggest advantages of ICP-MS compared to other techniques is its multi-element capability, allowing multiple elements to be measured simultaneously in a single analysis (Wilschefski, Baxter 2019). It also has a large analytical range, a low detection limit, a high sample throughput, needs a low sample volume, and the high resolution and tandem mass spectrometry offer a very high level of interference control (Wilschefski, Baxter 2019). Methane addition to the plasma was used to decrease formation of oxide-based spectral interferences, to improve sensitivity for elements with high first ionization potentials, and to minimize matrix effects (Rodushkin et al. 2005). A laboratory MARS5 microwave digestion system (CEM Corporation, Matthews, NC, USA) was used for sample digestions. A set of matrix-matched lyophilized test samples (trace elements in urine and trace elements in whole blood, SERO AS, Billingstad, Norway) were used for quality control and quality assurance (Rodushkin et al. 2005).



Illustration No. 22: ICP-MS instrument ELEMENT XR used for sample analysis. Source: <https://chemistry.olemiss.edu/icp-sfms/>

The laboratory materials used during sample preparation were soaked in 0.7 M nitric acid (HNO₃, Sigma-Aldrich Chemie GmbH, Munich, Germany) for 24h at room temperature and rinsed with de-ionized Milli-Q water (Millipore, Bedford, MA, USA) prior to use (Rodushkin et al. 2005). A sample volume of 0.5 mL was added into Teflon vials followed by 1 mL of HNO₃ and 0.01 mL hydrogen fluoride (HF). The vials were then capped and placed on a carousel, which was placed in a microwave digestion system and a pre-programmed digestion cycle was initiated (30 minutes of increasing temperature up to 170°C followed by 30-minute holding time at that temperature). The digests were diluted to 10 mL with water and concentration of the twelve elements were determined by ICP-SFMS (Rodushkin et al. 2005). Matrix-effect correction was accomplished by internal standardization (indium added to all solutions at a concentration of 2.5 µg/L) and quantification was done by external calibration with synthetic, concentration-matched standards (Rodushkin et al. 2005). Potential contamination of the samples from the sampling equipment was evaluated by leaching test with weak acid at room temperature (Rodushkin et al. 2005). The concentrations of the essential minerals are expressed in µg/L. No elemental outlier values were excluded.

5.4 Statistical analysis

384 blood samples were collected from the jugular vein of 164 different free-ranging Scandinavian brown bears in south-central Sweden for this study, in the years 2010, 2013, and 2017 to 2022 during spring, summer and winter captures. Some of these 164 bears got captured several times over the period of time, adding up to a total of 322 capture events. To sum up, 322 captures of 164 different free-ranging Scandinavian brown bears were made in the years 2010, 2013 and 2017-2022, where 384 blood samples were taken, of which 131 were sampled in EDTA tubes, 232 in TE tubes and 21 in HE tubes. The 21 blood samples using HE tubes were all taken during spring and summer 2021, all from different bears. The 131 blood

samples using EDTA tubes were taken during spring and summer in the years 2010, 2013, 2017, 2018 and 2019. No bears were sampled more than once for the EDTA tubes during each capture. The 232 blood samples using TE tubes were taken during spring, summer and winter in the years 2019, 2020, 2021 and 2022.

The studied bear population was first characterized based on age, sex, body weight, area distribution, and the seasons and years the blood samples were collected (see 6.1 Descriptive statistics).

Normal distribution and homogeneity of variances tests were conducted firstly with all the samples pooled together (i.e., from the three sampling tubes EDTA, TE and HE) with the season, year and bear variables tested for significance.

Reference intervals were established from the 384 samples for each type of tube.

Normal distribution, homogeneity of variances and non-parametric ANOVA tests were then conducted comparing the mean values and variances among tubes, to see if all the samples could be analyzed together if the differences found were not significant. Post-hoc tests including the Wilcoxon and Dunn tests were established in order to determine which groups were responsible for the significant differences observed among groups.

Boxplots and scatter plots were performed firstly with all 384 samples together to assess annual and seasonal variations in essential mineral concentrations, and in a second part only with the 232 samples collected from TE tubes because of the significant differences in values found according to the type of tube.

6. RESULTS

6.1 Descriptive statistics

This study included 384 blood samples collected from 164 free-ranging Scandinavian brown bears in the years 2010, 2013 and 2017 to 2022, in south-central Sweden. Some of the 164 individuals were captured several times over the years, and some bears were even recaptured several times a year from one season to the next. The total number of bears, or captures in the years 2010, 2013 and 2017 to 2022 was 322 (Figures 23 and 24).

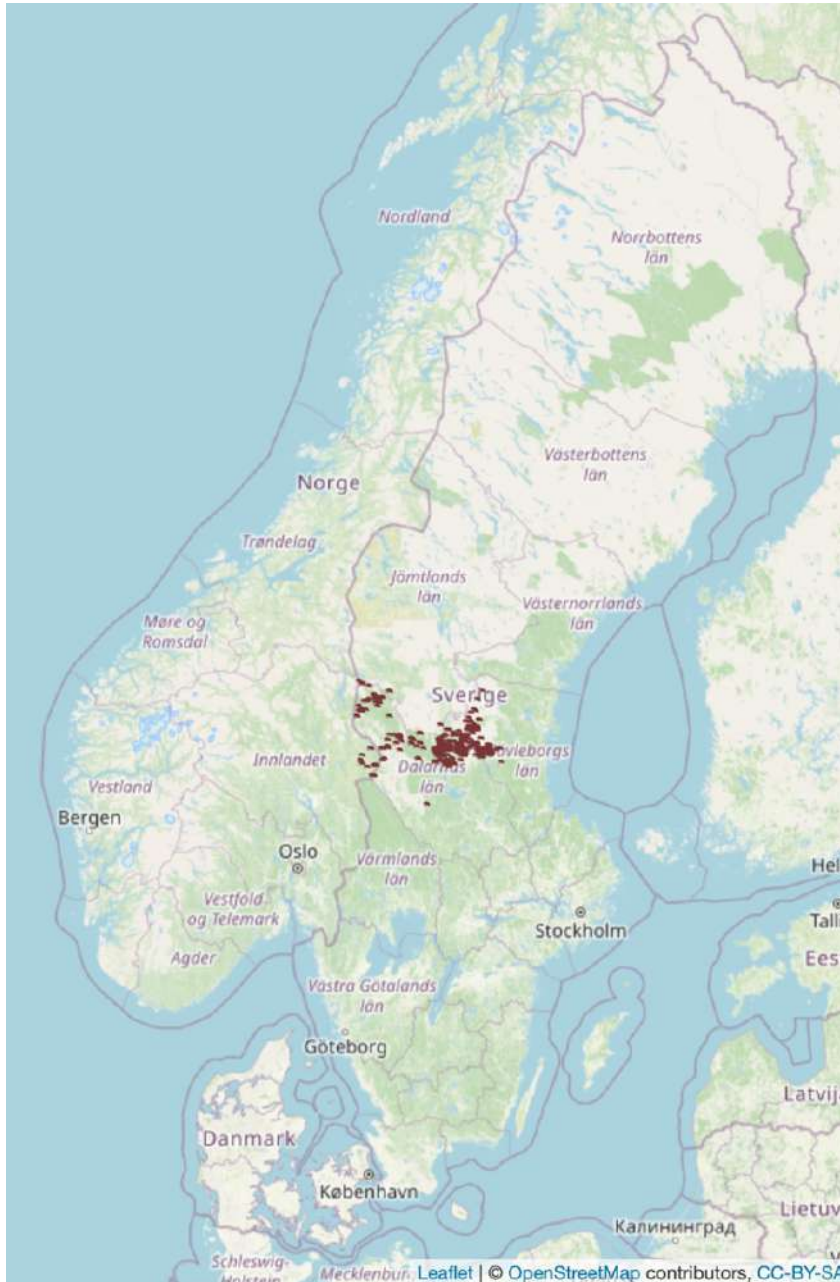
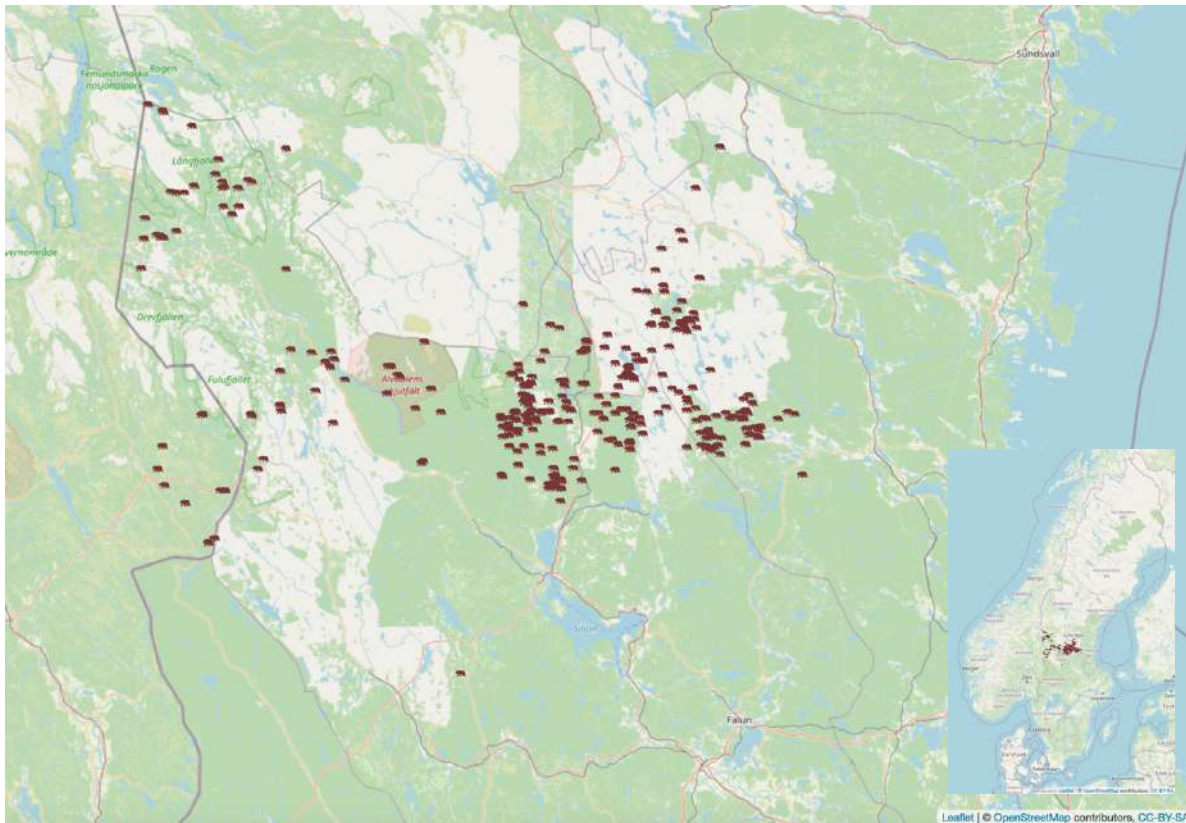


Figure 23: Distribution of the capture positions for blood sampling of brown bears *Ursus arctos* ($n=322$) in south-central Sweden in the years 2010, 2013 and 2017-2022. Source: Rstudio, by author.



*Figure 24: Zoom-in of the distribution of the capture positions for blood sampling of brown bears *Ursus arctos* (n=322) in south-central Sweden in the years 2010, 2013 and 2017-2022. Source: Rstudio, by author.*

Out of the 322 captured bears, 227 were females and 95 were males (Table 13).

Table 13: Number of bears (or capture events) and gender distribution for the years 2010, 2013 and 2017-2022. Source: Rstudio, by author.

Year	2010	2013	2017	2018	2019	2020	2021	2022	Total
Number of bears	13	9	36	54	51	67	56	36	322
Number of females	4	6	27	38	38	46	40	28	227
Number of males	9	3	9	16	13	21	16	8	95
Percentage of females (%)	30.77	66.67	75.00	70.37	74.51	68.66	71.43	77.78	70.50
Percentage of males (%)	69.23	33.33	25.00	29.63	25.49	31.34	28.57	22.22	29.50

Apart from the year 2010 when only four females were captured compared to nine males, all years had a majority of females captured. The years 2010 and 2013 had a significantly lower number of bears captured compared to years 2017-2022 so their values found for the gender proportions might not be as representative. We see however that in the years 2013 and 2017-2022, the percentage of female bears captured ranges from 66.67 up to 77.78%. In total, a mean percentage of 70.50% were females and 29.50% were males. The females were thus more represented in this study and should be taken into consideration when interpreting the results.

Table 14: Number of samples ($n=384$) and gender distribution for the years 2010, 2013 and 2017-2022 according to season (spring, summer and winter). Source: Rstudio, by author.

Year	2010			2013			2017			2018		
Gender	Males	Females	Total	Males	Females	Total	Males	Females	Total	Males	Females	Total
Number of samples	9	4	13	3	6	9	9	27	36	16	38	54
Spring	9	4	13	3	6	9	7	24	31	14	32	46
Summer	0	0	0	0	0	0	2	3	5	2	6	8
Winter	0	0	0	0	0	0	0	0	0	0	0	0

Year	2019			2020			2021			2022		
Gender	Males	Females	Total	Males	Females	Total	Males	Females	Total	Males	Females	Total
Number of samples	21	61	82	22	49	71	21	57	78	8	33	41
Spring	13	43	56	19	41	60	21	49	70	5	19	24
Summer	5	13	18	1	5	6	0	8	8	2	9	11
Winter	3	5	8	2	3	5	0	0	0	1	5	6

In the years 2010, 2013, 2017, 2018 and 2021, no samples were taken during winter neither from males nor females. In addition, samples were only taken during the spring captures in 2010 and 2013. In the years 2017 and 2018 both males and females were sampled during spring and summer. Samples for each season and both genders were collected in the years 2019, 2020 and 2022.

Among the 164 bears, 93 were females and 71 were males. Several bears were captured several times during multiple years, and some even several times in the same year but during different seasons. Thus, a total of 322 captures were made in the years 2010, 2013 and 2017-2022, of which 227 were females and 95 were males, with ages ranging from 1 to 25 years old at the time of immobilization (Table 15).

Table 15: Age of the 322 captured free-ranging Scandinavian brown bears at the time of immobilization according to the years of capture. Source: Rstudio, by author.

Age/Year	2010	2013	2017	2018	2019	2020	2021	2022	Total
1	5 (38.46%)	2 (22.22%)	14 (38.89%)	21 (38.89%)	5 (9.80%)	15 (22.39%)	5 (8.93%)	1 (2.78%)	68 (21.12%)
2	0	0	3 (8.33%)	10 (18.52%)	11 (21.57%)	7 (10.45%)	9 (16.07%)	4 (11.11%)	44 (13.66%)
3	0	0	2 (5.56%)	3 (5.56%)	9 (17.65%)	9 (13.43%)	6 (10.71%)	3 (8.33%)	32 (9.94%)
4	0	1 (11.11%)	2 (5.56%)	3 (5.56%)	3 (5.88%)	10 (14.93%)	7 (12.50%)	1 (2.78%)	27 (8.39%)
5	0	0	1 (2.78%)	0	4 (7.84%)	4 (5.97%)	11 (19.64%)	5 (13.89%)	25 (7.76%)
6	0	2 (22.22%)	2 (5.56%)	3 (5.56%)	1 (1.96%)	3 (4.48%)	1 (1.79%)	6 (16.67%)	18 (5.59%)
7	2 (15.38%)	0	1 (2.78%)	1 (1.85%)	3 (5.88%)	1 (1.49%)	5 (8.93%)	1 (2.78%)	14 (4.35%)
8	1 (7.69%)	3 (33.33%)	1 (2.78%)	0	2 (3.92%)	4 (5.97%)	0	2 (5.56%)	13 (4.04%)
9	0	0	0	2 (3.70%)	0	1 (1.49%)	2 (3.57%)	0	5 (1.55%)
10	3 (23.08%)	0	1 (2.78%)	1 (1.85%)	1 (1.96%)	0	0	1 (2.78%)	7 (2.17%)
11	0	0	0	0	1 (1.96%)	1 (1.49%)	0	0	2 (0.62%)
12	0	0	2 (5.56%)	1 (1.85%)	0	1 (1.49%)	1 (1.79%)	0	5 (1.55%)
13	2 (15.38%)	1 (11.11%)	0	1 (1.85%)	1 (1.96%)	0	1 (1.79%)	0	6 (1.86%)
14	0	0	0	0	2 (3.92%)	0	1 (1.79%)	0	3 (0.93%)
15	0	0	0	0	0	1 (1.49%)	0	0	1 (0.31%)
16	0	0	1 (2.78%)	0	0	0	1 (1.79%)	0	2 (0.62%)
17	0	0	2 (5.56%)	1 (1.85%)	1 (1.96%)	0	0	0	4 (1.24%)
18	0	0	0	2 (3.70%)	1 (1.96%)	1 (1.49%)	0	0	4 (1.24%)
19	0	0	0	0	1 (1.96%)	0	2 (3.57%)	0	3 (0.93%)
20	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	1 (1.49%)	0	0	1 (0.31%)
24	0	0	1 (2.78%)	0	0	0	0	0	1 (0.31%)
25	0	0	0	1 (1.85%)	0	0	0	1 (2.78%)	2 (0.62%)
NA	0	0	3 (8.33%)	4 (7.41%)	5 (9.80%)	8 (11.94%)	4 (7.14%)	11 (30.56%)	35 (10.87%)
Total	13 (100%)	9 (100%)	36 (100%)	54 (100%)	51 (100%)	67 (100%)	56 (100%)	36 (100%)	322 (100%)

Some ages were non-available (NA), either because the age of the bear was unknown or the corresponding data was missing. The percentages in Table 15 correspond to the proportion of a certain age category within the year of capture. For instance, a majority of one-year-old bears were captured in 2010 (n=5/13, 38.46%), in 2017 (n= 14/36, 38.89%), in 2018 (n=21/54, 38.89%), and in 2020 (n=15/67, 22.39%). A majority of three-year-old bears were captured in 2013 (n=3/9, 33.33%), a majority of two-year-olds in 2019 (n=11/51, 21.57%), a majority of five-year-olds in 2021 (n=11/56, 19.64%), and if excluding the majority of bears with unknown ages in 2022 (n=11/36, 30.56%), six-year-olds were mostly captured in 2022 (n=6/36, 16.67%). Overall, the brown bears with the age of one year old were mostly represented in this study (n=68/322, 21.12%).

All bears that got sampled appeared healthy based on a clinical examination after being immobilized. Blood sampling and other tissues (i.e., muscle, skin, hair, feces etc.) were only sampled after assessing the bears' good health. At the time of immobilization and sampling, some females were single subadults or adults, some were lactating, some had cubs of the year or yearlings by their side. Their reproductive status could for instance be determined when their mammary glands were developed and sometimes milk could even be collected from their teats.

In order to understand better the studied population, the mean body weight (BW) of females, males, and both genders according to the year of captures are presented in Table 16.

Table 16: Mean body weight (BW, expressed in kg) of females, males and both genders in the years 2010, 2013 and 2017-2022. Source: Rstudio, by author.

Year	2010			2013			2017			2018		
Gender	Males	Females	Total	Males	Females	Total	Males	Females	Total	Males	Females	Total
Mean BW (kg)	149.14	56.00	121.20	68.67	69.50	69.22	42.20	55.85	52.06	31.17	53.50	48.35

Year	2019			2020			2021			2022		
Gender	Males	Females	Total	Males	Females	Total	Males	Females	Total	Males	Females	Total
Mean BW (kg)	75.27	62.18	66.55	72.77	65.15	67.30	121.41	71.04	90.10	118.71	78.08	87.26

The mean BW of females, males, and both genders according to season each year are presented in Table 17, to see the differences in weight for both genders depending on the season each year.

Table 17: Mean body weight (BW, expressed in kg) of females, males and both genders according to season in the years 2010, 2013 and 2017-2022. Source: Rstudio, by author.

Year	2010			2013			2017			2018		
Gender	Males	Females	Total	Males	Females	Total	Males	Females	Total	Males	Females	Total
Mean BW (kg) Spring	149.14	56.00	121.20	68.70	69.50	69.22	3600	55.85	52.13	31.17	53.22	47.71
Mean BW (kg) Summer	/	/	/	/	/	/	51.50	NA	51.50	NA	56.00	56.00
Mean BW (kg) Winter	/	/	/	/	/	/	/	/	/	/	/	/

Year	2019			2020			2021			2022		
Gender	Males	Females	Total	Males	Females	Total	Males	Females	Total	Males	Females	Total
Mean BW (kg) Spring	91.43	61.63	70.70	75.75	66.72	69.37	121.40	69.71	90.39	131.80	82.25	94.05
Mean BW (kg) Summer	53.00	84.14	80.23	NA	91.00	91.00	/	85.00	85.00	132.00	71.50	78.22
Mean BW (kg) Winter	45.00	42.33	43.67	37.00	41.33	40.25	/	/	/	40.00	48.50	46.80

The mean BW for each season (spring, summer and winter) for females, males and both genders are presented in Table 18.

Table 18: Mean body weight (BW, expressed in kg) of females, males and both genders according to season over the period of time 2010-2022. Source: Rstudio, by author.

Gender	Males	Females	Total
Mean BW (kg) Spring	88.24	64.36	76.84
Mean BW (kg) Summer	78.83	77.53	73.66
Mean BW (kg) Winter	40.67	44.06	43.57

6.2 Mineral concentration analysis in EDTA, HE and TE tubes

6.2.1 Annual variations in whole blood mineral concentrations

Boxplots and scatter plots are presented in Figures 25-36 for each essential mineral, displaying their values and annual variations. All the 384 samples from the three types of tubes (EDTA, TE and HE) are presented in the following scatter plots and boxplots.

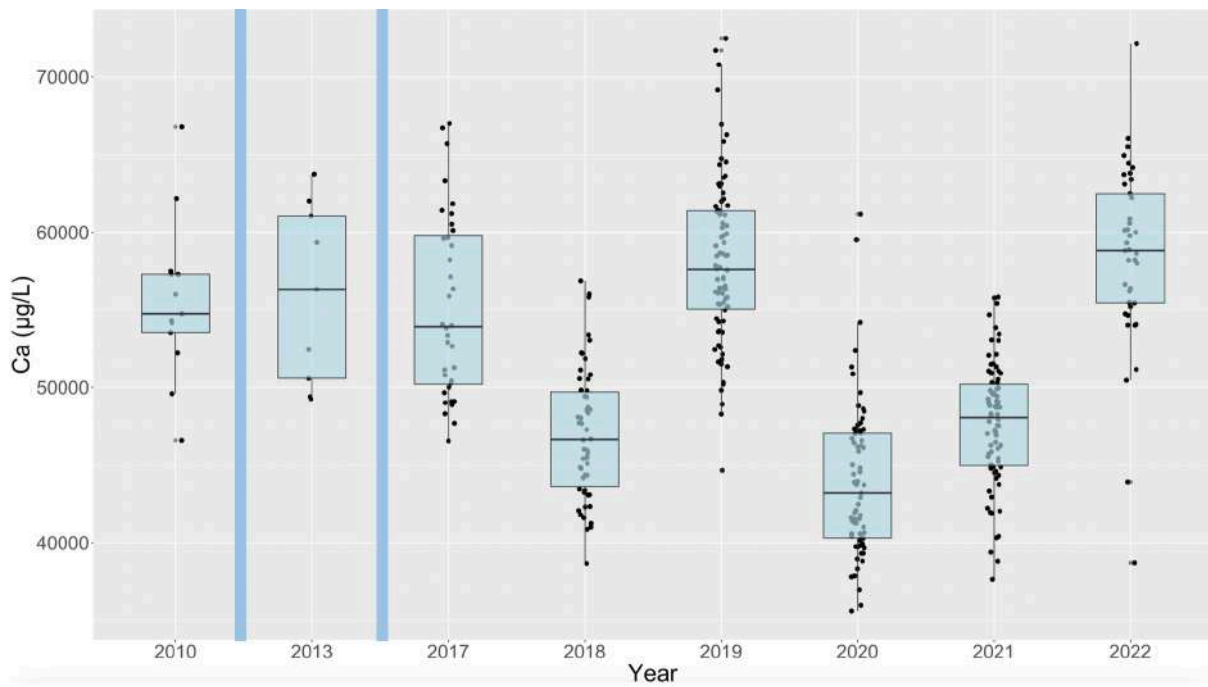


Figure 25: Annual variations in whole blood calcium concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2010, 2013, and 2017-2022, from 384 blood samples. Source: Rstudio, by author.

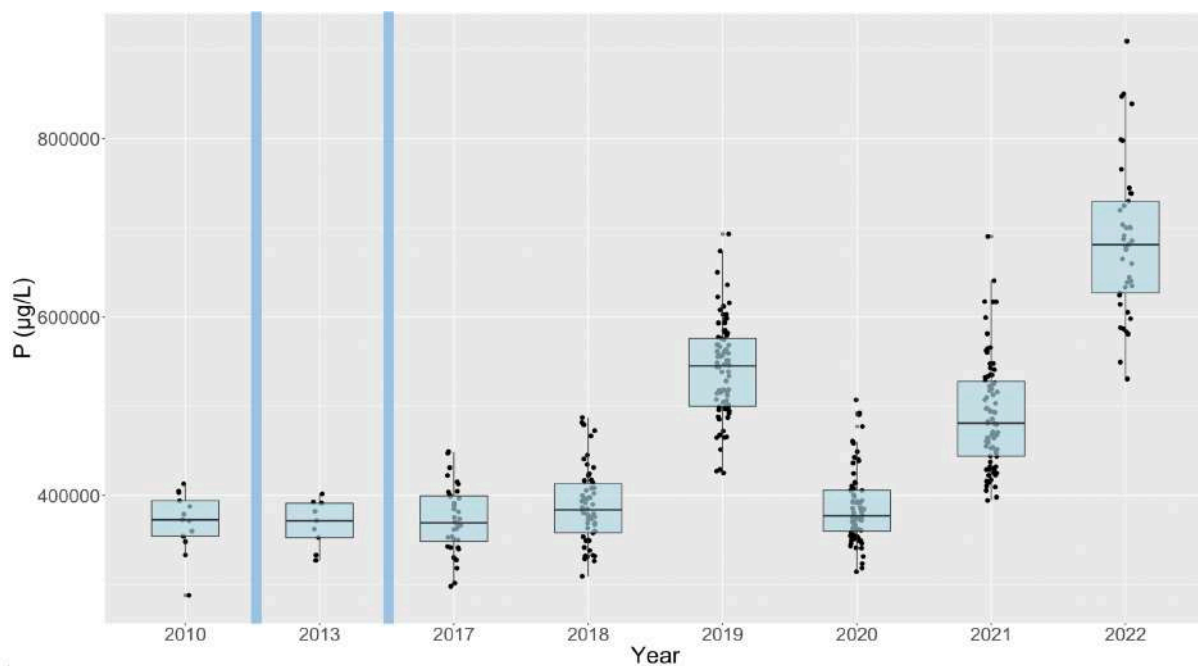


Figure 26: Annual variations in whole blood phosphorus concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2010, 2013, and 2017-2022, from 384 blood samples. Source: Rstudio, by author.

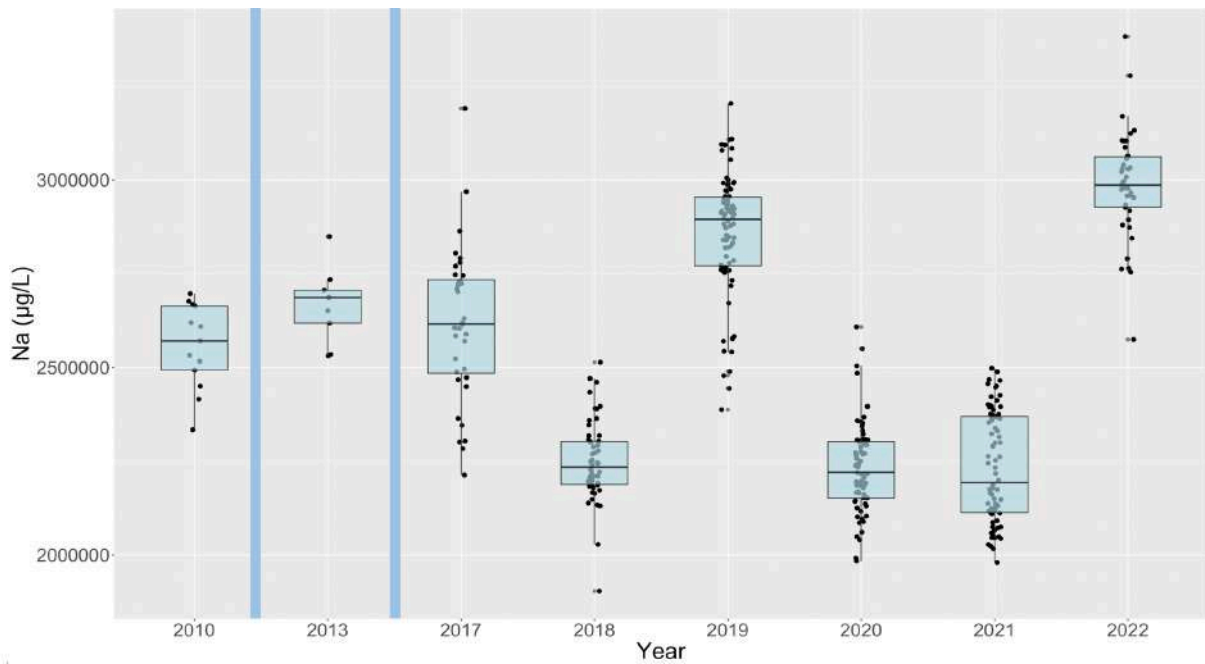


Figure 27: Annual variations in whole blood sodium concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2010, 2013, and 2017-2022, from 384 blood samples. Source: Rstudio, by author.

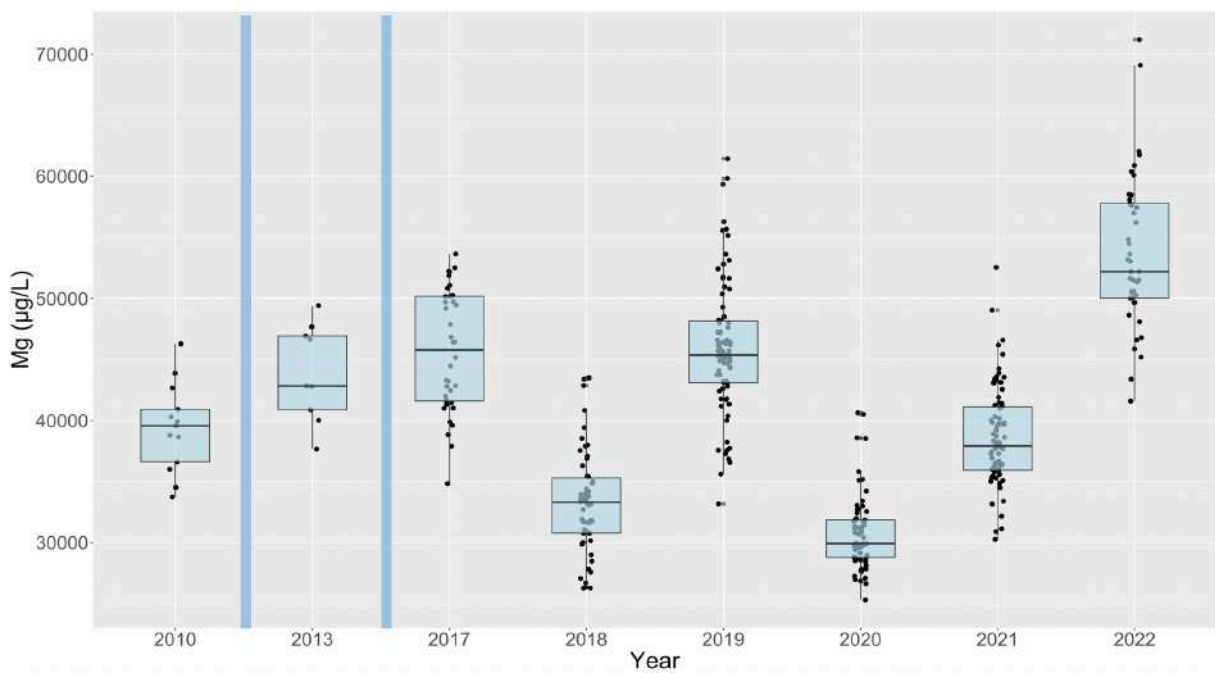


Figure 28: Annual variations in whole blood magnesium concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2010, 2013, and 2017-2022, from 384 blood samples. Source: Rstudio, by author.

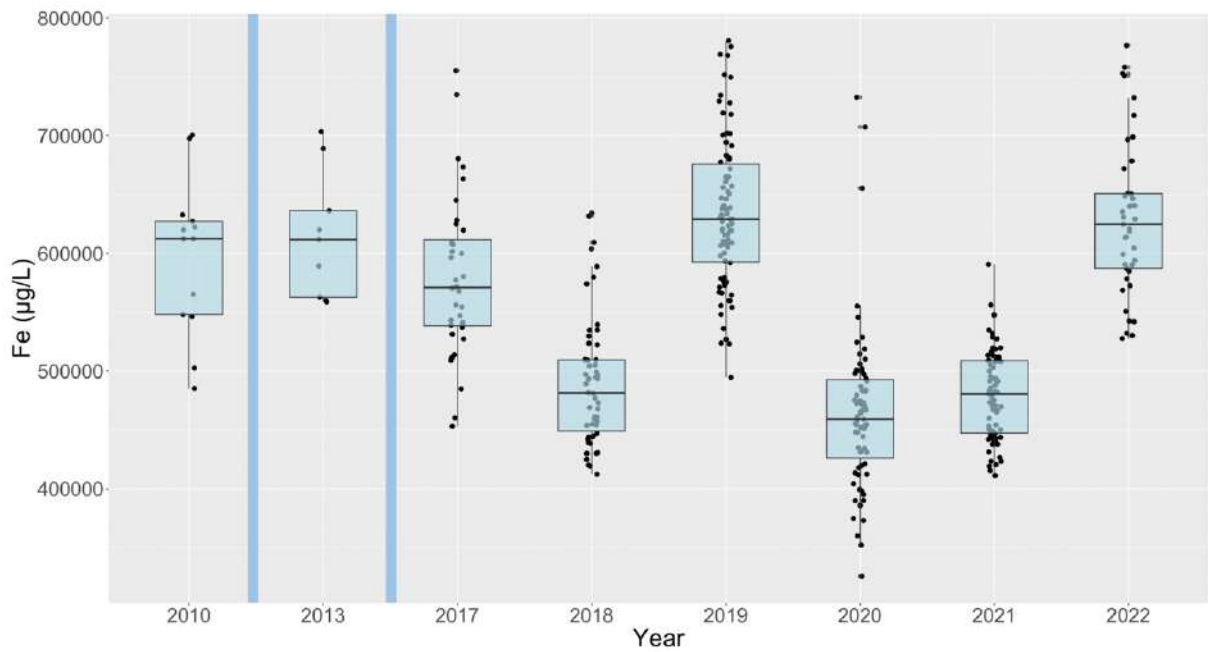


Figure 29: Annual variations in whole blood iron concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2010, 2013, and 2017-2022, from 384 blood samples. Source: Rstudio, by author.

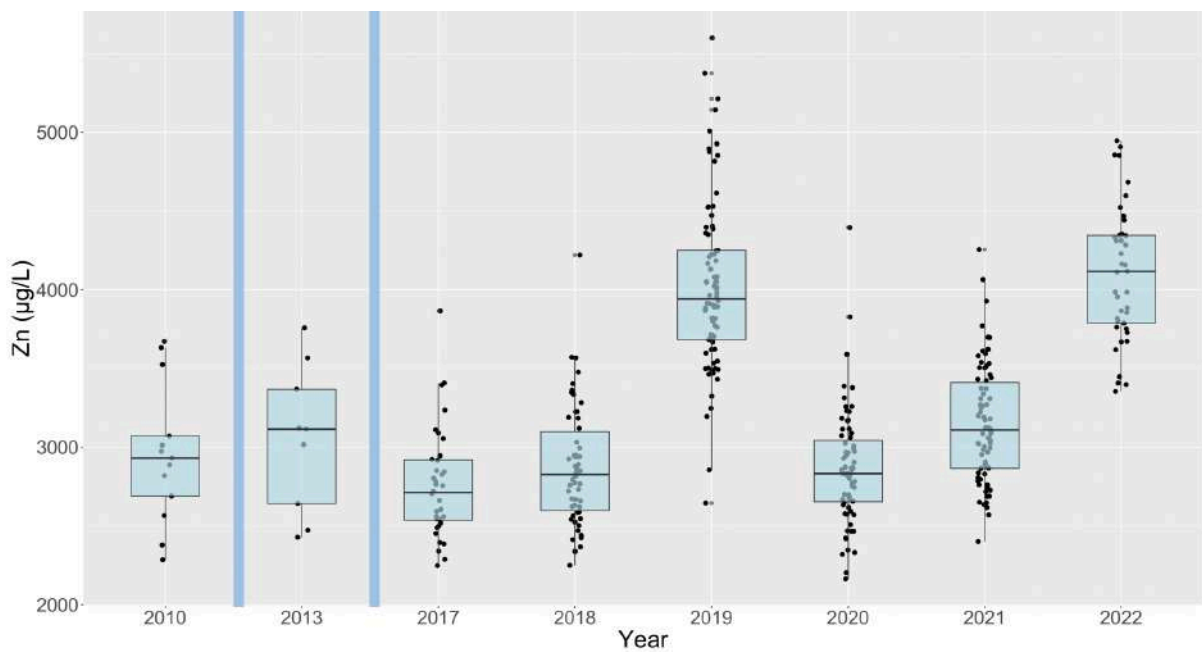


Figure 30: Annual variations in whole blood zinc concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2010, 2013, and 2017-2022, from 384 blood samples. Source: Rstudio, by author.

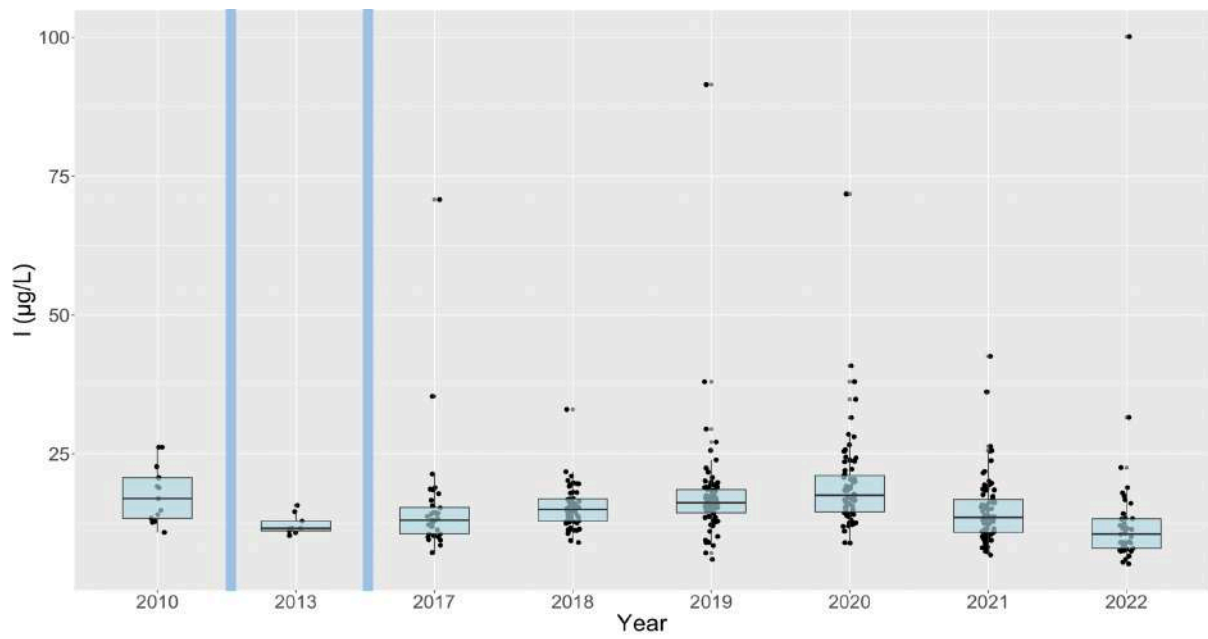


Figure 31: Annual variations in whole blood iodine concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2010, 2013, and 2017-2022, from 384 blood samples. Source: Rstudio, by author.

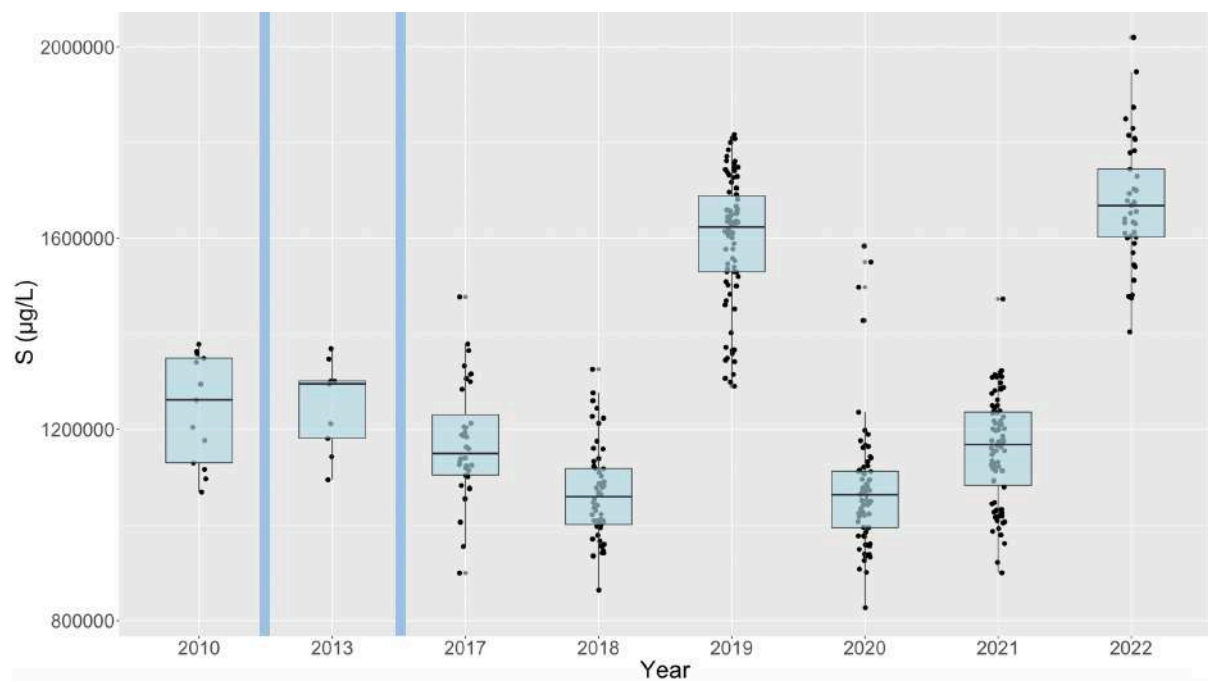


Figure 32: Annual variations in whole blood sulfur concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2010, 2013, and 2017-2022, from 384 blood samples. Source: Rstudio, by author.

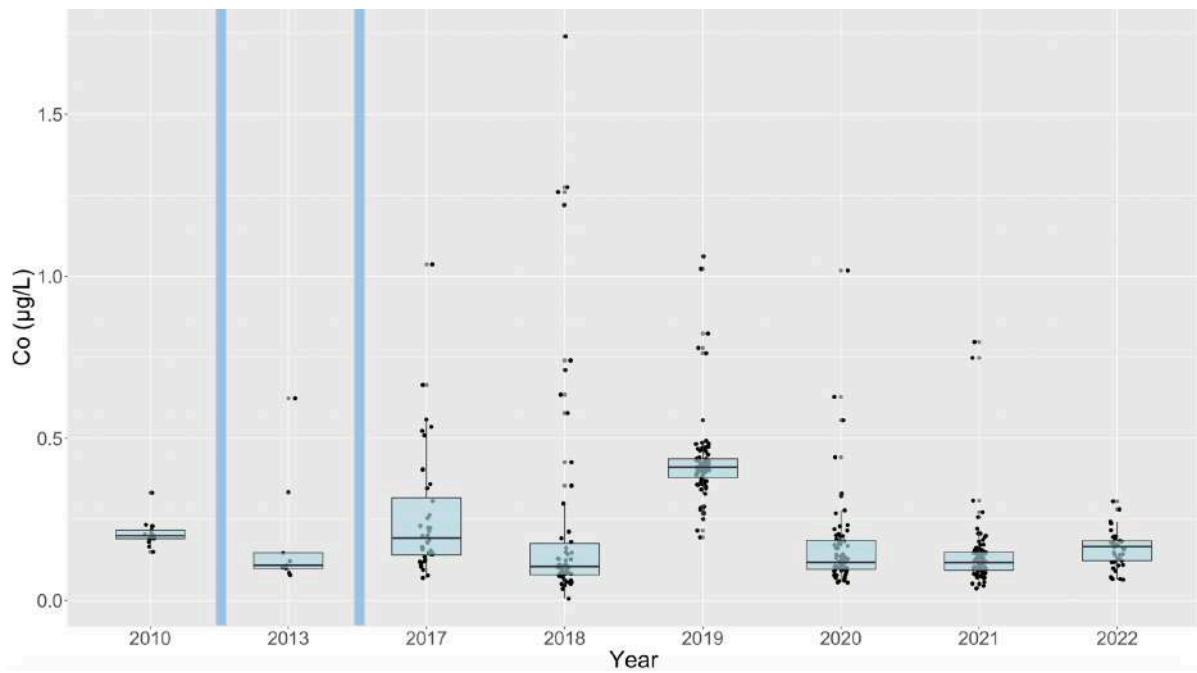


Figure 33: Annual variations in whole blood cobalt concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2010, 2013, and 2017-2022, from 384 blood samples. Source: Rstudio, by author.

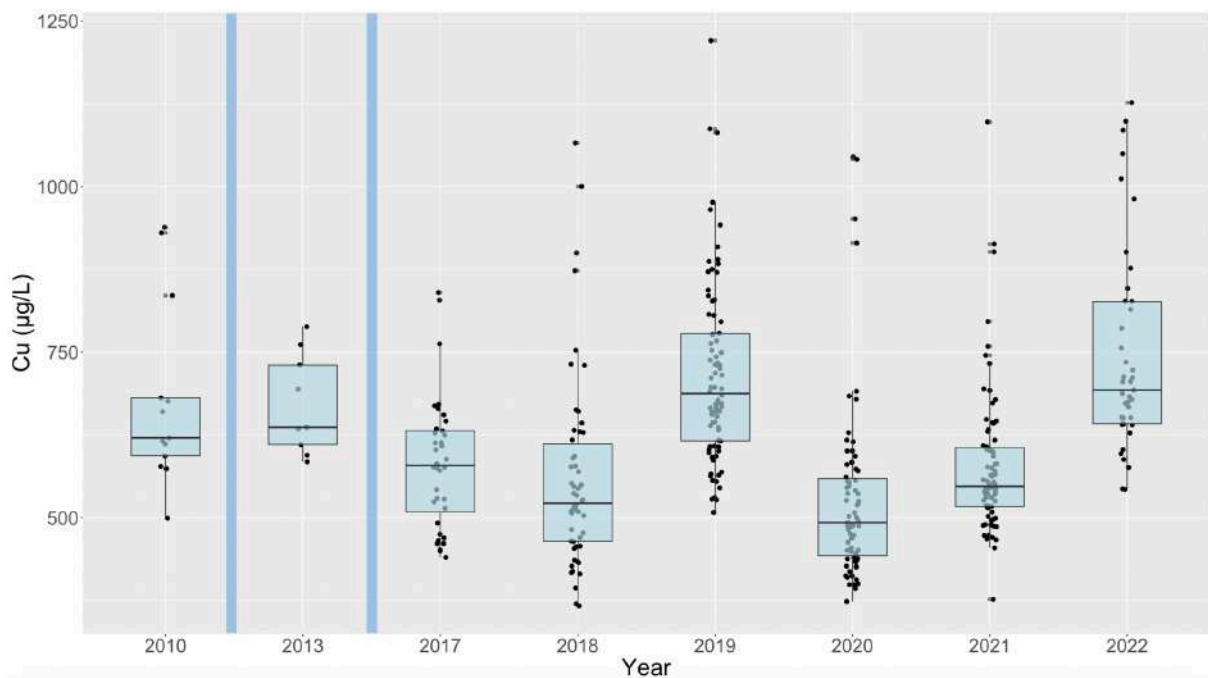


Figure 34: Annual variations in whole blood copper concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2010, 2013, and 2017-2022, from 384 blood samples. Source: Rstudio, by author.

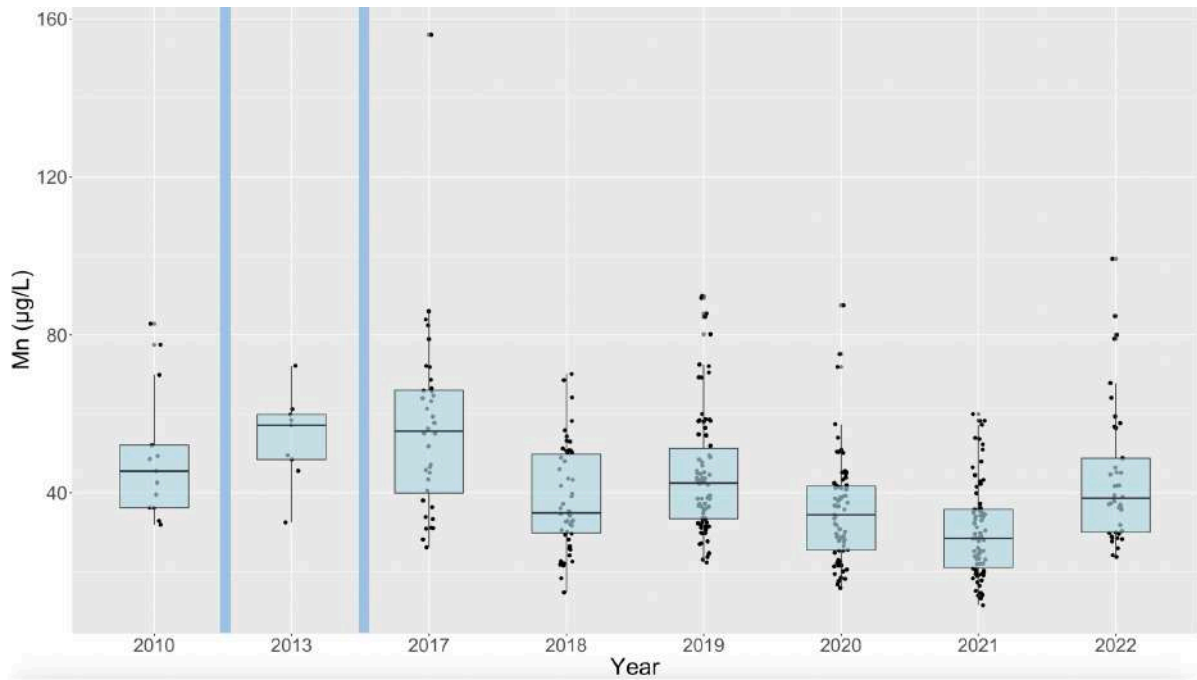


Figure 35: Annual variations in whole blood manganese concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2010, 2013, and 2017-2022, from 384 blood samples. Source: Rstudio, by author.

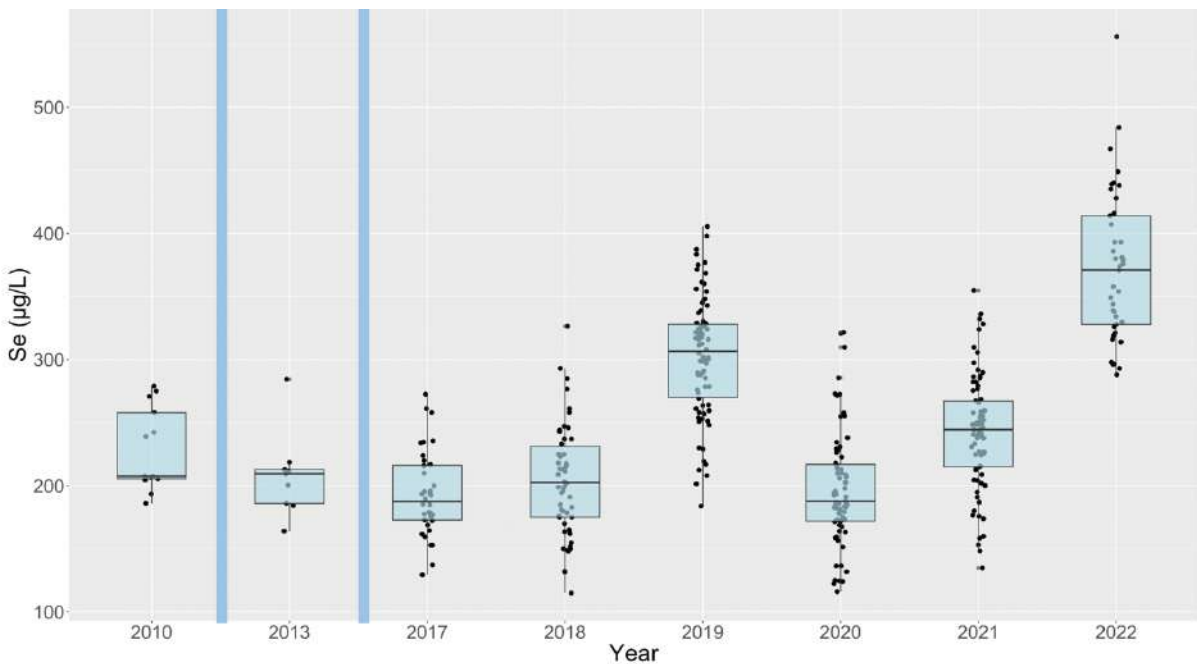


Figure 36: Annual variations in whole blood selenium concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2010, 2013, and 2017-2022, from 384 blood samples. Source: Rstudio, by author.

The boxplots displaying the mineral blood concentrations from the 384 blood samples for each essential mineral reveal variations among years. Not all minerals show the same trend in variation, for instance there is a trend observed for several essential minerals where the values seem higher in the years 2019 and 2022 compared to the other years. At first glance, P, Zn, S and Se show higher values in 2019 and 2022 than the other years. Other minerals, such as Ca, Na, Mg and Fe show lower concentrations in the years 2018, 2020 and 2021 compared to the other years. On the other hand, I, Co, Cu and Mn display a relatively homogenous distribution of concentrations according to the years.

6.2.1.1 Normal distribution test: the Shapiro-Wilk test

In order to compare and interpret the results of the values of the twelve essential minerals and their variances according to years, verifying normality and the homogeneity of variances is a requirement for running statistical tests such as parametric or non-parametric ANOVA tests. Testing for linearity was conducted to see whether the data followed a linear model or not. Linear regression and other linear modeling techniques assume that the relationship between variables is linear. If the data doesn't follow a linear model, using a linear model for analysis may produce incorrect or unreliable results. To test whether the values of the essential minerals followed a linear model with the year and bear variables, a Shapiro-Wilk normality test was used in Rstudio. The null hypothesis (H_0) states that the variable is normally distributed, and the alternative hypothesis (H_1) states that the variable is not normally distributed. Here, the chosen alpha level was 0.05 ($\alpha = 0.05$). All the p-values for each essential mineral were below 1.13×10^{-13} and therefore 0.05. We can state with a 95% confidence interval that the values of the twelve essential minerals did not follow a normal distribution.

6.2.1.2 Test for homogeneity of group variances: the Fligner-Killeen test

Knowing that the data did not follow a normal distribution, the Fligner-Killeen test was a suitable choice to check for homogeneity of variances with the year and bear variables. All the p-values for each essential mineral were inferior to 0.05, which was the chosen alpha level. We can state with a 95% confidence interval that the values of the twelve essential minerals had significant variances among groups.

6.2.1.3 Non-parametric ANOVA test: the Scheirer-Ray-Hare test

Since the mineral concentrations didn't follow a normal distribution, a non-parametric ANOVA test, the Scheirer-Ray-Hare test, was performed in Rstudio to investigate the significance of differences with the factors "year" and "bear". All p-values were well below 0.05, indicating significant differences in concentrations among years. This test does not identify however which specific groups differ from each other, but tells us that there are differences among groups as a whole. The p-values < 0.05 indicate that at least one group is different from the others. Therefore, post-hoc tests were performed, using the Wilcoxon rank-sum test with Bonferroni correction for multiple comparisons. For iodine, manganese, cobalt, and copper, the Wilcoxon rank-sum test was unable to return an exact p-value due to tied values and displayed approximations. The results of the Wilcoxon test for these four minerals were therefore based on approximations. An attempt to perform an alternative post-hoc test with the Dunn test was conducted but the results did not reflect the visual trend of concentrations according to the years.

6.2.2 Seasonal variations in whole blood mineral concentrations

Boxplots and scatter plots were performed for each essential mineral from all 384 samples, displaying the values and their seasonal variations within each year. For clarity reasons, only the boxplots and scatter plots from the TE tubes displayed in Figures 49-60 are presented in this study.

6.2.2.1 The Shapiro-Wilk and Fligner-Killeen tests

In order to compare and interpret the results of the values of the twelve essential minerals and their variances according to seasons, verifying normality and the homogeneity of variances was required. Here, the chosen alpha level was 0.05 ($\alpha = 0.05$). All the p-values for each essential mineral were below 1.79×10^{-10} and therefore 0.05. We can state with a 95% confidence interval that the values of the twelve essential minerals did not follow a normal distribution. Knowing that the data didn't follow a normal distribution, the Fligner-Killeen test was a suitable choice to check for homogeneity of variances with the season and bear variables. All the p-values for each essential mineral were superior to 0.05 except for manganese (p-value = 1.98×10^{-2}). We can state with a 95% confidence interval that the values of all essential minerals except for Mn didn't have significant variances among groups.

6.2.2.2 Non-parametric ANOVA test: the Scheirer-Ray-Hare test

Since the mineral concentrations didn't follow a normal distribution, a non-parametric ANOVA test, the Scheirer-Ray-Hare test, was performed in Rstudio to investigate the significance of differences with the factors "season" and "bear". All p-values were below 0.05, except for Ca (p-value = 0.35), indicating that all essential minerals except Ca had significant differences in concentrations among seasons. Some minerals had p-values well below 0.05 ($< 4.0 \times 10^{-7}$), which included Se, Cu, S, and Fe, displaying the most important seasonal variations.

6.3 Reference intervals according to EDTA, TE and HE tubes

The mean, sd, and reference interval for all twelve essential minerals according to the different tube types were calculated and shown in Tables 19 to 21.

Table 19: Mean, sd, and reference interval of the twelve essential minerals from 232 blood samples using trace element tubes. Essential mineral concentrations are expressed in $\mu\text{g/L}$. Source: Rstudio, by author.

Mineral	Mean	SD	Reference interval
Ca ($\mu\text{g/L}$)	5.12×10^4	8.03×10^3	$[5.02 \times 10^4 - 5.23 \times 10^4]$
P ($\mu\text{g/L}$)	5.12×10^5	1.18×10^5	$[4.96 \times 10^5 - 5.27 \times 10^5]$
Na ($\mu\text{g/L}$)	2.52×10^6	3.76×10^5	$[2.47 \times 10^6 - 5.57 \times 10^6]$
Mg ($\mu\text{g/L}$)	4.10×10^4	9.52×10^3	$[3.98 \times 10^4 - 4.22 \times 10^4]$
Fe ($\mu\text{g/L}$)	5.43×10^4	1.04×10^5	$[5.30 \times 10^5 - 5.57 \times 10^5]$
Zn ($\mu\text{g/L}$)	3.47×10^3	6.77×10^2	$[3.38 \times 10^3 - 3.56 \times 10^3]$
I ($\mu\text{g/L}$)	1.69×10^1	1.04×10^1	$[1.56 \times 10^1 - 1.83 \times 10^1]$
S ($\mu\text{g/L}$)	1.33×10^6	2.94×10^5	$[1.30 \times 10^6 - 1.37 \times 10^6]$
Co ($\mu\text{g/L}$)	2.30×10^{-1}	1.70×10^{-1}	$[2.09 \times 10^{-1} - 2.54 \times 10^{-1}]$
Cu ($\mu\text{g/L}$)	6.39×10^2	1.65×10^2	$[6.17 \times 10^2 - 6.60 \times 10^2]$
Mn ($\mu\text{g/L}$)	3.87×10^1	1.57×10^1	$[3.67 \times 10^1 - 4.07 \times 10^1]$
Se ($\mu\text{g/L}$)	2.68×10^2	8.03×10^1	$[2.58 \times 10^2 - 2.78 \times 10^2]$

Table 20: Mean, sd, and reference interval of the twelve essential minerals from 21 blood samples using heparin tubes. Essential mineral concentrations are expressed in $\mu\text{g/L}$. Source: Rstudio, by author.

Mineral	Mean	SD	Reference interval
Ca ($\mu\text{g/L}$)	4.67×10^4	4.56×10^3	$[4.46 \times 10^4 - 4.88 \times 10^4]$
P ($\mu\text{g/L}$)	4.38×10^4	2.38×10^4	$[4.27 \times 10^5 - 4.49 \times 10^5]$
Na ($\mu\text{g/L}$)	2.36×10^6	5.00×10^4	$[2.34 \times 10^6 - 2.38 \times 10^6]$
Mg ($\mu\text{g/L}$)	3.69×10^4	3.23×10^3	$[3.54 \times 10^4 - 3.83 \times 10^4]$
Fe ($\mu\text{g/L}$)	5.08×10^5	3.65×10^4	$[4.91 \times 10^5 - 5.25 \times 10^5]$
Zn ($\mu\text{g/L}$)	3.04×10^3	3.60×10^2	$[2.87 \times 10^3 - 3.20 \times 10^3]$
I ($\mu\text{g/L}$)	1.38×10^1	3.77×10^0	$[1.21 \times 10^1 - 1.56 \times 10^1]$
S ($\mu\text{g/L}$)	1.24×10^6	6.19×10^4	$[1.21 \times 10^6 - 1.27 \times 10^6]$
Co ($\mu\text{g/L}$)	1.31×10^{-1}	1.46×10^{-1}	$[6.45 \times 10^{-2} - 1.98 \times 10^{-1}]$
Cu ($\mu\text{g/L}$)	5.23×10^2	6.44×10^1	$[4.94 \times 10^2 - 5.53 \times 10^2]$
Mn ($\mu\text{g/L}$)	2.69×10^1	1.07×10^1	$[2.21 \times 10^1 - 3.18 \times 10^1]$
Se ($\mu\text{g/L}$)	2.44×10^2	3.57×10^1	$[2.28 \times 10^2 - 2.60 \times 10^2]$

Table 21: Mean, sd, and reference interval of the twelve essential minerals from 131 blood samples using EDTA tubes. Essential mineral concentrations are expressed in µg/L. Source: Rstudio, by author.

Mineral	Mean	SD	Reference interval
Ca (µg/L)	5.26×10^4	6.90×10^3	$[5.14 \times 10^4 - 5.37 \times 10^4]$
P (µg/L)	4.03×10^5	6.99×10^4	$[3.91 \times 10^5 - 4.15 \times 10^5]$
Na (µg/L)	2.51×10^6	2.77×10^5	$[2.46 \times 10^6 - 2.55 \times 10^6]$
Mg (µg/L)	4.00×10^4	7.29×10^3	$[3.87 \times 10^4 - 4.12 \times 10^4]$
Fe (µg/L)	5.51×10^5	7.86×10^4	$[5.38 \times 10^5 - 5.65 \times 10^5]$
Zn (µg/L)	3.05×10^3	6.15×10^2	$[2.94 \times 10^3 - 3.15 \times 10^3]$
I (µg/L)	1.55×10^1	6.43×10^0	$[1.44 \times 10^1 - 1.66 \times 10^1]$
S (µg/L)	1.21×10^6	2.10×10^5	$[1.17 \times 10^6 - 1.24 \times 10^6]$
Co (µg/L)	2.70×10^{-1}	2.70×10^{-1}	$[2.23 \times 10^{-1} - 3.16 \times 10^{-1}]$
Cu (µg/L)	6.02×10^2	1.31×10^2	$[5.79 \times 10^2 - 6.24 \times 10^2]$
Mn (µg/L)	4.62×10^1	1.90×10^1	$[4.30 \times 10^1 - 4.95 \times 10^1]$
Se (µg/L)	2.18×10^2	5.33×10^1	$[2.09 \times 10^2 - 2.27 \times 10^2]$

As stated in the introduction, in order to obtain population-based RIs, blood should be sampled from a large number of animals (a minimum of 120 ideally), with a mixture of ages and genders. In this study, according to the above definition, RIs were correctly obtained for the TE tubes (n=232) and EDTA tubes (n=131) but not enough samples regarding the heparin tubes (n=21) allow us to define the obtained intervals as RIs per se. The intervals using heparin tubes (Table 20) give us an idea of the values for each essential mineral but further larger-scale investigations are required to establish reliable RIs in free-ranging Scandinavian brown bears.

In Figures 25-36, annual variations in the twelve essential minerals were observed. One of the factors potentially playing a role in those variations were the types of tubes. In order to be able to compare the mean values for each mineral according to the different tube types (EDTA, HE and TE), statistical tests needed to be performed. A Shapiro-Wilk test was performed in order to assess if the data followed a normal distribution and a Fligner-Killeen test was performed to assess homogeneity of variances. For both tests, all the p-values were far below 0.05, which was the chosen alpha level. The values of the essential minerals did not follow a normal distribution and there were significant differences of variances between types of tube. The standard one-way analysis of variance (ANOVA) assumes that the data follows a normal distribution, which wasn't the case with the data used in this study. Therefore, the statistical test to be performed was a non-parametric ANOVA test, such as the Kruskal-Wallis test. The latter was performed to assess whether there were significant differences in the blood concentrations of the twelve essential minerals among the three types of tube (EDTA, HE and

TE). In other words, the investigation whether the choice of tube type had a significant effect on the essential mineral concentrations was performed. The Kruskal-Wallis test displayed a p-value far below 0.05, indicating significant differences in mineral concentrations among the three tube types. In order to know more precisely where these differences lied, a post-hoc analysis was performed, using pairwise Wilcoxon rank-sum tests which compared each pair of tube types. This allowed to see which essential minerals had significantly different concentrations from one tube type to another. The results of the Wilcoxon test are shown in Table 22.

Table 22: Results of the post-hoc Wilcoxon test. “” indicates a p-value < 0.05 and therefore significant. Source: Rstudio, by author.*

Tube	Mineral	Comparison	p-value EDTA	p-value HE
HE	Ca	vs.	$9.99 \times 10^{-4*}$	-
TE		vs.	2.56×10^{-1}	$3.54 \times 10^{-2*}$
HE	P	vs.	$1.24 \times 10^{-4*}$	-
TE		vs.	$2.41 \times 10^{-18*}$	$1.00 \times 10^{-2*}$
HE	Na	vs.	3.10×10^{-1}	-
TE		vs.	1.00×10^0	1.00×10^0
HE	Mg	vs.	2.13×10^{-1}	-
TE		vs.	1.00×10^0	2.06×10^{-1}
HE	Fe	vs.	5.03×10^{-2}	-
TE		vs.	6.34×10^{-1}	1.00×10^0
HE	Zn	vs.	1.00×10^0	-
TE		vs.	$2.39 \times 10^{-9*}$	$1.46 \times 10^{-2*}$
HE	I	vs.	6.46×10^{-1}	-
TE		vs.	6.82×10^{-1}	2.61×10^{-1}
HE	S	vs.	6.15×10^{-2}	-
TE		vs.	$2.06 \times 10^{-3*}$	1.00×10^0
HE	Co	vs.	$1.56 \times 10^{-3*}$	-
TE		vs.	1.00×10^0	$2.62 \times 10^{-4*}$
HE	Cu	vs.	$1.76 \times 10^{-2*}$	-
TE		vs.	2.58×10^{-1}	$1.19 \times 10^{-3*}$
HE	Mn	vs.	$2.23 \times 10^{-6*}$	-
TE		vs.	$1.37 \times 10^{-4*}$	$5.35 \times 10^{-4*}$
HE	Se	vs.	$1.28 \times 10^{-2*}$	-
TE		vs.	$6.94 \times 10^{-9*}$	6.20×10^{-1}

This table summarizes the differences in mean values for the twelve essential minerals and shows that there are significant differences from one tube type to another. The asterisks (*) next to the p-value have been added to indicate statistically significant differences in the mean values of each mineral between the respective tubes (EDTA vs. HE, EDTA vs. TE and HE vs. TE), with a chosen significance level of 0.05. Out of the twelve essential minerals, four didn't show any significant differences among the different tubes (highlighted in green in Table 22). Sodium, magnesium, iron and iodine are the four essential minerals that didn't show concentrations significantly different from one tube to another. On the other hand,

phosphorus and manganese showed significantly different concentrations between all three tube types (highlighted in orange in Table 22). Significant differences between EDTA and TE tubes include phosphorus, zinc, sulfur, manganese and selenium. Significant differences between EDTA and HE tubes include calcium, phosphorus, cobalt, copper, manganese and selenium. Significant differences between TE and HE tubes include calcium, phosphorus, zinc, cobalt, copper and manganese. We therefore can't regroup all the mean values together (EDTA, HE and TE together) in the following analysis since there are significant differences in the mean values between tube types. In order to simplify the analysis, the seasonal and annual variations of the twelve essential minerals were analyzed in the following sections with the TE tubes only.

6.3 Mineral concentration analysis in trace element tubes

Boxplots and scatter plots are presented in Figures 37-48 for each essential mineral, displaying their values and annual variations. The blood mineral concentration analysis according to years and seasons included 232 samples from TE tubes (169 samples from females, 63 samples from males), from 117 different individuals (74 females, 43 males).

6.3.1 Annual variations in whole blood mineral concentrations in TE tubes

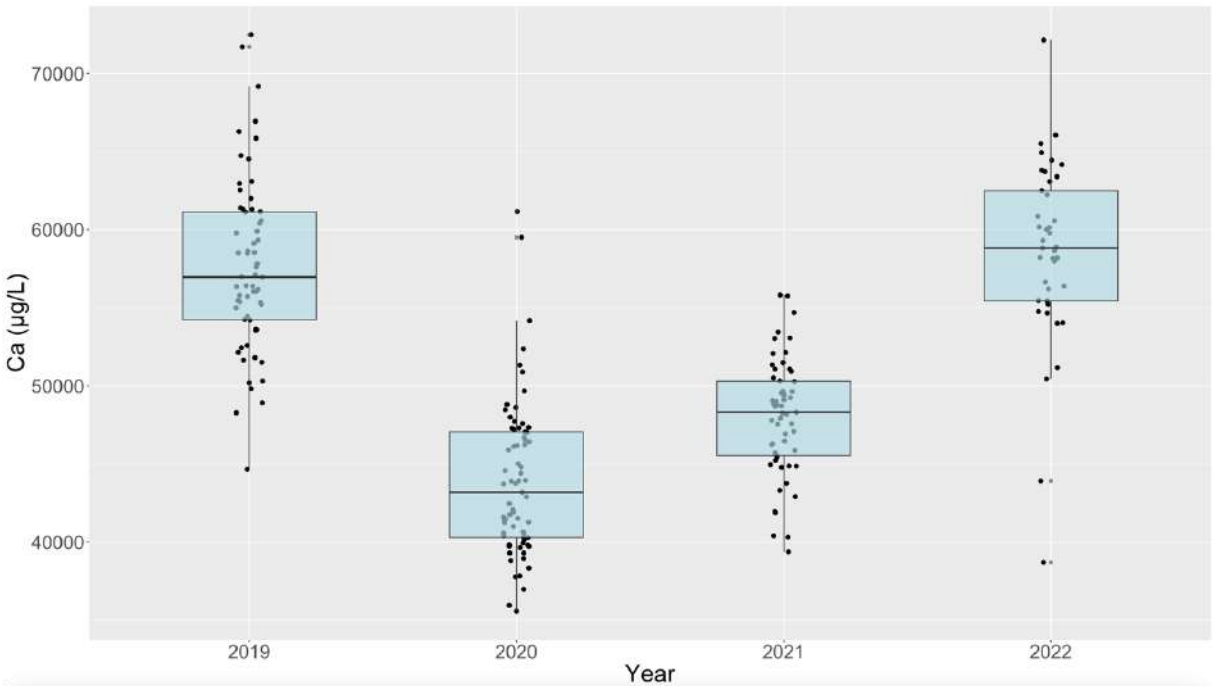


Figure 37: Annual variations in whole blood calcium concentrations (µg/L) in Scandinavian brown bears in the years 2019-2022, from 232 blood samples collected in trace element tubes. Source: Rstudio, by author.

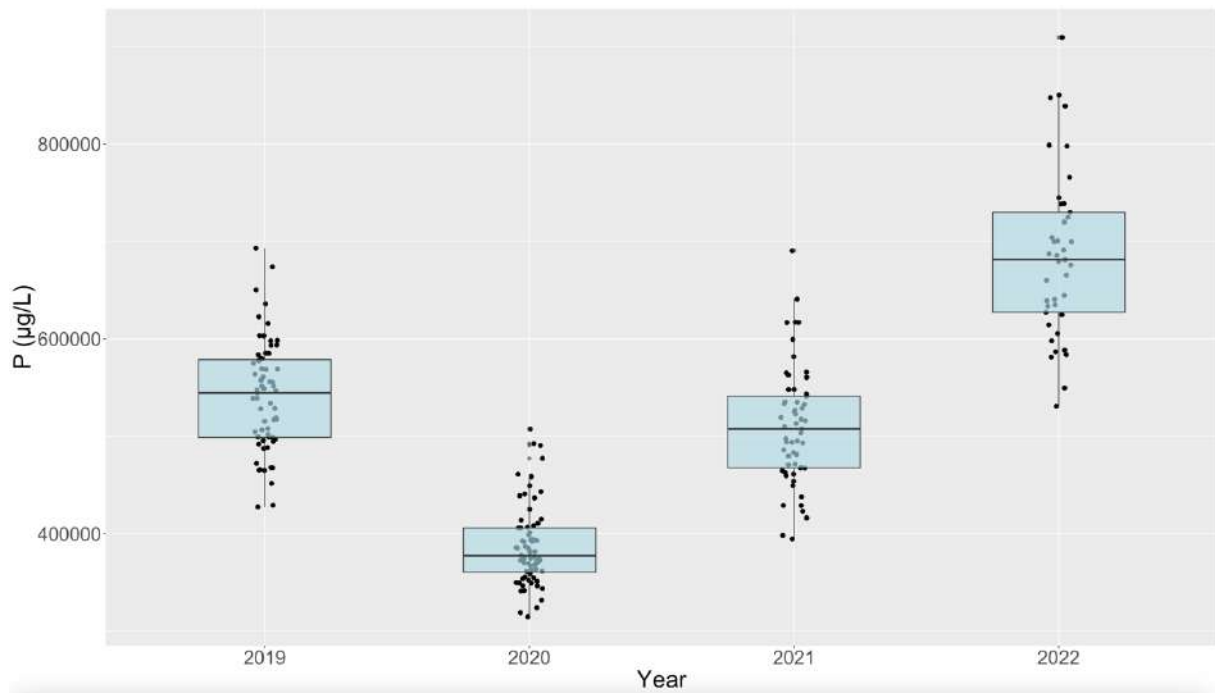


Figure 38: Annual variations in whole blood phosphorus concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2019-2022, from 232 blood samples collected in trace element tubes. Source: Rstudio, by author.

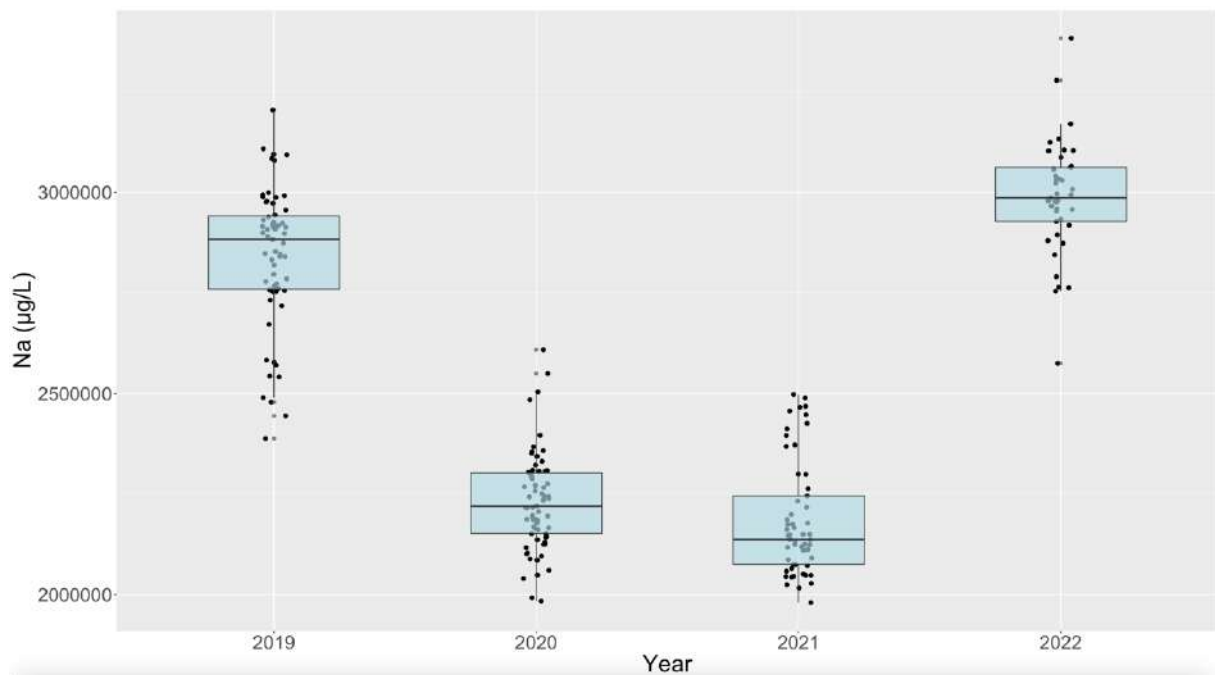


Figure 39: Annual variations in whole blood sodium concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2019-2022, from 232 blood samples collected in trace element tubes. Source: Rstudio, by author.

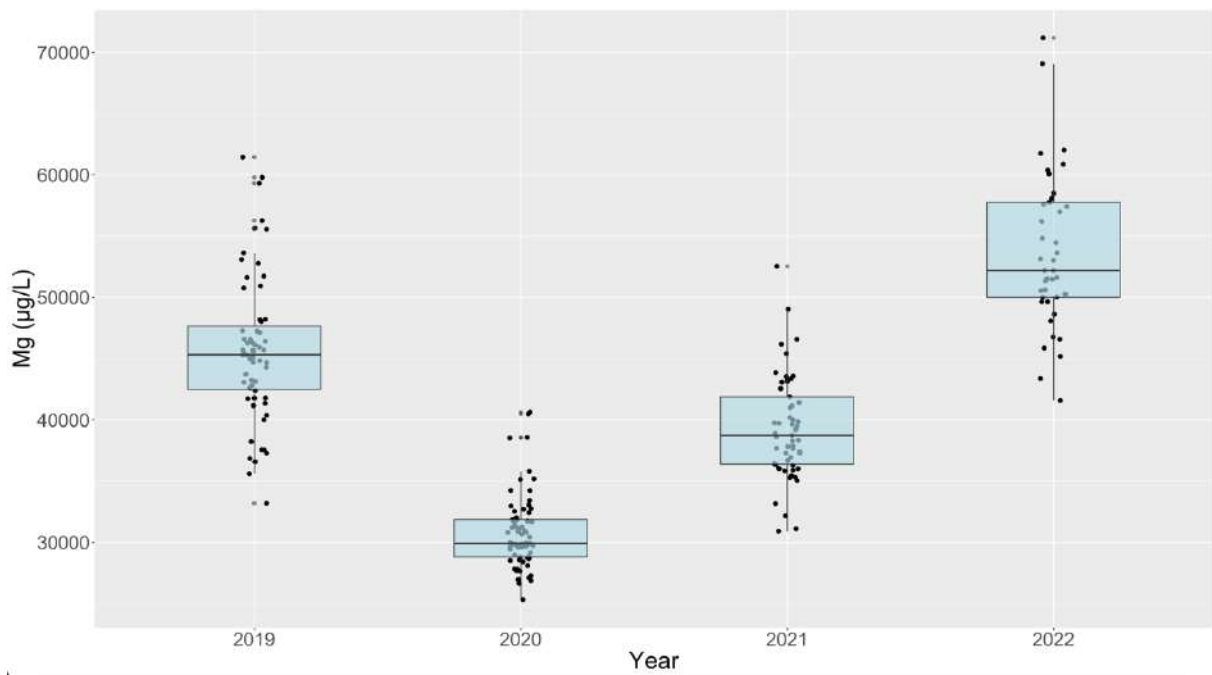


Figure 40: Annual variations in whole blood magnesium concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2019-2022, from 232 blood samples collected in trace element tubes. Source: Rstudio, by author.

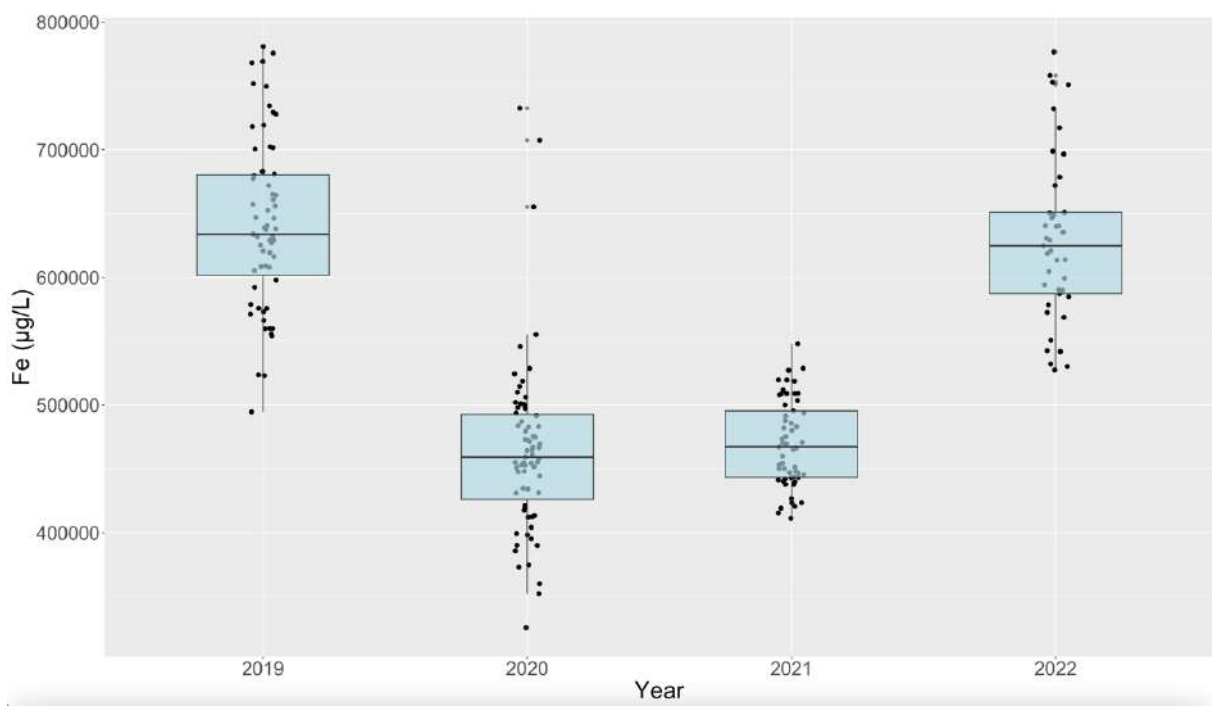


Figure 41: Annual variations in whole blood iron concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2019-2022, from 232 blood samples collected in trace element tubes. Source: Rstudio, by author.

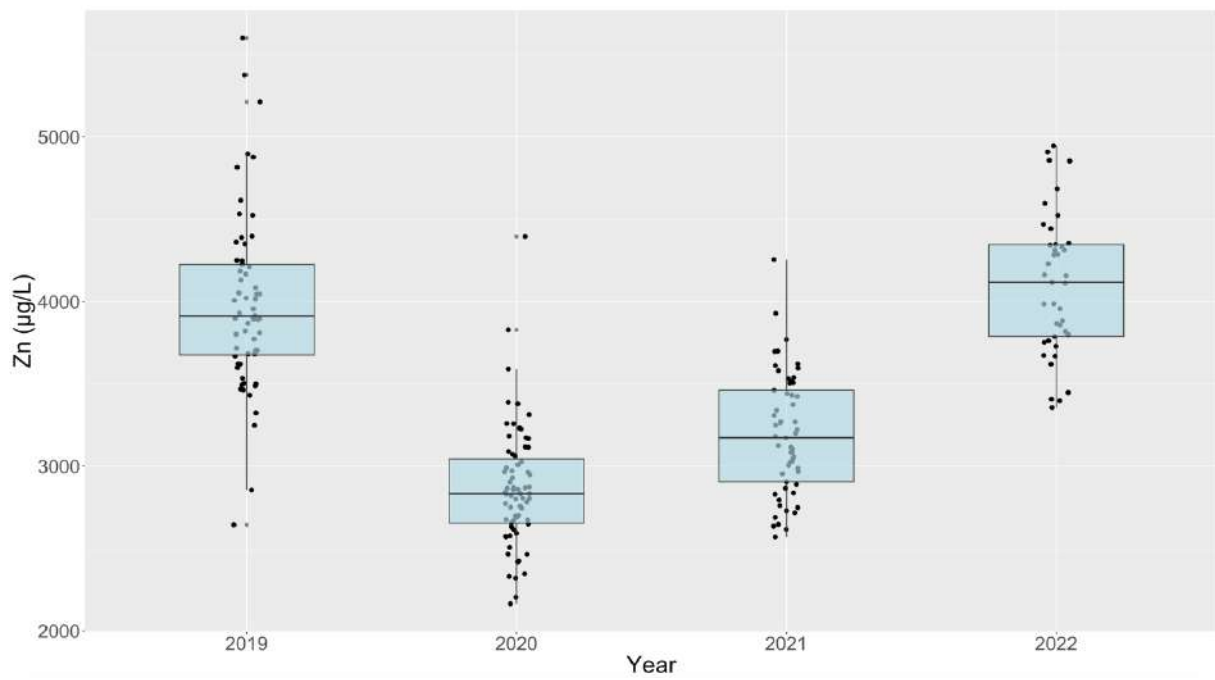


Figure 42: Annual variations in whole blood zinc concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2019-2022, from 232 blood samples collected in trace element tubes.
 Source: Rstudio, by author.

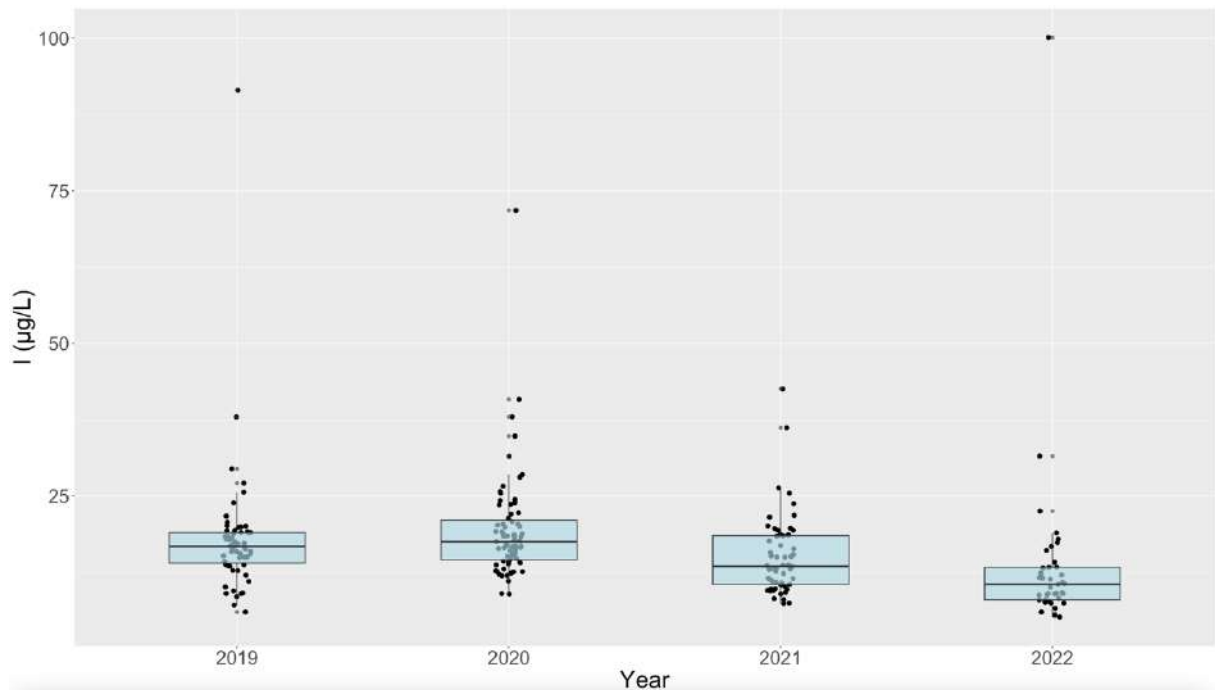


Figure 43: Annual variations in whole blood iodine concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2019-2022, from 232 blood samples collected in trace element tubes.
 Source: Rstudio, by author.

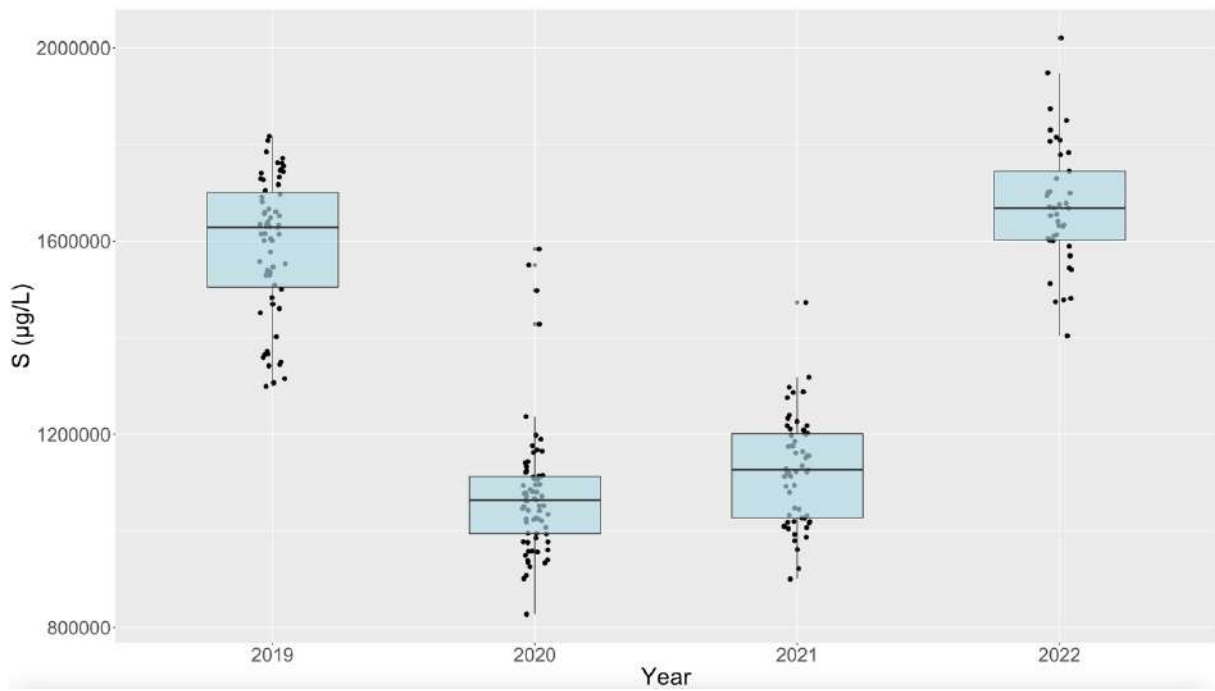


Figure 44: Annual variations in whole blood sulfur concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2019-2022, from 232 blood samples collected in trace element tubes. Source: Rstudio, by author.

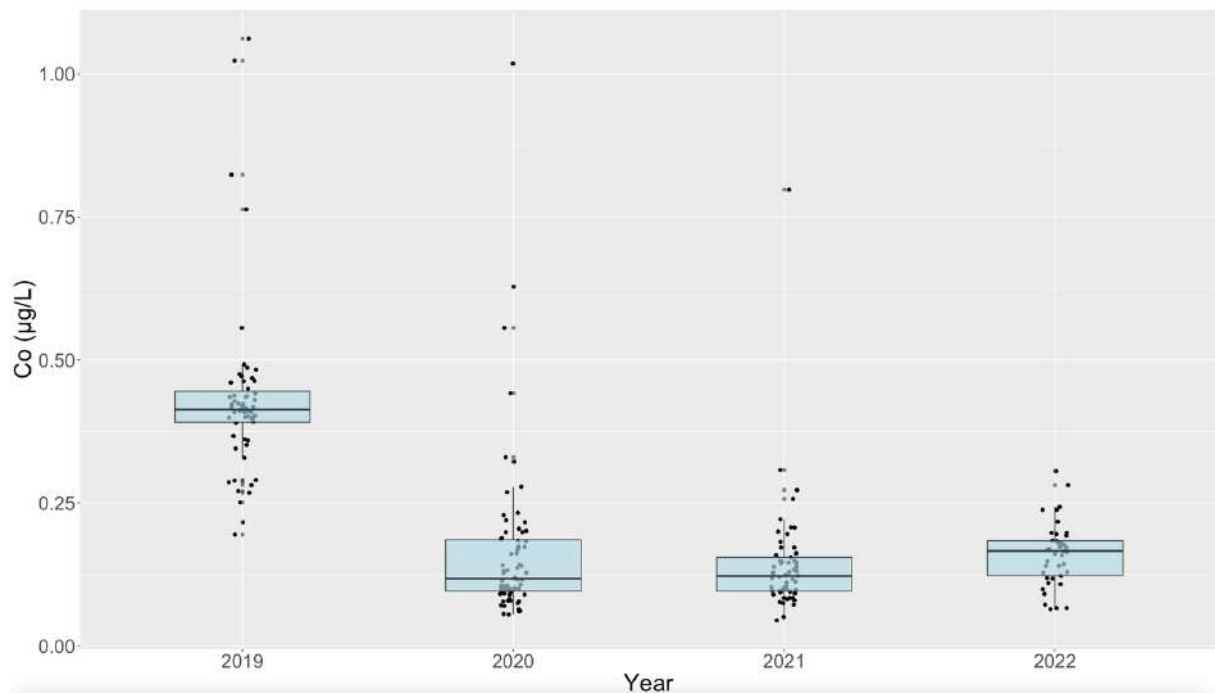


Figure 45: Annual variations in whole blood cobalt concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2019-2022, from 232 blood samples collected in trace element tubes. Source: Rstudio, by author.

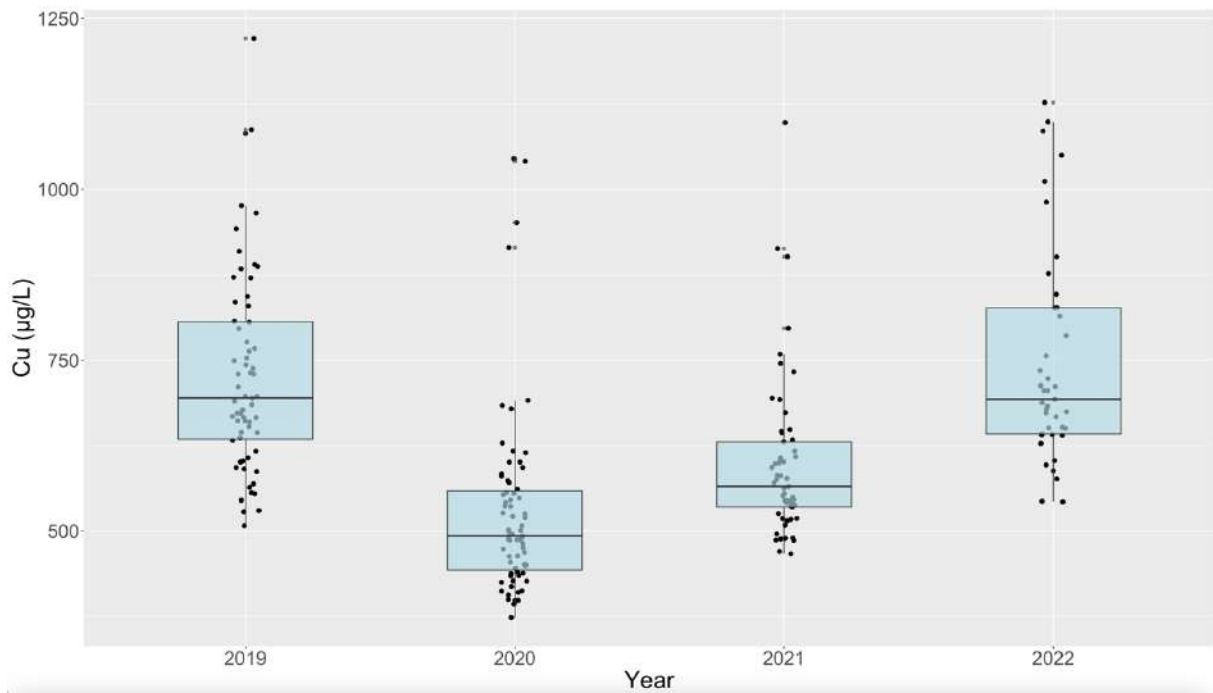


Figure 46: Annual variations in whole blood copper concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2019-2022, from 232 blood samples collected in trace element tubes.
Source: Rstudio, by author.

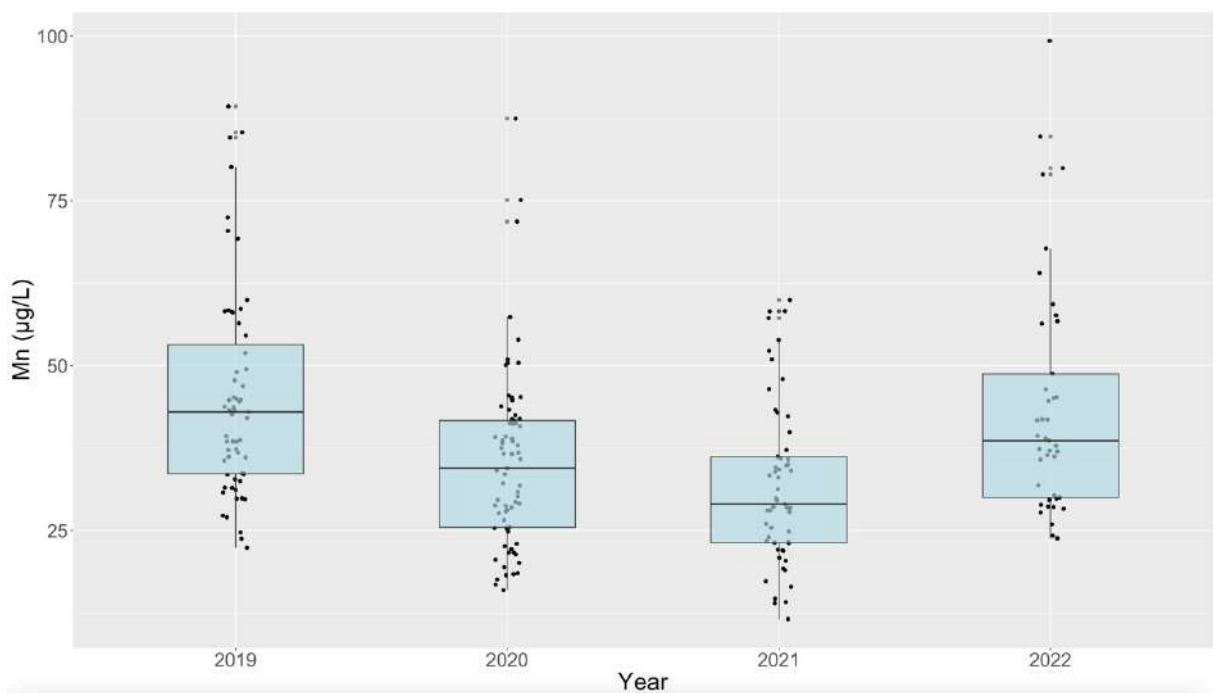


Figure 47: Annual variations in whole blood manganese concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2019-2022, from 232 blood samples collected in trace element tubes.
Source: Rstudio, by author.

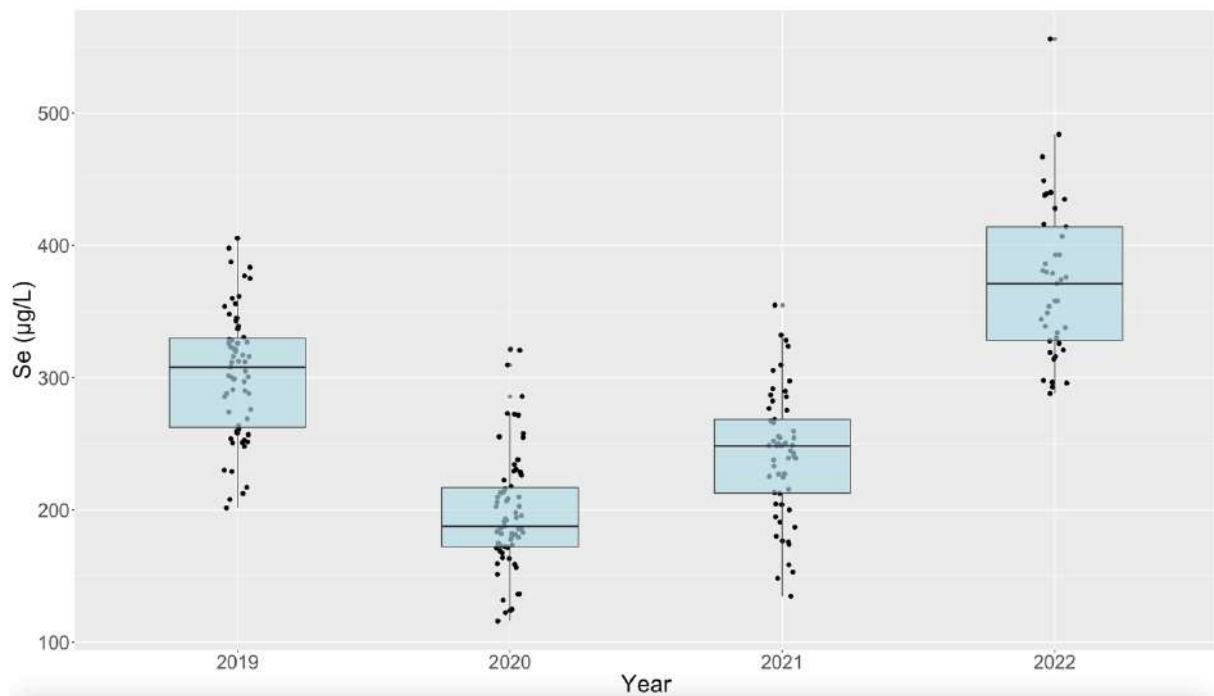


Figure 48: Annual variations in whole blood selenium concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2019-2022, from 232 blood samples collected in trace element tubes. Source: Rstudio, by author.

6.3.1.1 The Shapiro-Wilk and Fligner-Killeen tests

To test whether the values of the essential minerals sampled in TE tubes followed a linear model with the “year” and “bear” variables, a Shapiro-Wilk normality test was used. The chosen alpha level was 0.05 ($\alpha = 0.05$). All the p-values for each essential mineral were below 1.29×10^{-11} and therefore 0.05. We can state with a 95% confidence interval that the values of the twelve essential minerals sampled in TE tubes didn’t follow a normal distribution. Knowing that the data didn’t follow a normal distribution, the Fligner-Killeen test was a suitable choice to verify homogeneity of variances. The chosen alpha level was 0.05 ($\alpha = 0.05$). All the p-values of the Fligner-Killeen test were above 0.05, therefore there were no significant differences in variances across the groups.

6.3.1.2 Non-parametric ANOVA test: the Scheirer-Ray-Hare test

Since the mineral concentrations didn’t follow a normal distribution, a non-parametric ANOVA test, the Scheirer-Ray-Hare test, was performed in Rstudio to investigate the significance of differences with the factors “year” and “bear”. All p-values were well below 0.05, indicating significant differences in all mineral concentrations among years.

One particular boxplot that stood out was the boxplot displaying the annual variations of Na levels. 2019 and 2022 showed far higher means than the years 2020 and 2021. For instance, between 2021 and 2022, no values overlapped.

6.3.2 Seasonal variations in whole blood mineral concentrations in TE tubes

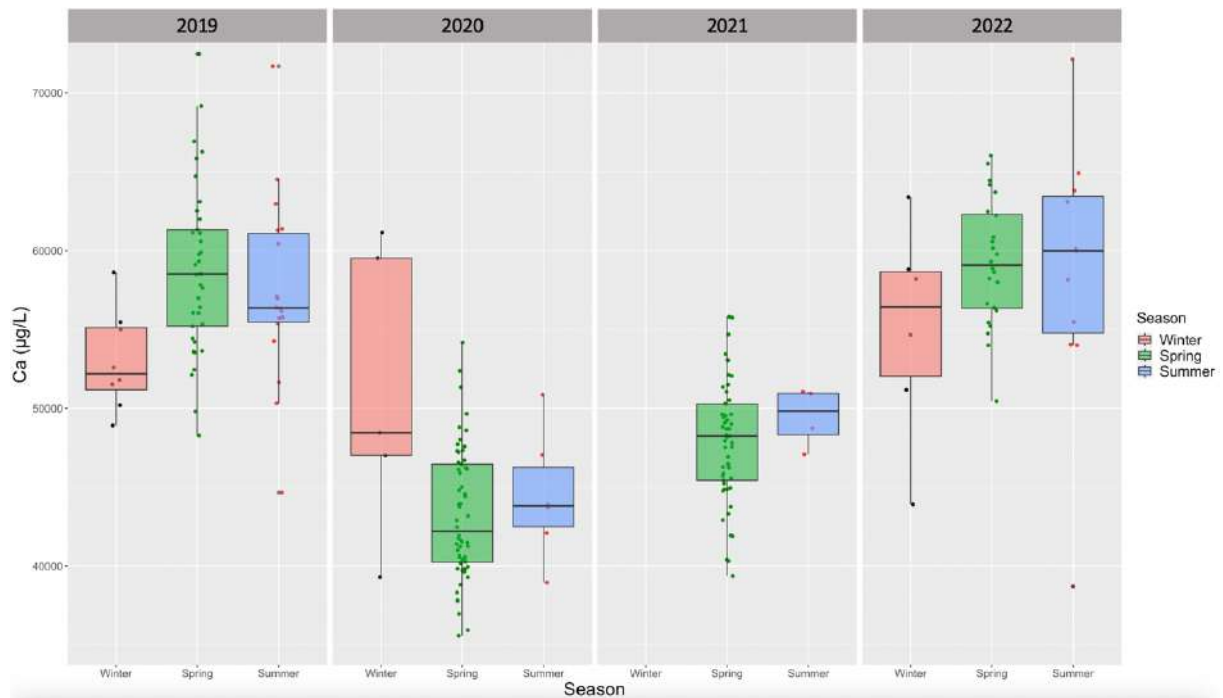


Figure 49: Seasonal variations in whole blood calcium concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2019-2022, from 232 blood samples collected in trace element tubes. Source: Rstudio, by author.

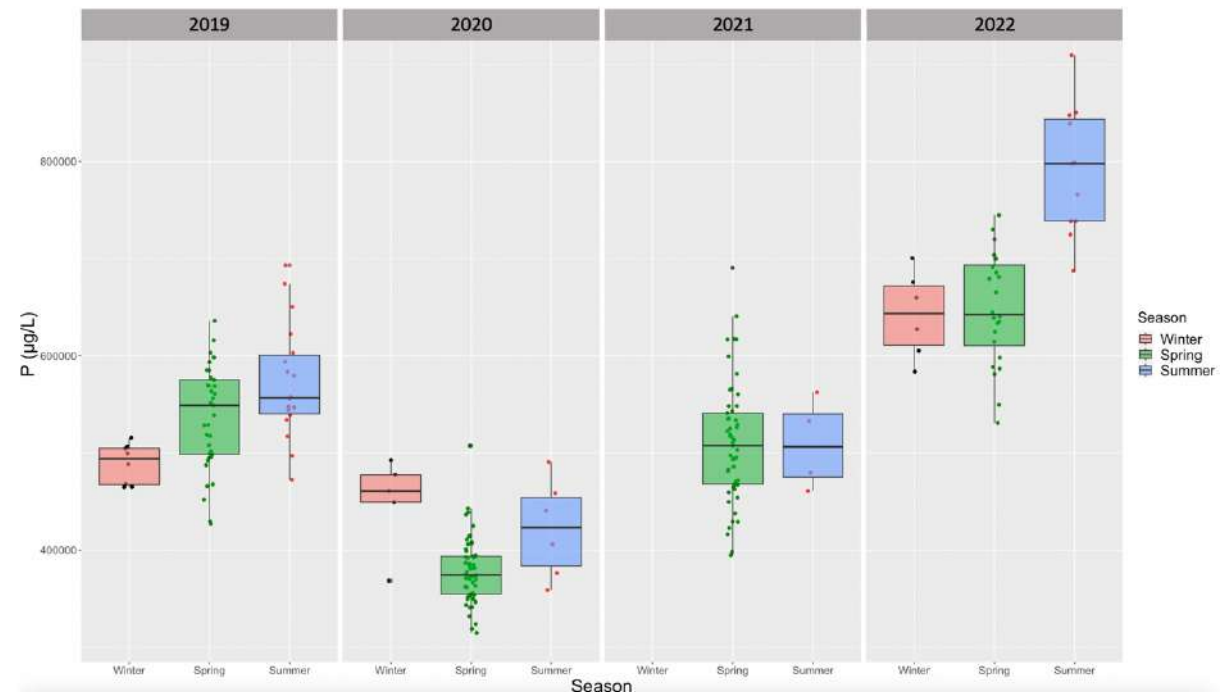


Figure 50: Seasonal variations in whole blood phosphorus concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2019-2022, from 232 blood samples collected in trace element tubes. Source: Rstudio, by author.

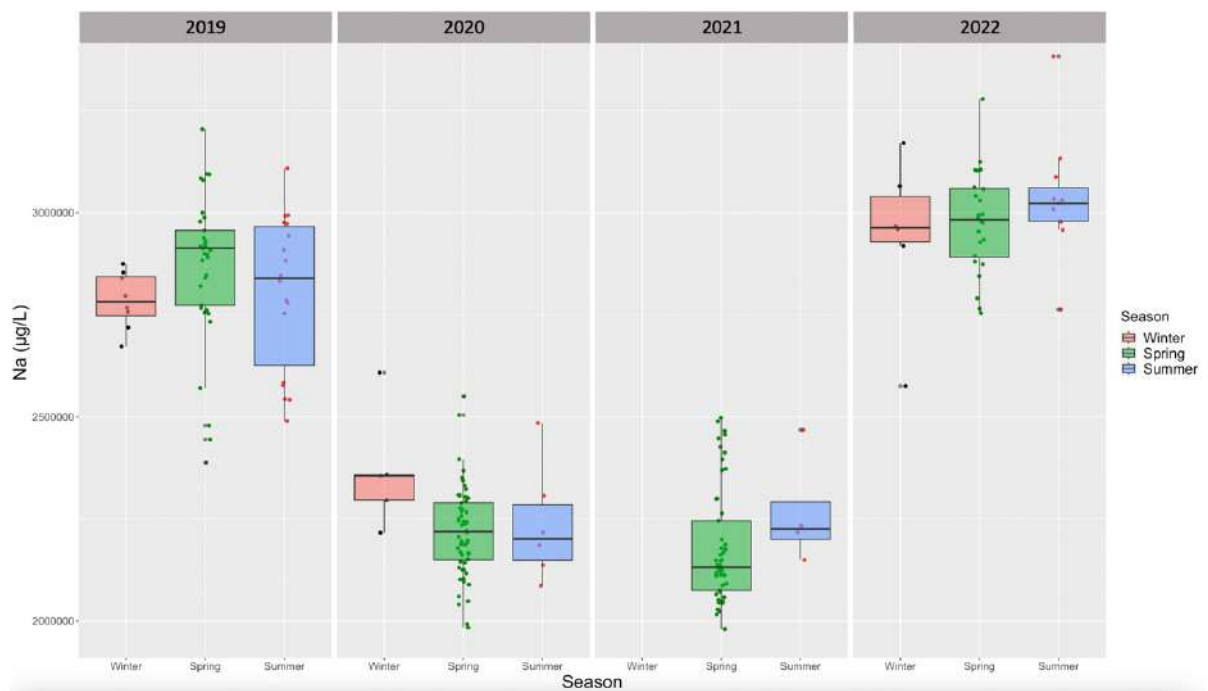


Figure 51: Seasonal variations in whole blood sodium concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2019-2022, from 232 blood samples collected in trace element tubes. Source: Rstudio, by author.

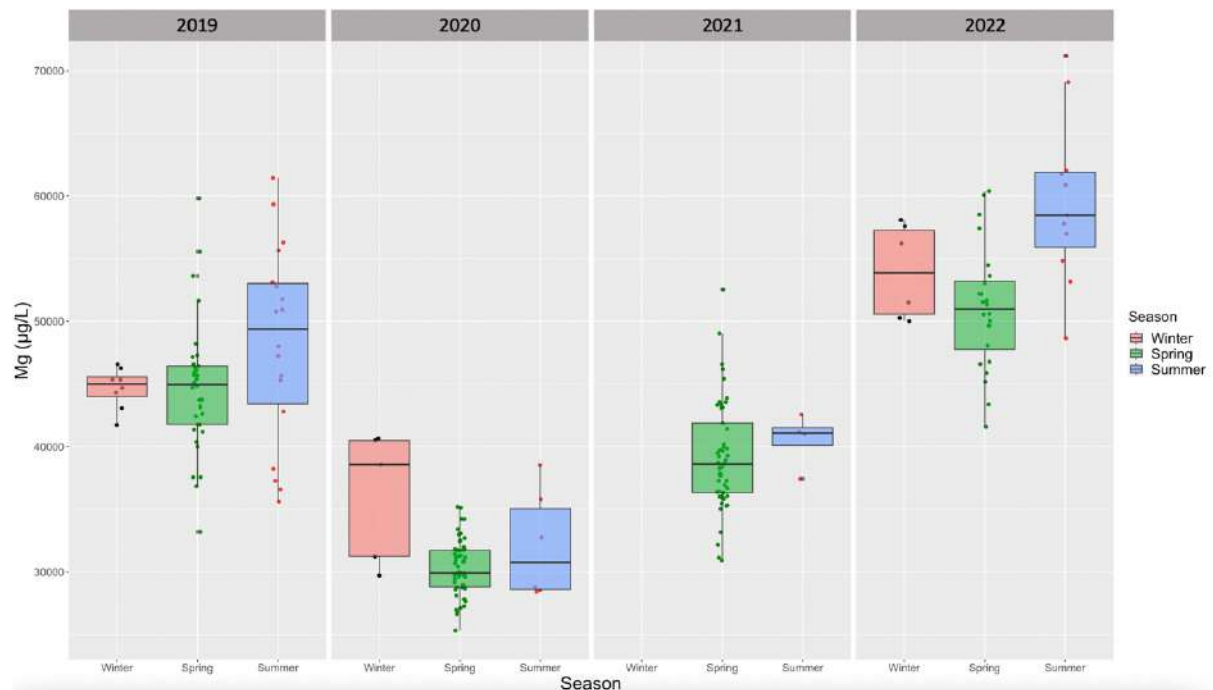


Figure 52: Seasonal variations in whole blood magnesium concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2019-2022, from 232 blood samples collected in trace element tubes. Source: Rstudio, by author.

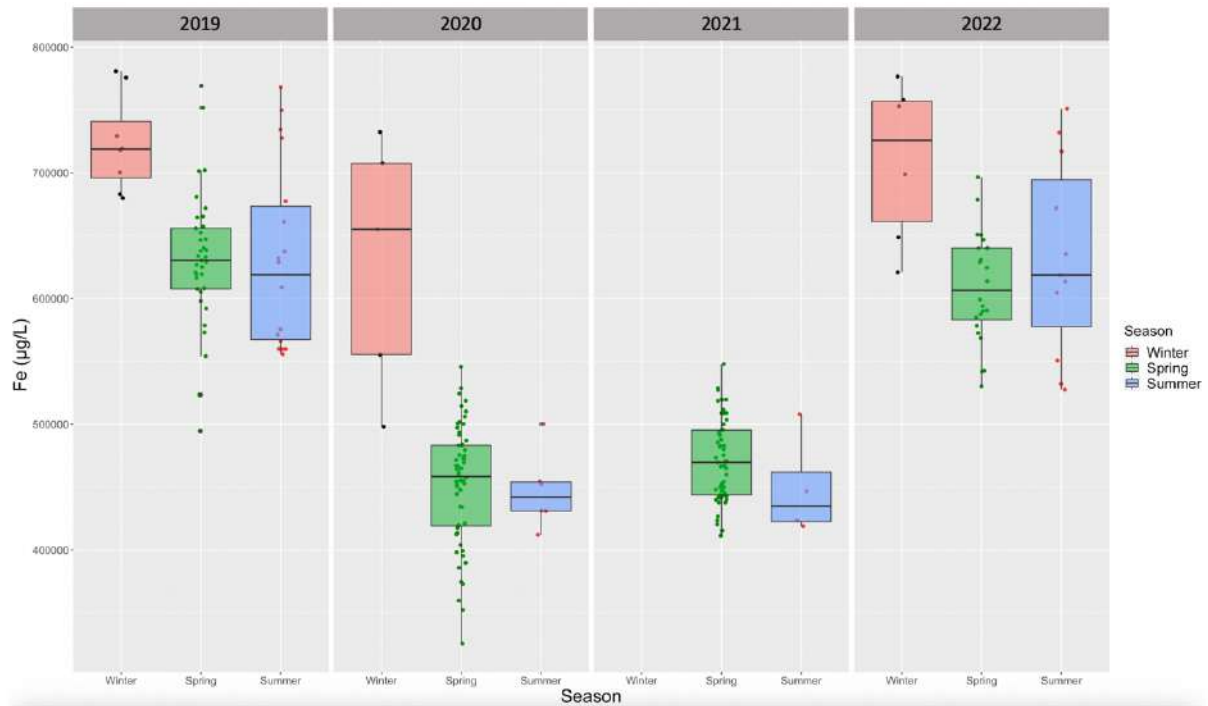


Figure 53: Seasonal variations in whole blood iron concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2019-2022, from 232 blood samples collected in trace element tubes. Source: Rstudio, by author.

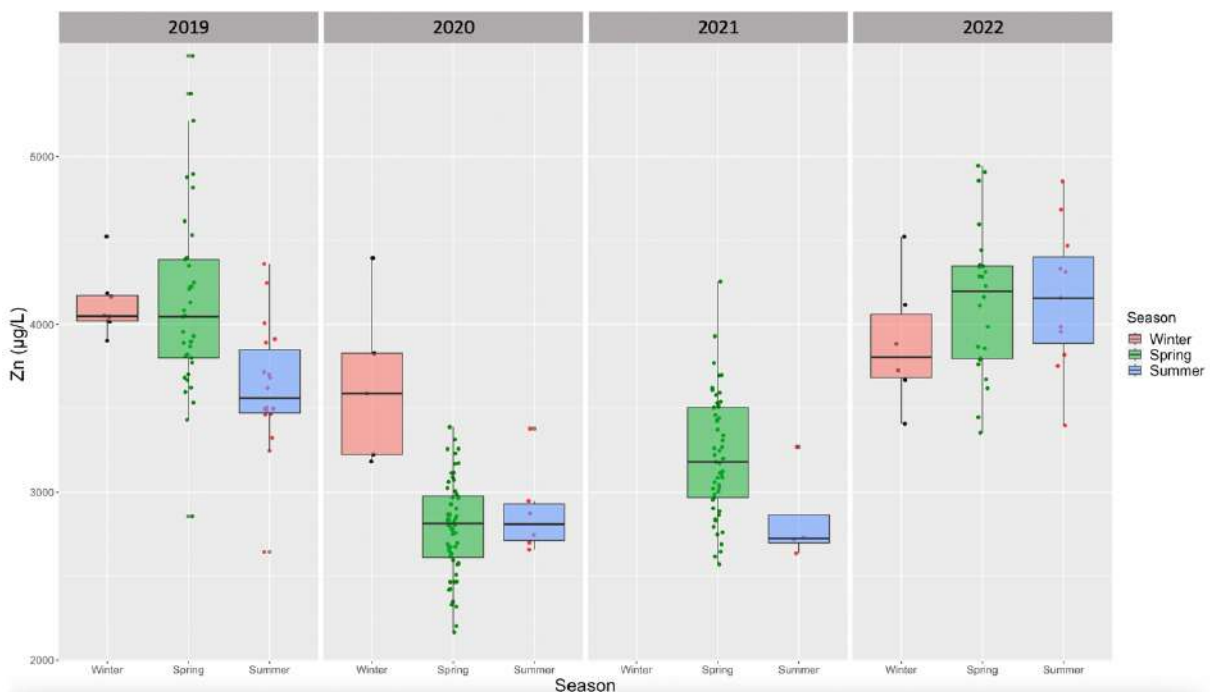


Figure 54: Seasonal variations in whole blood zinc concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2019-2022, from 232 blood samples collected in trace element tubes. Source: Rstudio, by author.

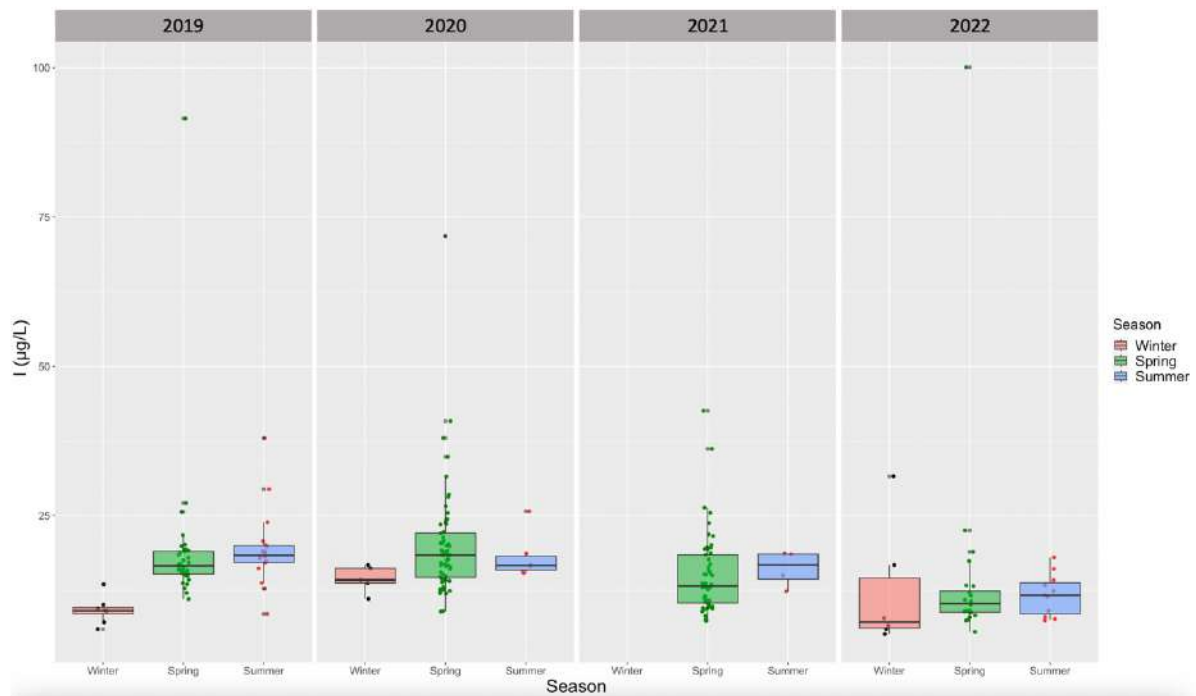


Figure 55: Seasonal variations in whole blood iodine concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2019-2022, from 232 blood samples collected in trace element tubes. Source: Rstudio, by author.

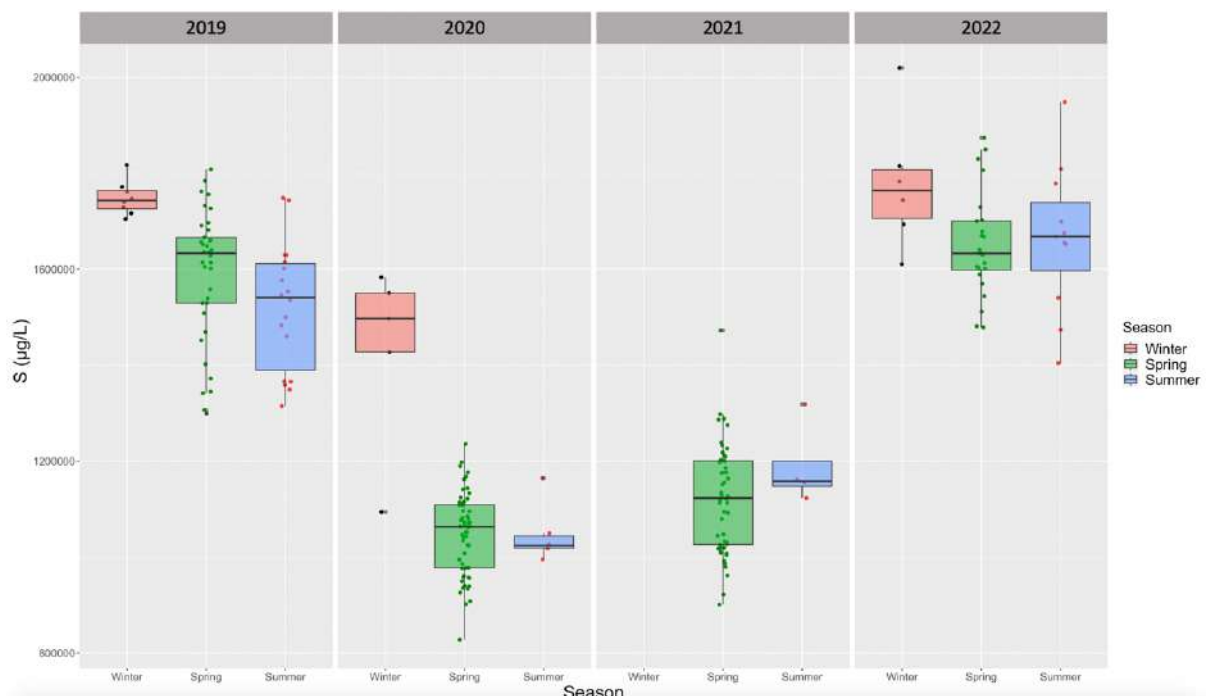


Figure 56: Seasonal variations in whole blood sulfur concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2019-2022, from 232 blood samples collected in trace element tubes. Source: Rstudio, by author.

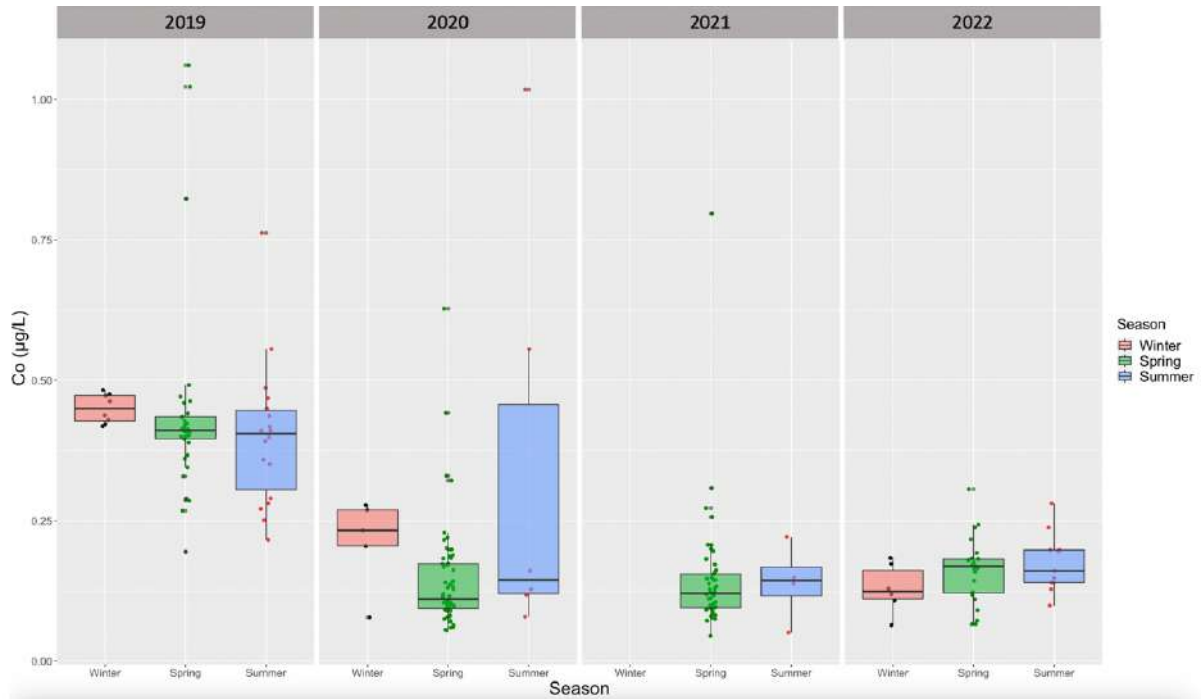


Figure 57: Seasonal variations in whole blood cobalt concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2019-2022, from 232 blood samples collected in trace element tubes. Source: Rstudio, by author.

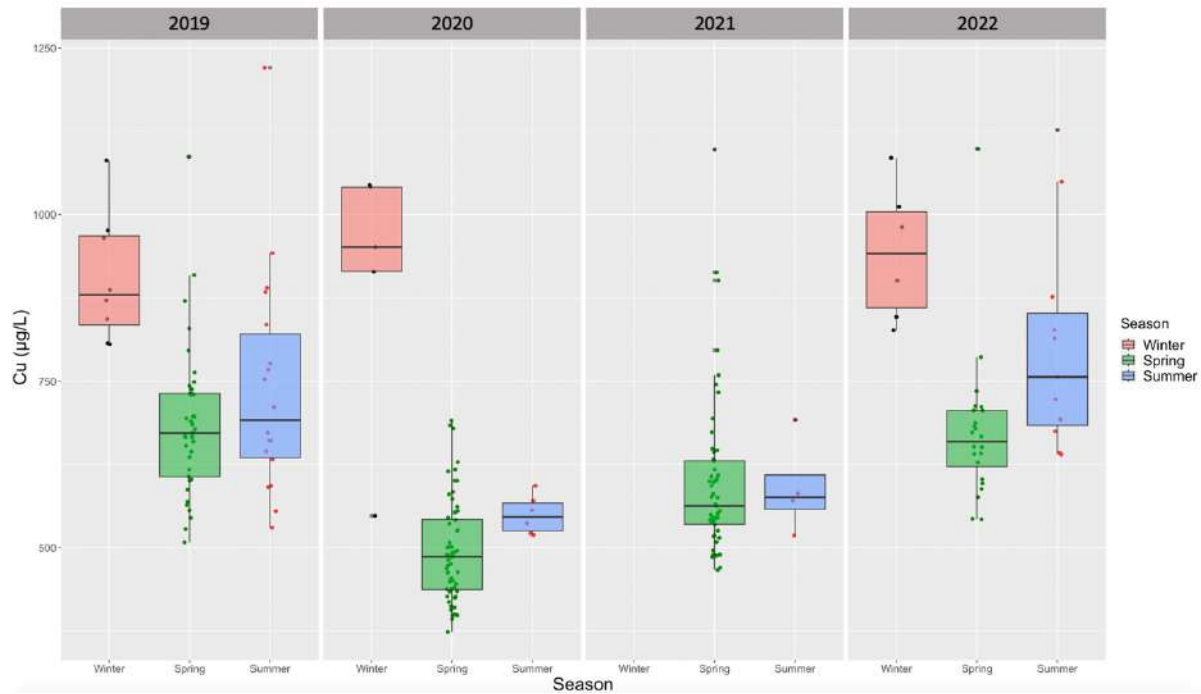


Figure 58: Seasonal variations in whole blood copper concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2019-2022, from 232 blood samples collected in trace element tubes. Source: Rstudio, by author.

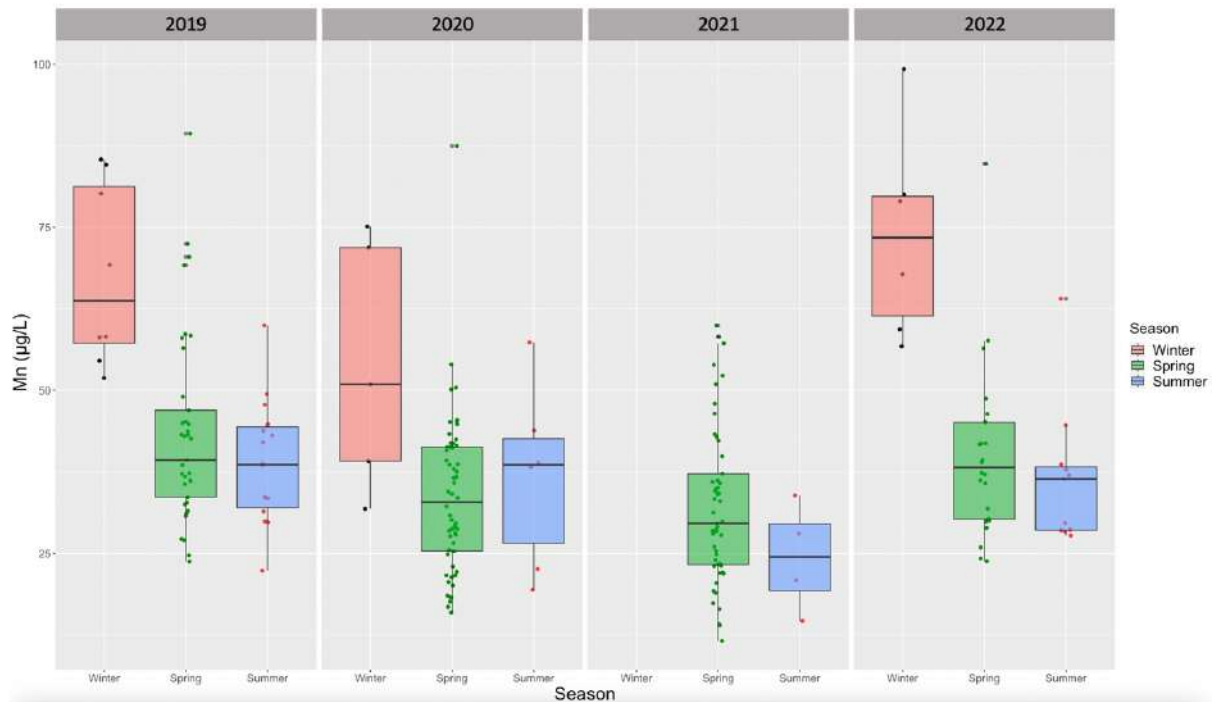


Figure 59: Seasonal variations in whole blood manganese concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2019-2022, from 232 blood samples collected in trace element tubes. Source: Rstudio, by author.

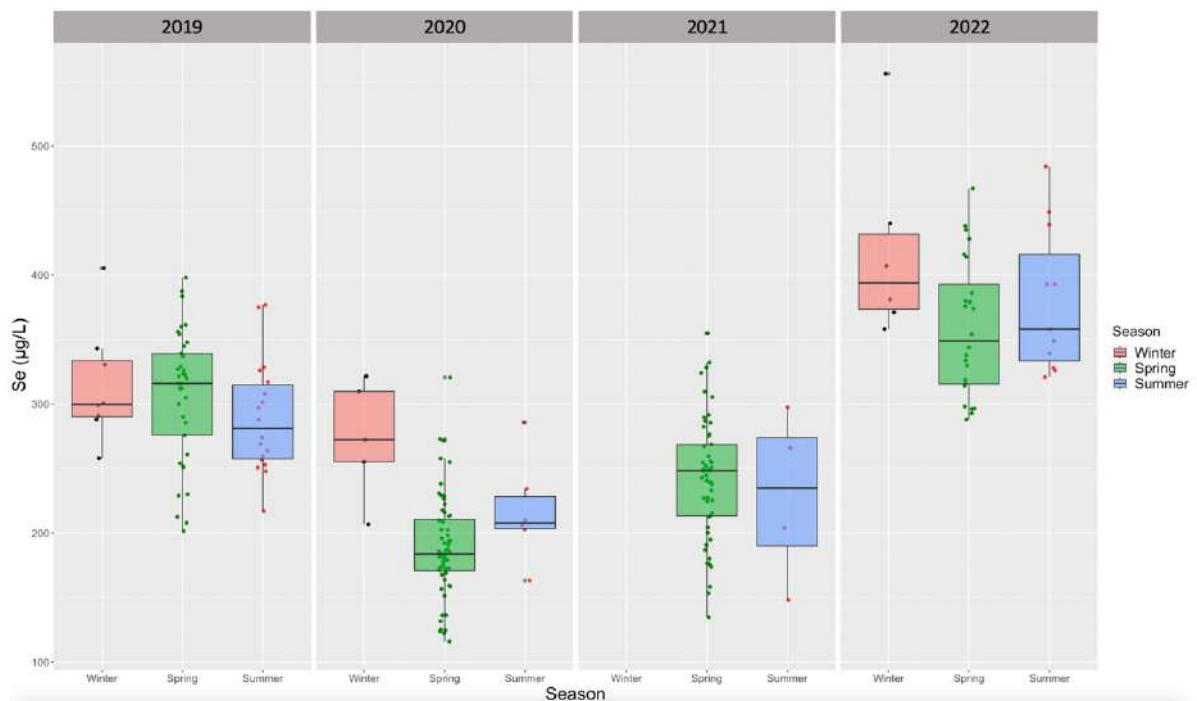


Figure 60: Seasonal variations in whole blood selenium concentrations ($\mu\text{g/L}$) in Scandinavian brown bears in the years 2019-2022, from 232 blood samples collected in trace element tubes. Source: Rstudio, by author.

6.3.2.1 The Shapiro-Wilk and Fligner-Killeen tests

To test whether the values of the essential minerals sampled in TE tubes followed a linear model with the “season” and “bear” variables, a Shapiro-Wilk normality test was used. The chosen alpha level was 0.05 ($\alpha = 0.05$). All the p-values for each essential mineral were below 1.24×10^{-8} and therefore 0.05. We can state with a 95% confidence interval that the values of the twelve essential minerals sampled in TE tubes didn’t follow a normal distribution. Knowing that the data did not follow a normal distribution, the Fligner-Killeen test was a suitable choice to verify homogeneity of variances. The chosen alpha level was 0.05 ($\alpha = 0.05$). All the p-values of the Fligner-Killeen test were above 0.05, therefore there were no significant differences in variances across the groups.

6.3.2.2 Non-parametric ANOVA test: the Scheirer-Ray-Hare test

Firstly, a non-parametric ANOVA test, the Scheirer-Ray-Hare test, was performed in order to assess potential interactions between the “year” and “season” variables. All the p-values reflecting significant differences for the interaction “season*year” were above 0.05, except for Zn which had a p-value of 0.46. Thus, with the exception of Zn, no interaction between the variables “year” and “season” was revealed. Knowing that, a second Scheirer-Ray-Hare test was performed in Rstudio to investigate the significance of differences with the factors “season” and “bear” (Table 23).

Table 23: Scheirer-Ray-Hare test to assess differences among groups according to the “season” and “bear” variables. “*” indicates p-value <0.05 and therefore significant. Source: Rstudio, by author.

Mineral	Variable	p-value
Ca	Season	1.18×10^{-1}
	Bear	9.68×10^{-1}
	Season*Bear	8.82×10^{-1}
P	Season	7.67×10^{-2}
	Bear	9.98×10^{-1}
	Season*Bear	4.94×10^{-1}
Na	Season	$8.67 \times 10^{-3} *$
	Bear	9.79×10^{-1}
	Season*Bear	8.68×10^{-1}
Fe	Season	$3.08 \times 10^{-7} *$
	Bear	9.56×10^{-1}
	Season*Bear	6.75×10^{-1}
Mg	Season	6.50×10^{-2}
	Bear	9.98×10^{-1}
	Season*Bear	4.93×10^{-1}
Zn	Season	$4.81 \times 10^{-2} *$
	Bear	7.62×10^{-1}
	Season*Bear	6.84×10^{-1}
I	Season	$2.02 \times 10^{-2} *$
	Bear	$4.41 \times 10^{-2} *$
	Season*Bear	9.87×10^{-1}
S	Season	$5.27 \times 10^{-5} *$
	Bear	8.85×10^{-1}
	Season*Bear	7.81×10^{-1}
Co	Season	$4.92 \times 10^{-4} *$
	Bear	6.91×10^{-1}
	Season*Bear	8.79×10^{-1}
Cu	Season	$3.13 \times 10^{-9} *$
	Bear	9.33×10^{-1}
	Season*Bear	9.93×10^{-1}
Mn	Season	$1.58 \times 10^{-4} *$
	Bear	$1.68 \times 10^{-2} *$
	Season*Bear	9.99×10^{-1}
Se	Season	$2.47 \times 10^{-4} *$
	Bear	9.47×10^{-1}
	Season*Bear	6.88×10^{-1}

There were significant differences among groups and thus a season effect for sodium, iron, zinc, iodine, sulfur, cobalt, copper, manganese and selenium. Only phosphorus, calcium and magnesium didn't show any significant difference according to season. In addition, there were significant differences among groups for manganese and iodine according to the bear variable. The fact that Zn revealed to have a significant p-value with the Scheirer-Ray-Hare test and therefore an interaction between the season and year variables prevents us to draw conclusions regarding the influence of the season on the Zn concentrations.

Table 24: Results of the post-hoc Wilcoxon test. “a” indicates a p-value < 0.05 and therefore a significant difference between spring and summer mean values. “b” indicates a p-value < 0.05 and therefore a significant difference between summer and winter mean values. “c” indicates a p-value < 0.05 and therefore a significant difference between spring and winter mean values. Source: Rstudio, by author.

Mineral	Season	Mean	SD
Ca (µg/L)	Spring	5.02×10^4 ^a	7.97×10^3
	Summer	5.49×10^4 ^a	8.03×10^3
	Winter	5.31×10^4	6.15×10^3
P (µg/L)	Spring	4.89×10^5 ^a	1.05×10^5
	Summer	6.04×10^5 ^{a b}	1.41×10^5
	Winter	5.27×10^5 ^b	8.91×10^5
Na (µg/L)	Spring	2.45×10^6 ^{a c}	3.69×10^5
	Summer	2.73×10^6 ^a	3.41×10^5
	Winter	2.72×10^6 ^c	2.66×10^5
Mg (µg/L)	Spring	3.89×10^4 ^{a c}	8.32×10^3
	Summer	4.82×10^4 ^a	1.12×10^4
	Winter	4.53×10^4 ^c	7.72×10^3
Fe (µg/L)	Spring	5.17×10^5 ^{a c}	8.92×10^4
	Summer	5.84×10^5 ^{a b}	1.04×10^5
	Winter	6.94×10^5 ^{b c}	7.44×10^4
Zn (µg/L)	Spring	3.39×10^3 ^c	6.98×10^2
	Summer	3.58×10^3 ^b	6.03×10^2
	Winter	3.92×10^3 ^{b c}	3.86×10^2
I (µg/L)	Spring	1.76×10^1 ^c	1.13×10^1
	Summer	1.67×10^1 ^b	5.90×10^0
	Winter	1.15×10^1 ^{b c}	6.14×10^0
S (µg/L)	Spring	1.27×10^6 ^{a c}	2.79×10^5
	Summer	1.45×10^6 ^{a b}	2.52×10^5
	Winter	1.67×10^6 ^{b c}	1.92×10^5
Co (µg/L)	Spring	2.10×10^{-1} ^{a c}	1.66×10^{-1}
	Summer	3.01×10^{-1} ^a	1.98×10^{-1}
	Winter	2.86×10^{-1} ^c	1.55×10^{-1}
Cu (µg/L)	Spring	5.92×10^2 ^{a c}	1.29×10^2
	Summer	7.13×10^2 ^{a b}	1.69×10^2
	Winter	9.15×10^2 ^{b c}	1.27×10^2
Mn (µg/L)	Spring	3.63×10^1 ^c	1.35×10^1
	Summer	3.63×10^1 ^b	1.08×10^1
	Winter	6.60×10^1 ^{b c}	1.70×10^1
Se (µg/L)	Spring	2.54×10^2 ^{a c}	7.59×10^1
	Summer	2.98×10^2 ^a	7.59×10^1
	Winter	3.37×10^2 ^c	7.96×10^1

Fe, S and Cu showed mean values significantly different among all three seasons. The other essential minerals also showed differences between seasons but not between all three seasons. Since Ca, P and Mg didn't show any significant p-values in the Scheirer-Ray-Hare test in Table 23, the differences found between seasons in the Wilcoxon test in Table 24 are to be handled with caution. Among the essential minerals displaying significant seasonal variations in Table 23, Na, Fe, S, Co, Cu, Mn, and Se had the lowest mean values during spring. Iodine was the only essential mineral with the highest values during spring. Fe, S, Cu, Mn and Se had

the highest values during hibernation. It is however delicate to compare the mean seasonal values for each mineral because the sample size per season was unequal. For instance, the sample sizes during winter were a lot smaller than the sample sizes during spring which was the season with the most samples.

For Ca and Mg, no significant differences between seasons were revealed with the Scheirer-Ray-Hare test even though there seemed to be a seasonal difference in values when looking at the boxplots (Figures 49 and 52). On the contrary, Na doesn't seem to have a seasonal influence on the values when looking at the boxplot (Figure 51) but showed a significant p-value with the Scheirer-Ray-Hare test. This could partly be explained by the fact that the sample sizes among seasons were uneven, with far less samples taken during winter than spring.

7. DISCUSSION

The annual variations in the twelve essential mineral concentrations displayed on the boxplots in Figures 25-36 may be related to several factors. They could be linked to the fact that not all samples were collected the same way from one year to another. Annual variations in mineral concentrations could first of all be observed due to the different sample size among years, as shown in Table 11. The sample sizes range from $n=9$ in 2013 to $n=82$ in 2019. The brown bear population is therefore not equally representative among years. With fewer samples collected in 2010 and 2013, the population is not equally representative as in the years 2019, 2020 and 2021 with more than 71 samples. In addition, the seasons may have an influence on the annual variations since not all years had a similar sample size for each season within the same year. As shown in Table 14, the number of samples collected according to season varies from one year to another. For example, samples collected during winter were only gathered in the years 2019, 2020 and 2022. In the years 2010 and 2013, samples were only collected during spring, with a small sample size compared to the other years. Not all years had samples collected for each season and in the same amount. In relation to the season variable, brown bears undergo physiological changes in metabolism throughout the year and since not all years have the same number of samples collected for each season, some years didn't reflect the variability in physiological changes while some did. For instance, in the years 2010 and 2013 when no samples were collected during winter or summer, only the bears' physiological state during spring were represented whereas the years 2019, 2020 and 2022 displayed values reflecting the physiological states of the bears during the three seasons (winter, summer and spring). Dehydration is one example of physiological changes observed among seasons. The dehydration state of the bears can hence influence the mineral concentrations, which is especially true during wintertime (Welinder et al. 2016) and if high ambient temperatures or intense chasing occurred. These biases are reduced in the analysis of the blood mineral concentrations from TE tubes only, displayed on the boxplots in Figures 37-48. These variations in blood mineral levels are expected to be less affected by the sample size ($n = 63$ in 2019, $n = 71$ in 2020, $n = 57$ in 2021 and $n = 41$ in 2022) and seasonal factors because samples were collected in all three seasons (except in 2021 when only samples were taken during spring and summer).

Gender might also influence the annual variations displayed on the boxplots in Figures 25-36 and 37-48. As shown in Tables 13 and 14, the percentage of males and females sampled differed more or less importantly each year. 2010 was the only year with a majority of males sampled, whereas the other years had a majority of females (more than 66%). Apart from 2010, the years showed a ratio (r) females:males between 2 and 3.5 ($r = 4/9 = 0.44$ in 2010; $r = 6/3 = 2$ in 2013; $r = 27/9 = 3$ in 2017; $r = 38/16 = 2.38$ in 2018; $r = 38/13 = 2.92$ in 2019; $r = 46/21 = 2.19$ in 2020; $r = 40/16 = 2.5$ in 2021; $r = 28/8 = 3.5$ in 2022). If the ratio females:males would have been similar from one year to the next, the gender factor would have potentially had less impact on the differences in mean values among years. A study conducted in 2015 in free-ranging Scandinavian brown bears revealed significant differences in serum concentrations according to the bears' gender for Na, where serum Na levels were significantly higher in females than males (Græsli et al. 2015). Although the mineral levels were evaluated in plasma and not serum in this study, it supports the hypothesis that gender might have an influence on plasma mineral concentrations in free-ranging brown bears. Further studies with statistical analysis of the influence of gender on whole blood mineral concentrations in Scandinavian brown bears would be interesting to explore (Annex 8).

The age difference in bears among years might also come into play in the annual variations shown from the boxplots in Figures 25-36 and 37-48. As shown in Table 15, the age categories vary from one year to another. A majority of one-year-old bears were captured in 2010, 2017, 2018 and in 2020, a majority of three-year-old bears were captured in 2013, a majority of two-year-olds in 2019, a majority of five-year-olds in 2021 and six-year-olds were mostly captured in 2022. Age is known to have an influence on elemental blood concentrations, in particular for toxic elements (Fuchs et al. 2023), and might therefore impact the annual mineral variations in this study since the age categories vary from one year to another. Young and older individuals may have different mineral requirements and absorption rates. A study conducted on red deer in 2013 showed significant differences in serum mineral concentrations between young and adult individuals (Kučer et al. 2013). For instance, they found a significant increase in serum levels of Na with age. In our study, plasma Na levels in TE tubes showed the highest values in the year 2022, when the major age category of the captured bears was six years-old, which was higher than the other years (Table 15). A study conducted in 2015 in free-ranging Scandinavian brown bears revealed significant differences in serum concentrations according to the bears' age for Fe, Mg, and P (Græsli et al. 2015). Further studies with statistical analysis of the influence of age on whole blood mineral concentrations in Scandinavian brown bears would be interesting to explore. What is more, investigating the influence of both age and gender factors on whole blood mineral concentrations in Scandinavian brown bears would be of great interest (Annex 8). In addition, in relation to the age and gender factors, the reproductive status may influence the annual variations in mineral concentrations. The percentages of single or lactating females varied among years and since lactating females may have altered mineral metabolism, this could come into play in the annual and seasonal variations.

More importantly, essential minerals in animals are provided through diet, thus variations in food availability between years may impact the annual mineral concentrations. Indeed, blood mineral analysis is frequently used in animals as a means of assessing mineral nutrition (Herdt, Rumbelha, Braselton 2000). Both variations in food quality and quantity can influence the mineral levels in brown bears. Some years might have shown more abundant yields in berries (which is one of the most important food items in the brown bear's diet), or more carrion abundance during spring. These annual variations in food availability and composition can be influenced by environmental conditions such as temperature, precipitation, snow cover etc. Significant seasonal and annual variations have been objectified in dietary components such as insects, vertebrates, plants and berries (Stenset et al. 2016). The variations found among the years in essential mineral blood concentrations in Scandinavian brown bears may strongly be linked to the variations in food availability. Indeed, the levels of essential minerals found in whole blood depend partly on the food items containing such minerals brown bears ingest. The seasonal and annual shifts may coincide with changes in food-resource availability, more specifically ripening of berries, birth of moose calves and seasonality of plants. While some years are "good years" for bilberries, this is not necessarily the case for lingonberries or crowberries and vice versa. Since the components in essential minerals in the various food items eaten by bears are different, the annual variations objectified in essential minerals in this study may thus be partly linked to the annual variations in berry availability. In this study, seasonal variations in mineral concentrations in TE tubes were found in Na, Fe, I, S, Co, Cu, Mn and Se. No seasonal variations were statistically revealed for Ca, P and Mg, and the results for Zn were inconclusive since an interaction between year and season was objectified. These seasonal variations found in Na, Fe, I, S, Co, Cu, Mn and Se may strongly be linked to the physiological changes in metabolism from one season to another in Scandinavian brown

bears, especially the differences found during wintertime, when the bears are in a hypometabolic state. A study conducted on African goats in 2012 revealed no significant differences in serum calcium and phosphorus levels between the dry and wet seasons (Rumosa Gwaze, Chimonyo, Dzama 2012). Of course, the mineral concentrations in this study were evaluated with plasma of free-ranging wild omnivorous species and not serum of domesticated herbivores. Even though brown bears undergo physiological changes in metabolism among seasons and some mineral blood concentrations could be expected, the fact that no significant differences in blood calcium, phosphorus and magnesium levels were found supports the hypothesis that these minerals are regulated homeostatically and maintained within narrow limits (Rumosa Gwaze, Chimonyo, Dzama 2012). A study conducted in 2015 in free-ranging Scandinavian brown bears revealed significant differences in serum concentrations among seasons for chloride, iron, magnesium, phosphorus and sodium (Græsli et al. 2015). An interesting area of research would be to dig more into other factors than dietary components affecting the values of essential minerals in bears, and also how these variations affect the brown bear's biology and behavior (how do these variations affect the hibernation cycle, their reproductive success and cycle, survival, etc.). Some experimental studies have already objectified the impact of climate change on food resource availability. Winter thaws occurring later in the year cause major reductions in berry production, going up to 52-95% in bilberries and 11-75% in crowberries (Bokhorst et al. 2011). Berries represent the main food source during hyperphagia prior to hibernation, but with this reduction in berry availability, Scandinavian brown bears can be expected to switch to other foods during hyperphagia, as it is happening in northern Spain (Rodríguez et al. 2007). This could be one of the explanations to the annual variations in the essential mineral reference intervals found in this study, with the brown bears gradually switching the ratios of their food items during hyperphagia.

Another factor that might affect the results is the animal's last meal status. Knowing when an animal last ate can be useful for nutrient blood analysis. After food consumption, the body starts to digest and redistribute the nutrients to the body via the bloodstream. This results in a spike in circulating nutrients and minerals in the body when they enter the bloodstream for redistribution to the peripheral tissues. Nutrient redistribution depends on the nutrient in question, and some are digested quicker than others. Therefore, the best way to avoid nutrient spikes and differences in returned values from one animal to the next is to take the blood sample from a fasting animal. Doing captures on wild animals makes it impossible to fast the animal before blood sampling, so the sampled bears all had a different status regarding the timing of their last meal (except in winter, when fasting begins at den entrance in October-November). Having different status regarding the bear's last meal may therefore add a bias in the essential mineral analysis.

Another important factor that may have affected the annual variations is the type of tubes used for blood sampling which may have led to a variability in quantification of some minerals and trace elements in the blood. Improper design or use of blood collection devices can adversely affect the accuracy of laboratory test results (Bowen, Remaley 2014). Not all years had the same number of samples collected from different types of tube (EDTA, HE and TE). Table 11 sums up the number of samples collected each year according to the type of tubes. For instance, only EDTA tubes were used for blood sampling during the years 2010, 2013, 2017, 2018. In 2020 and 2022, only TE tubes were used and HE tubes were only used in the year 2021. Most of the blood samples were taken using TE tubes (n= 232), fewer were taken using EDTA tubes (n=131) and even fewer were taken using HE tubes (n=21). Therefore, for

HE tubes, with a sample size more than ten times smaller than the sample size for TE tubes, the values were therefore less significant. The Kruskal-Wallis and Wilcoxon tests performed showed that there were statistically significant differences for some mineral concentrations according to the type of tubes. P and Mn were the two minerals that showed significant differences in blood concentrations between all three tubes. Some minerals showed significant differences between two types of tubes while other minerals showed no significant differences between all three tubes (i.e., Na, Mg, Fe and I). Furthermore, K₂ and K₃-EDTA blood tubes contain anticoagulants that chelate Ca (and other minerals such as Fe, Zn and Mg ions) (Staff 2013; Imafuku et al. 2002; Nkuna, Ijoma, Matambo 2022). Knowing that, decreased values could have been expected for these ions in the EDTA tubes, but this wasn't the case in this study. In humans, it has been shown that elements present in blood/serum at concentrations higher than 10 ng/mL i.e., Na, Ca, Mg, P, Fe, Br, Si, Zn, Cu, Rb, Se and I, contribution of contamination from the tube sampling devices was negligible, but for the majority of trace and ultra-trace elements it may significantly affect accurate concentration determination (Rodushkin, Odman 2001). In this study, the concentrations of ions potentially impacted by the chelating agents within the EDTA tubes (i.e., Ca, Mg, Fe and Zn) didn't show significantly decreased values compared to the ones found in the HE and TE tubes.

The transportation method, the storage conditions and temperature of the blood samples, the time between sampling and storage, the laboratory analysis (i.e., different people in charge of running the analysis among years, change of analysis equipment and /or method, etc.) also have to be considered, adding a human error factor. With such important annual variations as the ones displayed in Figures 25-36 and 37-48, the "operating/laboratory errors" factor could have had a strong influence. For instance, Figure 39 displaying the annual variations in Na showed significant differences between years 2019 and 2022 with the years 2020 and 2021. No Na values overlapped in the boxplots and scatter plots between years 2021 and 2022. Even though studies on red deer and Scandinavian brown bears show that age and gender affect Na concentrations in individuals (Kučer et al. 2013; Græsli et al. 2015), such big differences seem unlikely to only be due to age and gender factors. Therefore, one of the biggest factors influencing such annual variations in this study is hypothesized to be due to operating and handling errors in the analysis of the minerals. There may have been different protocols, analyzers, transportation and storage conditions that differed greatly between years. Further studies with assurance that identical methods used between years would be interesting to pursue.

To sum up, the annual variations in the twelve essential minerals may be influenced by annual sample size, gender, age and reproductive status, season and physiological metabolic changes associated, environmental conditions impacting the quality and quantity of dietary components, tube type, transport, storage and laboratory analysis methods. In order to eliminate the influence of the choice of tube in the mineral concentration variations seen among years, only the concentrations collected in TE tubes were analyzed in a second section of the results part. Annual and seasonal variations were also objectified but with smaller ranges of values than the ones observed in the analysis with the three types of tubes. Common factors having the potential to interfere with the biochemical variables from the collected samples include: stress and increased physical activity due to the chase, the anesthetic drugs (Arnemo JM, Caulkett N 2007), starvation, dehydration, diet, diseases, genetic factors, reproductive status (pregnant, lactating, female in heat), daily and seasonal variations, etc. (Irrjala, Grönroos 1998). All these factors need to be taken into consideration and should be avoided to a maximum, with a consistency in the sampling and protocol procedures. While

some extrinsic factors may be more or less easy to avoid or at least controlled in some ways, intrinsic factors potentially interfering with the blood variables are challenging to manage. Bears of different ages and gender show differences in metabolism, diet, body weight, behavior and physiology, which may be reflected in the biochemical variables. This was however the purpose in this study, to have a population with a mixture of ages, reproductive status and gender in order to get RIs as representative as possible.

Further studies would be interesting in analyzing the concentrations in whole blood of these twelve essential minerals in other species to compare the results found in brown bears with. For instance, choosing animal species from the same environment as the brown bear would be insightful. More particularly, a herbivorous species, such as the moose *Alces alces* and a carnivorous species, such as the wolf *Canis lupus*. Moose and wolves constitute studied species at the Inland Norway University of Applied Sciences at Evenstad, and so like the bears, captures of these species take place in order to conduct ecological studies, radio collaring and marking. The positions of some individuals have been taken, showing a proximity with the brown bear's habitat range (Annex 9).

At a larger scale, studies comparing the concentrations of essential minerals found in free-ranging Scandinavian brown bears with other subspecies of *Ursus arctos* would be interesting as well. Brown bears have different diets across the world depending on their geographical location. Spatial variation in brown bear diet is the result of environmental conditions (e.g., climatic factors), which affect the local availability of food and the nutritional and energetic requirements of brown bears (Bojarska, Selva 2012). Therefore, depending on the brown bears' location, the proportions of food components in their diet vary from largely herbivorous to highly carnivorous (Bojarska, Selva 2012). For example, comparing these values with the ones found in the bear populations in Europe (e.g., Pyrenean, Cantabrian, Carpathian, Karelian, Dinaric-Pindos, and Balkan populations; The IUCN Red List of Threatened Species 2018; IUCN/SSC 2023), North America (e.g., Canadian population, Alaskan population, Yellowstone National Park population; *Bears of North America* 2023), Japan (e.g., Hokkaido population; Endo et al. 2021) or Russia (Kudrenko et al. 2022) would be beneficial in assessing the impact of diet on essential mineral concentrations. Since different food components contain different concentrations in essential minerals, we can expect the values and reference intervals in other bear populations to differ from the blood concentrations found in the Scandinavian brown bears.

8. CONCLUSION

This study aimed to establish reference intervals for twelve essential minerals in the whole blood of free-ranging Scandinavian brown bears (*Ursus arctos*), chemically immobilized with a combination of medetomidine, tiletamine and zolazepam during spring and summer, and ketamine in addition during winter. The analysis revealed seasonal and annual variations in mineral concentrations. The variations were likely influenced by several variables, including gender, age, sampling tube type, operating/handling errors, the physiological shift in metabolism from active phase to hibernation, and environmental conditions impacting the bear's dietary components and availability. This study contributes data on whole blood essential mineral concentrations, which enables us to broaden our knowledge on their physiology and the potential impacts of environmental changes in their diet. Further larger-scale and comparative studies with other wildlife species and/or brown bear subspecies could enhance our understanding of the broader ecological dynamics.

9. ANNEXES

Annex 1: Links describing the Scandinavian Brown Bear Research Project:

- 1) <https://www.inn.no/forskning/forskningsnyheter/pa-sporet-av-bjornens-hemmeligheter/index.html>
- 2) <https://www.youtube.com/watch?v=TdNNBuwnuQY>

Annex 2: Different designs of trap sites based on the Aldrich-type foot snare for brown bears used in Croatia. Source: Huber et al. 1996.



Fig. 3. "American cubby" type of set (Flowers 1977).

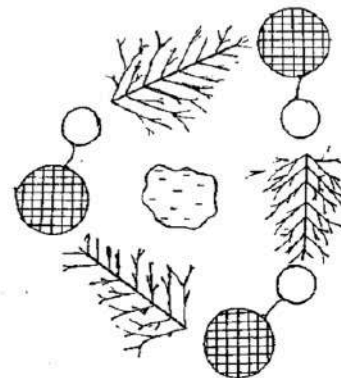


Fig. 4. "European cubby" type set.



Fig. 6. "Passage" type of trail set (Flowers 1977).

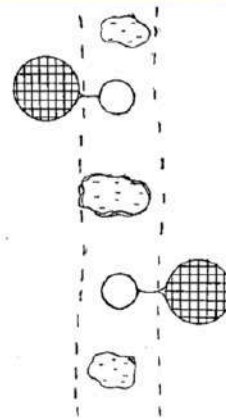


Fig. 5. Trail set.

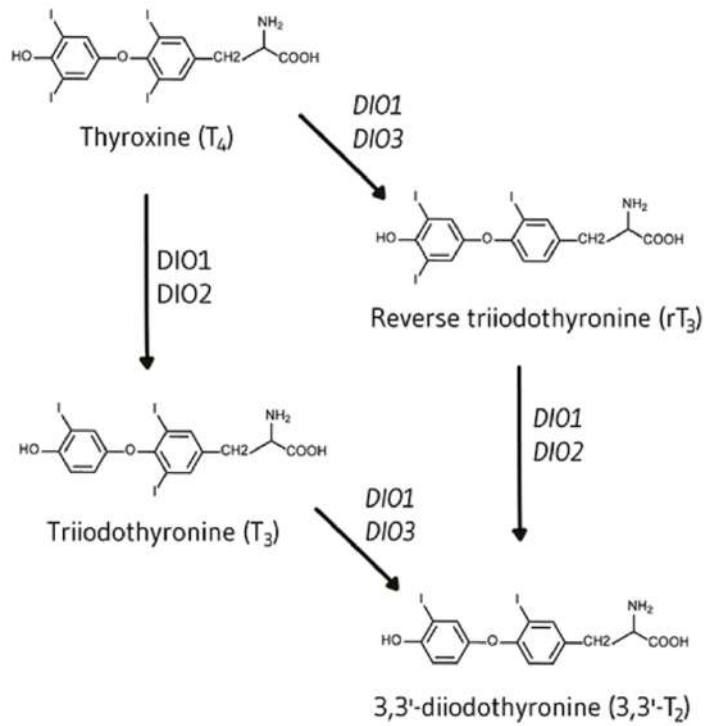
Annex 3: Guedel's classification of all four stages of general anesthesia. Source: <https://www.pharmacy180.com/article/stages-of-anaesthesia-1125/>

STAGE	Respiration		Ocular movem.	Pupil size	Reflexes	SK.mus. tone	B. P.	H. R.	USES
	Thor.	Abd.							
I ANALGESIA			NORMAL						Labour, Incisions & Minor ops.
II DELIRIUM			ROVING EYE BALLS						NIL
SURGICAL ANAESTHESIA III	1		ROVING EYE BALLS						Most of the surgical operations
	2		ROVING EYE BALLS						
	3		FIXED EYES						Occasionally reached now
	4		FIXED EYES						Never attempted
IV MEDULLARY PARALYSIS									

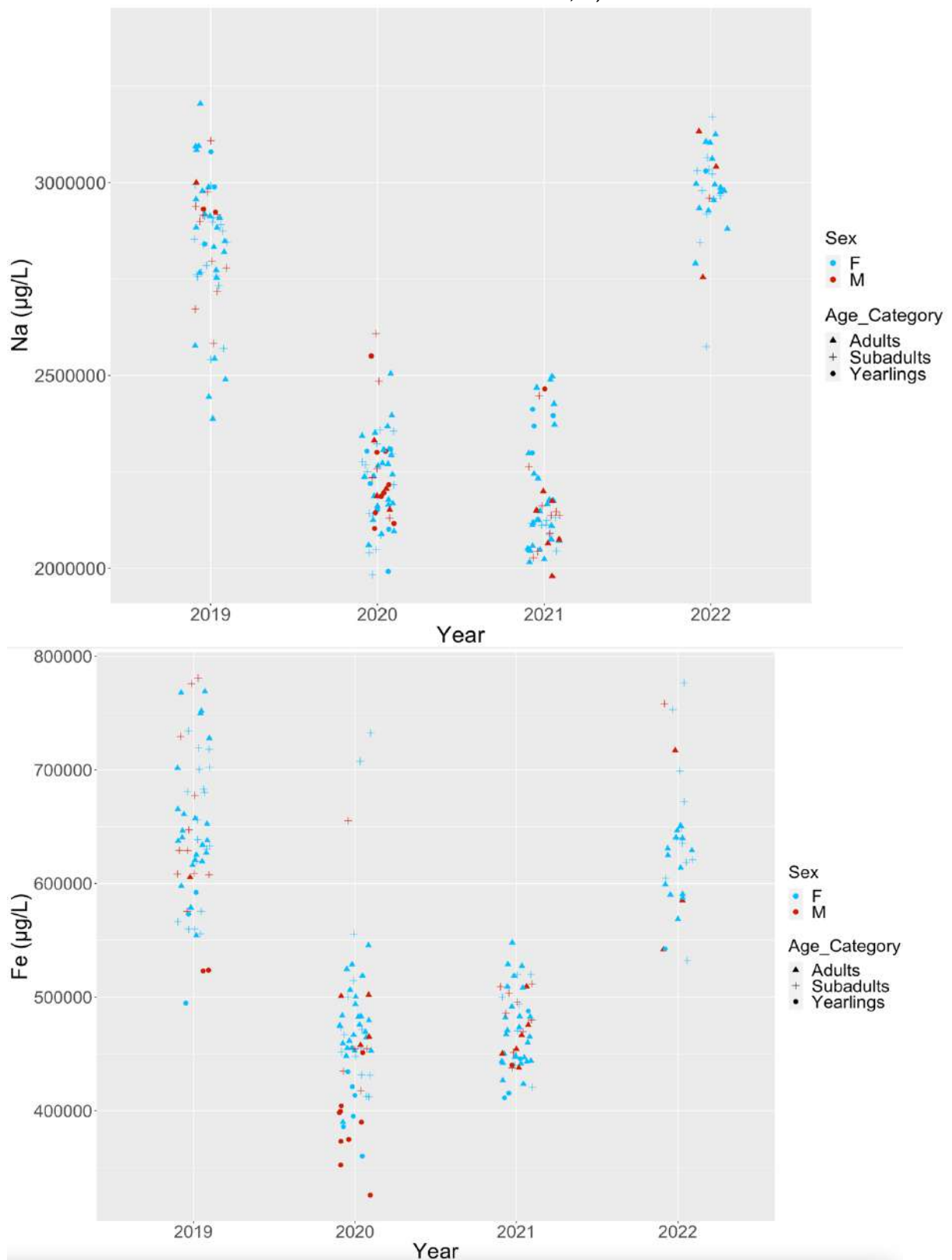
Annex 4: Inorganic constituents of bone. Source: <https://faculty.ksu.edu.sa/sites/default/files/smch36.pdf>

Constituent	Percentage of Total Body Content Present in Bone
Calcium	99
Phosphate	86
Carbonate	80
Magnesium	50
Sodium	35
Water	9

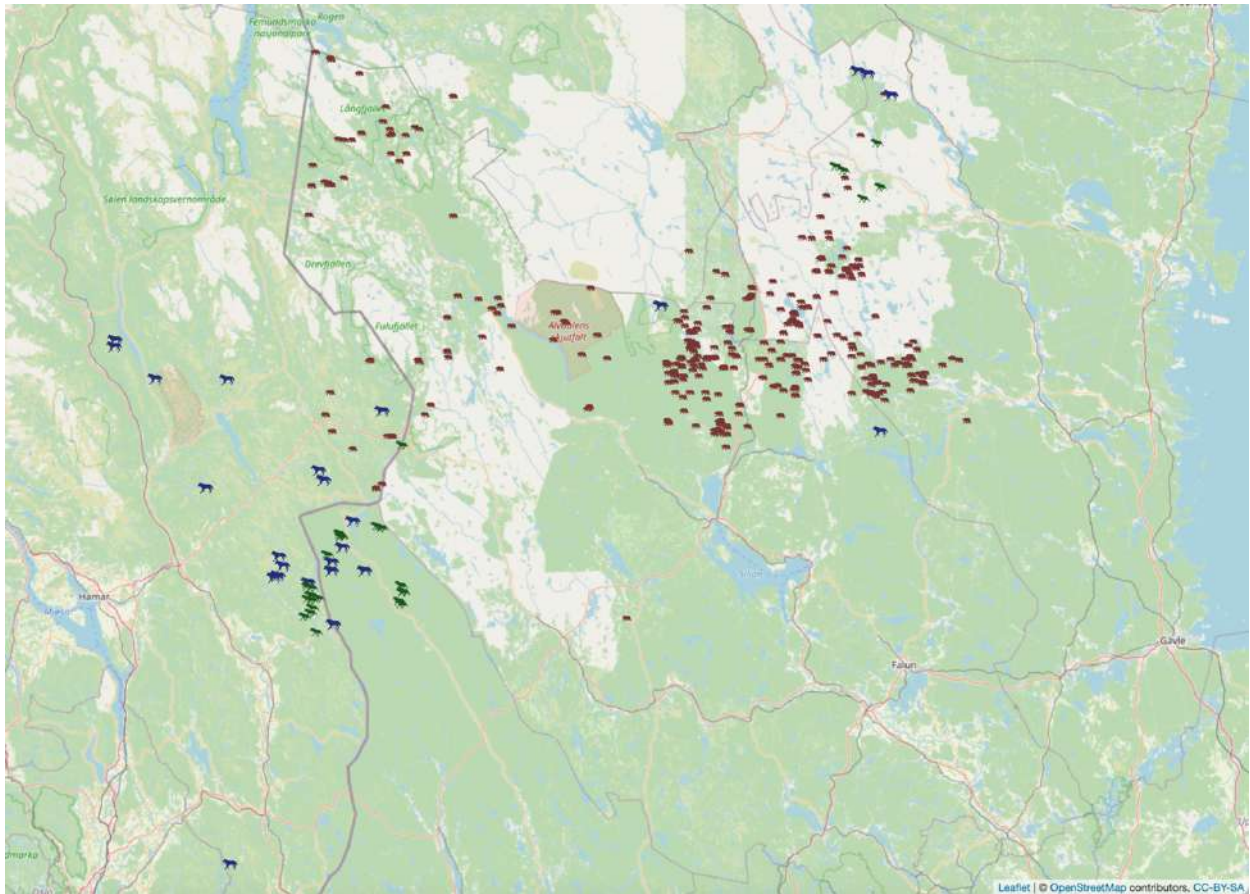
Annex 7: Deiodination of thyroid hormones by iodothyronine deiodinases (DIOs). Source: Oregon state University website <https://lpi.oregonstate.edu/mic/minerals/selenium#reference6>



Annex 8: Whole blood sodium and iron concentrations ($\mu\text{g/L}$) according to age and gender in Scandinavian brown bears in the years 2019-2022, from 232 blood samples collected in trace element tubes. Source: Rstudio, by author.



Annex 9: Distribution of capture positions for blood sampling of brown bears (*Ursus arctos*, $n=322$, in the years 2010, 2013 and 2017-2022), wolves (*Canis lupus*, $n=26$, in the years 2014-2016 and 2018-2022), and moose (*Alces alces*, $n=31$, in the years 2018-2020). Source: Rstudio, by author.



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Title: Annual and seasonal variations of essential minerals in free-ranging Scandinavian brown bears (*Ursus arctos*)

Abstract: Exploring blood chemistry values has the potential to improve our understanding of physiological changes in wildlife. However, it has been sparsely investigated in brown bears (*Ursus arctos*). The objectives of this study were to determine reference intervals for twelve blood chemistry variables and to evaluate differences between seasons and years. To establish reference values for biochemical variables in whole blood for free-ranging Scandinavian brown bears, 384 blood samples were collected from 164 different bears over a period of twelve years, from 2010 to 2022. The samples were collected during winter (February-March), spring (April-May) and summer (June), in the county of Dalarna in southcentral Sweden. The bears were chemically immobilized inside their den during winter, while being darted and immobilized from a helicopter during spring and summer. The drugs used for the chemical immobilization are a combination of tiletamine, zolazepam and medetomidine, with ketamine in addition in winter. Essential minerals are one of the important nutritional components having a significant role in animal health, reproduction, immune system, metabolism, etc. The biochemical variables included in this study are the following twelve essential minerals: calcium, cobalt, copper, iodine, iron, magnesium, manganese, phosphorus, selenium, sodium, sulfur and zinc. Seasonal and annual variations of the mineral values were established for the brown bears.

Key words: Scandinavian brown bear, *Ursus arctos*, whole blood, essential minerals, reference intervals, seasonal and annual variations

Titre : Variations annuelles et saisonnières des minéraux essentiels chez l'ours brun (*Ursus arctos*) sauvage scandinave

Résumé : L'exploration des concentrations sanguines en minéraux essentiels permet d'améliorer notre compréhension des changements physiologiques rencontrés dans la faune sauvage. Cependant, il s'agit d'un sujet peu étudié chez l'ours brun (*Ursus arctos*). Les objectifs de cette étude sont d'établir des intervalles de référence en concentrations sanguines de douze minéraux essentiels et d'évaluer leurs variations entre saisons et années. Afin d'établir ces valeurs de référence pour les ours bruns sauvages scandinaves, 384 échantillons sanguins ont été prélevés sur 164 ours bruns sur une période de douze ans, de 2010 à 2022. Les échantillons ont été prélevés en hiver (février-mars), au printemps (avril-mai) et en été (juin) dans le comté de Dalarna, dans le centre sud de la Suède. Les ours ont été immobilisés chimiquement à l'intérieur de leur tanière pendant l'hiver, tandis qu'ils ont été téléoanesthésiés à partir d'un hélicoptère lors des captures au printemps et à l'été. Les molécules anesthésiques utilisées pour l'immobilisation chimique sont une combinaison de tilétamine, de zolazépan et de médétomidine, avec de la kétamine en plus en hiver. Les minéraux essentiels sont des composants nutritionnels importants qui jouent un rôle significatif dans la santé animale, la reproduction, le système immunitaire, le métabolisme, etc. Les variables biochimiques incluses dans cette étude sont les douze minéraux essentiels suivants : calcium, cobalt, cuivre, iode, fer, magnésium, manganèse, phosphore, sélénium, sodium, soufre et zinc. Chez chaque ours, des prélèvements sanguins veineux ont permis d'analyser ces douze minéraux.

Mots clés : Ours brun scandinave, *Ursus arctos*, concentrations sanguines, minéraux essentiels, intervalles de référence, variations saisonnières et annuelles.