THESE DE DOCTORAT DE L'ETABLISSEMENT UNIVERSITE BOURGOGNE FRANCHE-COMTE PREPAREE A L'UNIVERSITE DE FRANCHE-COMTE

Ecole doctorale n°554

Environnements - Santé

Doctorat de Biologie des Populations et Ecologie

Par

Quentin Devalloir

Effects of multi-stressors (pollution, nutritional quality) on the immunocompetence of wood mice

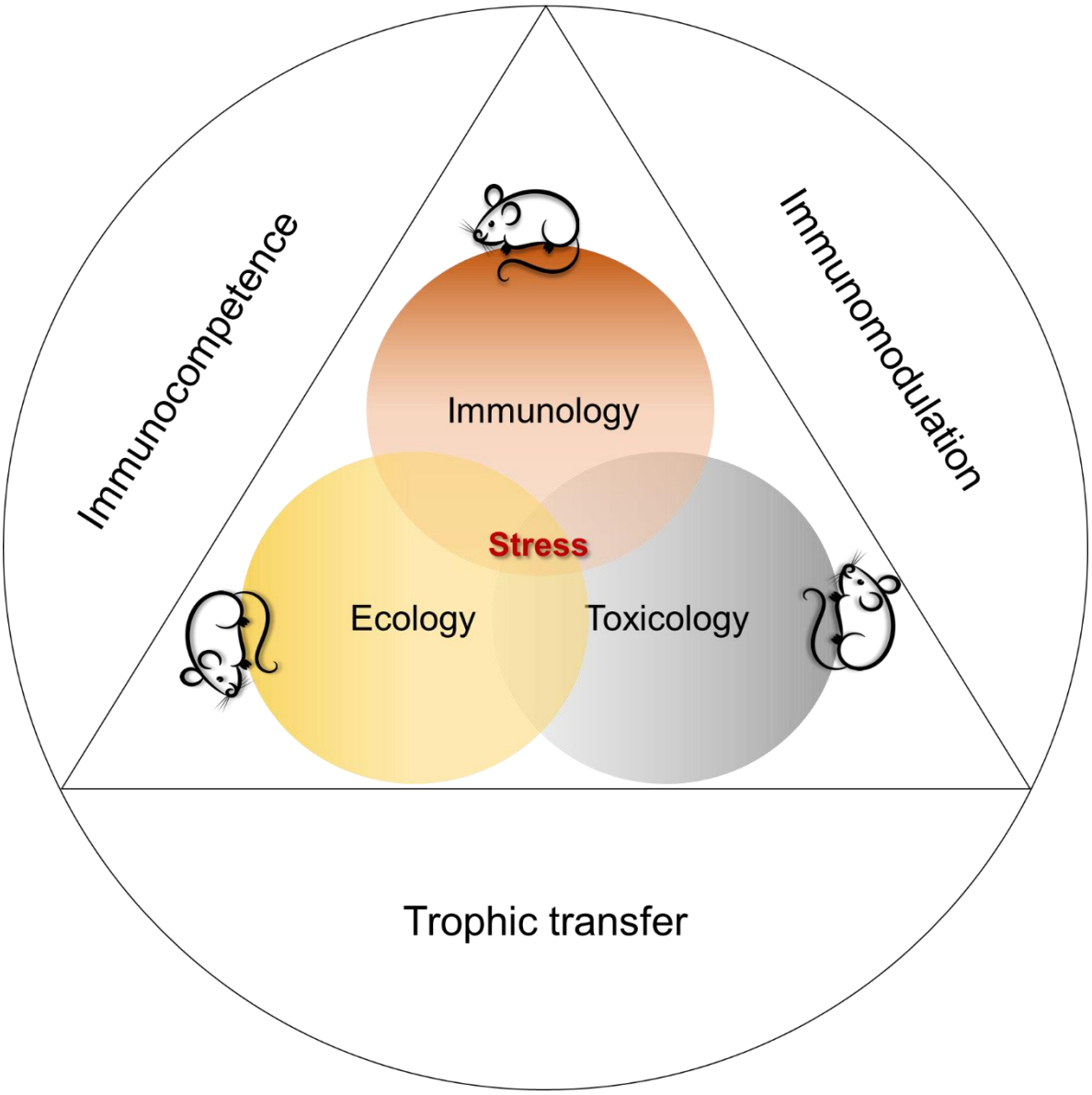
Effets de stresseurs multiples (pollution et qualité nutritionnelle) sur l'immunocompétence du mulot sylvestre

Thèse présentée et soutenue à Besançon, le 22 juin 2023

Composition du Jury :

Monsieur Bruno Faivre	Professeur des Universités, Université de Bourgogne	Président
Monsieur Frédéric Angelier	Directeur de Recherches, CNRS	Rapporteur
Madame Christy Morrissey	Professeur des Universités, Université du Saskatchewan, Canada	Rapporteur
Madame Aurélie Goutte	Maître de Conférences, EPHE	Examinatrice
Monsieur Renaud Scheifler	Professeur des Universités, UBFC	Directeur de thèse
Monsieur Nico van den Brink	Associate professor, Wageningen University	Co-directeur de thèse
Madame Clémentine Fritsch	Chargée de Recherche, CNRS	Membre invitée
Madame Cécile Grand	Ingénieur, ADEME	Membre invitée

Effects of multi-stressors (pollution, nutritional quality) on the immunocompetence of wood mice



Quentin Devalloir

2023

Acknowledgments

I deeply thank the member of the jury Bruno Faivre, Frédéric Angelier, Christy Morrissey and Aurélie Goutte for evaluating the present work.

Many thanks to my thesis supervisors Renaud Scheifler and Nico van den Brink. I would like to particularly thank Renaud Scheifler for his unswerving support during my thesis. Despite many ups and downs, he always knew how to get things right. He always trusts in my intuitions and I hope that my work will pay the tribute of his teachings. Thanks a lot to Nico van den Brink for his precious scientific advises. I am really glad to see that we managed to achieve despite the numerous setbacks linked to the COVID-19 epidemic that cancelled all the experiments in the Netherlands. I would also like to thank my thesis co-supervisor, Clémentine Fritsch. I learned a lot from her, both in my Master 2 internship and during my PhD. She was always here to get me back on track. I would not be where I am without her and I am very proud of the work I have been able to accomplish thanks to her. Many thanks to Francis Raoul for his precious advices and his help all along my master and PhD studies. I also particularly would like to thank Shinji Ozaki for all the help he gave me during my PhD in significantly improving all this work. Thank you very much to Vincent Driget for his support and the essential role he played during field and lab works. It has been a real honour to work with this research team, which has given me so much professionally and personally.

Many thanks to the PhD steering committee, Anne-Pauline Bellanger, Rafael Mateo, Gabriele Sorci, Stephane Garnier and Bruno Faivre who examined the relevance of my work and also helped to add new perspectives to my research.

I would like to thank the members of the doctoral school Environnement-Santé for their dynamic support to doctoral students.

I also thank my master students and co-authors, Yara Alchammas and Cloé Hadjadji for their internships. Many thanks for your help and all the work you did. I am really proud to see by the end of my PhD that Yara Alchammas is protecting biodiversity in Lebanon and that Cloé Hadjadji is at the beginning of a promising scientific career.

I specifically would like to thanks Eve Afonso and Anne-Claude Goydadin for the knowledge they gave me in molecular biology. Also, many thanks to Coralie Barrera who teach me a lot in immunological analyses.

I am deeply grateful to all the people who contributed to my work in the field and in the laboratory: Nadia Crini, Caroline Amiot, Benoit Valot, Michaël Coeurdassier, Dominique Rieffel, Loic Angonin.

I am very gratefull to Anabelle Sequeira-Le Grand, Serge Monier and Nicolas Pernet from the Plateforme d'imagerie & Cytometrie of Dijon.

I would like to thank the University of Bourgogne - Franche-Comté and the Agence De l' Environnement et de la Maîtrise de l'Energie, notably their coordinator Cécile Grand, for their financial support to this work.

I thank many teachers I met during my long studies in France. They aroused my interest in ecology and oriented me towards research. I am deeply grateful to Eve Afonso, Frédéric Gimbert, Annette de Vaufleury, François Gillet, Patrick Giraudoux, Gabriele Sorci, Stephane Garnier, Bruno Faivre and Loïc Bolache for their teaching.

I sincerely thank the two directions of the laboratoire Chrono-environnement I met during my thesis, as well as all the staff who welcomed me at the laboratory.

I would specially thank my two faithful colleagues Charles Henriot and Chloé Godeau for being there during every moments of my PhD and for having the patience to put up with me.

Also, thanks to Jimmy Fedna for the good times we have during this PhD. I hope we can have another beer time before you return to Haiti.

Many thanks to the PhD students with whom I shared good times and several activities Chloé Godeau, Honorine Gauthier-Manuel, Alexandre Lhosmost, Romain Colpaert, Milva Druguet Dayras, Clémence Kavira.

Thanks to my dear friends who deal with my long discussion about science and the functioning of research institutes Ramette, Chloé, Charles, Camille, Remi, Nico, Raphy, Audrey, Louis et le petit Camille. Thank you very much to my parents, my dear sister, my grandparents and my stepfamily for their support during that long and cloudy period. All my thoughts go to those who are no longer here to read these words and to whom I address all my thanks for what they brought me in particular to my dear grandmother who gave me the strength to finish this work. And obviously, many thanks to Pauline who supported me for so long and without whom nothing would have been possible. You have enlightened my life and made me walk on a straight line where I found the joy and happiness that our two wonderful children give us.

Table of contents

1 GENERAL INTRODUCTION

1.1 Introduction

1.1.1 Connecting stress to ecology, immunology and toxicology

1.1.1.1 Crossing borders between ecology and immunology

Introduction to Ecoimmunology

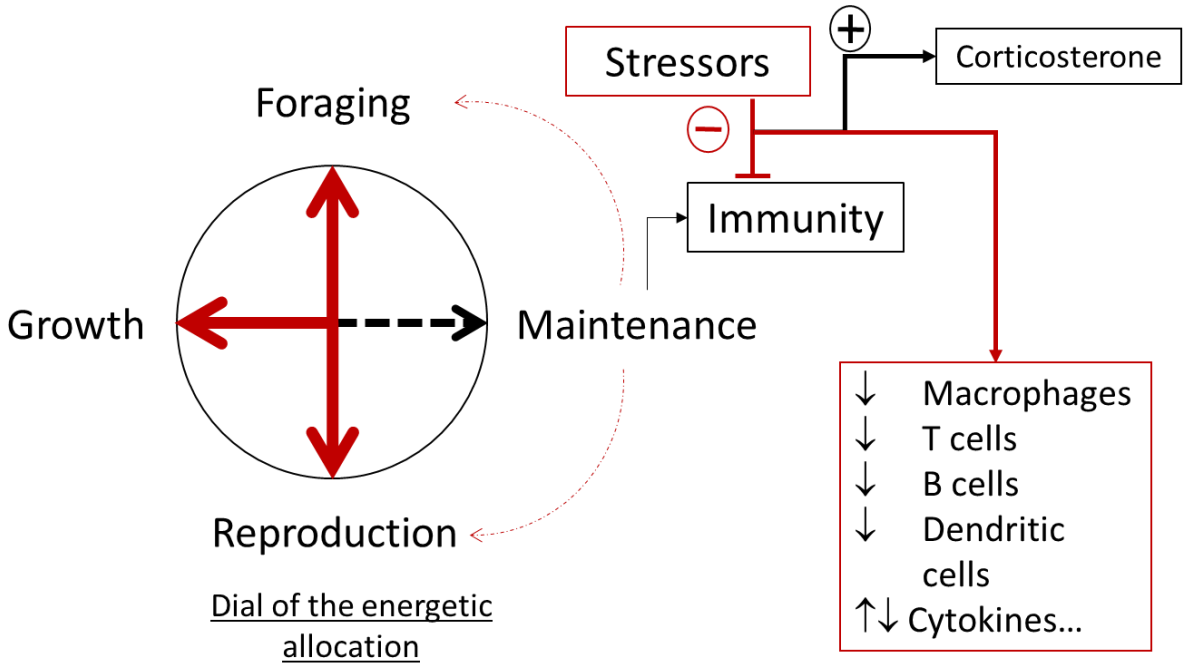
Ecology is a branch of biological sciences that was initially defined as the study of the relationships of organisms with their environment (Haeckel, 1866). The term broadly evolved to the study of structures and functioning of ecosystems entering Haeckel 's definition in hierarchical levels of organisations (Odum and Barrett, 1971). In opposition to reductionism, ecology is a holistic science that addresses all biological organization levels from atoms to the biosphere (Odum, 1977). Ecological immunology or ecoimmunology emerged from the convergence of the holistic approach in ecology and reductionist methods and concepts in immunology. According to (Martin *et al.,* 2011) ecoimmunology aims "[…] to describe and explain natural variation in immune functions, specifically why and how biotic and abiotic factors contribute to variation in immunity in free-living organisms.". Indeed, ecoimmunology engulfs pieces of knowledge from the fields of immunology, ecology, evolution and endocrinology to understand the selective advantages and the origins of the immune response in wild organisms (Downs and Stewart, 2014). A pioneering approach originates from the Hamilton-Zuk's handicap hypothesis (Hamilton and Zuk, 1982), which postulates that sexually selected attributes could mediate a honest signal of resistance to pathogens (Schoenle *et al.,* 2018). In response to this approach, Folstad & Karter (1992) proposed the immunocompetence handicap hypothesis (IHH), which suggests that androgens (*e.g.* testosterone) could favour both the expression of secondary sexual attributes in males (*e.g.* ornaments) and suppress immune response towards parasites. The IHH brought the central term of immunocompetence that corresponds to the ability of an organism to resist to a pathogen or a parasite through an immune response. The IHH, however, did not address the real complexity of variations in immunity found in nature. The seminal paper of Sheldon & Verhulst (1996) conceptualised that variation in immunity can be caused by trade-offs in the allocation of resources between competing costly processes like investment in reproduction or immune responses (Schoenle *et al.,* 2018). Sheldon and Verhulst, (1996) introduced a functional interpretation of the immune response as a mediator of long-term trade-offs between reproduction and survival. From an ecological point of view, the principle of energetic allocation predicts that a limitation in resources will induce a trade-off between the energetic investment in crucial processes like reproduction, survival, or immune response (**Figure 1**). This principle came out of the life history theory on the energetic cost trade-offs that considers that not all

traits can be invested at the same time. During infection, the temporality of life history traits is thus constrained by fitness, commonly determined by reproductive success along the lifetime, and the investment in immune responses (Graham *et al.,* 2011). To this end, fitness was redefined in terms of energetic cost by Brown, Marquet & Taper (1993) as "[…] the rate of conversion of energy into useful work for reproduction.". Thus, one issue of ecoimmunology is to determine the cost/benefit ratio of the energetic investment in either fitness or immune responses and their consequences. Host fitness, parasite density, and relevant immune responses may be measured to address this complexity as detailed in Graham *et al.* (2011), which stipulate that:"[…] neither the strongest immune responses nor the lowest parasite densities necessarily maximize host fitness". Energetic or food restrictions may compromise individual fitness in promoting an energetic trade-off between allocation to immunity or reproduction (Downs and Stewart, 2014). In mammals, recent studies showed that the characterisation of energetic trade-offs (*e.g.* in response to food restriction) should integrate maternal immunity as a processes providing immunoglobins (IgG, IgA, and IgM) to offsprings through milk (Albery *et al.,* 2020; Landete-Castillejos *et al.,* 2002). In response to this complexity, new approaches in the field of ecoimmunology use a systemic approach of immune heterogeneity in populations and communities. Indeed, addressing immune heterogeneity need to integrate different layers of complexity as measuring immunocompetence of individuals from a given or several populations would integrate variations in immunity mediated by life traits (*e.g.* maternal transfer), location (*e.g.* pathogen hot spot), hormonal status, season… (Schoenle *et al.,* 2018). Furthermore, the measurement of an immune parameter such as immunocompetence was refined to optimal immunity that integrates the complex equilibrium between an efficient immune response against pathogens and a viable offspring as a component of fitness (Graham *et al.,* 2011). It is noteworthy that measuring immunocompetence and the way free-ranging animals trade this cost to maximize (or not) their fitness could allow to understand how they maintain an optimal immunity in natural environment (Viney *et al.,* 2005). Recent findings focus on comparative immunology between wild and captive animals, which display relevant discrepancies not only in their parasitic loads but also in the proportion and levels of expression of an immune marker, found lower in the wild (Abolins et al., 2017a; Viney and Riley, 2017a). This new trend produced fascinating experimental designs of rewild animals, which consists in exposing lab animals like mice (*Mus musculus domesticus*) to natural constraints promoting immune response toward pathogenic infections in conditions of environmental stress (Graham, 2021a; Flies and wild comparative immunology consortium, 2020).

Stress in Ecoimmunology

A stressor is a general term for an external stimulus often related to an aversive condition that could enhance stress if not endured or avoided (Martin, 2009a; Apanius, 1998). Thereby, stress responses correspond to compartmental (*e.g.* escape) or physiological (*e.g.* hormonal) mechanisms allowing an individual to recover or survive from the action of a stressor (Martin, 2009a). Lochmiller (1996) set stress as one of the indirect factors inducing immunosuppression, which by extension may decrease individual resistance to infections and alter population dynamics. This functional approach of down-regulating immune response (*i.e.* immunosuppression) mediated by stress is intended to promote a reallocation of resources towards physiological processes connected to fitness (*i.e.* reproduction, growth and/or foraging). Glucocorticoids (GCs) are key physiological mediators of stress whose increasing production aims to avoid self-damages from defence mechanisms (*e.g.* autoimmunity) threatening both homeostasis and immunity (Robert M. Sapolsky et al., 2000). Four ecological interpretations involving the influence of GCs production (in response to stress) on immunity were proposed to depict an energetic trade-off (**Figure 1**).

Figure 1. Illustration of the energy saving hypotheses on the relationship between stress and immunity proposed by Sapolsky *et al.* (2000). In red: framework of the response to a stressor (*e.g.* predation, resource scarcity, infection…).



One is the energy saving hypothesis, proposed by Sapolsky *et al.* (2000), which posits that immunity declines during stress response to reallocate resources towards functions immediately implied in survival to the detriment of maintenance (**Figure 1**). This hypothesis

mainly relies on the ability of glucocorticoids (GCs) to promote anti-inflammatory activity, which can inhibit or reduce immune cell proliferation (*e.g.* lymphocytes, macrophages…) and decrease cytokines (*i.e.* a major mediator of the immune response) gene expressions (Dhabhar, 2002). However, the production of cytokines was also found to be enhanced by acute stress such as pro-inflammatory cytokines (*e.g.* TNF-α or Il-6). A second alternative to this hypothesis also proposed by Sapolsky *et al.* (2000) considered that immune cells may be sacrificed (*i.e.* apoptosis) to turn them into valuable energetic compounds like proteins or glucose promoting more energy for the maintenance of physiological processes such as homeostasis. A third interpretation of the energetic trade-offs mediated by stress was proposed in Råberg *et al.* (1998) as the autoimmune avoid hypothesis. The study of Råberg *et al.* (1998) considered an optimum between resistance and immunopathology when an organism endures stress. Indeed, the secretion of GCs and other hormones of stress may favour the appearance of self-antigens produced by the degradation of tissues. In that way, the immune response has to be diminished to avoid self-damage also known as autoimmunity. Such a mechanism of immunosuppression aims to prevent the first lineage of immune cells from their activity of "seek and destroy" of antigens. This ultimately translates that enduring stress may outweigh the benefit of investing energy into a non-selective immune response against both host and pathogens. The interpretations detailed above omit that both reallocation of resources or apoptosis is energetically costly. Furthermore, as detailed in Martin, (2009), suppressing the immune system would finally suppress an evolved system integral to the recovery from pathogenic infections.

Take-home messages

1) Immunoecology provides relevant approaches that aim to study how wild animals respond to stressors.

2) Trade-offs in the energetic allocation to physiological function are prone to be under the control of the hormonal response to stress.

3) GCs are key elements of inflammation whose prime role is to down-regulate immune defence once infections are controlled.

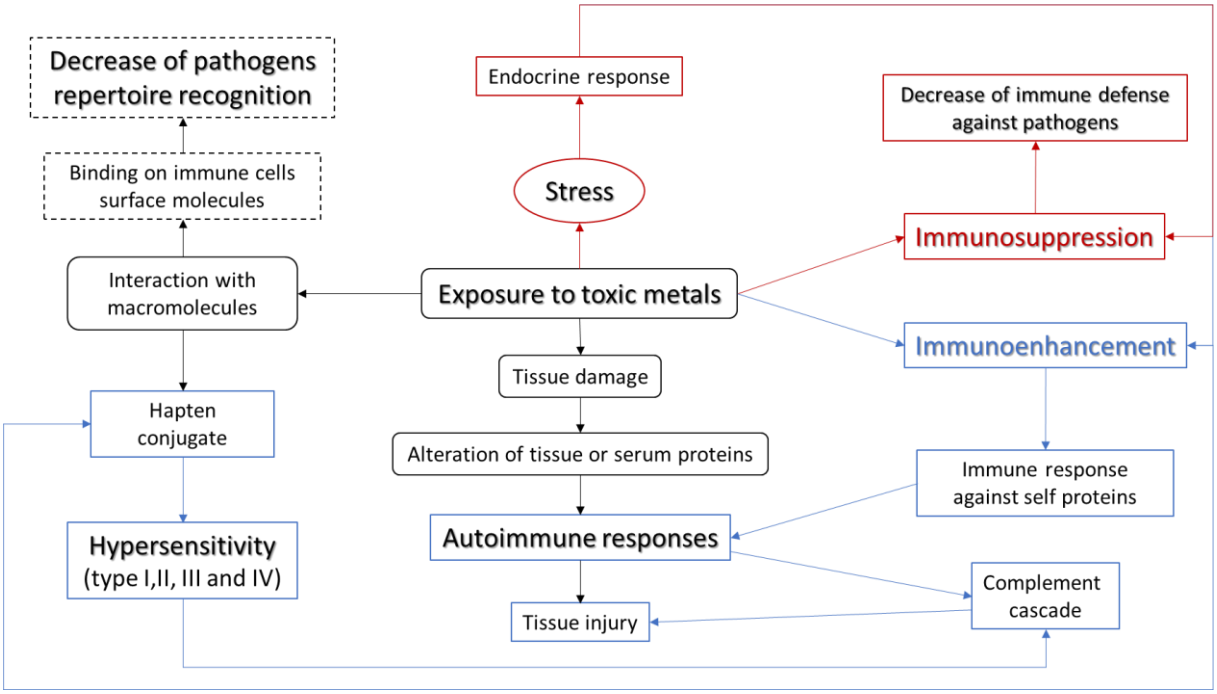
4) The relative influence of stress on immunity results in a protective function of organisms to either reallocate energy to essential functions or avoid auto-immune damage.

1.1.1.2 Crossing borders between immunology and toxicology

Immunotoxicology is a very functional approach of the fate and effects of a compound on host's (mainly humans) immune system (Brousseau *et al.,* 2012). The discipline emerged in the 70's with the discovery that the administration of some compounds may enhance immunosuppression (Germolec *et al.,* 2017). While some toxic compounds can suppress the immune response, others were found to enhance abnormal immune responses against self (*i.e.* autoimmune disorders). The preferred term to qualify alteration whether up (immunoenhancement) or down (immunosuppression) activation of the immune system is therefore immunomodulation. Both stimulation and suppression can be measured with a wide range of immunomarkers, whose specific properties will target immune alterations. The close connection of immunotoxicology with clinical uses allowed the development of several immunoassays enabling diagnosis on the immune compartments affected by a specific compound (Luster & Gerberick, 2010). Those techniques and assays have been widely developed and standardized in laboratory rodent species to characterise the immunotoxicity of a given compound (Luster and Gerberick, 2010; US EPA, 1998; Luster *et al.,* 1993). The very methods to assess the effects of a toxic compound rely on the Tiers approach proposed in Dean, Padarathsingh & Jerrells (1979). Tier 1 corresponds to the screening of the effects of a given toxic compound on the immune system. Tier 2 aims to define which component of the immune system is affected. Tier 3 is attached to understand the mode of action of a toxic compound. Tier 1 can be assessed with the measurement of haematological parameters (e.g. a decrease of the leukocyte count) or of histological disorders such as lymphoid cell depletion in the spleen red pulp. Tier 2 would require further methodological approach to determine if cellular (*e.g.* cytokine measurement) and/or humoral (*e.g.* plate forming colony assays) immunity are affected by a toxic compound. Then, the combination of Tier 1 and 2 would provide knowledge to determine the mode of action of a given toxic compound. Among the common modes of action found in the literature, Zelikoff *et al.* (1994) illustrated the mechanism involved in the exposure to a toxic compound, here adapted in **Figure 2** to toxic metals, with the addition of findings of Lawrence & McCabe (2002). Exposure to contaminants like metals at toxic levels may modulate the immune response (immunomodulation) towards activation (immunostimulation or immunoenhancement) or deactivation (immunosuppression) of the immune system. Both patterns of immunomodulation can be mediated or not by hormonal stimulation in response to the stress of exposure to metals. Immunosuppression corresponds to a down-regulation of the immune system that may lead to a decrease in immune defence against pathogens and thus may favour the development of diseases and cancers (Zelikoff *et al.,* 1994). Gallucci, Luckett-Chastain & Yucesoy (2020) defined an immunostimulation as an "inappropriate and nonspecific activation of the immune system, which can lead to

hypersensitivity and autoimmunity". Immunostimulation or immunoenhancement results in an up-regulation of the immune system enhancing a defensive response like an increase of the phagocytic activity or the inflammatory-mediators proliferation (Luster *et al.,* 1993; Luster and Gerberick, 2010). This mechanism can be associated with hypersensitive reactions of the immune system like in asthma or flulike symptoms and in some cases leads to autoimmune responses against "self" proteins leading to tissue injuries (Brousseau *et al.,* 2012). Metal-induced hypersensitivity corresponds to an allergic reaction due to the disturbance of homeostatic mechanisms implied in the prevention of inappropriate immune response against non-pathogenic compounds (McKee and Fontenot, 2016).

Figure 2. Immunological (in red and blue), and toxicological (in black) consequences of toxic metal exposure. The patterns of immunosuppression (in red) and immunoenhancement (in blue) were adapted from Zelikoff *et al.* (1994) with the implementations of Lawrence and McCabe (2002) in dotted lines.



This mechanism mainly relies on the sensitization by hapten conjugates produced by the interaction with toxic metals and carrier proteins of "self", also called haptenization (Zelikoff *et al.,* 1994). Haptens are small molecules that elicit an immune response (*i.e.* production of antibodies) occurring only when they are bound to a carrier (*e.g.* proteins) to be recognised as an immunogen. Hypersensitive reactions associated with metal exposure involving hapten conjugates are mainly of type-I (or immediate hypersensitivity), which is mediated by IgE antibodies in humans, and of type-IV (or delayed hypersensitivity), which is mediated by macrophages and T-lymphocytes (Woolhiser *et al.,* 2005; Zelikoff *et al.,* 1994). Toxic metals were also found to activate the complement system through hypersensitivity of type II mediated

by a cytotoxic reaction involving IgG and IgM in humans and type III that more directly enhances immune complexes. The four types of hypersensitivity initially presented by Gell and Coombs, (1963) are thus potential modes of action of toxic metals to enhance inflammatory immune responses. However, Lawrence & McCabe (2002) proposed another mechanism of hapten inhibition, which considers that toxic metals can bind to peptides or proteins (*i.e.* immune cell receptors) disturbing antigen recognition among the repertoire of an immune cell (*e.g.* T cells).

Take-home messages

1) Assays and protocols have been developed to evaluate the immunotoxicity of a compound to a living organism.

2) Toxic compounds including trace metals elicit several immunotoxicological effects leading to either immunosuppression or immunoenhacement

3) The Tiers approach allows to evaluate and understand how a specific component of the immune system is affected by a toxic compound.

4) Most of procedures and measurements rely on laboratory rodent and human studies.

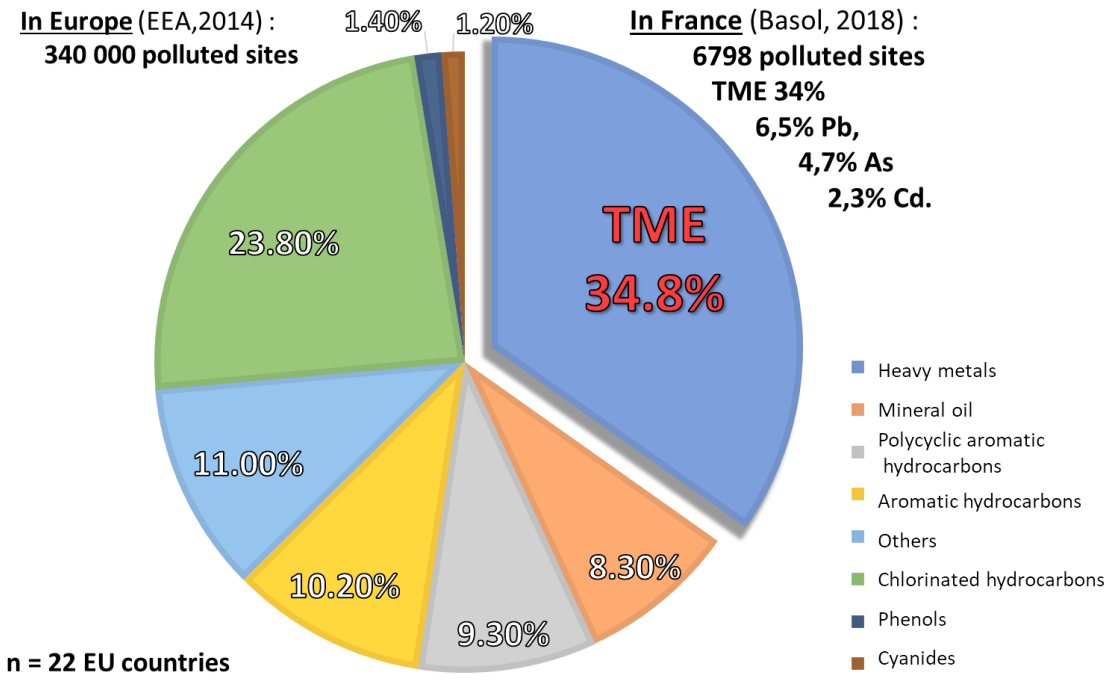
1.1.1.3 Crossing borders between ecology and toxicology

Fundamentals of ecotoxicology

Ecotoxicology is a reductionist discipline at the intersection of ecology and toxicology, which emerged during the 70's with the arising consideration that an increasing number of pollutants are contaminating the biosphere (Ramade, 2007). The whistleblowing book « *Silent spring* » of Rachel L. Carson (1962) targeted the intensive use of post-WWII synthetic pesticides (*e.g.* dichlorodiphenyltrichloroethane, abbreviated as DDT) and their harmful consequences on wildlife. Concomitant observations that environmental contaminants can be transferred from the abiotic compartment to living organisms (along the food chain or not) and that these contaminants were associated with adverse effects, conducted to the formalisation of ecotoxicology as the study of the ecological effects of pollutants (Truhaut, 1977). Ecotoxicology can also be defined as the study of the behaviour and the effects of pollutants, either from artificial or natural sources, on the structure and the functioning of communities and their

ecosystems (Gillet, 2018; Ramade, 2007; C H Walker et al., 2012). Several ecological disasters from nowadays can be quote as relevant examples of the need of ecotoxicology in our rapidly changing world. For example, the *Lancet* Commission on pollution and health considers that “Pollution is the largest environmental cause of disease and premature death in the world today.” (Landrigan *et al.,* 2017). The study was widely spread over the media, which retained a scary estimate number of 9 million premature deaths (16% of total global mortality) attributed to pollution-related diseases in 2015, among which 0.5 million was attributed to soil, heavy metals, and chemicals pollution, plus 0.5 million only from lead exposure. By now, pollution is widely considered as one of the resulting factors of anthropogenic activities causing deep damage to ecosystem and the biodiversity it sheltered (Reid *et al.,* 2005). Indeed, pollution is among the threats (*e.g.* intensive fishery, habitat loss, climate change, *etc.*) touching the widest and longest spatial and temporal scales (Bonebrake *et al.,* 2019). The **Figure 3** illustrates the proportion of polluted sites (soil and groundwater) by their types of contaminant in 22 European countries. Among the 340 000 polluted sites recorded in 2011, 34.8 % are polluted by trace metals, 23.8% by chlorinated hydrocarbons and 11% polycyclic aromatic hydrocarbons (EEA, 2014). In France, 34% of the polluted sites are contaminated by trace metals mainly by arsenic (As), cadmium (Cd) and lead (Pb) (Commissariat général au développement durable, 2019). Each year the management of polluted sites costs an estimated € 6.5 billion to Europe private companies (mainly) and public institution (European Commission, 2014). The European Environment Agency (EEA) targeted agriculture and industrial activity as the main sources of the actual trace metal emissions in Europe (EEA, 2020). It thus the challenge of governmental and nongovernmental organisations to raise public policies solving this environmental and human health issues.

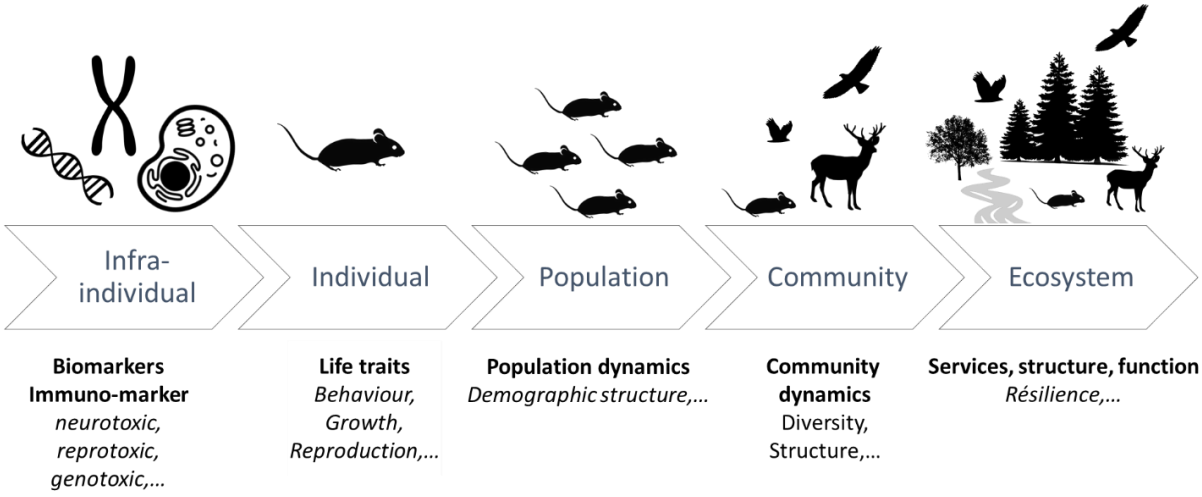
Figure 3. Proportion of contaminants affecting the solid matrix (soil, sludge, sediment) in European polluted sites reported in 2011 (EEA, 2014; source: https://www.eea.europa.eu/data-and-maps/daviz/overview-of-contaminants-affecting-soil#tab-chart_3) and the proportion of trace metals (TME) in French polluted sites (Commissariat général au développement durable, 2019).



The close connection of ecosystem health with animal and human health has promoted the development of a systemic approach presented under the concept of “One health”. Ecotoxicology addresses many of the issues of the One Health concept considering the potent ability of toxic compounds to disturb endocrine and immune systems (Acevedo-Whitehouse and Duffus, 2009a). For example, toxic compounds display immunotoxic effects that can directly affect human or animal health but also indirectly promote disturbance in ecosystem functioning or in the transmission of zoonoses by increasing pathogen prevalence in reservoirs or intermediate hosts (Destoumieux-Garzón et al., 2018a). Among the numerous cases of anthropogenic pollution, the disease of Itaï Itaï refers to a cadmium (Cd) poisoning in human and fish in 1912 at Toyoma, Japan (Aoshima, 2016). At the origin, an increasing demand in weapon production led to a release of high levels of Cd in the Jinzu River, which brought fish and drinking water for people, and irrigation water for crops. The strong dependency of local inhabitants and dwelling species to the river ended in an observed increasing mortality in both fishes and humans. Another example is the reactor meltdown of Chernobyl in 1986, leading to severe loss in wildlife species in the exclusion zone where elevated levels of radiation are still emitted by caesium 137 (^{137}Cs) and strontium 90 (^{90}Sr)(Beresford *et al.,* 2020). Both ^{137}Cs and ^{90}Sr exhibit similar properties as calcium and potassium, respectively, which allows them to

incorporate bones and tissues, promoting radiotoxic effects from β and γ radiations (Ramade, 2007). However, a study of Fritsch *et al.* (2008) using *ex-situ* mesocosms with polluted soil by ^{137}Cs showed that the presence of earthworms increased by 2 to 3-fold ^{137}Cs concentrations in snails. The ecology of the earthworms may have increase bioavailability of ^{137}Cs or simply get back on surface deep ^{137}Cs promoted by casts which were more accessible to snails. Here, the "eco" in ecotoxicology brings a comprehensive framework on the direct and indirect mechanisms underlying the contamination of an ecosystem and, on the transfer and the impact of toxic compounds to target and non-target species (Hoffman *et al.,* 2002; Cairns, 1988). The approaches have steeply evolved towards the development of new tools to measure, characterise or evaluate the effects of pollution on organisms. For instance, bioindicators are model species regrouping an assembly of features sufficiently relevant to characterise toxicological effect to organisms. For example, land snails (*e.g.* the garden snail *Cantareus aspersus*) are good indicators of soil pollution as they are ubiquitous, they can accumulate contaminants in their tissues (bioaccumulator), they have an intermediate trophic position (*i.e.* saprophagous and herbivorous) and they can easily be bred in the lab. Bioindicators can serve to measure biomarkers, which, according to Walker *et al.* (2012), represent "any biological response to an environmental chemical at the individual level or below demonstrating a departure from the normal status.". The relationship between bioindicators and biomarkers made them relevant tools to characterise exposure to and effects of pollutants at different levels of organisation (Amiard-Triquet *et al.,* 2012; Walker *et al.,* 2012). Indeed, biomarkers can be used to measure, for example, the effects of radiations on DNA of snails, the effect of DDT on eggshells of birds or the impact of Cd or lead (Pb) on liver or kidneys of small mammals, which may all have impacts on individual fitness and population and community dynamics. The cursor of the biological level of organisation as illustrated on **Figure 4** depends thus on the hypothesis tested to define the level of precision of the measurement (*i.e.* measurement of biomarkers*,* life traits, *etc.*). From an ecotoxicological perspective different levels of biological organisation imply different levels of spatial and temporal organisation (Lidicker, 2008).

Figure 4. Biological organisation levels in ecotoxicology.



According to Kapustka *et al.* (2001), there is a significant need to integrate a spatial dimension to environmental risk assessment in order to characterize the duration, the intensity and the sources of exposure to contaminants. Landscape heterogeneity in terms of both structure and composition represents the foundations of the ecological niche of the local biodiversity (Hutchinson, 1965, 1957). A landscape is commonly described as a mosaic of patches characterized by its composition, structure and land uses (Wiens *et al.,* 2006; Urban *et al.,* 1987; Forman and Godron, 1981). Anthropogenic activities have shaped the actual landscape for example the set-up of buildings, roads and facilities can divide (*i.e.* fragmentation) or take up (*i.e.* loss) the habitat of free-ranging animals (Houghton, 1995; McNeely, 1992). In this context, landscape ecotoxicology is a recent discipline based upon the observation that responses to chemical stress could vary with the study range and the spatial heterogeneity (Cairns and Niederlehner, 1996). Both spatial and temporal scales need to be considered as exposure may be affected by the expand of the emissions and the persistence of pollution (Devalloir et al., 2023). For instance, the physical distance to a source of pollution may create a gradient of exposure. The availability of the pollutant may be modulated by the land uses and the structure of patches which can display different physico-chemical properties. Indeed, the presence of a gradient of pollution may persist over time due to repeated emissions or to the persistence itself of the pollutant. All these patterns of exposure rely on the scale at which their studied as pollution exposure can change depending on the time and the localisation. Scale changes over time and space brought a holistic approach of the effects of pollutants on the ecological processes inside and between organization levels.

From ecotoxicology to stress ecology

Several parameters have been developed to characterise the effects of toxic compounds on lab species and bioindicators, such as the effective concentration (EC50) or the no effect

concentration (NOEC). The basic strategy of ecotoxicology aimed to extrapolate data from one species to another, thus promoting relevant tools to evaluate the risk on species sharing the same sensitivity to a given compound or "cocktail" (*i.e.* mixture of pollutants) (Posthuma *et al.,* 2001). Though this strategy relies on reproducible and reliable measurements under control conditions, the main limit relies on environmental constraints which cannot be simulated in the lab (van Straalen and van Gestel, 2008). In natural conditions, wild animals have to constantly cope with stressors (as defined above in 1.1.1.1.), which can be declined as environmental (*e.g.* predation*,* inclement weather, *etc.*) or anthropogenic (*e.g.* pollution*,* habitat loss, *etc.*) stressors (Acevedo-Whitehouse and Duffus, 2009a). Both types of stressors are closely connected. For example, habitat loss caused by human activities can dampen quality and/or limit the access to resources resulting in an elevated stress response (Halbrook *et al.,* 2003). Field study is thus an integrative methodology allowing obtaining a "real" measure of stress in natural conditions. However, the transfers and the effects of contaminants may vary with the environmental constraints among wild animals which not the case of laboratory studies done in controlled environment. It thus necessary to define a reference values under natural conditions, such as the normal operative range which defines the boundaries of a given system in absence of stress (Van Straalen, 2003). The steady state of a given population study is often obtained from a control unexposed population. Measurements of the effects of stressors on wild species driven out of their normal operative range could provide relevant overview of stress in natural environment. It thus the challenge of stress ecology to propose valuable extrapolation of results which must address the complexity of ecosystem (Van den Brink, 2008). Stress ecology promotes an integrative approach of the natural and anthropogenic factors modulating exposure to a contaminant. This can be connected to the following quotation of J. Cairns (1988):

"Ecotoxicologists who study the effects of stress on individual species and natural communities are ideally situated to play a major role in this undertaking if more eco is put in ecotoxicology.".

Among the advantages of field studies, biomonitoring promotes an efficient and relevant tool to evaluate the chronicity of the exposure to a contaminant among a given population. Nonetheless, these methods often required the development of non-lethal tools such as measurements in fur, feathers, blood, *etc* (*e.g.* Powolny *et al.,* 2019). The development of this techniques has given some insights over the last decades and now a large panel of proxies can be used to monitor the concentrations of toxic compounds (*e.g.* Bustnes *et al.,* 2013; Fossi *et al.,* 2003). The integration of field approach among wild animals have promoted the inclusion of nutrition in the scope of ecological disturbance that may threat biodiversity. Stoichiometric ecotoxicology was recently proposed by Peace *et al.* (2021) introducing toxic compounds as a

factor that could modulate food nutrient quality, availability, assimilation and transfer in the whole supply chain of food web.

Take home messages

1) Ecotoxicology is the study of the behaviour and fate of pollutants in environmental matrices.

2) Stress ecology displays a relevant framework integrating both anthropogenic and environmental stressors as either regulative or disturbing factors of ecological interactions.

3) Ecotoxicology was a reductionist discipline that is gaining more insight within a more holistic approach promoted by the “eco” of ecology.

1.1.1.4 Towards a field of ecoimmunotoxicology

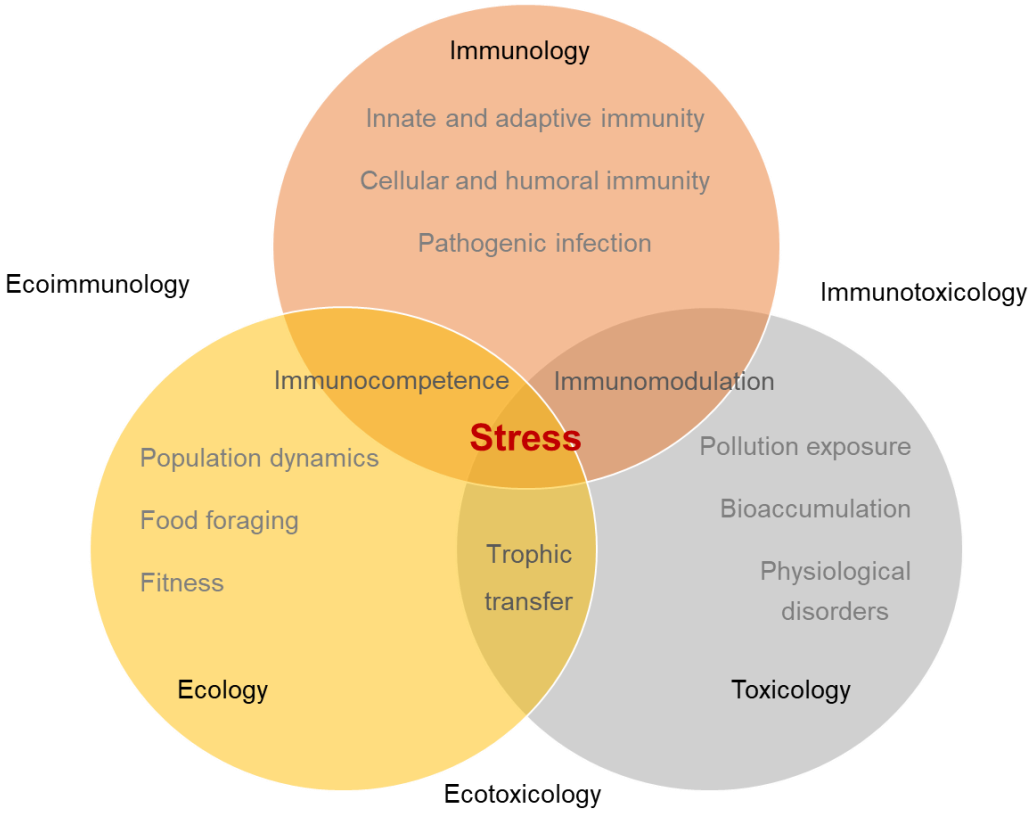
The study of the impacts of toxic compounds, including metals, on the immune system has conducted to the development of a field of immunotoxicology (Brousseau *et al.,* 2012). Anthropogenically induced immunomodulation in wildlife is basically a recent approach calling for further investigation (Woolhiser *et al.,* 2005). As described above in 1.1.1.2., unintended immunomodulation may have severe consequences in increasing pathogen susceptibility (immunosuppression) or increasing host autoimmune damages (immunoenhancement). On one hand, most of the immunotoxicological studies used laboratory raised animals such as mice or rats to extrapolate their results to humans (Luster and Gerberick, 2010). On the other hand, many immunological studies are now moving out from the paradigm of mice and men because of the discrepancies found in immune responses between free ranging and laboratory animals to e.g. immune challenge (Graham, 2021a; Abolins et al., 2017a; Jackson, 2015). Indeed, immunomodulation also occurs in wild animals that are constantly challenged by different stressors including pathogens, pollution and nutrition (Acevedo-Whitehouse and Duffus, 2009a). On the basis of these observations, Ecoimmunotoxicology was proposed by Woolhiser *et al.,* (2005) as a new approach overlapping ecology, immunology and toxicology to understand how toxic compounds may affect immune system of wild animals at different organization levels (**Figure 4**). Ecoimmunotoxicology intends to use wild animals as sensitive markers of environmental stress to measure their immune status, based upon knowledge and methods of ecology and immunotoxicology. Hence, ecoimmunotoxicology promotes an integrative approach connecting the individual methodology from human risk assessment to

population or community methodologies from ecological risk assessment on (Sample and Suter, 2002). To this purpose, the adaptation of immunotoxicology on wild animals is set up upon the observation that immunological organization levels are highly conserved among vertebrates (Woolhiser *et al.,* 2005). Equivalent of immune system components can also be found in invertebrates. Hemocytes (the equivalent of white blood cells in mammals) are able to produce corticoid molecules, which are sensitive to the effect of stress on the immune system (Galloway and Depledge, 2001). The absence of controlled conditions introduces a tremendous source of variability that implies to integrate measurement related to field studies. For instance, spleen size (commonly used in Tier 1 to realise a first screening of the immunotoxicological effects, see 1.1.1.2) may not be relevant in field studies as stressors such as food restriction may decrease its size. It is thus a priority in ecoimmunotoxicology to adapt (*i.e.* cross-reactive biomarkers) or develop methods to monitor immunotoxicological effects using non-lethal procedures regarding the ethical issues on wild animal conservation. This topic have gained insight into wildlife studies relating the detrimental effect of contaminants in artic ecosystems including trace metals, polychlorobiphenyls (PCB), organochlorines (OC)… (Dietz *et al.,* 2019). Large screening on immunomodulation and an increasing consideration on immune deficiency have been done since the 70's with first studies on marine mammals (Dietz *et al.,* 2019, 2013; Desforges *et al.,* 2016). General findings on marine mammals highlight a significant trophic transfer of pollutants in mammal tissues associated with immunotoxicological effects, like trace metal hypersensitivity (Kakuschke *et al.,* 2006). Specific cases of immunomodulation are given in Chapter 1 (see 3.1.).

In this PhD research, I tried to combine stress ecology to ecoimmunotoxicology by placing "stress" as the "consensual overlapping area" of three disciplines that are ecology, immunology and toxicology. As detailed above, stress may influence all physiological compartments, and thus promote disturbance at individual level that could affect populations and communities. The framework presented in **Figure 5** aims to display how interdisciplinarity has developed relevant tools to evaluate the response to stress and their impacts on individual fitness and population (or higher levels of organization) dynamics. Indeed, response to stress can be enhanced by exposure to a stressor (*e.g.* trace metals) leading to physiological disorders (*e.g. immune* cell damage), which may modulate immune (*e.g. immunosuppression*) and endocrine (*e.g. increase of circulating glucocorticoids*) systems in increasing or decreasing individual immunocompetence. Then, an immune alteration mediated or not by endocrine response to stress would then implied variation in the energetic demand toward functions either valuable to fitness or either to maintain an efficient immunocompetence. Ultimately, a decrease in individual immunocompetence may affect the structure of wild population in for example

increasing risk developing infectious diseases which could increase the virulence of a pathogen (Brousseau et al., 2012).

Figure 5. Setting stress at the crossroad of ecology, immunology and toxicology.



To this end, the framework detailed in **Figure 5** aims at connecting three disciplines addressing the following conclusion of Acevedo-Whitehouse & Duffus (2009):

"The increasing rates of disease (both infectious and non-infectious) in wildlife is of great concern since disease may be an indication that populations are approaching a state of stress which is negatively affecting immune function, and it is unknown how close this is to the upper limits of their tolerance."

Indeed, mammal immune system is constantly under the pressure of environmental factors (*i.e. nutrient reduction, pollutant, habitat loss…*), originating from anthropogenic sources or not, which may dampen host resistance to infectious disease. In this purpose, I propose within the research undertaken in my PhD to revisit the concept of "ecoimmunotoxicology" into a "real" world adaptation of ecoimmunology, stress ecology and immunotoxicology, which could be gathered under the term of stress immunoecology. The meltdown of these three disciplines under a stress based approach aims to understand (i) the effect of stressors on the immunocompetence in considering the energetic cost allocated to the response to these stressors (Martin, 2009a), (ii) the ability of stressors to interfere with physiological processes

and to larger extent ecosystem functioning (Van Straalen, 2003); and (iii) the methods and procedures of immunotoxicology to characterise the impact of toxic compounds on the immune system at different biological organisation levels (Woolhiser *et al.,* 2005).

Take home messages

1) Ecoimmunotoxicology is a promising field that integrates three disciplines (*i.e.* ecology, immunology, toxicology) to determine the influence of toxic compounds on the immune system of wild animals.

2) The “Eco” in Ecoimmunotoxicology aims to move out from the paradigm of mice and humans studies when addressing immunomodulation by pollutants in wild animals.

3) A new framework is proposed to disentangle the influences of multiple-stressors on immunocompetence.

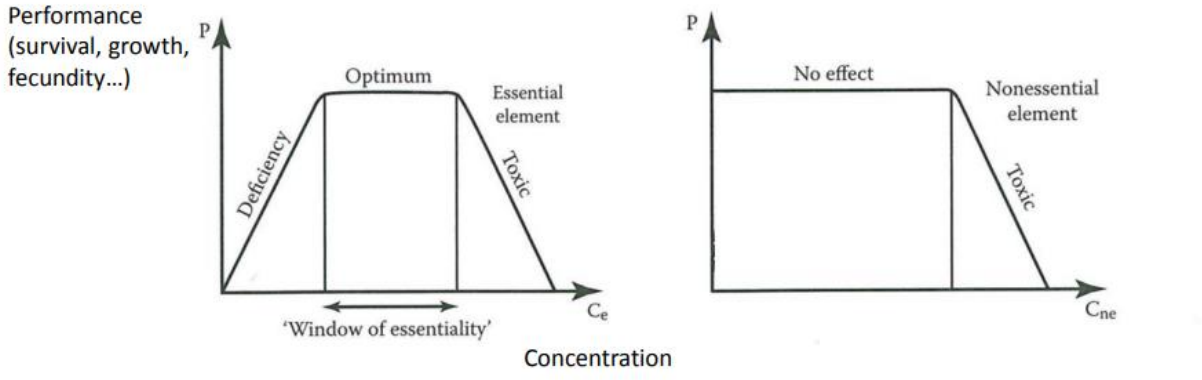
1.1.2 Environmental pollution by trace metals

1.1.2.1 Description, sources, fate and global consequences of trace metals

In our industrial societies, metal(loid)s display several advantages in terms of economy, uses and services. A metal is a lustrous element that can be a precious element (*i.e.* gold), a good electric conductor (*i.e.* copper), or provide nuclear energy (*i.e.* caesium 137). All metals are classified in the periodic table according to their similar chemical properties (same column) or their ability to share electrons with other elements. Most of metals are naturally occurring in earth surface (water and soil) among which mono-or di-valent cations like calcium (Ca), magnesium (Mg), potassium (K) and sodium (Na) are found in abundant quantity. Carbon (C), hydrogen (H), nitrogen (N), oxygen (O), phosphorus (P) or sulfur (S) are basic atoms of living organisms and belong to non-metal elements. Metalloids, like arsenic (As), boron (B), or selenium (Se), have the same characteristics of both metals and non-metals. The other elements of the periodic table are called transition metals like chromium (Cr), copper (Cu), iron (Fe), lead (Pb), manganese (Mn), mercury (Hg) and zinc (Zn). Arsenic, Cd, Hg and Pb, formerly considered as “heavy” metal considering their high density, have been targeted by European policies regarding their toxicity to biota (EEA, 2020). The actual preferred term is “trace metals “or “trace metallic elements” as it engulfs all elements which proportion on the earth crust is

below 0.1% (Kabata-Pendias and Szteke, 2015). Among trace metals, cobalt (Co), copper (Cu), chromium (Cr), iron (Fe), manganese (Mn), molybdenum (Mo), nickel (Ni), selenium (Se), and zinc (Zn) are essential elements required to maintain physiological processes involved in the immune system and homeostasis (Mehri, 2020). Nieboer & Richardson (1980) proposed a classification of essential and non-essential metals according to their affinity with oxygen (class A: Ca, K, Mg, *etc.*), or with sulfur or nitrogen (class B: Cd, Cu, Hg, silver), or their borderline affinity with both (borderline: As, Cr, Fe, Pb, Zn, *etc.*). As presented in **Figure 6**, essential elements are efficient on individual performance at an "optimum" level below which individuals may suffer from deficiency and above which the element turns into a toxic compound (Walker *et al.,* 2012). Non-essential elements have no (known) effect on biological functions (*i.e.* homeostasis, immune system) until a species-specific threshold where they become toxic to the exposed organism. Metalloids like As and Hg can bind to organic molecules to form organometallic lipophilic compounds such as methylated arsenic or methyl mercury. Transition metals such as Fe and Cu are prone to electron transfer reactions with oxygen leading to the production of oxygen radicals involved in cellular damages (*i.e.* oxidative stress). Further information on the mechanisms by which some essential elements promote beneficial effects and As, Cd, Hg and Pb exert toxicological effects is detailed in Chapter 1 (see 3.1.).

Figure 6. Relation between individual performance (survival, growth, fecundity, *etc.*) and the concentration of an essential element (left) and a non-essential element (right) taken by an organism (source: Walker *et al.,* 2012).



Nearly all of these metals, with the exception of laboratory man-made (*e.g.* radioisotopes), are naturally present on Earth. Natural trace metal occurrence is often related to cycles whose emissions is the result of weathering of ore bodies or volcanic activity or wild fires (Ramade, 2007). However, the global emissions of these elements are disturbed by human activities, among which industrial activities such as mining or smelting release trace metals like Cd, Pb or Zn initially hold in the bedrock (Walker *et al.,* 2012). Many examples associating industrial activities and environmental pollution around world can be found in press journal (see:

https://ejatlas.org/) or scientific papers and here in this thesis manuscript with the iconic site of Metaleurop Nord SAS (see 2.1). The anthropogenic sources of trace metal emissions often surpass the natural sources defining a background value (Pacyna and Pacyna, 2001; Nriagu, 1996). In example, Pb anthropogenic source display an anthropogenic enrichment factor of 97% with an anthropogenic source of 300 against 10 million of kg per year from the natural sources in the 1980's (Walker *et al.,* 2012). This very high value of Pb global emission was mainly attributed to lead-based additives to gasoline. In Europe, lead emissions have since steeply decrease with the application European policies against Pb additives of 2001, hence a sharp decrease record in tawny owls feathers as presented in Bustnes *et al.* (2013). Indeed, organisms assimilate a part of trace metal emission from their environment. The uptake of trace metals is dependent of many factors such as their mobility in the environment, exposure routes, transfer through biological membranes, and potential antagonisms with other metals (Kabata-Pendias & Szteke, 2015; Walker *et al.,* 2012). In terrestrial environment the uptake is conditioned by environmental availability, which are the physicochemical processes enabling trace metals to reach the liquid phase of soil, and the environmental bioavailability that correspond to the physiological processes enabling the absorption of trace metals (Ramade, 2007). Those two environmental processes determine the contamination of plants through foliar deposit or roots, and vertebrate or invertebrate *via* oral principally, respiratory or cutaneous uptake (Kabata-Pendias & Szteke, 2015; Walker *et al.,* 2012; Halbrook *et al.,* 2003). Once assimilated trace metals are either excreted of accumulated in organs and tissues (bioaccumulation) exerted or not toxicological effect at different level of biological organization.

Take home messages

1) Trace metals are constitutive elements of earth crust that can surpass the natural background value when emitted by human activities.

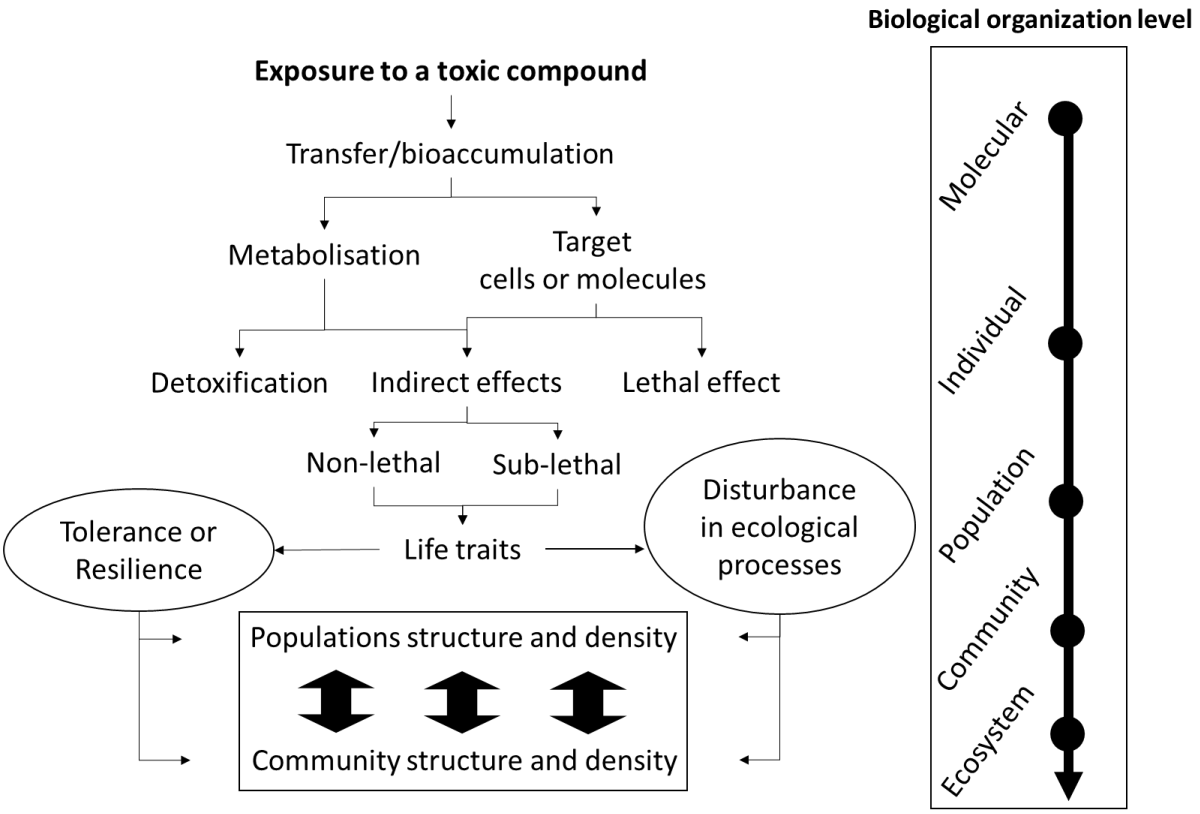
2) Essential elements are required by organisms, at a given range of concentration, to maintain metabolic processes (*i.e.* homeostasis, immune system, *etc.*).

3) Non-essential elements became toxic to living organism until they reach a given threshold of internal concentration.

1.1.2.2 Impacts of trace metals at different levels of biological organization

At the individual level (**Figure 7**), the exposure to trace metals can induce a transfer to organism that is then transported in the circulation (*e.g.* blood stream) and accumulate in target organ or tissues affecting physiological processes towards direct (*i.e.* detoxification or lethal) or indirect effects (Lagadic *et al.,* 1997). Trace metals are not biodegradable and are detoxified *via* proteins are enzymes (*e.g.* metallothionein) which inactivate them allowing their storage or excretion (Walker *et al.,* 2012). Mechanism of detoxification are part of homeostasis that is dependent of individual parameter like age, sex or nutritive status (Nordberg *et al.,* 2014). Indirect effects can be non-lethal and organism can tolerate or even be resilient after an exposure to a toxic compound. Though tolerance or resilience may translate no observed effects on life traits, they can be the result of an evolutive adaptation leading to an induced tolerance to a given toxic compounds (*e.g.* Hua *et al.,* 2015). Sub-lethal effects are commonly defined as biological, behavioural, physiological, or demographic effects on individuals or populations which survive exposure to a toxicant at lethal or sublethal dose/concentration (França *et al.,* 2017). Hence, sub-lethal effects may modify life traits (*e.g.* behaviour, reproductive success, *etc.*) and thus disturb ecological process at higher biological levels of organisation (**Figure 7**).

Figure 7. Ecotoxicological effects of exposure to a toxic compound from the individual to the community scale.



The exposure of trace metals may occur at different levels but the transfer can be influenced by trophic levels as accumulation in consumers and predators would depend on trace metal concentrations of their resources. The processes of biomagnification correspond to an increase of contaminant with the trophic levels and principally relies on persistence of contaminant along food chain, ability of a specific organism to accumulate trace metals and diet preferences (Ramade, 2007). However, transfer may be modulated by diet content as different accumulation of trace metals may occur at the same trophic level. For instance, higher accumulation of trace metals was found in insectivorous and carnivorous than in herbivorous small mammals caught on polluted sites by Cd, Pb and Zn mainly (Fritsch *et al.,* 2011). However, environmental and biological factors may modulate exposure of wild animals and the transfer along food chain towards variation in foraging processes such as food selection. Indeed, exposure to Pb and accumulation of Cd in liver and kidneys was found to decrease along a gradient of plant richness in the diet wood mice (*Apodemus sylvaticus*) population inhabiting polluted sites (Ozaki *et al.,* 2019).

Take home messages

1) Trace metals exposure can affect every level of organisation via direct (*i.e.* lethal) and/or indirect (*i.e.* sub-lethal) effects.

2) Accumulation of trace metals can increase along food chain.

3) Disturbances in foraging processes, such as food item consumption, can modulate Cd and Pb exposure and accumulation.

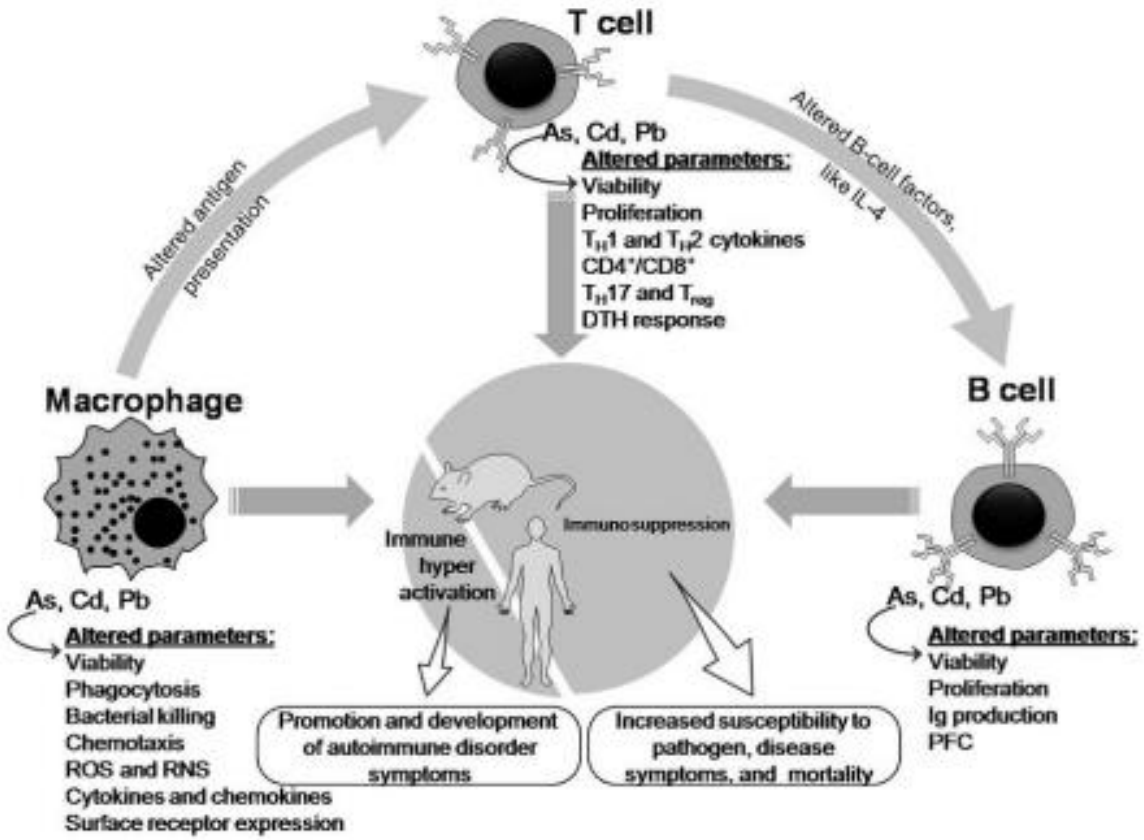
1.1.2.3 Immunotoxicity of trace metals

This part is a general summary of immunotoxic effects of trace metals presented in Chapter 1 (for more details see 3.1.).

Trace metals exposure such as Cd and Pb are commonly associated to immunosuppression (Gera *et al.,* 2015). Few studies highlighted hyper reactive immune response in a context of toxic metals exposure. However, immunotoxicity of trace metals is mainly documented in laboratory animals (*i.e.* mice and rats) and humans, while immunotoxicity in wild populations is rare among the literature (see 3.1.).

Two mechanisms can be distinguished to describe the multiple immunotoxic effects of traces metals. One relies on the ionic mimicry of toxic metals such as Cd^{2+}, which cationic form has a higher affinity to nucleophilic groups of organisms' macromolecules (Bridges and Zalups, 2005). The other is the ability of toxic metals to enhance reactive oxygen species (ROS), oxidative stress and weakened antioxidant defence, in damaging immune cells (Balali-Mood *et al.,* 2021). Through these mechanisms, trace metals can affect immune cells functioning, structure, proliferation and communication (**Figure 8**).

Figure 8. Immune parameters affected by As, Cd and Pb exposure related immune hyper-activation of immunosuppression. The figure is from Gera *et al.* (2015).



Among toxic metals, As, Cd, Pb and Hg can decrease macrophages and T cells proliferation to modulate immune cell(s) reactivity (*i.e.* immunostimulation and immunosuppression) against pathogens (Zelikoff *et al.,* 1994). Disturbance mediated by As, Cd and Pb was found to affect the secretion of a large panel of cytokines involved in both inflammatory response and regulation of the immune system (Gera *et al.,* 2015). For instance, Pb exposure was found to both stimulate cellular and humoral immune response *via* the secretion of pro-inflammatory (*i.e.* TNF-α*,* Il-6) and regulatory (*i.e.* Il-4) cytokines, respectively (Fenga *et al.,* 2017).

As detailed on **Figure 8** from (Gera *et al.,* 2015) As, Cd and Pb can altered the functioning and the structure of antigen presenting cells (APC) to recruit T cells which can be themselves affected by toxic metal exposure (*e.g.* decrease of T cells viability). Disturbance in T cells population like abnormal recruitment of T cells lineage populations (see 1.1.2.5.) which can promote self-damage, also called autoimmunity (Bjørklund *et al.,* 2017). The exposure to toxic metal can also interfere in the viability or proliferation of B cells and lead to a lower or higher production of immunoglobin (Ig) which can reduce immune defence or enhance autoimmune damage, respectively (Fenga *et al.,* 2017; Gera *et al.,* 2015; Mishra, 2009).

Take home messages

1) Immunotoxicity of Cd and Pb is mainly studied in laboratory mammals and humans and is often associated to immunosuppression.

2) Both Cd and Pb are susceptible to compromise organism's immune response to pathogens.

3) The exposure to Cd and or Pb was found to either promote hyper-reactive or immunosuppressive immune response.

1.1.3 Physiological compartment affected by stress in small-mammals

1.1.3.1 The hormonal response to stress

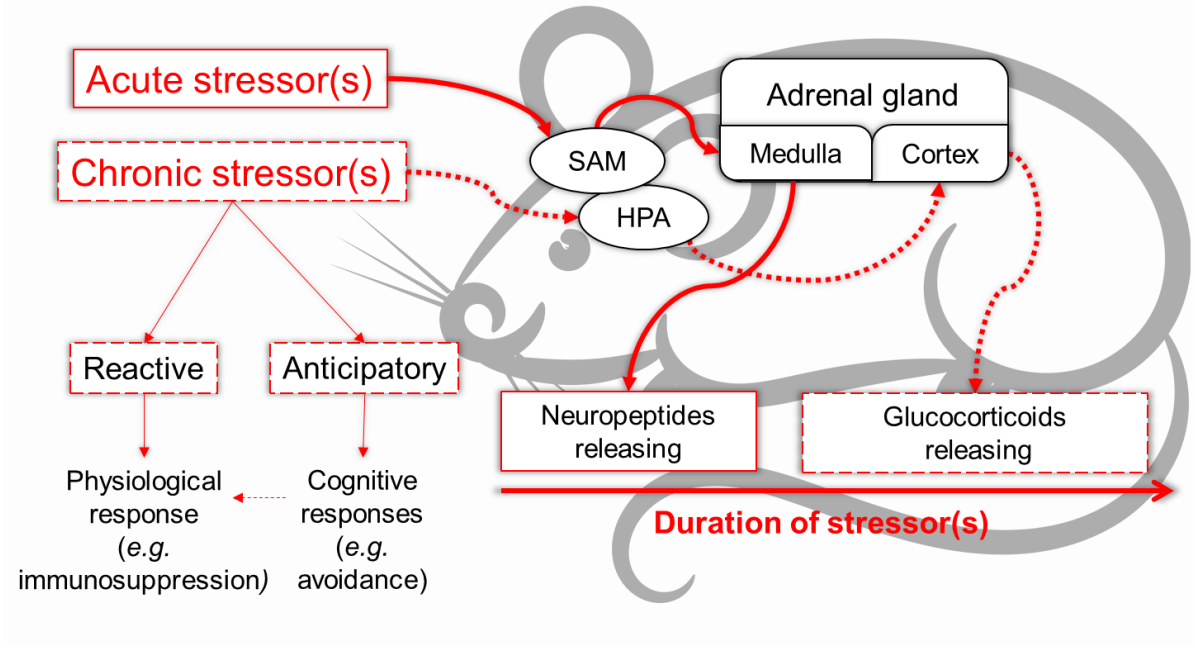
Robert Sapolsky described stress response as "incredibly ancient evolutionarily" as many classes of vertebrates secrete the same stress hormones (Shwartz, 2007). Stressors are commonly separate into acute and chronic and stress responses are monitored through physiological and/or behavioural measurement. In example, in wildlife an acute stress can be enhance by the presence of a predator promoting a stress response of escape in a prey which, if its success to flee from the predator, can recover from its stress in a short period.

As displayed in **Figure 9**, acute stress activates the sympathetic nervous system, which stimulate the adrenal gland to release neuropeptides (*e.g.* catecholamines, epinephrine, norepinephrine, *etc.*) in the blood. In the case of a pathogenic invasion, the peripheral nervous system enhance an innate immune response at local inflammatory sites and promote a vasoactive reaction to allow immune cell recruitment enabling the clearance of the pathogen (Sternberg, 2006).

According to (Boonstra, 2013), chronic stressors can be divided into either reactive (*i.e.* a direct response without cognitive processing), or anticipatory (*i.e.* an indirect response dependent from cognitive processing). A reactive response to a chronic stressor can be a direct physiological challenge (*e.g.* food scarcity). While an anticipatory response may be related to a cognitive perception of a stressor (*e.g.* intense threat of predation) that indirectly, affect physiological function of animals over a long period. Chronic stressors activate a hormonal cascade from the hypothalamus to the pituitary gland, which stimulate the adrenal cortex to release glucocorticoids (*e.g.* corticosterone in rodent and cortisol in humans). The anti-inflammatory activity of glucocorticoids affects cells in binding to glucocorticoid receptors and relies on two mechanisms, which are transactivation and transrepression of gene involved in the regulation of the immune system (van der Velden, 1998). Transactivation is produced when glucocorticoid/glucocorticoid receptor complex activates the expression of anti-inflammatory protein, while transrepression represses the expression of proinflammatory proteins by transcription factors (Vreugdenhil *et al.,* 2001). Those two mechanisms allow regulating pro- and anti-inflammatory molecules including cytokines, chemokines, and adhesion molecules.

Stressors can affect both the hormonal and immune system as a close linked between the two (*i.e.* regulation of the inflammation) influence stress induced physiological changes. Even some cell mediators like IL-6 acts as both promotion of promotion of pro- and anti-inflammatory response (Qing *et al.,* 2020).

Figure 9. Impact of acute and chronic stressor(s) on the activation of the sympathetic adrenal medullary (SAM) axis and the hypothalamic–pituitary–adrenal (HPA) axis.



Take home messages

1) Hormonal response to stressor depends on the duration of stressor(s) (*i.e.* acute or chronic)

2) Acute stressor could enhance innate immunity during inflammation

3) Reactive response to chronic stressors promotes direct physiological response including immunosuppression mediated by glucocorticoid release.

1.1.3.2 Stress and the immune system

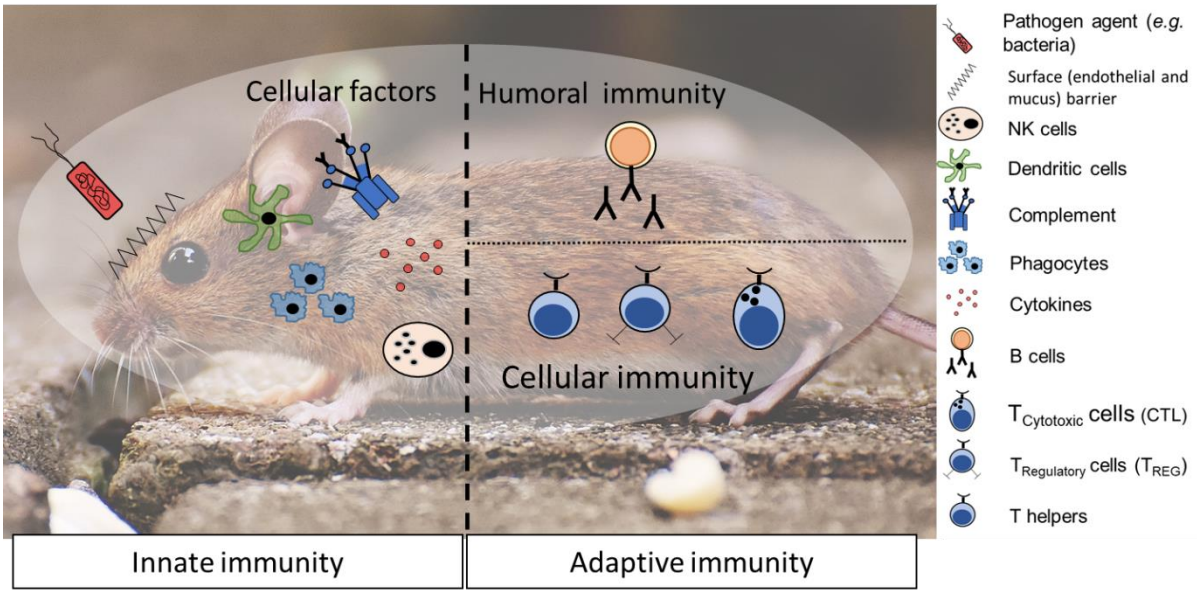
1.1.3.2.1 Component of the immune system in brief

The immune system of mammals has evolved to protect animals from parasites/pathogens which are in constant co-evolution with their hosts (Chaplin, 2010). A complex array of mechanisms is held behind the protective function of the immune system that have to respond to a tremendous type of pathogens or immunogenic compounds present in the environment. The aim of an efficient immune response is to rapidly protect the "host" against foreign invaders, which are pathogens (i.e. bacteria, virus, parasites or prions), or immunogens (*i.e.* contaminants or toxic compounds). The main complexity of immune system is held in recognition mechanisms allowing or not to tolerate self-antigens (Woolhiser *et al.,* 2005). Antigen recognition occurs at several sites of an organism involving different compartments of the immune system. However, immunogens such as toxic compounds can interfere in the recognition of constitutive element from "self" and "non-self" such as pathogens. The recognition of "non-self" molecules enhance a stress response of the immune system that can lead to inflammation. According to Medzhitov (2008), inflammatory response aims "to remove or sequester the source of the disturbance, to allow the host to adapt to the abnormal conditions and, ultimately, to restore functionality and homeostasis to the tissue". Inflammatory response will activate, recruit and produce immune cell lineages belonging to different compartment of the immune system enabling the elimination of an antigenic compounds. As display on **Figure 10**, the first compartment involved is the innate immune system, which is prone to either react rapidly in recognising pathogens or directly eliminate them. The second occurring compartment is the adaptive immune system that is made of a small number of cells,

in standard condition, which specifically react with antigenic compounds in proliferating at sufficient amount to eliminate them.

As displayed on **Figure 10**, immune cells can be distinguished by their hematopoietic stem lineage that are myeloid or lymphoid progenitors. Myeloid progenitors give erythrocytes, granulocytes, megakaryocytes and platelets. The granulocyte lineage produces key cellular effector of the immune system, which are neutrophils, monocytes, macrophages, eosinophils, basophils, and mast cells. Lymphoid progenitor are lymphocytes with B cells and T cells and natural killer (NK) cells. Lymphocytes are discriminated by their surface receptor protein promoting different antigen recognition repertoire like T cell receptor (TCR) and B cell receptor (BCR). Indeed, subpopulations of T cells can be distinguished with the presence of cluster of differentiation (CD) on their surface: with CD8 on cytotoxic T cells (CTL) and CD4 on T helper (Th) and T regulatory (Treg) cells.

Figure 10. Compartment of the immune system of a small-mammal.



Innate immune system

Innate or non-specific immune system is common among vertebrates and mainly relies on physical barriers and cellular factors (**Figure 10**). Physical barriers to pathogen such as epithelial cell layers or the mucus secreted on epithelium (*e.g.* respiratory, gastrointestinal and genitourinary tracts) avoid pathogens to easily enter in the body (Chaplin, 2010). Cellular factors that are constitutive small proteins or molecules including complement proteins and immune cells involved in the elimination and recognition of antigenic compounds. The role of small proteins and molecules is to marked or destroyed pathogens (*i.e.* cell wall destruction) and for cytokines to attract other immune cells (Alberts *et al.,* 2002). Cytokines are key immune

mediators which according to Chaplin, (2010) “regulate the function of other cells, chemokines that attract inflammatory leukocytes, lipid mediators of inflammation, reactive free radical species, and bioactive amines and enzymes that also contribute to tissue inflammation.” Cytokines such as interleukins (IL) and tumour necrosis factors (TNF), are small protein able to cross cell lipidic barrier to ensure cell signalling such as pathogen detection (Secombes et al., 2016).

Pathogen detection is ensuring *via* antigen-presenting cells (APC, *i.e.* dendritic cells, B cells, macrophages and monocytes) that displays antigen bound by major histocompatibility complex (MHC) proteins on their surface. The MHC class I and II genes are shared by vertebrates to encode surface proteins that present antigen peptides to T cells (Mak et al., 2014; Piertney and Oliver, 2006). The MHC I occurs on all nucleated cells, platelets, but not on red blood cells to display antigen peptide to CD8 on CTL. While MHC II is expressed by APC to present peptide to CD4 on Th cells.

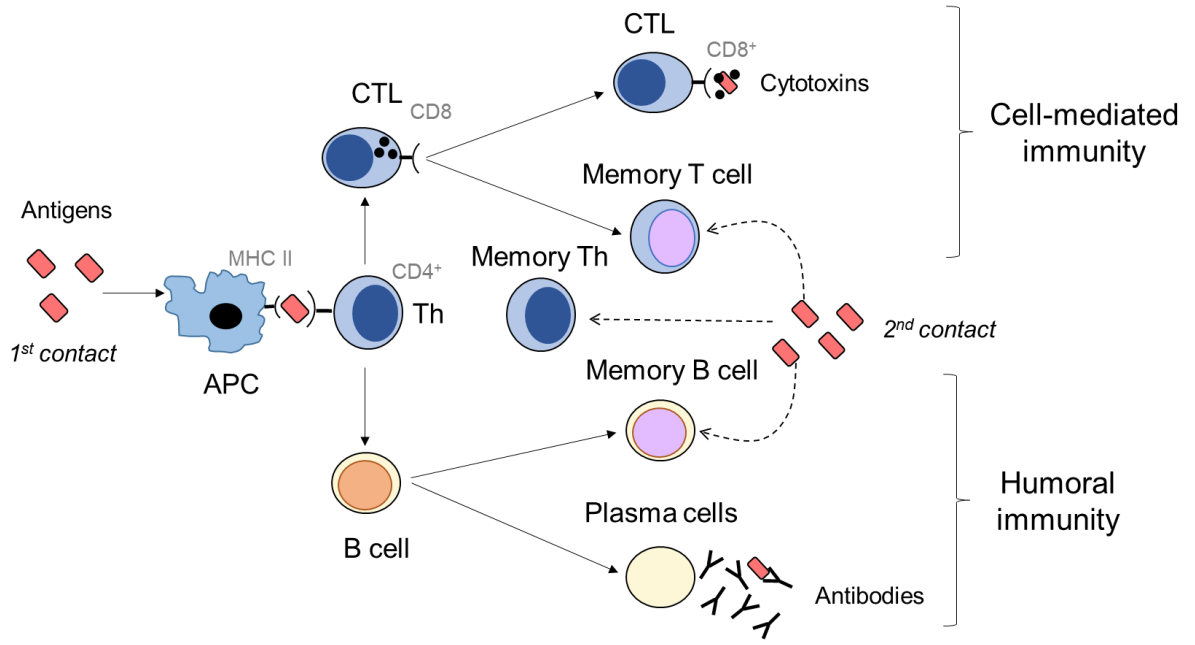
Two types of cells are associated with innate immunity that are natural killer (NK) cells and phagocytic cells (Woolhiser *et al.,* 2005). The NK cells are lymphoid cells developed in the bone marrow (mainly stimulated by IL-2 and IL-15) and are activated by T cells. No antigen-specific receptors are present in NK cells whose cytotoxicity is inhibited by recognition of the MHC I molecules enabling identification of cell from self (*i.e.* tumor, virally infected cells, *etc.*). Phagocytic cells are neutrophils, macrophages and monocytes whose principal defence mechanism aims to engulf pathogens in intracellular vacuoles where they are exposed to toxic molecules (*e.g.* enzymes, nitric oxide, *etc.*) and destroy. Pathogen recognition relies on Fc and complement receptors to eliminate particles already marked by innate or adaptive immune cells. Phagocytic cells can be activated by cytokines to exert their pathogen killing activity. For instance, Il-1, Il-6, or TNF- α can enhance nitric oxide production by macrophage promoting oxidative damage (Chaplin, 2010).

The complement system is part of the innate immune system which represent a cascade of cleavage of small proteins (the complement) allowing to activate host defences and inflammatory response to pathogen (Janeway *et al.,* 2001). Indeed, the activation of the complement promote 3 immune functions conventionally associated to three pathways. The classical pathway consists in attacking membrane of bacteria complexed with antibodies in rupturing cell wall. The alternative pathway mainly relies on phagocytosis which occurs through opsonisation (*i.e.* use of opsonin protein to tags the target antigen of phagocytic cells) and does not use antibodies. The lectin pathway enhances an inflammatory response in attracting macrophages and neutrophils *via* lectin (*i.e.* use lectin and mannose-binding lectin or ficolin proteins to allow pathogen recognition by immune cells).

Adaptive immunity

Lymphocytes are crucial element of the adaptive immunity as subpopulation orchestrates the immune response to a specific pathogen. The specificity and the diversity of lymphocytes' receptor allow the recognition of a large mapping of antigen. As displayed on **Figure 11** adaptive immunity is enhance after an APC (*e.g.* a macrophage) engulfs a pathogen and present the pathogen's antigen to a Th cell. This process involves MHC II proteins to allow the activation of $CD4^+$ Th cells which either present the antigen to CTL, to enhance cell-mediated immunity, or B cells for humoral immunity (Murphy and Weaver, 2016).

Figure 11. Illustration of mechanisms of the cell-mediated and the humoral immunity (APC: antigen presenting cell, CTL: cytotoxic T lymphocyte, Th: T helper cell).



As presented on **Figure 11**, cell-mediated immunity occurs when Th cells complexed with antigen encounter CTL. The recognition of the pathogen by Th cells enhance cytokines release that allowing activated T cells to bind to the infected cells *via* MHC II-antigen complex and permit the differentiation of T cells into CTL and memory T cells. The activation of CTL ($CD8^+$) enhance a release of cytotoxins (perforin, granzymes, and granulysin), which produce apoptosis. Memory T cell are then release in the circulation system and remain inactivated until they encounter the antigen. Humoral immunity relies on constitutive elements of the humors (*i.e.* body fluids) including antibodies, complement system and antimicrobial peptide to eliminate pathogen. As displayed on **Figure 11,** humoral immunity starts with B cells activation which occurs when antigen-Th cell complex binds to B cells. Once activated, B cells turn into plasma cells to release antibodies to component of the innate immune system through phagocytosis or opsonisation (*e.g.* complement system). When Th cells bind to B cells,

cytokines are release by Th cells enhancing plasma cells and memory B cells. Memory B cells corresponds to inactivated B cells which circulate in the blood and turn activated in cases of reinfection. Another pathway not present in **Figure 11** consists on a direct activation of B cells without T cell activation.

Take home messages

1) The component of the immune system works in harmony to ultimately eliminate pathogens from an organism.

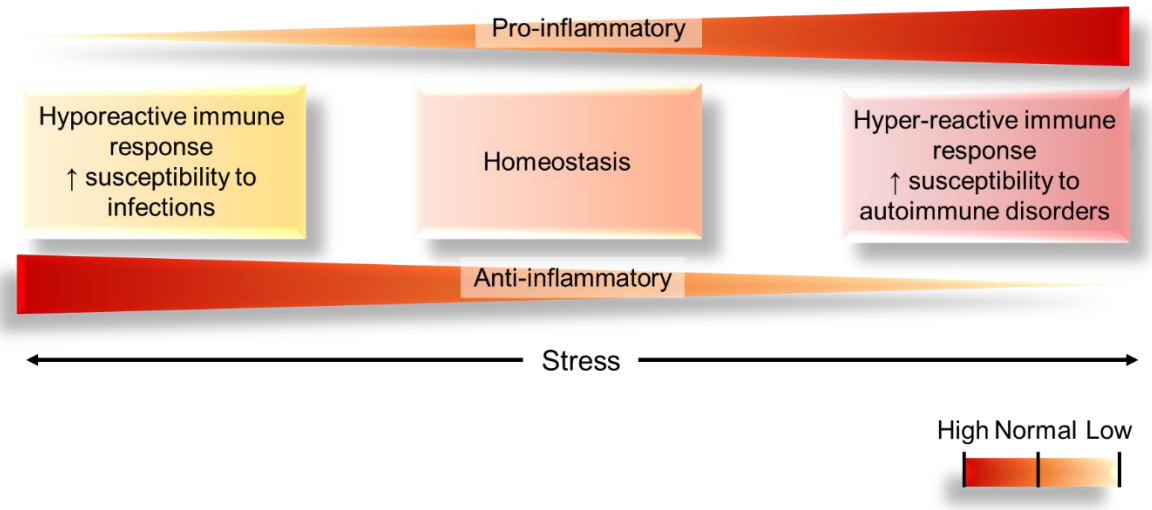
2) Cytokines are small proteins/molecules that orchestrate the activation, regulation and recruitment of immune cells.

3) Immune responses depend on the nature of infection and the underlying mechanisms to regulate it.

1.1.3.2.2 Stress and immunity

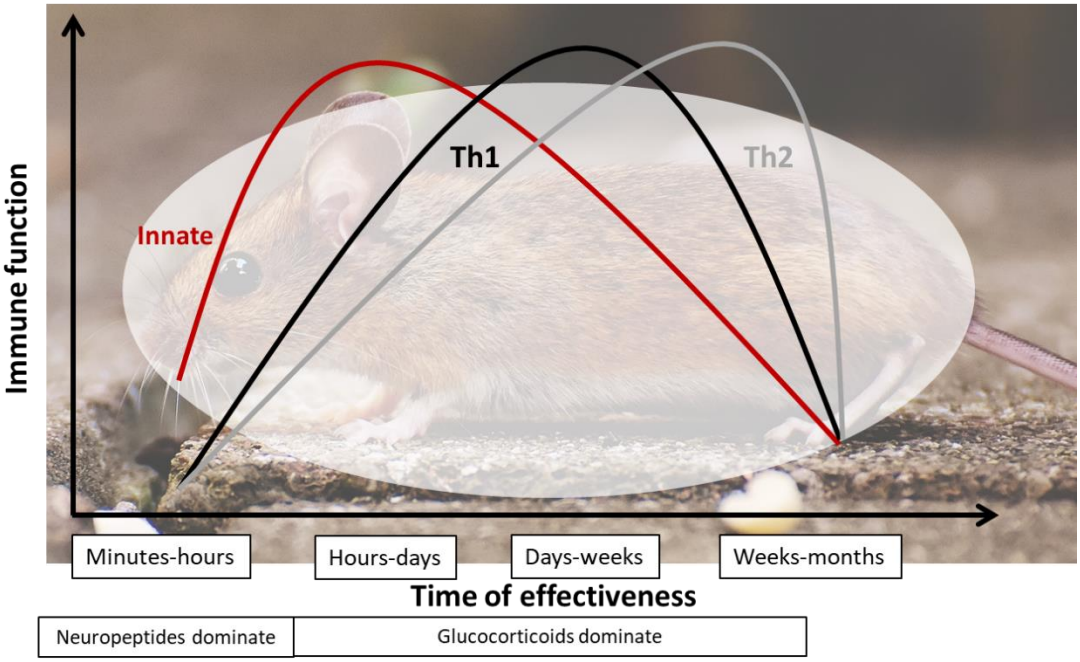
The immune system aims to protect host from pathogens while the endocrine system maintains homeostasis (Wensveen *et al.,* 2019). Both systems ensure an equilibrium between pro-(immune) and anti-(endocrine) inflammatory response to a stress, avoiding autoimmune damages or an increasing susceptibility to infections (**Figure 12**). In the context of an infection, host organism will display a pro-inflammatory response (*e.g.* T cell activation *via* cytokines) which is then regulated by an anti-inflammatory response (*e.g.* glucocorticoids release) in a purpose of maintaining homeostasis. However, in abnormal conditions, as may do stress, high anti-inflammatory mediators can be produced which dampens the pro-inflammatory response favouring hyporeactive immune response promoting higher susceptibility to infectious diseases. In opposition disturbances in anti-inflammatory mechanisms, as may do a toxic compound, can enhance higher pro-inflammatory response producing a hyper reactive immune response which increase the risk of autoimmune disorders (**Figure 12**).

Figure 12. The equilibrium between pro-(immune) and anti-(endocrine) inflammatory response to a stress.



Martin (2009) proposed a framework crossing hormonal and immune response to stress allowing to disentangle acute from chronic stresses with the type of stressor, its intensity and its duration (**Figure 13**). A stress is not always intensive or can last a few times and a rapid recovery from an immune response to a stressor may occur within minutes to hours. For instance, the stress of predation is known to enhance hormonal responses like GCs production and enhance briefly an immune response (*e.g.* increase of leukocyte counting) (Gelling *et al.,* 2009). When animals have to endure a more severe and/or longer period of stress such as habitat loss, food restriction or pollution, stress hormones may turn down the immune system (*i.e.* immunosuppression) towards patterns related to energy saving towards essential functions (McEwen and Wingfield, 2010). Stress hormones are crucial inflammatory responses as detailed in **Figure 13**, adapted from Martin (2009), a first hormonal response to stress is dominated by the release of neuropeptides which enhance the immune response within minutes or hours followed by a GCs dominant phase that regulates immune cells' activity. In parallel, the inflammatory cascade of immune function involved innate immune (already present at the site of infection) cells and activate T cells (Th1) and B cells (Th2) which to summarize aims to recognise, sequester or eliminate pathogens. A second enhancement of the innate immune system has to be considered when long-term stressors enhance autoimmune disorders. All these components of the immune system are thus susceptible to hormonal-induced immunosuppression.

Figure 13. Responses of immune functions to stressors in the wood mouse (*Apodemus sylvaticus*) copy from Martin (2009). Red line: innate immunity, black line: T helper 1 (Th1) are mainly (pro-)inflammatory T cells, grey line: T helper 2 (Th2) humoral response mainly mediated by B-cells.



Take home messages

1) Stress could modulate immune response toward hypo- *vs* hyper-reactive immune response

2) A close link exists between hormonal and immune response to stress as one can regulate the other

1.1.3.3 Nutritional stress

Nutritional quality represents a major challenge against a multi-stressor environment whose further insight may enable an immunomodulatory profiling. As part of the ecological niche nutrient supplies and composition is inherent to species maintaining towards metabolism and immune system functioning. In a conservation issue many species fitness have proven to be intrinsically connected to their diet content (Strandin *et al.,* 2018). A parallel consideration can be drawn out from human Hidden Hunger index which monitored deficiencies in populations suffering from poor micronutrients intake in a purpose of global caring and children nutritional health status (Ruel-Bergeron *et al.,* 2015). Species specific micronutrients requirement is highly dependent on resource availability and define the boundaries of animal conservation

regarding their benefit towards health and immunocompetence (Birnie-Gauvin et al., 2017a). For example, Vitamin E found its major dietary sources in vegetable oil of nuts (Lee and Han, 2018). Different adaptation of species as artic fox in extreme environment promoting mechanism of vitamin A and E conservation in tissues in response to seasonal decrease of their availability (Ilyina and Baishnikova, 2019). Same trends were observed in bird species experimenting micronutrients supplementation to identify species specific ability of vitamin withholding (Calle *et al.,* 1989). As illustration, the Kakapo is an emblematic example in conservation biology as it is a terrestrial endangered large parrot, which highly relies on Rimu fruit to maintain its fitness. Indeed, its breeding periods is essentially dependent on a quintennial fruit tree extremely rich with calcium and vitamin D which provide it sufficient amount of energy for reproduction (von Hurst *et al.,* 2016). More recently, a review of Espin and Sanchez-Virosta (2021) get insight on the key role of vitamins in bird species. The potencies of micronutrient to alleviate the burden of toxic model exposure may reduce the energetic cost of an efficient immune response against non-self and should get more insight in mammals. Nonetheless, knowledge about interactions between toxic element and micronutrients remains scarce, and would require further insight in both terrestrial and marine environment. Indeed, significantly higher levels of Hg and Hg:Se ratio was record in the liver harbour and whales which died from various infectious disease in comparison with animals which died from physical trauma ("Exposure to heavy metals and infectious disease mortality in harbour porpoises from England and Wales," 2001). Also localisation should be integrated as cofounding factor as geography have proven to module nutrient content and food content in free ranging marine mammals (Bluhm and Gradinger, 2008).

Living in a stressful environment may thus end in a direct or indirect impairment of the different compartment of the immune system stimulating innate and/or adaptive immunity and their component (*i.e.* th1/th2)(Martin, 2009a). However, the common framework associating toxic metals exposure to a direct immunosuppressive response have proven its limit over studies (Zelikoff *et al.,* 1994). The effects of micronutrient deficiency on metal toxicity can result in an aggravation or to an appearance of new symptoms due to the exposure (Peraza *et al.,* 1998). In captive animals the potential effect of immunomodulators have proven to improve individual self-maintaining both in poultry and aquaculture (Dawood *et al.,* 2018; Fritts *et al.,* 2004). While of the studies considered immune system on mice and human models, the immune system of wild mammals is poorly documented. This observation could be in touch with Strandin *et al.* (2017) who also deplore a lack of study investigating on the effect of food provisioning in the immunity of wild species.

Physiological, environmental or lab-controlled factors such as food content and supplies may promote alternative individual responses of the immune system. Environmental disturbance in

individual physiological processes (*i.e.* homeostasis) may modulate ecological interactions which shape community ecology (Warne *et al.,* 2019). A second order of consideration should be held on nutrient deficiency of organisms as minerals and vitamins are key element of immunity. Optimal food supplies promote efficient response against environmental stressors including pathogens and contaminants. For terrestrial ungulates, the deficiency of Se and vitamin E was associated with the White Muscle disease highlighting the essential role of these micronutrient for mammals health (Flueck *et al.,* 2012). Despite more laborious methodology of capture, wild ungulates constitute valuable biological models in various ecoregions (tundra, savanna, fjord…), allowing long term monitoring of their immune or nutritional status during their life (Jolles *et al.,* 2015). Another issue relies on the microbiome alterations due to host minerals availability for pathogens also known as nutritional immunity (Malavia *et al.,* 2017). Once assimilated toxic metal(oid)s may induce alteration in the microbiote community and in some terms conduct to energetic trade-offs in favouring metal(loid) elimination and/or hypersensitive reaction which could enhance autoimmune disorders. This bias in energetic allocation also called allostasis implied increasing energy consumption might lead to a decrease of individual immunocompetence. In opposition, oligo-elements may thus alleviate toxicity of metal(loid) favouring homeostasis. This will consequently maintain an equilibrium in the energetic budget allowing to allocate energy to immunity and even to attributes related to individual fitness (Lochmiller and Deerenberg, 2000).

Take home messages

1) Free-ranging animals can suffer from deficiencies due to a decrease of available food items in their environment

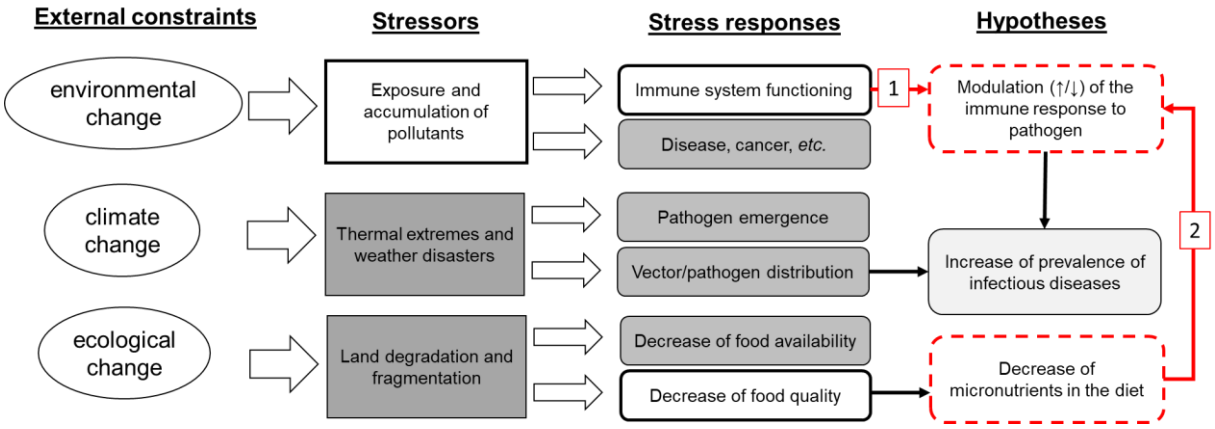
2) Nutritional quality and quantity are key elements of the homeostasis

3) Several components of the immune system relies on micronutrients to display an efficient immune response

1.1.4 Hypotheses and objectives

External constraints such as environmental, climate and ecological changes identified by Acevedo-Whitehouse and Duffus (2009) are the sources of the emergences of the multiple stressors affecting free ranging animals health (**Figure 14**). Indeed, pollution exposure and accumulation in animals' tissues may originate from environmental changes promoted by anthropogenic activities (*e.g.* industry, waste management, *etc.*). This environmental stressor may thus (as detail above) lead to stress responses for which pollution exposure could affect the immune system and promote the development of diseases and cancers (Acevedo-Whitehouse and Duffus, 2009a). Climate change was found to promote increase of temperatures, and now becomes obviously responsible of thermal extremes and weather disasters. Variations in thermal and weather conditions was found to modify pathogen emergence as well as pathogen or vector distribution (Destoumieux-Garzón et al., 2018b). Ecological changes occurring in the vicinity or even in inhabiting area of free-ranging animals enhance land degradation and even fragmentation of their habitat (Bonebrake et al., 2019).

Figure 14. Illustration of the context of living in a stressful environment adapted from Acevedo-Whitehouse and Duffus (2009). In red: links studied during this PhD; in dark grey: topics not studied during the phD; in light grey: open perspective connected to the hypotheses; items 1 and 2 represent the "Main objectives of the PhD" detailed below.



Most of knowledge acquired on the immunotoxicity of toxic metals can be found in laboratory studies on rat or mouse models. However, few is known about the effect of toxic metal exposure in free-ranging mammals and, even a few studies address the effect of micronutrients (*i.e. essential elements and vitamins*) on mammals immunocompetence. To this, the first part of the results (Chapter 1) aims to review all the immunotoxic effect of toxic metals *vs* the beneficial effect of micronutrients and their interaction studied in both laboratory and free-ranging mammals. For this work we hypothesised that the detrimental effect(s) of toxic metals (*e.g.* Cd, Pb) can be counteract, or at least attenuate, by beneficial effect(s) of micronutrients on free-ranging mammals immunocompetence.

Wood mouse populations from a highly polluted site with Cd and Pb, called Metaleurop Nord (see 2.1), was chosen to study these two stressors. A contrast design was held in the second part of the results to figure out the main effects of pollution and nutritional quality (*i.e.* Se-deficient diet) for 'high' *vs* 'low' exposure to Cd and Pb. Toxicological, hormonal and immunological parameters were measured on immune challenged individuals in natural and in experimental conditions testing essential element deficiency (*i.e.* Se), to determine if any differences occurred between 'high' *vs* 'low' exposure to Cd and Pb (Chapter 2). The main hypothesis behind this work was that immune response to challenge of wood mice is modulated (*i.e.* hyper or hypo-reactive, see1.1.3.2.2.) by their chronic exposure to Cd and Pb and/or the effect of a naturally occurring compound of their diet (*i.e.* Se).

Many tools have been developed to determine the immune status of animals and human among them complete blood count (CBC) is a relevant asset for veterinary and medicine diagnosis. Nonetheless, in wild mammals the absence of reference values constrains the relevant interpretation of CBC results as control populations need to be measured to determine any discrepancies. To this end, a long-term monitoring of CBC in wood mouse populations from several parts of France was used to compute reference intervals allowing an accurate diagnosis of their immune status (Chapter 3). For this we hypothesised that developing haematological reference intervals for the CBC of wood mouse in considering external (*e.g.* season, location, soil pollution, *etc.*) and biological features (e*.g.* age, sex, mass, *etc.*) will allow investigating wood mouse population health status.

Measurement of immunocompetence of free-ranging animals often relies on proxies (*e.g.* observational measurement of carotenoid coloration, spleen/bone-marrow ratio…) and immunological (*e.g.* ELISA assays of immunomarkers, phagocytic activity…) measurements. In an ethical concern, each approaches and related technics displayed pros and cons to which developmental procedure tends to reduce invasiveness and avoid lethal ones. Cross-reactivity with commercially available reagents and related products (*i.e.* antibodies) often constrains the use of assays often developed in mouse and rat laboratory models. Furthermore, parameters relating the immune response to immune challenges commonly found in laboratory, such as Lipopolysaccharide (LPS) solution, is rarely present among taxa. In this purpose, the fourth part of the results (Chapter 4) is dedicated to development of immunological measurements of immune parameters a wood mouse population living in the urbanised area of the campus of La Bouloie (Besançon). We hypothesised that the commercially available kits developed in mouse and rat can cross react with the wood mouse cytokines regarding their phylogenetic proximity (both belonging to the subfamily of *Murinae*).

The present PhD manuscript aims to determine the effect of two stressors, which are pollution exposure and nutritional quality on the wood mouse immunocompetence. Among the several kinds of pollution exposure found in the environment the present manuscript focuses on toxic metals regarding their ubiquity and particular immunotoxic effect. The stress of nutritional quality was refined to micronutrients uptake regarding their particular involvement in mammal immunocompetence and the growing concern on their availability to free-ranging animals in a changing world. The refinement on these two stressors allow shedding some light on two main objectives detailed here after.

Main objectives of the PhD

1) Determine how Cd and Pb exposure may modulate immune response to pathogen in the wood mouse.

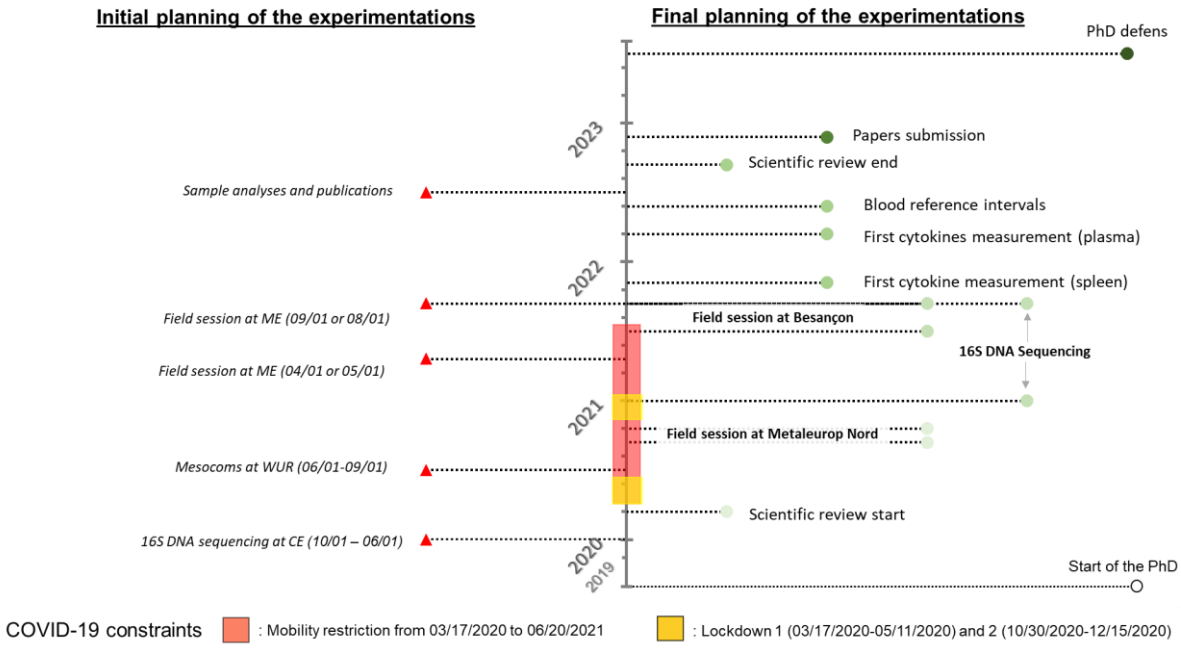
2) Understand how nutritional quality could interfere on Cd and Pb immunotoxicity.

2 MATERIALS AND METHODS

2.1 COVID-19 constraints

The present PhD thesis occurred during the pandemic of COVID-19. The initial program detailed (**Figure 15**) was constrained by the mobility restriction and the ability of suppliers to provide materials required for the experiments by the time of the PhD.

Figure 15. Initial and final planning of the PhD after COVID-19 constrains.



Initial planning of the experimentations

The initial framework of the PhD was organized in three work packages (WPs):

- WP 1: Unravel co-exposure to pollutants and pathogens of wildlife in natural and polluted habitats.
- WP 2: Decipher the role of food quality on responses to stress: immunomodulation
- WP 3: Explore immunoticoxicology in the real world

The WP1 aimed to determine if microbial community found in wood mouse tissues is affected by different levels of pollution with Cd and Pb. We hypothesized that the composition of microorganism communities (Bacteria) varied in response to host exposure to contaminants. To do so, the experimentation consist in realising a screening of pathogens (and after, a clustering of pathogen community) found in different tissues of wood mice trapped in 2013 at Metaleurop Nord and Plateaux (another polluted site, mainly by Cd, in The Netherlands). The principle of the methods employed was to amplify and sequence several copies of the V3-V4

hypervariable region of the 16S rRNA gene, which is common to a bacterial taxonomic set. This part would have occurred during the first year (October 2019 to June 2020) of the PhD in Besançon. Then, WP2 experiment was planned during summer 2020 (June to September 2020) at the Wageningen University in The Netherlands. The hypothesis behind WP2 was that wild rodents in mesocosms (*i.e.* a semi-natural environment) under controlled conditions will depict trade-offs in the investment in immune function between contrasted levels of pollution and different diets. The WP2 experiments consisted in maintaining in captivity wood mice from contaminated and non-contaminated sites and feeding them with different diets (i.e. standardversus selenium-deficient diet) to evaluate the immunomodulatory potential of Se. Finally, knowledge acquired from both WP1 and WP2 was expected to be use for designing of field experiments in Metaleurop Nord by spring (April/May) or autumn (September/October) 2021.

Final planning of the experimentations

The succession of mobility restriction and lock downs led us to postpone or abandon the experiments described above, and to start a bibliographic review presented in Chapter 3.1. The first lockdown in France, from 17 March to the 11 May 2020, followed by one year of mobility restrictions (ended in summer 2021), finally obliged to cancel the WP2 experiments at the Wageningen University. For this reason, WP2 experiments were adapted to WP3 in designing a “field / lab” experiment. Indeed, in this work, a part of the wood mice captured in high and low levels of soil pollution on Metaleurop Nord site were maintained in captivity for a short period (5 days) with standard or Se-deficient diet (see Chapter 3.2.). The field session at Metaleurop (28 September to 13 October 2020) occurred just before the second lockdown (30 October to 15 December 2020). The uncertainty on further mobility restrictions and lock downs led us to start with WP3 and to delay WP1 experiment (as samples were already present at the lab) to summer 2021. However, the difficulties met to order consumables (*i.e.* taq polymerase, tips with filter…), which were also required for COVID-19 PCR analyses, and technical problems (breakdown of the electrophoresis device) did not allow to get results on PCR amplicons (*i.e.* absence of amplifications at 450pb), which were supposed to be analysed for 16S RNA sequencing. In parallel with WP1 experiments, a field session was organised in the campus of La Bouloie (Besançon, France) during summer 2021. This field session aimed to provide haematological parameters including leukocyte counts and sample used to measure immune response to LPS challenge. A part of the data acquired in this session was used to fill a long-term monitoring of haematological parameter in wood mice populations. Haematological parameters from several locations in France was compiled in a data base and analysed with the involvement of a Master 1 student and colleagues. The objective of the data analyses was to propose reference intervals as the ones used in medical or veterinary sciences, to estimate

an individual's health status (Chapter 3.3.). Tissues samples collected during la Bouloie field session were used to develop methods and techniques able to measure cytokines in the plasma and the spleen of wood mice, and this has finally led to a scientific article (Chapter 3.4.). The development of measurements of at least one cytokine in plasma samples (*i.e.* the tumor necrosis factor alpha) took approximately one year.

The final framework of the PhD is thus organized in four WPs, each of them corresponding to one of the technical Chapters of the present PhD:

- WP 1. The effect of toxic elements versus micronutrients on mammal immunity (Chapter 3.1.)
- WP 2. Environmental pollution and nutritional quality modulate immune response of the wood mouse (*Apodemus sylvaticus*) through hormonal disturbances (Chapter 3.2.)
- WP 3. Moving from haematological parameters analysed at population level to individualised health assessment: reference intervals and blood profiles in small mammals (Chapter 3.3.)
- WP4. Developing non-lethal tools to assess immune response to challenge in the wood mouse (*Apodemus sylvaticus*) (Chapter 3.4.)

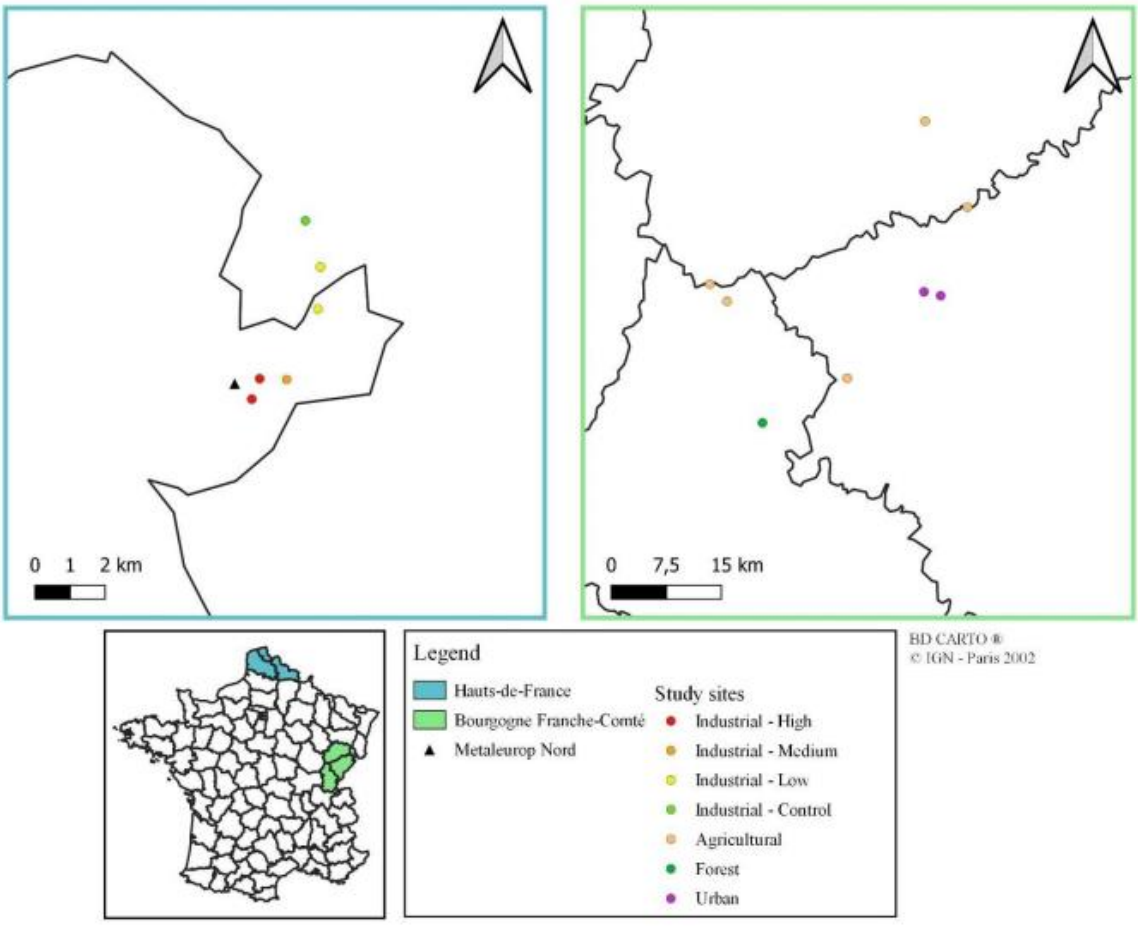
2.2 Study sites

The main study site of the present PhD was the former Pb and Zn smelter of Metaleurop Nord (Hauts-de-France, northern France) because of the elevated levels of soil pollution by Cd and Pb, and the long-term monitoring (since 2006) of the site by our team.

The development of non-lethal techniques to measure immune response in the wood mouse could be done locally, and this was performed at the campus of la Bouloie (Bourgogne Franche-Comté, eastern France) in a wood mouse population inhabiting a moderately urbanized area.

To relevantly address patterns that may shape biological variations in haematological and immunological (leukocyte counts) heterogeneity in blood counting, several sites from both regions (Hauts-de-France, and Bourgogne Franche-Comté) were studied and included in Chapter 3. The study sites present a diversified panel of land uses with urban, agricultural, forest and industrial areas.

Figure 16. Maps of the study areas in the Hauts-de-France (in blue) and in Bourgogne - Franche-Comté (in green). (Credits C. Hadjadji).

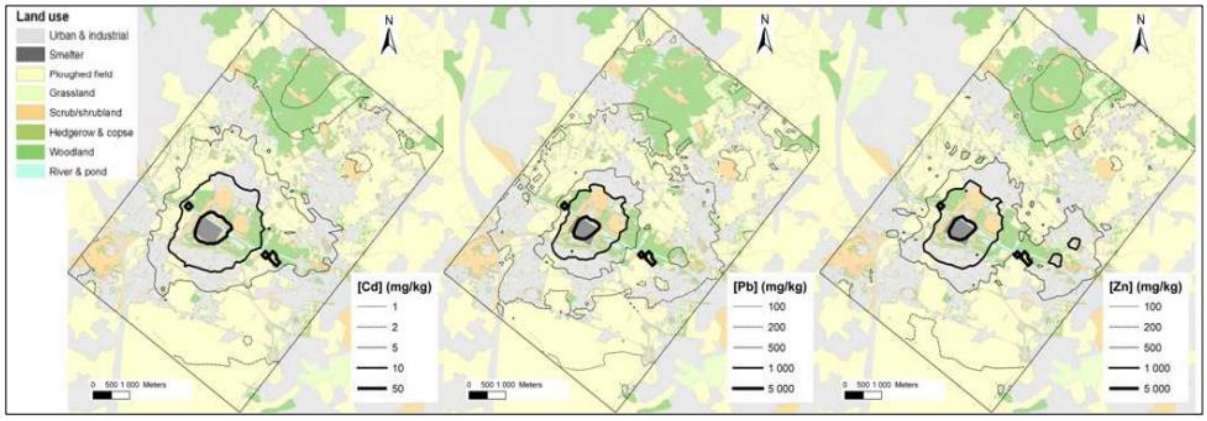


2.2.1 Metaleurop Nord (Hauts-de-France, FRANCE)

The effect of the two environmental stressors (*i.e.* pollution and nutritional quality) on wood mouse immunocompetence was measured in the surroundings of the former smelter Metaleurop Nord (Hauts-de-France, northern France, 50°25'42N, 3°00'55E). Between 1894 and 2003, the activity of the smelter generated very high levels of pollution by trace metals (mainly Cd, Pb and Zn) sourcing from pyro-metallurgic dust. Even if trace metal emissions were reduced by environmental regulation and technical improvement from the 70's, the plant was still releasing 1 ton of Cd, 17 tons of Pb and 31 tons Zn in 2002 (DRIRE, 2003). A total of 120 km² has been contaminated by the plant and another one named UMICORE nearby(Douay et al. (2008)). The site ranks as one of the most contaminated by trace metals across Europe (Traullé, 2022). Measurement of several land uses (*i.e.* agricultural, urban, *etc.*) recorded highly elevated trace metal concentrations in the top soil (Douay *et al.,* 2008; Sterckeman *et al.,* 2002a). Soils from woody habitats (*i.e.* hedges or small woods corresponding to the wood mouse preferred habitat) reached total concentrations of 236, 7,331 and 7,264 mg/kg of dry soil for Cd, Pb and Zn, respectively (Fritsch *et al.,* 2010b). In

comparison, background values of different soil types not affected by known contamination sources (*e.g.* traffic transport, industries…) were of 0.4, 30 and 67 mg/kg of dry soil for Cd, Pb and Zn, respectively for the upper part of the Hauts-de-France (Sterckeman *et al.,* (2002b). Spatial analyses from Fritsch *et al.,* (2011) computed iso-concentration lines of predicted Cd, Pb, and Zn in soils around the smelter show a decrease with the distance to the former smelter and a directional pattern of contamination dominated by SW to NW prevailing winds (**Figure 17**).

Figure 17. Land cover map of the Metaleurop Nord study area with the projection of iso-concentration lines of predicted total Cd, Pb and Zn concentrations (mg/g dry soil). The figure is from Fritsch *et al.,* 2011.

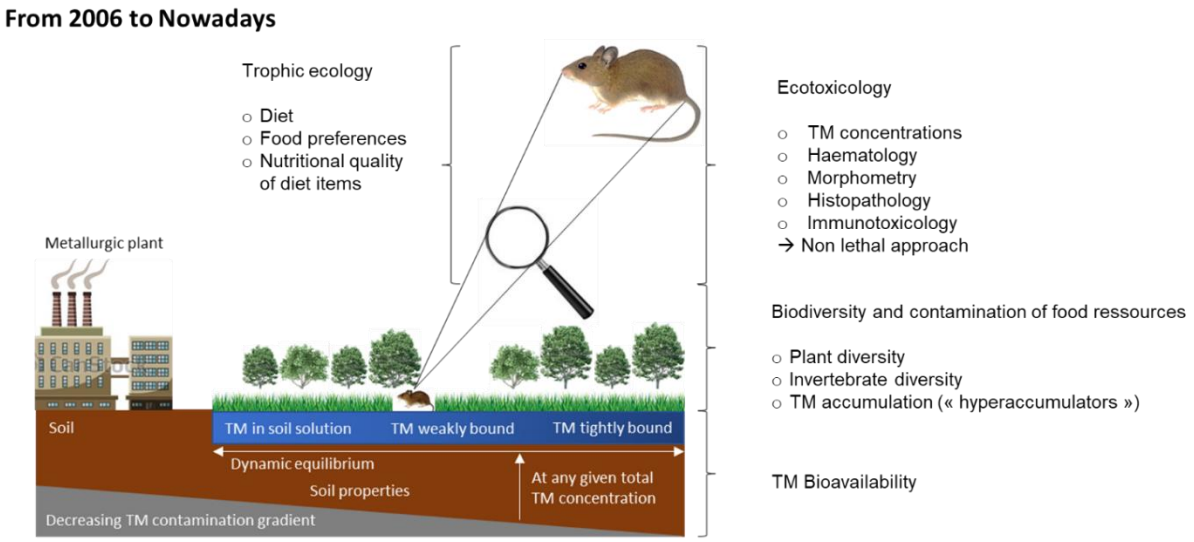


Monitoring of soil pollution and its impacts

Since 2006, an ecotoxicological monitoring is maintained in the surroundings of Metaleurop Nord (**Figure 18**). The investigation on Cd and Pb showed a transfer of these toxic elements from soils to vegetation, and then to animals (*e.g.* Fritsch *et al.,* 2010b, 2010a). The transfer and accumulation of Cd and Pb were found to be modulated by the proximity to the former smelter (*i.e.* forming a gradient of contamination) and landscape features (*e.g.* different physio-chemical soil properties in woody habitats) (Fritsch et al., 2012, 2010b). Wild animals, including the wood mouse, chronically exposed to high levels of Cd and Pb displayed morpho-physical disturbances like a decrease of somatic indices (*i.e.* proxies of individual health status based on morphometric parameters) in response to Cd hepatic concentrations (Tête et al., 2013). Physiological alterations were also found in sympatric communities of small mammals with an increase of the metallothionein concentration in response to Cd and Pb soil concentrations, mainly in shrews (Fritsch *et al.,* 2010a). Histological damages mediated by Cd and Pb exposure were found in the liver and in kidneys of wood mice inhabiting the vicinity of the former smelter (Tête et al., 2014a). The environmental pollution by Cd and Pb at this study site also was able to shape ecological interactions in promoting variations in food selection (Ozaki

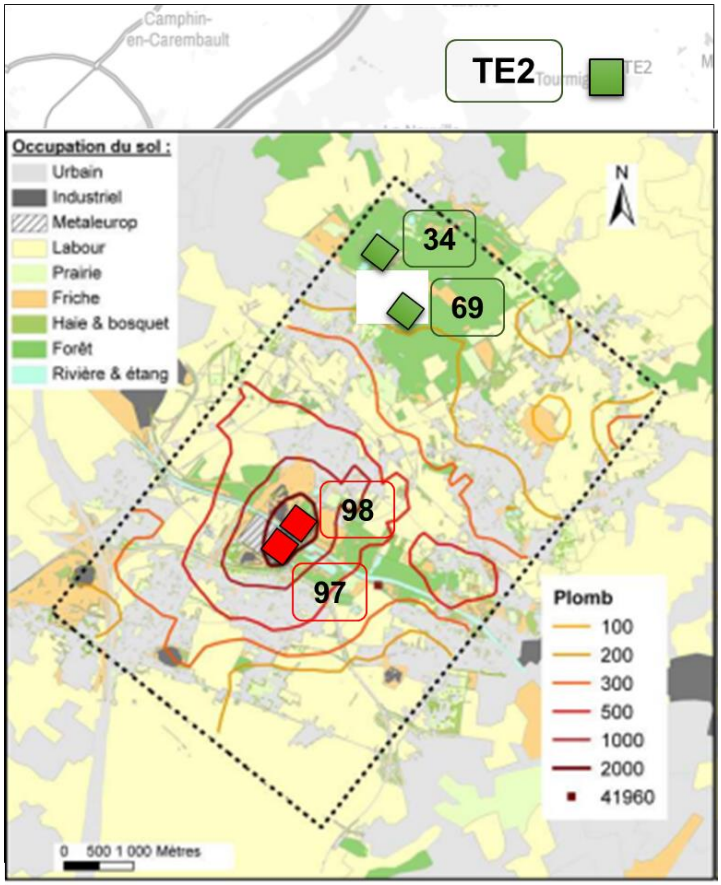
et al., 2018). The trophic transfer of Cd and Pb was found to be modulated by the diet content (*i.e.* presence of Cd hyper-accumulator plants in the diet) where exposure to Cd decrease with the diet richness of wood mice (Ozaki et al., 2019). A decrease of the nutritional quality in terms of minerals and micronutrients content (C, N, Mg…) was found in food items consumed by wood mouse in high polluted sites (unpublished data). Furthermore, non-lethal procedures were applied to monitor Cd and Pb pollution in wood mice and blackbirds (Tête et al., 2014a; Fritsch et al., 2012).

Figure 18. Current framework of the ecotoxicological monitoring of pollution and its impacts at Metaleurop Nord (Hauts-de-France, northern France, France). (Credits: R. Scheifler).



The study area, spanning 40 km^2 (5x8 km) around the former smelter, was subdivided into a meshing 160 squares of 500x500 m (25 ha) that constituted the sampling units of the monitoring since 2006 (**Figure 19**). The “squares” used for this thesis were selected according to their distance to the former smelter with 097 and 098 as “High” polluted sites (0.6 km far from the smelter) and 034, 069 and TE2 as “Low” polluted sites (more than 3 km far from the smelter). The site TE2 referred to as a control area located at 11 km north from the smelter provides “reference values” for studies or a starting point when a gradient of trace metal contamination was wished. The sites 034 and TE2 belong to the Communauté de Commune de Pévèle-Carembault (CC Pévèle-Carembault) presented in Chapter 3.4. Soil trace metal concentrations used to enable a contrast of soil pollution levels (*i.e. “High” vs “Low”*) rely on measurements from 2006 (Fritsch et al. (2010)). Unpublished data from new soil samplings performed in 2017 showed that total concentrations of Cd, Pb, and Zn in soils did not change significantly between 2006 and 2017 (see 3.2 – supplementary materials).

Figure 19. Landcover map of the Metaleurop study area with the iso-concentration lines of predicted Pb total concentrations in soil (mg/kg dry soil) with the location of study sites (High in red and Low in green). Note that TE2 is placed in the figure using a different scale for the purpose of readability (the site is actually 11km north to the former smelter).



2.2.2 Study sites in Bourgogne - Franche-Comté

The Campus of la Bouloie is located in the city of Besançon (47° 14′ 35″ N, 6° 01′ 19″ E). The site depicts all the features of a French intermediately urbanized environment with the presence of buildings, roads, a highway, dense human traffic during school periods and also some open areas with grass and woody habitats (shrubs, groves). Soil trace metal concentrations, organic matter and pH were measured by master 2 students during a practical training in 2019 (see 3.4.). The site of “Forêt de Chaux” (47° 04′ 46″ N, 5° 40′ 58″ E) is a large forest mainly made of sessile oak (*Quercus petraea*). Some other sites, dominated by agricultural landscapes were presented in Chapter 3, They belong to one of the French sites (https://zaaj.univ-fcomte.fr/)of the Integrated European Long-Term Ecosystem, critical zone and socio-ecological Research (eLTER, https://elter-projects.org/).

2.3 Animal studied

The wood mouse (*Apodemus sylvaticus* Linnaeus 1758, **Figure 20***)* was used as the animal model for this study, considering its relevance as bioindicator of metals from soil and/or trophic transfer (*e.g.* (Fritsch et al., 2011, 2010a), its ubiquity and its ability to cope with captivity (see 3.4). The wood mouse is a free-ranging small mammal widely spread in the Eurasian continent with a limit of distribution between North Scandinavia and North Africa (Butet and Paillat, 1997).

Figure 20. Female wood mice released at the site of capture.

Its presence in European countries suggests a good acclimatisation since a long-term settlement in Western palearctic regions last from the Pleistocene (1.5–1.6 My ago) (Michaux et al., 2005). Sexual dimorphism of wood mice is slight with females being smaller than males at equivalent age. Studies reported a biased sex ratio in favour of males (Butet and Paillat, 1997). The weaning occurs at 1 month and adults stop their growth around 7 and 9 months for an average adult mass of 23g and a stabilisation of growth at 20g (Saint-Girons, 1967). The life expectancy in the wild is around 2 to 4 months with a maximal value of 12 to 15 months (Butet and Paillat, 1997). During gestation, females host an average of 4.5 embryos and the reproduction period ranges from March to October, with specific cases reported during winter (Saint-Girons, 1967). The wood mouse is typically a nocturnal animal with an average home range in deciduous wood land of North-East Scotland of 2.0 ± 0.8 km² during breeding season and of 0.4 ± 0.1 km² out of the breeding season (Corp et al., 1997). Wood mice is principally granivorous and forages mainly on oleaginous seeds (*e.g.* Chestnuts) but also fruits (*e.g.* elder berries) and several invertebrates (Ozaki et al., 2018). Among the most consumed items are hazel, elm, oak, beech, ash, wild rose, bramble, elder, plum and some crop seeds like wheat

and oats, and many others (Butet and Paillat, 1997; Jensen, 1993). The caloric demand of wood mice metabolism remains stable across seasons with a daily energetic budget of 12.4 and 11.1 kcal/days for summer and winter respectively, suggesting a good acclimatisation to climatic variations (Henderson, 1970). As an aside, wood mice display an insightful physiological adaptation of spermatozoid cooperation in forming aggregates called “train of sperm”, which improves their mobility in female reproductive tract (Moore et al., 2002).

2.4 Animal trapping, housing, welfare and ethics

Trapping usually consists in 10 non-lethal INRA traps (with dormitory boxes) spaced 3 m apart and set for at least 3 consecutive nights. Number of trap lines (around 30 m long), and thus number of traps, then depend of the scientific objectives, the logistical effort that can be done, and the surface of the sturdy area. Dormitory boxes are filled with apple, hay, sunflower seeds, cat food and peanut butter. They ensure well-being and survival of the animals for at least 24 h. Trapping methods used for this PhD were not exclusive to wood mice and other small mammals were captured (*i.e.* voles and shrews). Individuals from other species than *A. sylvaticus* were immediately released, after checking their health status (no injury, not wet, apparent normal behaviour). Pregnant or suckling females (*i.e.* females exhibiting large abdomen and/or visible nipples), as well as young individuals (i.e. below 15g, (Gelling *et al.,* 2009)), of *A. sylvaticus* were also released. Only adult males and non-pregnant females were kept for these experiments.

Housing

Captive animals were housed individually (to avoid inter-individual aggressive interactions) in transparent boxes (see 3.2) filled with a solid hemp litter (allowing the collection of faeces), a wooden parallelepiped shelter containing natural cotton and a paper roll as enrichment. Boxes were placed in shelves in a room with no heating system (i.e. day and night temperatures followed roughly the natural ones and were of 18-20°C during days and of 15-18°C at night for all the duration of the experiment). Light also followed nychthemeral regime. Animals had access to water and food *ad libitum*. Individuals were weighed before captivity, then at each cleaning of the box (i.e. at day 2, 4 and 5 of the period of captivity) and at the end of captivity. At the end of their period of captivity challenged individuals received an intraperitoneal injection with a solution of bacterial lipopolysaccharides. Faecal samples and food remain were collected and weighed during each cleaning days. Faecal samples were stored at -20°C until analysis.

Ethics

Capture, handling, blood sampling, immune challenge and euthanasia were performed under gaseous anaesthesia using isoflurane. Experiments were performed under the authorization of the French National Ethical Committee (Project APAFIS N°5340) by skilled and experienced investigators from Chrono-environnement research department (EU0592), following directive 2010/63/EU on the protection of animals used for scientific purposes.

2.5 Technical procedures, samples collection and storage

Immune challenge

All challenged individuals received an intraperitoneal injection of a solution of bacterial lipopolysaccharides (LPS O111:B4, Merck) at 500 µg/kg, which is sufficient to enhance TNF-α production as found in several laboratory rodent models (Batista *et al.,* 2019). Two hours after LPS challenge, a blood sampling was performed for analyses. This period of two hours after the LPS injection and the dose of LPS are sufficient to enhance a systemic inflammation in lab rodents (Batista *et al.,* 2019).

Blood sampling, sub-sampling and storage

After anaesthesia with inhalation of isoflurane, a Pastor pipette was inserted behind the eyes (with an angle of 45°) to notch the retro-orbital sinus. Then, blood was allowed to flow by capillary action into Pastor pipettes and right after collected in plastic tubes coated with an anti-coagulant, the EDTA (Microvet EDTA K3). A small volume of blood (~50µL) was used fresh (i.e. stored at +5°C until analysis, which was performed within the 8h following the sampling) for blood counting (see below). The rest of the blood (~150 µL) was centrifuged at 2000 rpm for 15 minutes to collect plasma. After centrifugation, plasma was transferred in an Eppendorf tube. Plasma and red blood cells were immediately frozen at -20°C before being transferred to the lab for long-term storage at -80°C.

Tissue sampling

After immune challenge and blood sampling, whatever was the experimental group (i.e. with or without captivity), individuals were sacrificed by cervical dislocation under anaesthesia following the American Veterinary Medical Association ethical guidelines (Sikes and the Animal Care and Use Committee of the American Society of Mammalogists, 2016). The liver and eyes were extracted to measure trace metal concentrations in the liver and to determine age with crystalline lens mass (Vandorpe and Verhagen, 1979).

2.6 Measurement of trace metals and metalloids

The concentration of metallic elements was measured in the liver of wood mice and in soils. The liver was selected to estimate accumulation of Cd and Pb as both increase with soil pollution levels in this organ (Fritsch et al., 2010). The liver is also a relevant candidate to estimate Se metabolic storage, which mainly occurs in the liver (Avery and Hoffmann, 2018). Livers were lyophilised until constant mass, and then acid digested with nitric acid (69% HNO_3, ultratrace, Optima) and then diluted with ultra-pure water (Elga, 18.2 MV/cm). Soils were measured on dried samples after digestion with nitric (65% HNO_3, ultratrace, Optima) and hydrochloric acid (32% HCl, Optima). Concentrations of metals and metalloids in the liver and in soils were measured by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Thermo Fisher Scientific X Series 2). The quality of measurement was assessed by regularly measuring calibration solutions and blanks (ultra-pure water). Reproducibility was checked by using internal standards of the ICP-MS. Repeatability and accuracy of the analytical measurement were checked using standard reference materials for liver (National Research Council Canada TORT-3 Lobster hepatopancreas, National Institute of Standards and Technology SRM® 1577c Bovine liver) and for soils (Loamy Clay 1, Sigma Aldrich CRM052). The precise metrology is detailed on each Chapter (see 3.2, 3.3, 3.4), and recovery rates range between 70% and 102%. Concentrations of metals and metalloids were reported in µg/g dry mass for the liver and in mg/kg dry mass for soils.

2.7 Complete blood count

Haematological parameters were measured on fresh blood within a maximal period of 8 hours (*i.e.* end of the day during field sessions) and store in a fridge at 4°C. The limit of 8 hours guarantees a good quality of measurement after trials at different times post-sampling (result not shown). Complete blood counting was done with a Haematology Analyser automate (Horiba ABX Micros ES60). The automate is able to measure 14 blood parameters among which the following ones were used in this thesis: total WBC counts ($10^3/mm^3$), total counts and percentage of lymphocytes (LYMn and LYM%, respectively), of monocytes (MONn and MON%), and of granulocytes (GRAn and GRA%), total RBC ($10^6/mm^3$), HB (g/dL), HT (%), MCV (μm^3), MCH (pg), MCHC (g/dL), and RDW (%). Repeatability is automatically assessed by the analyser in measuring 3 times each blood sample. Moreover, each individual sample was measured 2 to 3 times in cases of non-repeatable measurements (high deviance within sample measurements). Reproducibility and accuracy were determined with an everyday automatic blank control and a measurement of blood quality control “low”, “medium” and “high” (MINOTROL 16, Horiba SAS).

2.8 Measurement of immunological and endocrine markers

This section only describes the kits displaying acceptable measurements and the methods used to evaluate metrology (repeatability, accuracy and precision of measurements) on wood mice samples. The details of kits and samples preparation are presented in Chapters 3.2 and 3.4.

Cytokines measurement

Different commercially available kits have been tested in Chapter 4 (section Materials and Methods) to measure cytokine concentrations in spleen cells and plasma. Only a high sensitivity Mouse TNF-α quantikine ELISA kit (R&D MH), originally developed in lab mouse species, was found to cross-react and provide acceptable results (*i.e.* sufficient sensitivity) with wood mouse plasma samples. Repeatability and accuracy of the measurements were assessed with positive controls (*i.e.* sample with a known concentration of TNF-α). Linearity (*i.e.* ability of a kit to detect increasing/decreasing concentration of TNF-α across dilution fold) could not be performed as low volume of plasma generally allowed to perform measurements in duplicate only (50µl each), and the few samples with high volume of plasma (>150µl) were used to control inter- and intra-assays precision. Spiked samples with a known quantity of TNF-α were used to evaluate if any inhibitors would disturb measurement of TNF-α concentration (*i.e.* test if the addition of a TNF-α solution with a known concentration to a given sample is additive or not). The measurement of non-challenged individuals whatever their sites of capture displayed TNF-α concentrations below detection limits.

Corticosterone measurement

The measurement of corticosterone was performed in plasma and faeces of individuals from Metaleurop to evaluate their level of stress and potent anti-inflammatory patterns. The method used to prepare faecal sample is detailed in Chapter 3.2. Determination of CORT concentrations were done with a commercially available enzyme immunoassay rat/mouse serum/plasma kit from DEMEDITEC Diagnostics (Kiel, Germany). Linearity was tested with a serial dilution (1:32, 1:16, 1:8, 1:4, 1:2, 1:1) of plasma and faecal extract regarding the low quantity of sample required (*i.e.* 10 µL). The precision of the measurement was evaluated with inter- and intra assay measurements, and the accuracy with spiked samples.

3 RESULTS

3.1 Chapter 1 (manuscript in preparation)

The effect of toxic elements *versus* micronutrients on mammal immunity

Quentin Devalloir[1,*], Clémentine Fritsch[1], Francis Raoul[1], Shinji Ozaki[1,3], Yara Alchammas[1], Nico van den Brink[2], Renaud Scheifler[1]

[1] UMR 6249 Chrono-environnement, CNRS / Université Bourgogne Franche-Comté, USC INRA, 25030 Besançon Cedex, France

[2] Division of Toxicology, Wageningen University, 6700 EA Wageningen, The Netherlands

[3] UK Centre for Ecology & Hydrology, Environment Centre, Library Avenue, Bailrigg, Lancaster, LA1 4AP, United Kingdom

*Corresponding author: quentin.devalloir@gmail.com

Authors' Contributions

QD and RS designed the study. QD and RS, with the help of CF, FR, SO, YA, and NvdB, wrote, reviewed, and edited the manuscript. All co-authors commented on the manuscript and accepted the final version. RS acquired the financial support leading to this publication.

Abstract

Over the last decades, wild animals have been affected by multiple stressors, which often source from anthropogenic activities, including habitat loss, food restriction, pollution, pathogen (re-)emergence, or climate changes. Mining and smelting are known to release persistent and hazardous pollutants, such as toxic elements and metalloids (*e.g.*, arsenic, cadmium, mercury and mercuric compounds, lead), into the environment. Many laboratory studies have reported negative immunotoxicological effects of these compounds on small-mammals raised in captivity, particularly laboratory rodents. However, Immunotoxicological studies in wild mammals are much less available in the literature, whilst they also display the negative effects of toxic elements on their immune system. In both cases (captive and wild mammals), many stressors including nutritional deficiency have been shown to interfere with toxic elements in the modulation of immunity. Among component of the nutrition threaten by anthropogenic activities, micronutrients (*i.e.* essential elements, sometimes called minerals, and vitamins) are involved the fitness of wild animals. Though laboratory studies highlight beneficial effects of micronutrients alone and against toxic elements in captive mammals, a few is known about their wild counterpart. The present review aims to fill gaps in knowledge about the ability of toxic elements, micronutrients, and the interaction of toxic elements and micronutrients to affect the immune system and modulate the immune response in wild and captive animals. Toxic elements have been found to affect all components of the immune system, with a decrease in pathogen recognition by immune cells and lymphocyte proliferation, which was often related to the production of reactive oxygen species. Micronutrients have been shown to contribute to immune system functioning (*e.g.,* iron, zinc, vitamin D) and to protect immune cells (*e.g.,* selenium, vitamins A, C, E). When acquired in sufficient quantity by a mammal, micronutrients alleviate the damage induced by exposure to toxic elements, mainly through reactive oxygen species. Beneficial effects of micronutrient intake have been mainly reported on laboratory or captive mammals, while studies on micronutrients are scarce in wild mammals. Most studies on the interactions between toxic elements and micronutrients in wild mammals have highlighted the beneficial effects of micronutrients on the immune system, such as reducing metal toxicity and immune cell damage. A combination of approaches from nutritional ecology, eco-immunology, and stress ecology was used to draw research perspectives about the characterisation of the immunological status of wild mammals exposed to toxic elements in the function of nutritional context. Methods for field studies have been proposed to assess how diet quality (*i.e.,* micronutrients) might modulate exposure to stressors (*i.e.* toxic elements) on mammal immunocompetence. In particular, we compiled the advantages and limits of immunological methods (*i.e.,* immunoassays) that could characterise immunomodulation in wild mammals. Further insight should be given to both the nutritional

status and the exposure to immunotoxic compounds such as toxic elements for a better understanding of the immunological mechanisms underlying wild animals' responses to pathogen infections.

Key words: stress ecology, immune system, immunomodulation, nutrition, non-essential metals, essential metals, vitamins

Table of contents

1. Introduction

Pollution, habitat fragmentation, resource depletion and many other environmental stressors are the current drivers of the sixth mass extinction (Birnie-Gauvin *et al.*, 2017a). In the context of rapid environmental changes, wildlife health is threatened by the adverse effects of anthropogenic activity. Among these threats, human activities like smelting and mining have polluted the environment with trace metals and metalloids, contaminating the biosphere for millennia (Ali *et al.*, 2019; C H Walker *et al.*, 2012; Duruibe *et al.*, 2007). Trace metals and metalloids are constitutional elements of the earth's crust and naturally occur in the environment (Alloway, 2013). Some of them, like arsenic (As), cadmium (Cd), mercury (Hg) and lead (Pb), are considered as non-essential elements because they do not exert any biological functions in organisms, except for some cases (*e.g.,* As is involved in the metabolism of methionine (Uthus, 2003)). Even if each organism can tolerate very low exposure to non-essential elements, trace elements in the environment chronically affect the whole environment, even at a low level. The toxicity of a given non-essential metal depends on its concentration in the environment but also its chemical form (*e.g.,* free cation, organometallic molecules). The chemical form of metals, named speciation, determines their bioavailability in the environment, influencing their transfer to organisms (Alloway, 2013). Organisms are exposed to trace elements through several routes by food, air, soil/sediment, or water, depending on their ecology (C H Walker *et al.*, 2012). Other metals are considered as essential because they exert various crucial physiological and biochemical functions. Both essential and non-essential metals can be toxic at a certain dose for a given organism. At toxic levels, metals can act on the immune system through two types of modulation: immunosuppression (*i.e.* hyporeactive response: a decrease of the ability of the immune system to respond to a challenge down from a basal level given a "normal" state) and immune hyperactivation (*i.e.* hyper-reactive response: an over increase of the immune response to a challenge up from a basal level which could led to self-mediated damages), also known as autoimmunity (Anderson and Shane, 2018; Zelikoff *et al.*, 1994).

Essential metals and vitamins constitute micronutrients that are required by organisms to maintain physiological processes and health. Micronutrients are ingested through food and water, and the requirements of organisms vary among species, sex, and life stages (Barboza *et al.*, 2009). They contribute to all components of an individual's fitness, like growth, maintenance, and reproduction, by maintaining homeostasis and immunity (Wang *et al.*, 2020; Harrison *et al.*, 2011; Speakman, 2008). The role of essential elements in homeostasis takes place in several metabolic processes as enzymatic co-factors (*e.g.,* copper (Cu)-zinc (Zn) superoxide dismutase) or structural constituents of proteins (*e.g.,* iron (Fe) in haemoglobin) (Higdon and Drake, 2012). Vitamins fulfil diverse functions in metabolic pathways, DNA

transcription, antioxidant activities, and growth and differentiation of tissues (Mora *et al.*, 2008). Among vitamins, retinol (*i.e.* a compound derived from vitamin A) ensures both vision and immunity functioning by developing epithelial cells as a skin barrier (Higdon and Drake, 2012). Micronutrients (*i.e.* essential elements and vitamins) can also counteract the impact of toxic elements like Cd, Hg, or Pb. For instance, selenium (Se) and selenoproteins can unbalance pro-oxidant processes with glutathione peroxidase (GPX) enzymes (Avery and Hoffmann, 2018; Schweizer *et al.*, 2016). Physiological functions of organisms can be affected when essential metals are in too low concentrations in the tissues due to the lack in the environment (a phenomenon called deficiency). Physiological and biochemical functions are ensured when tissue concentrations of essential metals are within an optimal range (called the window of essentiality). Organisms maintain these metals at the right concentration. At very high concentrations, even essential metals can become toxic to organisms (Newman, 2015). As part of micronutrients, vitamins are naturally occurring organic compounds required for the growth and nutrition of organisms. Acquisition from the diet is crucial to maintain the metabolic process involved in homeostasis and the immune system. Either alone or in synergy, vitamins A, B (*i.e.,* B12), C, D, and E, are essential in the functioning and regulation of mammal immune responses (Gombart *et al.*, 2020; Smith *et al.*, 2018). Vitamins are also considered as potent immunomodulators for innate and adaptive immunity (O'Brien and Jackson, 2012).

The role of metals in the immunity of wild organisms is therefore linked to a balance between essential ones, which maintain homeostasis, and non-essential ones, which lead to immune system impairments (Acevedo-Whitehouse and Duffus, 2009a). The balance between essential and non-essential elements depends on the quantity, quality, and availability of food resources because ingestion of food and water is supposed to be the main uptake route of both. Experimental approaches have also highlighted the need for heterogeneity and the quality of nutrients to maintain efficient immunity (Strandin *et al.*, 2018). Diet quantity (*i.e.* sufficient supply for the requirement of an organism) and quality (including the presence of essential metals at too low concentrations and/or of non-essential metals at toxic concentrations) have an outstanding ability to modulate the immune system of mammals (Uchio *et al.*, 2019; Nunez *et al.*, 2018; Hennigar and McClung, 2016). In contrast with micronutrients, the macronutrients (proteins, carbohydrates, fatty acids) are required in a large amount to mainly provide energy to the functioning of organisms (Tourkochristou *et al.*, 2021). Both macro- and micro-nutrients are involved in the regulation of the immune system of mammals, ensuring an immune homeostasis (Downs and Stewart, 2014). For instance, laboratory mice set in an outdoor enclosure and fed with a low-protein diet showed a reduction of immune parameters (*i.e.,* immunoglobulin (Ig)G1, interleukin (IL)-13) in response to *Trichuris muris* infection, but not a decrease in the parasite load (Budischak *et al.*, 2018). The study also

found that mice receiving a low protein diet presented a higher foraging behaviour, which might increase exposure to pathogens. Another study on wood mice (*Apodemus sylvaticus*) set in outdoor captivity showed that a diet rich in fat and micronutrients (Fe, Se, Zn, and vitamins A and E) promoted a higher efficiency of anthelmintic treatment and increased antibody responses to *Heligmosomoides polygyrus* infection (Sweeny *et al.*, 2021).

The immune system is constantly challenged by various natural and anthropogenic factors, which potentially affect the host. In wildlife, the immune system may be intrinsically regulated upward ("immunosuppression") or downward ("immunosuppression") by a plethora of external factors (food provision and composition, exposure to contaminants and/or pathogens, life stage…) (Poulsen and Escher, 2012; Jackson *et al.*, 2009). Immunostimulation refers to an enhancement of defensive response, like an increase in phagocytic activity or inflammatory-mediator proliferation (Luster and Gerberick, 2010; Luster *et al.*, 1993). Immunostimulation can be mediated by substances called immunostimulants, which correspond to "a naturally occurring compound that modulates the immune system by increasing the host's resistance against diseases that in most circumstances are caused by pathogens" (Bricknell and Dalmo, 2005). Immunostimulants are usually given as (pre-/pro- or synbiotic) feed additives (*e.g.,* in cattle, fish) and correspond to several types of compounds, including micronutrients (*i.e.*, minerals and vitamins), amino acids, proteins, organic acids, etc. (Dawood *et al.*, 2018). However, immunostimulation can also result in inappropriate and nonspecific immune system activation (Gallucci *et al.*, 2020). Intraperitoneal injection of As, Cd, and Pb is also related to immunostimulation (*i.e.,* increase of the phagocytic activity) in laboratory mice and rats. This mechanism can be associated with hypersensitive immune system reactions like asthma or flu-like symptoms and, in some cases, an abnormal immune response against oneself, known as an autoimmune disorder (Brousseau *et al.*, 2012). Metal-induced hypersensitivity corresponds to an allergic reaction due to the disturbance of homeostatic mechanisms implied in the prevention of inappropriate immune response against non-pathogenic compounds (McKee and Fontenot, 2016). Hypersensitive reactions associated to metal exposure mainly involve type-I or immediate hypersensitivity mediated by IgE in humans and, type-IV or delayed hypersensitivity mediated by macrophages and T-lymphocytes, which for type-IV can also be associated with inflammatory reactions (Woolhiser *et al.*, 2005; Zelikoff *et al.*, 1994). In contrast, immunosuppression is commonly associated with exposure to toxic elements. Zelikoff *et al.* (1994) introduced the term 'metalloimmunosuppression' to describe immune downregulation induced by toxic elements on host defence against intracellular pathogens. For instance, Pb and Hg suppress Th1 (*i.e.* involved in cellular immunity) in favour of Th2 (*i.e.* involved in humoral immunity) cell proliferation, which is promoted by overexpression of IL-4 and IgE and a lower expression of IFN-γ in laboratory mice plasma (Heo *et al.*, 1996).

The present review focuses on immunomodulation in mammals involving micronutrients and non-essential metals across various disciplines (veterinary, medicine, ecotoxicology, ecology). The main objective was to fill the gap between knowledge acquired in laboratory raised animals (Chapters 3 and 4) and their wild counterpart (Chapter 5). Indeed, Jolles *et al.* (Jolles *et al.*, 2015) highlight that almost everything we know about the mammalian immune system is based on studies in mice and humans. Within a large scope, including humans and humanised rodent models, we expect to get more insight into the immunomodulatory potency of non-essential metals at toxic levels (Chapter 3.1 of the present review), the beneficial effects of essential metals (Chapter 3.2) and vitamins (Chapter 3.3) on the immune system, and the interaction between non-essential metals and micronutrients (Chapter 4). Chapter 5 is dedicated to bibliographic research on studies in wild and captive mammals, including studies with food restriction/supplementation. In the wild, sentinel species or bioindicators have proven their efficiency in monitoring wildlife state and health (*e.g.,* (Desforges *et al.*, 2016; Acevedo-Whitehouse and Duffus, 2009a), but confounding factors cannot be controlled. Captivity, on the other hand, promotes an environment where the food supply is controlled, proving its ability to modulate the immune system and even gut microbiota (Kumar *et al.*, 2019; McKenzie *et al.*, 2017; Young *et al.*, 2017). By comparing both fields (wildlife studies and studies in captivity), we expect to get knowledge from captive mammals in the hope of setting important research perspectives on wild mammals, which are likely to suffer from micronutrient deficiencies and/or pollution *in natura*. From all of this, Chapter 6 draws perspectives in the field of immunoecotoxicology to better handle the impact of micronutrient nutritional balance on immunomodulation in mammals.

2. Materials and methods

Relevant reviews on the immunotoxicity of metals at toxic concentrations, the beneficial effects of essential elements and vitamins on the immune system, and the interaction between toxic elements and micronutrients were retrieved from classical scientific databases (Google Scholar, Web of Science (WoS)…). A literature search for study cases on wild and captive mammals was performed on the WoS within “Environmental Sciences & Ecology” and “Veterinary sciences” research areas using 60 combinations of the following full or truncated keywords: “immunity” (or immune function, system, defences / defenses), “immunomodulation”, “nutrition” (or diet, food intake, energy, metabolism), “vitamins”, “metals” (or heavy metals, toxic metals/metalloids, essential elements, minerals), and “wild” / “captive”, “terrestrial” / “marine mammals”. This search brought out 1,382 articles. Using the Boolean combination of keywords and reading article titles, human medical studies and articles dealing with other taxa than mammals were excluded. This step ended up with 432 articles. Then, a trimming was manually done by reading the title and abstract, ranking corresponding articles

from less (1) to most (3) relevant as follows: (1) if they at least considered immunological impacts of toxic metal(oid)s, (2) if they dealt with both toxic metal(oid)s and micronutrients, and (3) if they reported interactions of micronutrients with metal(oid)s toxicity. The number of articles retained for this review was 164. Introductory elements and perspectives were drawn upon reviews and opinion papers found during the bibliographic research and our experience in the area.

3. Effects of trace metals and vitamins on the immune system of mammals

3.1. Effects of non-essential metals and metalloids (As, Cd, Pb and Hg)

Exposure to non-essential metals and metalloids may severely affect the immune system, including autoimmune damage and immunosuppression. Arsenic, Cd, Hg and Pb are toxic elements considered as concerns regarding the threat they represent to ecosystems and public health (Rahman and Singh, 2019). Multiple environmental and biological parameters enhance their transfer from the environment to the biota (bioavailability). In organisms, toxic elements are transported by the circulating system and reach target organs or tissues. The effects of toxic elements on the immune system vary with species, sex, dose, duration, and exposure route (Gallucci *et al.*, 2020). Toxic elements can react with biological systems by losing one or more electrons, turning them into cations, which possess a higher affinity to nucleophilic groups of organisms' macromolecules. Cadmium and Hg share the same affinity to sulfuric-rich compounds, including proteins like metallothioneins, while As and Pb can bind to oxygen-containing compounds. Arsenic, Cd, Pb and Hg disrupt cell functioning, proliferation, repairment and apoptosis. All those toxic elements display similar abilities to enhance reactive oxygen species (ROS), oxidative stress and weakened antioxidant defence (Balali-Mood *et al.*, 2021). Depending on their binding sites, toxic elements can be stored or metabolised toward detoxification processes. They also exert their toxic action on endogenous compounds (*e.g.,* DNA) or macromolecules of structural elements (*e.g.,* membranes). Knowledge of how As, Cd, Hg and Pb impact the immune system remains limited (especially in wildlife) and often relates to physiological disorders without shedding light on underlying mechanisms. Several haematological (*e.g.,* decreasing haematocrit) or immunological (*e.g.,* decreasing lymphocyte proliferation) markers have been associated with clinical features in laboratory animals exposed to non-essential metals. Non-essential metals at toxic concentrations have either a direct effect on immune cell(s) reactivity (*i.e.,* immunostimulation and immunosuppression) or indirectly impact their regulation (Zelikoff *et al.*, 1994). The production of ROS by toxic elements may enhance immune cell damage, suppressing the immune system's defence mechanisms against pathogens. Toxic elements can also interfere with the regulation of the

immune system towards overstimulation of the immune response, leading to autoimmune disorders or allergic reactions. The mechanisms of immunomodulation mediated by toxic elements are not well known, and principal endpoints are yet to be discovered. Table 1 presents different immunotoxicological endpoints, which are consistent in different experimental designs (*i.e.*, *in vivo, in vitro* and *ex vivo*) and mammal models, including the most studied ones (*i.e.*, humans and laboratory mice and rats). Arsenic, Cd, Hg, and Pb are susceptible to reduce or even suppress delayed-type hypersensitivity (DTH), also known as cell-mediated immunity.

Table 1. Haematological and immune markers of toxicity for As, Cd, Hg and mercuric compounds, and Pb in mammalian studies. Acronyms are defined in Table footnotes, and ↑ and ↓ stand for increase and decrease, respectively, of a given parameter.

Metal	Target organs	Blood count	Cell-surface proteins	Inflammatory response	Humoral response	Oxidative stress	Ref.
Arsenic (As)	CNS Heart GI Liver Skin	↓ RBC ↓ Hb ↓ Hct	↓ MHCII ↓ CD69 ↓ CD4/CD8	↓ T-cells proliferation and phagocytic activity ↓ PBMC ↑ Pro-inflammatory cytokines (TNF-α, Il-1β) Inflammatory cytokines involved in clearance of: ↓ Intracellular pathogens (Il-2, IFN-γ) *i.e. T cell proliferation, macrophage activation…* ↓ Extracellular pathogens (Il-17) *i.e. neutrophils recruitment*	↑ IgE	↑ ROS in several immune cell types (*e.g.* macrophages, lymphocytes) ↓ ROS production by PBMC (NO^-, O_2^-)	[1] [3] [9]
Cadmium (Cd)	Bone Blood GI Kidney Liver Lung	↓ RBC ↓ Hb ↓ Hct ↑↓ WBC	↓ MHCII ↓ $CD4^+/CD8^+$	↓ T-cells proliferation and phagocytic activity ↓ NK cells count and activity ↑ T-cell apoptosis ↑ Pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) Inflammatory cytokines and chemokines involved in clearance of: ↓ Intracellular pathogens (Il-2, IFN-γ) *i.e. T cell proliferation, macrophage activation…* ↑ Extracellular pathogens (Il-8, Il-17, MIP-2) *i.e. neutrophils recruitment*	↓ IgE	↑ ROS in several immune cell types (*e.g.* macrophages, lymphocytes) ↑ NO^- in macrophages and splenocytes culture	[3] [5] [8] [10]
Mercury (Hg)	CNS GI Kidney Liver	↑↓ RBC ↑↓ Hb ↑↓ WBC	↑↓ MCHII ↑ CD4+	↓ T-cells proliferation and phagocytic activity ↓ NK cells count and activity ↑ T-cell apoptosis ↑ Pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) Inflammatory cytokines involved in clearance of: ↑ Intracellular pathogens (IFN-γ)	↑ IFN-γ ↑ IL-4 ↑ IgE	↑ ROS in mast cells	[4] [6] [7] [12]

				↑ Extracellular pathogens (Il-17) *i.e. neutrophils recruitment*			
Lead (Pb)	Blood CNS Lung Liver Heart	↓ RBC ↓↑ WBC	↓ MCHII ↓ $CD4^+/CD8^+$	↓ Lymphocyte proliferation and phagocytic activity ↑ Pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) Inflammatory cytokines involved in clearance of: ↓ Intracellular pathogens (Il-2, IFN-γ) *i.e. T cell proliferation, macrophage activation…* ↑ Extracellular pathogens (Il-17) *i.e. neutrophils recruitment*	↑ IL-4 ↑ IgE	↑ ROS *via* ↑ δ-ALA or ↑ direct cell membrane damage (lipids) in several immune cell types (*e.g.* macrophages, lymphocytes)	[2] [3] [7] [11]

δ-ALA: δ-aminolevulinic acid, CNS: central nervous system, CD: cluster of differentiation, GI: gastrointestinal tract , GSH: glutathione, Hb: haemoglobin, Hct: haematocrit, IFN-γ: interferon-gamma, Ig: immunoglobulin, IL: interleukin, LT: T lymphocyte, MIP: macrophage inflammatory protein-2, MHCII: major histocompatibility complex II, NO: nitric oxide, NS: nervous system, PBMC: peripheral blood mononuclear cells, RBC: red blood cells, ROS: reactive oxygen species, TNF-α: tumor necrosis factor alpha, Th: T helper lymphocyte, Treg: T regulator lymphocyte, WBC: white blood cells.

[1] (Dangleben *et al.*, 2013); [2] (Fenga *et al.*, 2017); [3] (Gera *et al.*, 2015); [4] (Pollard *et al.*, 2019); [5] (Hossein-Khannazer *et al.*, 2019); [6] (Havarinasab and Hultman, 2005); [7] (Lawrence and McCabe, 2002); [8] (Thévenod and Lee, 2013); [9] (Bellamri *et al.*, 2018); [10] (Wang *et al.*, 2021); [11] (Mishra *et al.*, 2009), [12] (Vas and Monestier, 2008).

DTH are reactions mediated by CD4+ T cells, which are characterised by abnormal inflammatory responses (*i.e.* increase of pro-inflammatory cytokines), and damage is caused directly by cytotoxic T cells. These toxic elements may decrease or even suppress delayed-type hypersensitivity challenge in response to immune challenge (*i.e.,* sheep erythrocytes), increasing susceptibility to autoimmune disorders (Bjørklund *et al.*, 2017; Gera *et al.*, 2015). Toxic elements may thus affect the different subpopulation of T cells involved in pro-inflammatory (Th1), non-inflammatory (Th2), anti-inflammatory (Th3), anti-microbial (Th17), and regulatory (Treg) activities (Gera *et al.*, 2015). In immunotoxicology testing, haematology is considered as the second level of changes leading to immunosuppression after disturbance in organ weights and histological alteration (Gallucci *et al.*, 2020). Haematological outcomes are associated with a decrease in red blood cells for As, Cd and Pb. Arsenic and Cd decrease blood's haematocrit, red cell count, and haemoglobin concentration. Cadmium, Hg and Pb also disturb white blood cell count, depending on species, dose, and duration of exposure to these metals.

Arsenic toxicity adversely affects different immune system compartments: monocyte activity and differentiation, dendritic cells, and phagocytic proliferation (Bellamri *et al.*, 2018). Oxidative stress *via* cell apoptosis, inflammation, and ROS production are the major mechanisms associated with As immunotoxicity. Exposure of mice and humans to As reduces class II major histocompatibility complex (MHCII), which is a cell surface protein mainly present on antigen-presenting cells (APC, *i.e.,* macrophages) (Dangleben *et al.*, 2013). Arsenic is also known to decrease the cluster of differentiation (CD) CD69 that is expressed on the surface of T cells and natural killer (NK) cells. (Dangleben *et al.*, 2013) highlighted the suppression of IL-2 secretion, involved in T cell proliferation, by exposure to As *in vitro* studies. Arsenic can also decrease inflammatory response *via* IL-1β and TNF-α (pro-inflammatory cytokines), whose inhibition can even lead to immune suppression. For example, Immunosuppression mediated by As decreased laboratory mice's immune response against influenza A (Kozul *et al.*, 2009). In humans, As is given as chemotherapy treatment for specific leukaemia to control the immune-mediated inflammatory disease. For instance, human CD4+ T cells exposed to As showed a decrease of Th17 *via* IL-17 inhibition involved in inflammatory response against bacterial or fungal extracellular pathogens (Morzadec *et al.*, 2012). The humoral immunity of animals seems to be unaltered by As. Only one study on Bangladeshis showed a consistent increase in IgE, which may be attributed to the direct inflammatory effects of As (Islam *et al.*, 2007)(Islam *et al.*, 2007). Arsenic is a well-known pro-oxidant forming complex with the thiol group of proteins increasing ROS production in different immune cells, including lymphocytes and macrophages (Bellamri *et al.*, 2018). Oxidative stress damage can be associated with an overproduction of ROS in peripheral blood mononuclear cells (PBMC). However, a decrease

in ROS production was also found in the PBMC of human and animal models (children, mice, chicken, molluscan), which could weaken PBMC defence against pathogens (Dangleben *et al.*, 2013).

Cadmium can severely impact immune cells' functioning, altering T cells and NK cells' proliferation and activity (Gera *et al.*, 2015). Cadmium and mainly Cd^{2+} particularly interfere with essential metal ions involved in the vital functioning of organisms (Thevenod and Lee, 2015). Indeed, the ionic form Cd^{2+} shares the same affinity to the protein thiol group as calcium, which can block calcium channels and disturb cellular homeostasis (Choong *et al.*, 2014). Cadmium can directly impact cells by entering inside cells *via* calcium channels and/or binding to cell surface proteins. Nevertheless, immune cell damage associated with exposure to Cd mainly comes from ROS, which can affect mitochondrial membranes and induce immune cell apoptosis (Wang *et al.*, 2020). The production of ROS mediated by Cd generates a decline in the CD4+/CD8+ ratio, inducing suppression of CD4+ cell subsets (helper T-cells) and an enhancement in CD8+ cells (cytotoxic T-cells). Indeed, a higher glutathione (GSH) depletion and apoptosis were found in the CD4+ cell population than in the CD8+ cell population in response to Cd. The decrease of CD4+ T cells exposed to Cd was also attributed to a reduction of IL-2 and IFN-γ secretion, which are cytokines involved in T cell proliferation, inflammation, and macrophage activation (Pathak and Khandelwal, 2008). García-Mendoza *et al.* (García-Mendoza *et al.*, 2019) found a higher GSH depletion in mast cells compared to macrophages in murine cell lineages cultured at different Cd concentrations. Hossein-Khannazer *et al.* ((Hossein-Khannazer *et al.*, 2020) proposed a framework where ROS production in neutrophils and macrophages by Cd activates the nuclear factor κB (NF-κB) promoting the expression of pro-inflammatory cytokines TNF-α, IL-6 and IL-1β, and chemokines IL-8 and MIP-2 involved in inflammation and the chemotactic recruitment of neutrophils. Cadmium also promoted the expression of pro-inflammatory cytokines IL-1b, IL-6, and TNF-α and inhibited anti-inflammatory cytokine IL-10 in macrophages by increasing ROS production (Wang *et al.*, 2021). A permanent expression of pro-inflammatory cytokines, such as IL-6 mediated, may trigger autoimmune damage and cancer (Thevenod and Lee, 2015). Cadmium primarily affects humoral immunity by inhibiting DNA and RNA expression and decreasing IgE production (Wang *et al.*, 2021). The mechanism for the affection of B cells by Cd is not well known in all species, as both intracellular and extracellular compartments are affected. However, *In vitro* studies on murine B cells and bone marrow-derived dendritic cells demonstrated a decrease in MHCII when cultures were exposed to $CdCl_2$ (Chakraborty *et al.*, 2014; Daum *et al.*, 1993).

Mercury displays severe immune system outcomes, which lead to inflammation and autoimmune disorders (Havarinasab and Hultman, 2005). Immunotoxicity of Hg has been associated with an imbalance in the polarisation of CD4+ T helper (Th) lymphocytes between

Th1 and Th2. The paradigm of Th1 and Th2 cells relies on a predominant cellular immunity (Th1) mediated by IFN-γ and on a predominant humoral or antibody-mediated immunity (Th2) mediated by IL-4 (Romagnani, 1997). Indeed, both *in vitro* and *in vivo* studies showed an increase of either or both IFN-γ (involved in macrophage activation) and IL-4 (promotes B cell activation and IgE production) (Pollard *et al.*, 2019). However, a study on gene deletion of mice exposed to Hg highlighted that T cell proliferation and B antibody production mediating autoimmune damage required IFN-γ but not IL-4 (Kono *et al.*, 1998). A mimicry of Hg-induced oxidative stress by hydrogen peroxide enhanced IL-4 production in rat mast cells, which suggests that Hg-induced IL-4 production would partially be due to ROS (Vas and Monestier, 2008). Mercury enhances CD4+ cell proliferation, which mainly interacts with the MHCII receptor of antigen-presenting cells and thus promotes MHCII expression. However, there is no clear variation in the MHCII levels of mice exposed to Hg (Table 1). Hu *et al.* (Hu *et al.*, 1997) found that MHCII was required for Hg-induced cytokine production and lymphoproliferation in mouse spleen cell culture. MCHII decreased in lymphoid organs (thymus and spleen) and increased in target organs for toxic elements (*i.e.,* kidney and liver) (Kim *et al.*, 2003).

Lead immunotoxicity can affect phagocytosis and lymphocyte proliferation and modulate humoral immunity (Mishra, 2009). The impact of Pb toxicity on immune cells is attributed to cell surface proteins with a decrease in MHCII and the CD4+/CD8+ ratio (Heo *et al.*, 1996; McCabe and Lawrence, 1991). Conventionally, Pb toxicity relies on its ability to produce ROS *via* inhibition of the δ-aminolevulinic acid dehydratase, accounting for an accumulation of rapidly oxidising δ-aminolevulinic acid, which produces free radicals (*i.e.,* hydroxyl radicals) (Lopes *et al.*, 2016). Liu *et al.* (Liu *et al.*, 2012) showed that an increase in antioxidant levels associated with Pb might decrease pro-inflammatory cytokines (IL-1β, TNF-α and IL-6), stimulating the activation of mitogen-activated protein kinases (MAPKs) and NF-κB. An increase in pro-inflammatory cytokines like IL-1β, TNF-α and IL-6 by Pb could enhance autoimmune damage in both *in vivo* and *in vitro* (Fenga *et al.*, 2017). A decrease in IL-2 and IFN-γ production and an increase in IL-4 production were found in response to Pb ingestion in mice (Iavicoli *et al.*, 2006). IL-4 is critical in humoral response as it is required to enhance MHCII expression in B cells and, indirectly, IgE and IgG production (Heo *et al.*, 1996). Whilst little is known about the effect of Pb on Treg and Th17, IL-17 secretion increases in human workers chronically exposed to Pb (Dobrakowski *et al.*, 2016). It has also been hypothesised that exposure to non-essential metals may enhance Tregs expression, which promotes immunosuppression (Lawrence and McCabe, 2002; McCabe and Lawrence, 1991).

Take home messages:

a) Toxic metal(loid)s can affect immune cells functioning, structure, proliferation and communication.
b) The immunotocicity of toxic elements mainly relies on the ionic mimicry (*i.e.* ability to bind to self-molecules) and oxidative stress (*i.e.* production of reactive oxygen species).
c) Arsenic, Cd, Pb and Hg can decrease macrophages and T cells proliferation to modulate immunocompetence.
d) Arsenic, Cd and Pb exposure affects the secretion of cytokines both involved in the inflammatory response and the regulation of the immune system.

3.2. Effects of essential metals (Cu, Fe, Mn, Se and Zn)

The macro- and micro-nutrient requirements of animal metabolism change along their life and development stages (Allen and Ullrey, 2004). Micronutrients are idiosyncratic components of the immune system. Their role in the system varies from homeostatic regulators to constitutive elements of immunity. The present review focuses on Cu, Fe, Mn, Se, and Zn from the list of essential elements because of their particular importance in the immune system and their relevance to wild animals' maintenance and reproduction (Gombart *et al.*, 2020; Allen and Ullrey, 2004). Vertebrates have developed mechanisms which to save minerals from pathogens during an infection. This phenomenon, called nutritional immunity, was defined by Hennigar and McClung (Hennigar and McClung, 2016) as "a process by which a host organism sequesters trace minerals in an effort to limit pathogenicity during infection". Nutritional immunity can also be presented as one example of the Red Queen Hypothesis on host/pathogen coevolution, where the host and pathogens have to constantly get the competitive advantage on the mineral resources of the host. This process is considered as one of the first lines of defence in case of pathogen infection as it relies on the ability of the host to withhold minerals to prevent pathogens from growing. Minerals are prone to have a beneficial impact on the epithelial barrier, cellular immunity, humoral immunity, and oxidative stress.

Copper is an enzymatic co-factor involved in antioxidant defence (superoxide dismutase), oxygen chemistry (*e.g.,* cytochrome C oxidase) and Fe metabolism (*e.g.,* ceruloplasmin), among others. Copper is mostly transported in the blood *via* a ferroxidase enzyme called ceruloplasmin (Cp). Ceruloplasmin has a strong antioxidant potency toward free Cu and Fe, which are both catalysts of free-radical damage *via* the Fenton reaction producing ROS (Kim *et al.*, 2008; Weiss, 2002). Free Cu can bind to Cp, promoting the Cu-Cp complex, whose

ferroxidase activity simplifies Fe^{2+} transport *via* transferrin (Johnson *et al.*, 1992). Recently, Culbertson *et al.* (Culbertson *et al.*, 2020) highlighted a change in Cu homeostasis during infection, where renal Cu levels decreased, and liver production of Cu-Cp increased to restore Fe levels in the serum. The regulation of Cu levels is determinant during infection as a host can either starve pathogens from Cu (sequestration) or take advantage of the antimicrobial properties of Cu against pathogens (Besold *et al.*, 2016). Infected macrophages (*Mycobacterium spp.*) treated with TNF-α and IFN-γ showed an accumulation of phagosomal Cu enclosed with pathogens, exploiting the intrinsic antimicrobial activity of Cu against unicellular microbial pathogens (Sheldon and Skaar, 2019; Palmer and Skaar, 2016; Wagner *et al.*, 2005).

Iron is involved in many metabolic processes, including oxygen transport and storage in haemoglobin. Iron is also a co-factor of several enzymes implied in DNA synthesis and repair (Nairz *et al.*, 2018). Immune cells like monocytes, macrophages, and T cells express transferrin receptors that control Fe uptake from blood circulation (Tourkochristou *et al.*, 2021). Indeed, transferrin receptor activation by Fe has been shown to amplify T cell development and proliferation (Gombart *et al.*, 2020; Cronin *et al.*, 2019). Elevated levels of Fe may trigger the Fenton/Haber-Weiss reaction promoting free radical production (Weiss, 2002). Iron is also a key component of enzymes involved in immunity, including myeloperoxidases, nicotinamide adenine dinucleotide phosphate oxidases, and NOx synthases (Smith *et al.*, 2018). By the last 40 years, a new insight has been given into the ability of host organisms to withhold Fe. During infection with extracellular (*e.g., Escherichia coli*) or intracellular (*e.g., Salmonella spp.*) bacteria, host macrophages will respectively incorporate Fe into ferritin (hypoferremia) and stimulate ferroportin transcription-controlling Fe^{2+} extracellular outflow. The regulation of macrophage Fe metabolism during bacterial infection can be stimulated by IL-6, amplifying a liver-derived hormone called hepcidin that is involved in intracellular Fe retention (Nairz *et al.*, 2018). Acute-phase haeme scavenger hemopexin mediated by IL-22 cytokines may counter Fe extraction from labile haeme in plasma by Gram-positive pathogenic bacteria (*e.g., S. aureus)*(Sakamoto *et al.*, 2017; Choby and Skaar, 2016).

Manganese (Mn) is an essential element in mammals' homeostasis as it is a co-factor of many enzymes involved in critical functions like glycogenesis (Kohlmeier, 2015). Manganese is also a component of metalloenzymes associated with antioxidant defence against ROS as Mn-SOD (Waldron *et al.*, 2009; Culotta *et al.*, 2006). Circulatory Mn is transported and distributed in the plasma by albumin, γ-globulin, and Mn-transferrin complex (Wang *et al.*, 2020). During bacterial infection, neutrophils release an antimicrobial protein called calprotectin that binds to Mn^{2+} and Zn^{2+}, making them unavailable for the bacteria (Lopez and Skaar, 2018; Brophy and Nolan, 2015). Host nutritional immunity on Fe withholding has driven pathogens towards

evolving strategies to maintain sufficient mineral intake for their development. For example, *Borrelia burgdorferi* has developed a strategy that consists in substituting Fe with Mn for the functioning of many metalloenzymes (Palmer and Skaar, 2016). Mn^{2+} is susceptible to enhance natural killer (NK) cell activity (in murine) mediated by Type 1 IFNs and is potentially involved in a pathogen signalling activity as danger-associated molecular patterns (DAMPs) (Wang *et al.*, 2020).

Selenium is crucial in physiological processes, immune responses, and the development of mammals. Selenoproteins and Se-dependent processes are involved in a wide range of functions implied in the regulation of ROS (Schweizer *et al.*, 2016). Among selenoproteins, glutathione peroxidases can reduce ROS like hydrogen peroxide by forming inoffensive compounds such as water (Avery and Hoffmann, 2018). Thioredoxin reductase regulates cell growth and viability and allows the regeneration of several antioxidants (Higdon and Drake, 2012). Selenium also regulates thyroid hormones in activating the iodothyronine deiodinases, which removes an iodine atom from thyroxine (T4) to form the active triiodothyronine (T3) (Higdon and Drake, 2012). Se supplementation promotes immunostimulatory reactions, including T cell proliferation and increasing NK cell activity (Avery and Hoffmann, 2018). Se deficiency is responsible for several pathologies like mulberry heart disease in pigs and boars (Pilarczyk *et al.*, 2019). Dietary Se is crucial to the mammals' immune response to bacterial, fungal, or viral pathogens. For example, supplementation with Se enhanced innate immune cell functioning, T cell proliferation, and NK cell activity in both human and laboratory mouse (Huang *et al.*, 2012). Experiments on laboratory mice showed that Se repletion promoted inflammatory cytokines expression (mainly IFN-γ and IL-6) (Tsuji *et al.*, 2015), while its deficiency aggravated inflammation, dampened selenoproteins expression and reduced adaptive immunity (Avery and Hoffmann, 2018).

Zinc is ubiquitous in organism cells whose nutritional importance requires daily intake to maintain homeostasis and immunity (Bonaventura *et al.*, 2015). Its regulation is ensured by Zn-binding proteins like metallothioneins, and its transport occurs through different Zn transporters and importers (Kehl-Fie and Skaar, 2010). The structural and catalytic roles of Zn are also implied in many transcription factors (around 50% in humans and mice). More than 300 enzymes and superoxide dismutase are involved in the production of hydrogen peroxide (Smith *et al.*, 2018). The impact of Zn deficiency on the immunity of wildlife has been poorly documented. However, Zn deficiency could cause an imbalance in the ratio of Th1/Th2 in humans. In fact, Zn deficiency decreases Th1 cytokines (IFN-γ, IL-2 and TNF-α) production but less affects the Th2 cytokine (IL-4, IL-6 and IL-10) production in humans (Prasad, 2000).

Take home messages:

a) Essential elements are constitutive elements of the immune system.
b) Copper, Fe, Mn, Se, and Zn are antioxidants involved in the regulation of reactive oxygen species.
c) Copper and Se directly (*i.e.* antimicrobial activity) and indirectly (*i.e.* enzymatic cofactor) contribute to innate immunity.
d) Dietary modulation of Cu, Fe, Mn, Se, and Zn displayed changes in cytokines secretion by immune cells.

3.3. Effect of vitamins (A, B12, C, D and E)

Vitamins are essential compounds taken from food. The pleiotropic activity of vitamins A, B12, C, D, and E is essential for metabolism, reproduction, and the immune system (Smith *et al.*, 2018; Shaik-Dasthagirisaheb *et al.*, 2013; Allen and Ullrey, 2004).

Vitamin E (VE) is a generic term for lipophilic compounds, which are declined in four types of tocopherols (α-, β-, γ-, and δ-) and four types of tocotrienols (α-, β-, γ-, and δ-). Among them, α-tocopherol represents the most biologically active form of VE and is abundant in vegetable oil (Atkinson *et al.*, 2013). The major beneficial function of VE relies on its antioxidant potency against ROS and NOx, which protect the polyunsaturated fatty acids from oxidation in membranes (Lee and Han, 2018). The supplementation of VE has positive influence on macrophages which pleiotropic roles (*i.e.* APC, NK cells regulator and cytokines production) may increase individual immunocompetence. A study in mice showed that VE reduced the macrophage the age-induced production of prostaglandin (PG)E_2 (*i.e.* a hormonal mediator of inflammation) preventing from the development of autoimmune diseases (Wu *et al.*, 1998). Another study also showed that VE inhibited the activity of cyclooxygenase (COX-2), which produces the PG$(E)_2$ preventing from the decline of T-cell-mediated function in mice macrophages (Beharka *et al.*, 2002). This study also showed that medium with VE increased the production of IL-2, which enhances T cell activation signal, in old mice. Vitamin E also positively impacted NK cell activity, antibody response, and dendritic cell maturation and functions (Gombart *et al.*, 2020; Lee and Han, 2018).

Vitamin A (VA) encompasses several compounds that commonly refer to as retinol (predominantly all-*trans*-retinol) and retinyl esters in mammals; both are precursors of active retinoids (Defo *et al.*, 2014). Retinyl esters refer to the fat-soluble form of VA mainly stored in the liver (Albalat, 2009). Retinol can be converted by an enzyme called retinol dehydrogenase

into retinaldehyde, which can be oxidised *via* retinaldehyde dehydrogenase into retinoic acids (RA), biologically active forms of VA (Theodosiou *et al.*, 2010). Vitamin A displays a pleiotropic activity involved in eyesight, skin and mucosal integrity, antimicrobial activity and regulates the immune system and gene expression. Vitamin A exhibits antioxidant protective activity in human peripheral lymphocytes (Alpsoy *et al.*, 2009). Retinoic acids can activate hetero- or homo-dimers of the retinoic acid receptors and the retinoid X receptors present in three isotypes (α, β, and γ) to form retinoic acid response elements, which can bind to consensus DNA regions controlling gene expression (Gombart *et al.*, 2020; Smith *et al.*, 2018; Eroglu and Harrison, 2013). Vitamin A and its precursor produced by plants, β-carotene, are described as the anti-infective agents of immunity (Goodwin, 1986). They are involved in B- and T-cells differentiation and regulation through cytokine expression, phagocytic cells protection against auto-oxidative damage, and activation of macrophages, cytotoxic T and NK cells (Smith *et al.*, 2018; Ross, 2012; Bendich, 1989; Green and Mellanby, 1928). In a general context, VA or RA limit Th1 (*i.e.,* IFN-γ decrease) and Th17 (*i.e.,* IL-6 decrease) processes, while they stimulate Th2 (*i.e.,* IL-4 increase) and Treg (*i.e.,* TGF-β increase) differentiation (Ross, 2012). Differentiation of Th0 cells into Treg and Th17 cells relies, respectively, on the balance between the transcription factors' expression of retinoid orphan receptor γ and forkhead box P3 (Foxp3) (Cook *et al.*, 2015). It has also been shown that dietary VA favours the generation of RORγt+ Tregs beside Th17 cell production (Ohnmacht *et al.*, 2015).

Vitamin C (VC) or L-ascorbic acid is a hydrophilic compound with low molecular weight and a potent electron donor. Vitamin C is required to produce key elements in organisms, including collagen (a major constituent of blood and tendons), norepinephrine (a neurotransmitter), and carnitine (an amino acid involved in the transport of fat into mitochondria) (Higdon & Drake, 2012). Vitamin C redox potentially confers organisms' major functions like antioxidant and enzyme cofactors (Linster & Van Schaftingen, 2007). Vitamin C also contributes to the protection of essential molecules against ROS, regenerates VE from tocopheryl radicals (in humans), and maintains transition metal ions in their reduced form (*i.e.,* regulation of Fe) (Gombart *et al.*, 2020; Carr & Maggini, 2017; Davies, 1992). Vitamin C mainly originates from fruits and promotes beneficial effects in both plants and mammals (Fenech *et al.*, 2019). Vitamin C requirements are species-dependent, as it is not synthesized in every mammal. For example, primates and guinea pigs cannot synthesize it (Hemilä, 2017). Sources of VC, as for humans, therefore rely on dietary intake. Beneficial outcomes from VC supplements in mammals have been widely reported (Hemilä, 2017). The antioxidant property of VC has a strong implication on immunity as it protects leukocytes from self-oxidative damage of ROS toxins (*i.e.,* superoxide radicals) released by phagocytic cells to kill invading pathogens (Carr and Maggini, 2017). The antioxidative function of VC is also to protect white blood cells (WBC)

from autoxidation by toxins (*e.g.* superoxide radicals) during pathogen infection (Higdon and Drake, 2012).

Vitamin D (VD) is a fat-soluble compound considered as the 'vitamin-hormone', which mainly comes from seeds and plants but, to a lesser extent, from endogenous production in some mammals (O'Brien and Jackson, 2012). In plants, ultraviolet B (UVB) sunlight radiation stimulates the production of ergocalciferol (VD_2), whereas, in animals, cholecalciferol (VD_3) can be obtained from the diet or also synthesized by exposure to UVB. In fact, VD can be synthesized by the skin in response to UVB radiation exposure, triggering photolysis of cholesterol compounds to produce a precursor of VD_2, which spontaneously isomerizes in cholecalciferol (VD_3) (Buffenstein and Pinto, 2009; Holick *et al.*, 2007). Both VD_2 and VD_3 are then converted in the liver into an inert circulating metabolite, calcidiol (25(OH)D), whose concentration enables measuring VD status (Hart *et al.*, 2006). Calcidiol is then hydroxylated in the kidney into the metabolically active VD, calcitriol (1,25-$(OH)_2$ D). Calcitriol then binds to the vitamin D receptor (VDR), enhancing gene regulation and the regulation of Ca and phosphorus (P) homeostasis in both the gastrointestinal tract and bones (O'Brien and Jackson, 2012; Holick, 2004). As the other vitamins presented here, the role of VD in the immune system has been mainly documented by extension from research in humans (*e.g.,* on rickets) and humanised mouse models (O'Brien and Jackson, 2012). Vitamin D has a strong immunomodulatory activity on antigen-presenting cells (*i.e.,* macrophage activation), T cells proliferation, cytokine production and regulation of B cells Ig synthesis, and the production of antimicrobial peptides (*i.e.,* cathelicidin, defensins) (Gombart *et al.*, 2020; O'Brien and Jackson, 2012; Buffenstein and Pinto, 2009).

Vitamin B12 (VB12), or cobalamin, is the heaviest vitamin in terms of molecular weight and presents the particularity of containing cobalt ions. Vitamin B12 can be distinguished by its prior metabolic role, as it is involved in folate metabolism and in succinyl-CoA synthesis. VB12 is involved in nutritional immunity (mostly in ruminants) considering that an important quantity of VB12 comes from bacteria colonies in the rumen (Rowley and Kendall, 2019). Nonetheless, it seems that VB12 may play a functional role in both innate and adaptive immunity, at least for humans (Gombart *et al.*, 2020).

Take home messages:

a) Vitamins A, B12, C, D, and E ensure the functioning of immune cells.

b) Vitamins D and E intervene in the hormonal regulation of the immune response.

c) Vitamins C and E protects immune cells from oxidative stress.

d) Vitamins A and D has an antimicrobial activity.

4. Interactions between non-essential metals and micronutrients in mammals

Interactions between non-essential metals and micronutrients have been poorly documented in mammals, apart from some laboratory studies dealing with classical laboratory animal models like rats and mice (Wren, 1986). Table 2 presents the interactions between micronutrients and toxic elements in mammals in the literature. An overview is given below for As, Cd, Hg, and Pb.

Table 2. Effects of micronutrients on toxic metal toxicity in mammalian studies. Acronyms are defined in Table footnotes, and ↑ and ↓ stand for increase and decrease, respectively, of a given parameter.

Metal	Vitamin B_{12}	Vitamin C	Vitamin D	Vitamin E	Copper	Iron	Selenium	Zinc
Arsenic (As)	↓circulating As (urinary excretion of dimethylarsenic acid) [1]	↓ ROS and NO [1] [2]		↓ ROS and NO [1] [2]			↓ ROS (via selenoproteins) ↓ toxicity *via* As:Se adduct (biliary excretion) [3]	
Cadmium (Cd)		↓ ROS [4] [5] ↑ Fe absorption in the intestinal tract (reducing the Cd-mediated anaemia) [6] [7] [8]	↑ Cd gastro intestinal absorption *via* substitution with Ca [9] [10]	↓ ROS [4] [5]	↓ available binding site on the metallothionein (antagonist of Cd) [11] [12] [13] ↓ Cu and ceruloplasmin levels [14]	↓ available binding site metallothionein (antagonist of Cd) [6] [11] [12] [13]	↓ ROS (selenoproteins) [4] [5] ↓ Cd toxicity *via* Cd:Se [4]	↓ available binding site metallothionein (antagonist of Cd) [11] [12] [13] [14]
Mercury (Hg)				↓ ROS[22] ↓ Met-Hg[23] [24]			↓ Hg toxicity *via* Hg:Se adduct [16] [17] ↓ ROS (selenoproteins) [18] [19] [20]	
Lead (Pb)		↑ Pb gastro intestinal absorption [29]	↑ Pb gastro intestinal absorption *via* substitution with Ca [23] [26] [27] [28]	↓ ROS [22]	↓ Pb inhibitory activity on δ-ALAD and ferochelatase involved in heme biosynthesis [23] [25]		↓ Pb toxicity *via* Pb:Se [15] ↓ ROS (selenoproteins) [23]	

δ-ALAD: delta-aminolevulinic acid dehydratase, NO: nitric oxide, ROS: reactive oxygen species.

[1] (Chi *et al.*, 2018); [2] (Mittal & Flora, 2007); [3] (Ponomarenko *et al.*, 2017); [4] (Gupta *et al.*, 2004); [5] (Karabulut-Bulan *et al.*, 2008); [6] (Fox *et al.*, 1979); [7] (Fox *et al.*, 1980); [8] (Hallberg *et al.*, 1989); [9] (Lorentzon & Larsson, 1977); [10] (Choong *et al.*, 2014); [11] (Bridges & Zalups, 2005); [12] ; (Belcastro *et al.*, 2005); [13] (Cousins, 1979); [14] (Funk, Day & Brady, 1987); [15] (Fu *et al.*, 2019); [16] (Khan and Wang, 2009); [17] (Yang *et al.*,2008); [18] Zwolak & Zaporowska, 2012; [19] (Qiu *et al.*, 2019); [20] (Vuksic *et al.*, 2018); [21] (Ropero *et al.*, 2016); [22] (Al-Attar, 2011); [23] (Peraza *et al.*, 1998); [24] (Ganther, 1978); [25] (Kwong, Friello & Semba, 2004); [26] (Goyer, 1995); [27] (Aub *et al.*, 1926); [28] (Moon, 1994); [29] (Yin *et al.*, 2022).

Arsenate (AsO_4^{3-}) has a substitutive chemical configuration to phosphate (PO_4^{3-}) in the molecular structure of ATP. Nonetheless, P uptake can be sufficiently abundant in a natural environment (*i.e.* plants, seeds, meat,…) in comparison with micronutrients and should be able to compensate for As substitution (Peraza *et al.*, 1998). Both As and Se are metalloids and share the same affinity to organic molecules, which supposes that both may compete with the same receptors. For example, selenite (Se^{4+}) and arsenite (As^{3+}) present the same electronic structure. Selenium and As are prone to react in the liver to form a conjugate As-Se compound (seleno-bis (S-glutathionyl) arsinium ion), which is excreted into the bile (Ponomarenko *et al.*, 2017). Indeed, concomitant increase of As and Se were found in biliary excretion of rats (Zeng *et al.*, 2005). Vitamin B12 and B9 (*i.e.* vitamin B9 or folic acid is involved in many metabolic processes) are directly implied in As metabolism and are prone to reduce As in the blood and increase the urinary excretion of dimethylarsenic acid (DMA) (Chi *et al.*, 2018). Vitamins E and C show their ability to act on NOx and ROS induced by As exposure (Chi *et al.*, 2018; Mittal and Flora, 2007).

Cadmium belongs to group 12 metals with Zn and Hg. These metals share the same ways of transport in mammalian cells towards active transport and passive diffusion (mainly Zinc transporters and metallothioneins) (Bonaventura *et al.*, 2015; Choong *et al.*, 2014). This process, called 'ionic mimicry', describes the potency of ionized toxic elements to interfere with essential elements under ionic forms like Zn^{2+}, Fe^{2+} and Ca^{2+} on receptor binding sites such as the thiol group of cysteine-rich metallothioneins (Belcastro *et al.*, 2005; Bridges and Zalups, 2005; Cousins, 1979). Zinc treatment can therefore be given as an antagonist of Cd to prevent the target organs from multiple pathologic lesions (*e.g.,* kidneys, liver, lungs, heart) (Peraza *et al.*, 1998). Cadmium enhances anaemia by decreasing haemoglobin and haematocrit levels due to its interference with Fe absorption in the intestine. This from of anemia can be attributed to the ability of Cd to bind to Fe transporters (*i.e.,* ferritin), as Fe supplementation can correct anaemia (Fox, 1979). Vitamin C can correct Cd-mediated anaemia by improving Fe absorption in the gastrointestinal tract (Hallberg *et al.*, 1989; Fox *et al.*, 1980). Moreover, Cd can also interplay with intestinal Ca absorption stimulated by VD in inhibiting the conversion of 25(OH)D into 1,25-$(OH)_2$D in the kidney (Choong *et al.*, 2014; Lorentzon and Larsson, 1977). Cadmium is also detoxified by metallothioneins, whose main activity is the homeostatic control of metabolic Cu and Zn (Funk *et al.*, 1987). Previous studies on pregnant rats have shown that Cd intoxication is associated with a decrease of circulating metallothionein and ceruloplasmin reducing the transport of Cu and Fe to foetus (Chmielnicka and Sowa, 1996). The antioxidant property of VC, VE and Se can reduce ROS due to Cd (Karabulut-Bulan *et al.*, 2008; Gupta *et al.*, 2004).

Mercury and Se interaction has been widely studied in immuno- and/or ecotoxicology studies on marine mammals (Yang *et al.*, 2008; Koeman *et al.*, 1973; Dietz *et al.*, 1996). The individual Se status and availability can be a relevant indicator of exposure to Hg compounds *via* selenide mercuric adducts (1:1 Hg-Se) (Kalisinska *et al.*, 2017). A large panel of Se-mediated (*i.e.,* Hg-Se complex, selonoprotein P) mechanisms may thus be involved in the detoxification of Hg, Cd, and Pb compounds (Fu *et al.*, 2019; Zwolak and Zaporowska, 2012; Khan and Wang, 2009; Yang *et al.*, 2008). It is noteworthy that growing interest has been given to Se potency against exposure to toxic elements in terrestrial ecosystems, notably its ROS scavenging activity and Pb and Hg detoxification (Qiu *et al.*, 2019; Vuksic *et al.*, 2018; Ropero *et al.*, 2016). Vitamins play a critical role in scavenging free radicals in response to toxic elements, such as VE *versus* Cd, Hg, and Pb (Al-Attar, 2011). The interaction between VE and methylmercury (Met-Hg) occurs in cell membranes, where VE breaks down Met-Hg and reacts with methyl radicals (Ganther, 1978; Peraza *et al.*, 1998).

Lead and Cd can interfere with homeostasis by mimicking the cationic form of physiological Ca, Zn, and Fe (Peraza *et al.*, 1998). Lead can compete with Ca binding sites, particularly disturbing Ca channels, Ca^{2+}/Na^{+} ATP pump and receptor proteins (*i.e.,* protein kinase C and calmodulin) (Goyer, 1995). Indeed, Aub *et al.* (1926) stipulated that the physiological Pb stream "follows the calcium stream". From this perspective, the interaction between Pb and VD makes sense. As detailed above, VD can stimulate intestinal Ca and P absorption and favour the co-absorption of Fe, Zn, and Pb (Peraza *et al.*, 1998; Moon, 1994). VD can also stimulate Pb 'mimicry' absorption in the kidney and bone by inducing Ca-binding proteins (Wasserman and Fullmer, 1995). Exposed of Wistar rats to Pb decreased serum 1,25-$(OH)_2$ D, which can be attributed to a Ca-dependent influence (Rahman *et al.*, 2018; Fullmer, 1997). Lead also disturbs haematopoiesis by inhibiting the delta-aminolevulinic acid dehydratase (γ-ALAD) and ferrochelatase enzymes involved in haeme biosynthesis. Recent findings highlighted that Fe supplementation and sufficient Fe stores could alleviate the effect of Pb toxicity in humans (Kwong *et al.*, 2004). A recent study by Yin *et al.* (2022) on *in vitro* gastrointestinal extracts highlighted that the presence of VC increased Pb absorption from 1.3 (gastric phase) to 3.1 (intestinal phase) fold.

Take home messages:

a) Interaction between toxic elements and essential element principally relies on ionic substitution.

b) Selenium, vitamins C and E principally decrease reactive oxygen species (mediated by toxic elements) damages to immune cells.

c) Vitamins C and D stimulate metabolic absorption of Pb.

d) Further research on the interaction between vitamin A and toxic elements may be done regarding its potent antimicrobial activity.

5. Study cases on wild and captive mammals

The rapid environmental changes due to anthropogenic activities have often led to a decrease in food quality (*e.g.,* linked to pollution), food quantity and/or food accessibility (*e.g.,* a decline of prey availability, a decrease in home range, or loss of connectivity) (Bonebrake *et al.*, 2019; Strandin *et al.*, 2018; Birnie-Gauvin *et al.*, 2017a; Acevedo-Whitehouse and Duffus, 2009a). These changes may affect food quality in terms of wild animals micronutrients requirements (Birnie-Gauvin *et al.*, 2017a). As detailed in Jackson *et al.* (2015), rodents have long been predilection laboratory model species for historical vectors of zoonotic diseases (*e.g.,* bubonic plague, echinococcosis) because of their easy breeding and relevance as biological models for pre-human and immune assays (*e.g.,* medicine, cosmetic). A comparative approach to the immune system of wild and captive domestic mice (*Mus musculus*) revealed an upper regulation of the immune system of wild mice, probably related to the diversity of pathogens met during their life (Abolins *et al.*, 2017a). This work and others (*e.g.,* Viney & Riley, 2017) also concluded that studies enabling the distinction between laboratory-controlled and natural conditions on small mammal health are scarce. In terms of haematological parameters, a comparative study on 1,574 wild and 186 captive field voles (*Microtus agrestis*) highlighted a significantly higher variance of WBC in wild individuals, including differences in lymphocytes and in monocytes (Beldomenico *et al.*, 2008a). Cytokine expression measured in comparative laboratory and field studies is too scarce to make a general observation (Viney & Riley, 2017). Studies on both wild and captive small mammals highlighted discrepancies in antibody titres in favour of wild animals (Abolins *et al.*, 2011). A decrease in blood haematocrit was observed in both wild shrews (*Crocidura russula*) and wood mice (*Apodemus sylvaticus*) exposed to toxic elements like Pb and Cd in their environment (Tête *et al.*, 2015; Sanchez-Chardi *et al.*, 2008) (Table 3). In captive pinnipeds, exposure to Al, Cr, Mo, Ni, Pb, Sn, and Ti increased lymphocyte proportion in total blood counting (Kakuschke *et al.*, 2011). Conversely, no effect was found in a spleen plaque forming assay for white-footed mice (*Peromyscus leucopus*) captured on sites contaminated with Cd, Cu, Hg, and Zn (Biser *et al.*, 2004). The laboratory study by García-Mendoza *et al.* (2019) reported an increase in murine macrophage TNF-α and nitrite and a decrease in mast cell TNF-α and Ig-E production in murine cell lines under exposure to Cd. Vitamin E has been shown to improve mouse embryonic cell viability exposed to Cd (*e.g.*, Warren, Patel & Kapron, 2000). Also, the studies by (Sugawara *et al.*, 1981;

Sugawara and Sugawara, 1979, 1978) showed a decrease in circulating VA in the plasma and an increase in VA in the liver of laboratory rats injected with Cd. No observed effects were found between vitamin A supplementation and Cu on laboratory rats (injection) and minks (diet supply) (Käkelä *et al.*, 1999).

Table 3. Immunomodulation by toxic metals and micronutrients in wild and captive (specified for laboratory raised animals and wild animals kept in captivity) mammals (phylogenetic orders: 1: murine; 2: ungulates; 3: carnivorous; 4: cetaceans). Acronyms are defined in Table footnotes, and ↑ and ↓ stand for increase and decrease, respectively, of a given parameter.

	Species	Living condition	Tissue	Micronutrient	Toxic metal	Measured endpoint	References
1	Mouse (*Mus musculus*)	Captive (laboratory) *in vitro*	Embryonic cells	Vit E	Cd	↑ cell viability (cytoprotective)	[1]
			Mast cells	-	Cd	↓ TNF-α, ↓ IgE	[2]
			Macrophages	-		↑ TNF-α, ↑ NO	
	White-footed mice (*Peromyscus leucopus*)	Wild	Spleen (*in vitro*)	-	Cd, Cu, Hg, Zn	No effect	[3]
	Shrew (C*rocidura russula*)	Wild	Blood	-	Pb	↓ hematocrit (%)	[4]
	Wood mouse (*Apodemus sylvaticus)*	Wild	Blood	-	Cd, Pb, Zn	↓ hematocrit (%)	[5]
	Rat (*Rattus rattus*)	Captive (laboratory)	Plasma	Vit A	Cd (injection)	↓ Vit A	[6] [7]
			Liver			↑ Vit A	[6]
			Plasma	Vit A	Cu (injection)	No Effect	[7]
			Liver				
2	Bison (*Bison bonasus*)	Captive *vs.* Wild	Liver	Se	Cd, Pb	Negative correlation between Se and Cd, and Se and Pb	[9]

Fallow deer (*Dama dama*)	Wild	spleen	Se (diet)	As, Cd, Pb	↓ As and Pb	[10]
Goats (*Capra hircus*)	Captive	Blood	Vit E (diet)	As (diet)	↓ As, ↑ humoral immunity	[11]
Red deer (*Cervus elaphus*)	Wild	Testis	Vit A	Pb	↓ retinyl palmitate ↓ retinol	[12]
		Liver	Vit A		↑ retinol ↓ retinyl esters	[13]
			Vit A, Se (bone)		↓ retinol	[12]
			1,25-OH-vitamin D		No effect	[14]
Wild boars (*Sus scrofa*)	Wild	Liver	Vit A	Pb	↑ retinol ↓ retinyl stearate	[12]
			Vit E	Pb (bone)	↓ tocopherol	
			Vit E ~ Se		↑ tocopherol	
				Cu	↑ retinol	[13]
			1,25-OH-vitamin D	Pb	No effect	[14]
Mink (*Mustela vison*)	Captive (laboratory) dietary exposure	Liver Plasma	Vit A	Cu (diet)	No Effect	[8]
	Wild and captive	Blood (*in vitro*)	-	MeHg	↑ IL-4, ↓ IL-2, ↓ TGF-β	[15]
					No effect	[16]

	Wild		Se (Na_2SeO_3) and SeMet	MeHg	No effect	[17]
Harbour seals (*Phoca vitulina)*	11B7501 B lymphoma cell line		-	As	↓ lymphocyte proliferation ↓ phagocytosis	[18]
			-	Cr	No effect	
			-	Zn	↑ lymphocyte proliferation ↓ phagocytosis	
	Wild	Blood	-	Mo, Ti, Ni, Cr, Al, Pb, Sn	↑ lymphocyte (%)	[19]
Grey seal (*Halichoerus grypus*)	Captivity	Blood (*in vitro*)	-	Cd	↓ lymphocyte proliferation	[20]
					No effect	[21]
			-	Hg	↓ lymphocyte proliferation	[22]
					↑ IL-2, ↑ IL-4	[21]
			-	Pb	↓ lymphocyte proliferation	[22]
Beluga whales (*Delphinapterus leucas*)	Wild	Blood (*in vitro*)	-	Zn	↓ lymphocyte proliferation	[22]
			Se (Na_2SeO_3)	Hg, MeHg	↑ lymphocyte proliferation	[23]
		Spleen (*in vitro*)	-	Cd	↓ lymphocyte proliferation	[24]
			-	Hg	↓ lymphocyte proliferation	
			-	Pb	No effect	
Bottlenose doplhin	Captive	Blood (*in vitro*)	-	Al	↓ phagocytosis	[25]
			-	Cd	↑ apoptosis, ↓ lymphocyte proliferation, ↓ phagocytosis	

(*Tursiops truncatus*)	-	Hg	↑ apoptosis, ↓ lymphocyte proliferation, ↓ phagocytosis
	-	Pb	↓ lymphocyte proliferation

NO: nitric oxid, TGF-β: Transforming growth factor beta, TNF-α: tumor necrosis factor alpha

[1] (Warren *et al.*, 2000); [2] (Garcia-Mendoza *et al.*, 2019); [3] (Biser *et al.*, 2004); [4] (Sanchez-Chardi *et al.*, 2008); [5] (Tête *et al.*, 2015); [6] (Sugawara and Sugawara, 1978); [7] (Sugawara and Sugawara, 1979); [8] (Sugawara *et al.*, 1981); [9] (Käkelä *et al.*, 1999); [10] (Durkalec *et al.*, 2018); [11] (Vukšić *et al.*, 2018); [12] (Mohanta *et al.*, 2014); [13] (Rodríguez-Estival *et al.*, 2011b); [14] (Rodríguez-Estival *et al.*, 2011a); [15] (Rodríguez-Estival *et al.*, 2012); [16] (Das *et al.*, 2008); [17] (Kakuschke *et al.*, 2009); [18] (Das *et al.*, 2016); [19] (Frouin *et al.*, 2010a); [20] (Kakuschke *et al.*, 2011); [21] (Frouin *et al.*, 2010b); [22] (Kakuschke *et al.,* 2006); [23] (Frouin *et al.*, 2012); [24] (Brousseau *et al.*, 2002); [25] (Pellisso *et al.*, 2008).

Interesting results have been provided by both free-ranging and captive ungulates studies. Several studies on livestock have reported the essential role of nutritional quality on the immune system. The review of Mc Clure (2008) suggested that Cu, Co, Fe, Mo, Mn, and Zn may directly act on, or at least regulate, immune functions in livestock. A study on goats exposed to As illustrated an improvement in humoral immunity and a decrease in As levels in blood with the addition of VE in the diet (Mohanta *et al.*, 2014). A similar scavenging effect of VA was found with a decrease in retinyl palmitate and retinol (VA compounds) in the testis of red deer exposed to elevated levels of Pb around a former mining area (Rodríguez-Estival *et al.*, 2011b). Another study on red deer and wild boars from the same site found a positive correlation between hepatic Pb and retinol, while negative correlation was found between hepatic Pb and retinyl compounds (Rodríguez-Estival *et al.*, 2011a). This was explained in Rodríguez-Estival *et al.* (2011a) by the mobilization of vitamin A storage, which releases retinol in response to Pb-induced oxidative stress. No effects were observed between Pb and 1,25 OH-VD in both red deer and wild boars (Rodríguez-Estival *et al.*, 2013). A negative correlation was found between VE levels and Pb bone concentrations, while a positive correlation was found with Se and VE concentration in wild boar liver (Rodríguez-Estival *et al.*, 2011b). A decrease in As and Pb concentrations in the spleen was also found in wild fallow deer fed with a Se-supplied diet (Vuksic *et al.*, 2018). The beneficial activities of both Se and VE was reviewed by Flueck *et al.* (2012) and considered critical for ungulate conservation. Indeed, VE can compensate for Se deficiency (and reciprocally) and may even alleviate the oxidative stress mediated by toxic elements. Severe deficiency in Se and VE in ungulates may result in white muscle disease, also called nutritional muscular dystrophy (*e.g.,* Brady *et al.*, 1978). Low levels of Se and Cu were also found in European bison in captivity and have been reported to reduce reproductive performance (Durkalec *et al.*, 2018). Negative correlations between Se and Cd and Se and Pb in bison liver were also found in Canadian bison (Macneil *et al.*, 1990). The scavenging role of Se against toxic elements remains poorly documented in the terrestrial environment, but its benefits have been widely documented in aquatic species. In cetaceans, an *in vitro* study on blood sampled collected from free-ranging beluga whales and exposed to Hg and Met-Hg showed an increase in lymphocyte proliferation in cultures supplemented with selenite (Na_2SeO_3) (Frouin *et al.*, 2012). *In vitro* studies on blood taken from both captive bottlenose dolphins and free-ranging beluga whales highlighted a decrease in apoptosis and phagocytosis and at least a decline in lymphocyte proliferation in response to Al, Cd, Hg, and Pb (Pellisso *et al.*, 2008; Brousseau *et al.*, 2003). A similar decrease in lymphocyte proliferation was observed in captive grey seals with Cd, Hg, and Pb stimulation of *in vitro* blood cell culture (Frouin *et al.*, 2010, Table 3). Another *in vitro* study on harbour seals 11B7501 B lymphoma cell line also observed a decrease in lymphocyte proliferation and phagocytosis with As stimulation, while no effect was observed with Cr (Frouin *et al.*, 2010a). On the contrary,

lymphocyte proliferation was observed after Zn stimulation in grey (blood isolation) and harbour (cell line) seals, while no effects were found after Cd stimulation in grey seal blood cells (Kakuschke *et al.*, 2006). The same study reported an increase in IL-2 and IL-4 production promoting a humoral response in grey seals. Indeed, the production IL-2 induces T-cell expansion and recruitments enhancing antibody synthesis and NK cell differentiation and proliferation. While, the production of IL-4 is to enhance Th2 cell differentiation and B cell production of IgG and IgE (Secombes *et al.*, 2016). An increase in IL-4 production and a decrease in IL-2 and TGF-β was found in harbour seal blood cells stimulated *in vitro* with MeHg (Das *et al.*, 2008). Both studies on grey and harbour seals showed increasing IL-4 production in response to Hg and Met-Hg, suggesting an impact on Th2 cell differentiation. A concomitant decrease in IL-2 and TGF-β, as found in harbour seals, suggests a disturbance in the regulation of the inflammatory response mediated by Hg and Met-Hg exposure. A study on free-ranging harbour seal blood cell culture did not show any beneficial effect of selenite and selenium methyl on Met-Hg (Das *et al.*, 2016).

6. Research perspectives on interactions between toxic elements and micronutrients in wildlife facing a changing world

The present review takes place in the broader context of understanding disturbances in the immune ecology of wild animals, particularly the factors that might contribute to immunomodulation. Among the key issues required to get a more comprehensive understanding of wildlife immunoecology are (i) studying the antigenic load to which wild animals are exposed, (ii) identifying immunoregulatory processes and their causes, and (iii) understanding the actual resource state of wild animals and its immunological consequences (Viney and Riley, 2017a). In the same paper, Viney & Riley (2017) stated that eco-immunology is characterised by three key concepts: (i) different species, different populations within species, and different individuals within populations may differ in their immune responses (immunoheterogeneity); (ii) individuals' immune responses may be constrained by resource availability (and more generally by their physiological state), and (iii) animals have a potentially very large and diverse antigenic load. The present review focuses on immunoregulatory processes and precisely looks at the role of exposure to toxic elements and the acquisition of micronutrients on immunomodulation. Relatively surprisingly -because the crucial role of micronutrients in immunity has been well established in humans and laboratory animal species-, this issue has been overlooked in wildlife compared to the role of macronutrients (*e.g.,* Vestey, Mcmurry & Lochmiller, 1993; Lochmiller, Vestey & Nash, 1992), which itself has received little attention up to now. Altogether, field studies that investigated the effects of food

quantity and quality on wildlife are scarce and are even rarer for micronutrients (present review, Espin & Sanchez-Virosta, 2021; Strandin *et al.*, 2018).

One major limitation for such studies, and that extends far beyond the present focus on immunomodulation by toxic elements and/or micronutrients, is the lack of "bespoke" immunological assays and/or reagents for wild species (Strandin *et al.*, 2018). One way of overcoming this technical barrier is to work on wild species identical or closely related to better-studied (*i.e.,* commonly used in lab work) species. A very elegant example illustrating this is the series of studies performed on the wild mouse *Mus musculus*, which is the very same species as the classical laboratory model (*e.g.* Abolins *et al.*, 2017). Taking advantage of the knowledge and availability of assays and reagents developed in the laboratory species, Abolins and colleagues (together with a few other studies on immunity in wildlife) showed that wild rodents generally exhibit highly activated humoral responses compared to laboratory animals and that their cellular compartment reveals extensive antigenic exposure (Abolins *et al.*, 2018, 2017a). A recent approach presented by Graham (2021) used "naturalised" mouse models to integrate abiotic (*e.g.,* seasonal photoperiod) and biotic (*e.g.,* diet, outdoor microbes) factors encountered by wild individuals in laboratory mice. The author argued that natural social stresses would directly modify the immune phenotype, as illustrated by the involvement of IL-6 in both inflammatory and hormonal stress responses (see Qing *et al.*, 2020). These authors, however, did not focus on immunomodulation through resources, even if they state that resource availability probably plays an important role in variations of the immune response within populations. Another way of overcoming the problem would be to develop species-specific reagents for a series of wild species chosen for their large geographic distribution, ubiquity, easiness of capture, handling, manipulation and rearing and/or other specific scientific and logistical reasons. This, however, would undoubtedly be time- and effort-consuming and costly but would for sure be of crucial interest in the development of wild immunoecology. A recent paper from Andrew S. Flies and Wild Comparative Immunology Consortium (WACI, www.wacimmuno.com) says that genomes of an increasing number of species (>200) have been fully sequenced, allowing rapid comparison of gene networks, and that *de novo* transcriptome assemblies could provide transcript sequences and expression patterns (Flies and wild comparative immunology consortium, 2020). Based on this, Flies *et al.* (2020) argue that recombinant proteins could be produced for proteins involved in the immune system of species of interest, even if their complete genome sequence is unavailable. This effort to develop antibodies, nanobodies or aptamers binding conserved protein motifs across taxonomic orders might overcome the development of species-specific reagents for many species, which would obviously be limited by funding constraints.

Apart from this general issue of limited availability of specific reagents for wild species, field studies would face the problem of finding sites where gradients (or at least contrasted habitats) of food quantity, availability, accessibility and/or quality (in terms of macro- or micronutrients and/or of pollutants). If such a site can be found, then, assessing diet is (at least qualitatively) relatively easy thanks to the development and democratisation of next-generation sequencing (molecular), isotopic, and other approaches (Bonin *et al.*, 2020; Sousa *et al.*, 2019; Alberdi *et al.*, 2019). Once the diet is characterised, sampling food items and assessing their quality (in terms of pollutants and/or nutrients) are also relatively easy by classical environmental chemistry. In parallel to such a descriptive approach, a more experimental and thus mechanistic approach can be developed by manipulating exposure to pollutants and/or food features. If artificially exposing natural populations to pollutants obviously poses ethical and technical issues, especially over a spatial scale adapted to the study animal model, supplementing locally individuals/populations with food naturally rich in (micro)nutrients (*e.g.,* natural salt licks) or artificially enriched by a single or a mixture of (micro)nutrients is easier. This has relatively commonly been performed in birds (especially those breeding in nest boxes, (Ruuskanen *et al.*, 2017; Espín *et al.*, 2016; Bańbura *et al.*, 2013) but, to our knowledge, rarely, if ever, been done in mammals. Checking that animals consume food provided to such an extent that the experiment changes the nutritional state of the target individuals/populations can be performed using classical (ideally little invasive) techniques.

Such a complementary approach offers other scientific opportunities for mammals in captivity. Captivity can take several forms. One is the capture in the wild of individuals from small species (typically rodents) that can be maintained in cages (< 1 m²) for a relatively short time (typically several days). Another is the use of medium size mesocosms (from several m² to a few hundreds of m²) in which a greater variety of species can be maintained and a higher diversity of experiments, including behavioural or reproductive (allowing the study of offspring) studies, can be performed. The last one is using large to very large (> 1,000 ha) outdoor enclosures, usually dedicated to studying large mammals. In all these relatively highly controlled settings, the manipulation of food availability, quality, and quantity (food supplementation or restriction) can easily be set up, as well as the artificial exposure of animals to single or mixtures of pollutants. Such captivity experiments require appropriate settings for the welfare of study animals and strict regulation by ethical rules (*e.g.,* the Three Rs), which makes experiments more challenging to handle than field studies from administrative and logistical viewpoints. Regarding immunology, captivity experiments also have to identify and quantify how stress associated with maintaining individuals in captivity might affect the observed results.

According to Luster & Gerberick (2010), the immune system is not fully operational until it is challenged. Thus, when possible, measurement of the immune response, should involve

procedures with appropriate antigen challenges. Different antigens commonly used in experimental procedures enable measuring functional responses to the challenge by targeting different immune system compartments. Sheep red blood cells (SRBCs) activate humoral immunity, while bacterial lipopolysaccharides (LPS) enhance cellular immunity by stimulating CD4 T cell response (Toll-like receptor 4). Other antigens can be used for more specific components, such as phytohaemagglutinin (PHA) to characterise DTH (*i.e., via* skin sensitisation), in monitoring swellings (Strandin *et al.*, 2018) or keyhole limpet haemocyanin (KLH), which induces a specific IgG-mediated immune response after immunisation (Martin *et al.*, 2007).

After the activation of a component of the immune system by a challenge, immunoassays enable estimating the amplitude and/or the intensity of the immune response. Whilst several immunological testing on the different compartments of the immune system can be performed on mammals, some disadvantages avoid their applications in wild animals (Table 4). The haematological profile provides a good overview of how leukocyte counting can be affected (or not) by toxic elements. Blood cell counts are commonly used to assess the health condition of individuals exposed to various pollution types, including toxic elements (Rogival *et al.*, 2006b).

Table 4. Advantages and limits of immunological assays on the components of the immune system of wild mammals exposed to toxic metals.

Immunological tests	Immunoassays and techniques	Advantages	Limits	Refs.
Haematological profile	Automatic / manual counting	Provides a good overview of how leukocytes can be affected (or not) by toxic metals.	Needs reference intervals to estimate abnormal values.	[1]
Immunophenotyping	Flow cytometry	Allows characterising, quantifying and defining the polarization (which refers to spatial differences in shape, structure, and function within a cell) of several immune cells alone or during the same run.	Needs species-specific or at least cross-reactive antibodies.	[2]
Cytokine profiling	Enzyme-linked immunosorbent assay (ELISA) Flow cytometry	Allows characterising production of cytokines by T cells to define their function as pro-inflammatory (Th1), non-inflammatory (Th2), anti-inflammatory (Th3), antimicrobial (Th17) and regulatory process (Treg).	Needs species-specific or at least cross-reactive antibodies.	[2]
Gene profiling	Real-time polymerase chain reaction (RT-PCR)	Estimates the level of expression of a specific component of the immune system (*e.g.* mRNA sequence of toll like receptors).	Needs at least an indexed genomic sequence of the target species.	[3]
Antibody mediated immunity	Plaque forming cell (PFC) Haemagglutination ELISA	Provides relevant information on the impact of toxic metals on humoral immunity.	ELISA needs species-specific or at least cross-reactive antibodies.	[4] [5] [6]
Cellular immunity	Delayed type hypersensitivity (DTH) *via* skin sensitization	Allows characterising the magnitude of the immune response against an intradermally introduced antigen (*e.g.* Sheep red blood cells).	Should be performed on living organisms.	[5] [7] [8]
	Cytotoxic T lymphocytes (CTL) assays Natural killer (NK) activity assays	Commonly used *in vitro* assays quantifying cytotoxicity of CTL and NK cells against viral infection or tumoral cells.	Often relies on a chromium (^{51}Cr) release assay, which requires equipment to measure radioactivity.	
C-Reactive protein (CRP)	ELISA	Often used as a proxy of the inflammatory immune response.	Needs species-specific or at least cross-reactive antibodies.	[9]

Phagocytic activity	Phagocytosis assays (*e.g.* Zymosane substrate)	Commonly used in immunotoxicity testing as it can be coupled with toxic metals at different concentrations in the medium.	Does not allow determining which phagocytic cells and / or components of the innate humoral innate immunity is involved.	[7]
	Microbial killing assays (MKA)	Allows measurement of innate immunity from antibodies and complement proteins (mainly in plasma or serum) for MKA and in addition from phagocytic cells (neutrophils and macrophages) for WBMKA.		
	Whole blood microbial killing assay (WBMKA)		WBMKA requires working on fresh blood.	[10]
Nitric oxides (NO)	Nitrite / nitrate assays (*e.g.* Test of Griess)	Allows quantifying NO compounds released by the immune system during an infection.	The production of NO can be done by most of immune cells. Its ubiquity unables to disentangle the sources of its production in cases of exposure to multiple stressors.	[11]
Physical measurement	Bone marrow / spleen weight ratio	Provides easy and efficient estimate of individual immune status on the field.	Requires sacrifice of the individuals.	[12]
	Bone marrow / thymus weight ratio			

[1] (Rogival *et al.*, 2006b); [2] (Abolins *et al.*, 2017a); [3] (Stordeur *et al.*, 2002); [4] (Lall and Dan, 1999); [5] (Dan *et al.*, 2000); [6] (Luster and Gerberick, 2010); [7] (Strandin *et al.*, 2018); [8] (Wonderlich *et al.*, 2006); [9] (Siwicki *et al.*, 1998); [10] (Fournier *et al.*, 2000); [11] (Bogdan, 2001); [12] (Luster *et al.*, 1993).

Leukocytes and derived white blood cells (granulocytes, lymphocytes, monocytes, neutrophils) offer the first sight into the immune status of individuals as they may be related to infection or diseases. However, reference intervals for most mammals are lacking in the literature, which makes it challenging to characterise abnormal values among the tested samples. Immunophenotyping enables us to characterise which specific cells are affected by toxic elements in terms of quantity and polarisation (which refers to spatial differences in shape, structure, and function within a cell). Nowadays, the main technic relies on flow cytometry analyses and requires species-specific, or at least cross-reactive, antibodies to fix to cell proteins *via* a labelled antibody allowing the counting of a specific type of cells (determined by its size and shape). Development of species-specific antibodies is time-consuming and costly, which often limits the use of this technique in wild mammals or may limit scientific research to cross-reactive antibodies (*e.g.,* wild *vs* domestic mouse, see (Abolins *et al.*, 2011). The same remark can be made on cytokine profiling, whose detection mechanism also relies on species-specific, or at least cross-reactive, antibodies. Cytokines are signal peptides produced by T cells, which allows us to define their function as pro-inflammatory (Th1), non-inflammatory (Th2), anti-inflammatory (Th3), anti-microbial (Th17) or regulatory (Treg) activity (Viney *et al.*, 2005). All these functions are major cell-mediated immunity types and can be affected in terms of production by exposure to toxic elements (Wang *et al.*, 2021; Dobrakowski *et al.*, 2016). An alternative immunotoxicity measurement can be done with the genic expression level, also called gene profiling. Cytokines mRNA detection and quantification with real-time PCR allow an efficient measurement of a cytokine's level of genic expression at the sampling time (Stordeur *et al.*, 2002). More components of the immune response can be quantified with this technique, such as transcription factors and toll-like receptors (Friberg *et al.*, 2011). This technique also enables obtaining a precise mapping of where toxic elements could interfere or not with the immune system. Nonetheless, the referenced genome is not sequenced and/or indexed for many wild mammals, which implies performing adjustment of probes and primers on fragments of sequences. To address the lack of reference genome, Flies & the wild comparative immunology consortium (2020) proposed to use transcript sequences isolated from *de novo* transcriptome assembly to produce recombinant proteins *via* protein-based immunology techniques. To overcome problems of cross-reactivity linked to species-specific reagents, the authors also call for another approach endeavouring a systematic effort to develop antibodies and other derivates (*e.g.,* nanobodies) able to bind on conserved motifs across taxonomic orders. Humoral or antibody-mediated immunity can be measured with several techniques, including plaque-forming cell (PFC) assays that are commonly used in laboratory *in vitro* studies to qualify immunosuppression by drugs or xenobiotics. Briefly, PFC assays measure the size of haemolytic plaques produced by antibodies in response to an antigen (*e.g.,* SRBC). Other techniques can also be performed on blood with

haemagglutination assays to titrate Ig in response to a specific antigen on repeated measurements a few days after a challenge (Lall and Dan, 1999). However, this approach sometimes is unable to distinguish cellular from humoral immunity, which requires completing a haemagglutination assay by directly measuring antibodies. Such measurement can be done by enzyme-linked immunosorbent assay (ELISA), which allows for quantifying the different antibodies produced by immune cells, such as IgG response to an antigen (Dan *et al.*, 2000). Cellular-mediated immunity can be characterised in living organisms with the measurement of DTH *via* a specific antigen, as detailed above. Other *in vitro* techniques allow estimating cytotoxic T lymphocytes or NK cells' activity against tumours or viruses, which, however, often relies on radioactive-based chromium (^{51}Cr) release assay (Wonderlich *et al.*, 2006). C-Reactive protein (CRP) is a very common inflammatory marker used in the clinical and epidemiological assessment of an individual's health status. Indeed, CRP represents a good proxy for estimating inflammation as it is an acute-phase protein found in high quantities in plasma during infection (Siwicki *et al.*, 1998). The production of CRP originates in the liver and is mediated by IL-6 secretion by macrophages or T cells. However, CRP alone does not allow us to estimate which compartment of the immune system is impacted by toxic elements. The activity of phagocytic cells provides a good proxy of the innate immune response towards pathogenic agents and can be cultured in the presence of toxic elements (*e.g.,* cadmium chloride). Several phagocytosis assays are employed to characterise the immunotoxicity of a compound promoting the functional ability of cells to engulf antigens or beads containing labelled particles such as the Zymosane substrate (Fournier *et al.*, 2000). Another functional assay is the microbial killing assay (MKA), which aims at quantifying the ability of innate humoral immunity (antibodies and complement) to eliminate a known bacterium (*e.g., Escherichia coli*) at a specific concentration. The whole blood microbial killing assay (WBMKA) focuses on phagocytic cell (neutrophils and macrophages) activity to quantify their ability to eliminate a bacterium (Strandin *et al.*, 2018). The main disadvantage of WBMKA is that it requires working on freshly drawn blood, while MKA can be performed on serum or plasma frozen at -80°C. Nitric oxides (NO) are a relevant proxy of the immune response to a pathogen. Macrophages produce a large amount of NO during infection to destroy bacteria (Bogdan, 2001). Nitrite/nitrate assays using the Griess test allow us to quantify both NO metabolites in biological fluids. Physical measurements such as weighing lymphoid organs (*e.g.,* bone marrow/spleen or bone marrow/thymus ratios) give a good estimate of the immune status of individuals. One major limitation, however, is that organ collection requires the sacrifice of individuals.

7. Conclusions

1. Both toxic elements and micronutrients can modulate mammals' immune response against pathogens.
2. Most laboratory experiments reported a positive influence of micronutrients on immune cell viability and functioning by antioxidant protective role.
3. Field studies also reported the benefit of micronutrients on the immune system of wild mammals but very few considered populations exposed to toxic elements.
4. Immunotoxicological approaches in wild mammals require further development of methods and techniques (including species-specific reagents).
5. Further research should integrate a framework of co-exposure to beneficial micronutrients and detrimental toxic elements to handle how micronutrients might alleviate the impacts of immunotoxic compounds.

Acknowledgements

The authors thank Anne-Pauline Bellanger, Bruno Faivre, Assif Friedman, Stéphane Garnier, Rafael Mateo, and Gabriele Sorci for their advice during the writing of this review. QD is funded by the Université of Bourgogne Franche-Comté ISITE-BFC programme. The authors acknowledge the French *Agence de la Transition Ecologique* (ADEME, ex-*Agence de l'Environnement et de la Maîtrise de l'Energie*) for funding the DYSPAT programme (contract n°1572C0309), which partly supported scientific ideas and researches leading to the present review.

3.2 Chapter 2 (manuscript in revision in Environmental Pollution)

Environmental pollution and nutritional quality modulate immune response of the wood mouse (*Apodemus sylvaticus*) through hormonal disturbances

Quentin Devalloir[1,*], Clémentine Fritsch[1], Yara Alchammas[1], Francis Raoul[1], Vincent Driget[1], Caroline Amiot[1], Shinji Ozaki[1,2], Nico van den Brink[3], Renaud Scheifler[1]

[1]UMR 6249 Chrono-environnement, CNRS / Université Bourgogne Franche-Comté, Université de Franche-Comté 16 route de Gray, 25030 Besançon Cedex, France
[2]UK Centre for Ecology & Hydrology, Lancaster Environment Centre, Library Avenue, Bailrigg, Lancaster, LA1 4AP, UK
[3]Division of Toxicology, Wageningen University, 6700 EA Wageningen, The Netherlands

*Corresponding author: quentin.devalloir@gmail.com

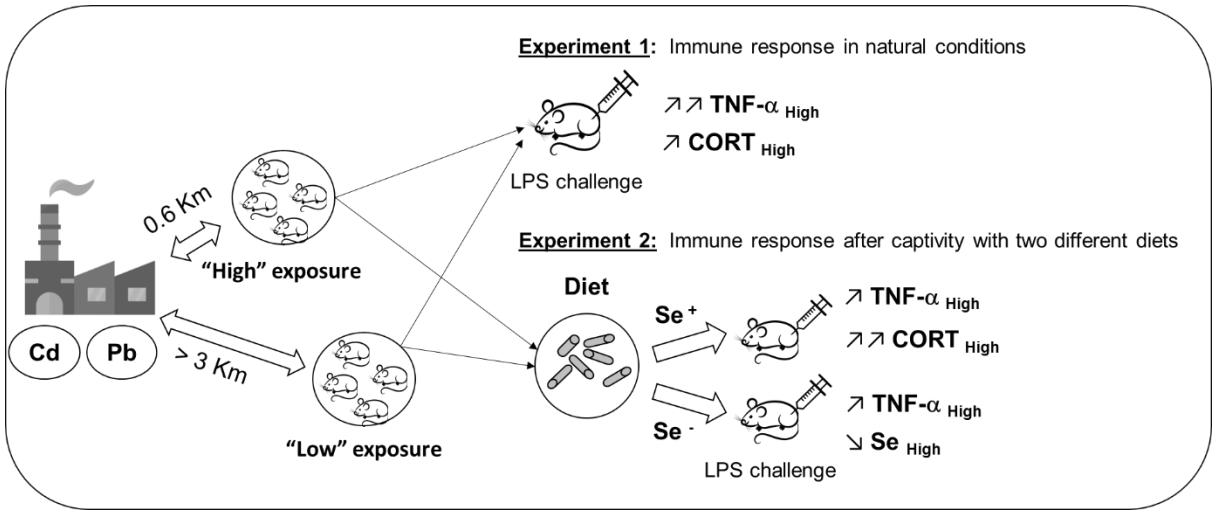
Abstract

Cadmium and Pb are known to enhance immune cell damages and to decrease cellular immunity, promoting higher susceptibility to infectious diseases. Selenium is an essential element involved in immunity and reactive oxygen species scavenging. This study aimed at evaluating how Cd and Pb and low nutritional (Se) quality modulate immune response to a bacterial lipopolysaccharide (LPS) challenge in wood mice (*Apodemus sylvaticus*). Mice were trapped near a former smelter in northern France in sites of High or Low contamination. Individuals were challenged immediately after capture or after 5 days of captivity, fed standard or Se-deficient diet. Immune response was measured with leukocytes counting and plasma concentration of TNF-α, a pro-inflammatory cytokine. Faecal and plasma corticosterone (CORT), a stress-hormone involved in anti-inflammatory processes, was measured to assess potential endocrine mechanisms. Higher hepatic Se and lower faecal CORT were measured in free-ranging wood mice from High site. LPS-challenged individuals from High site showed higher decrease of circulating leukocytes of all types, higher TNF-α concentrations, and a significant increase of CORT, compared to individuals from Low site. Challenged captive animals fed standard food exhibited similar patterns (high decrease of leukocytes, significant increase of CORT, and detectable levels of TNF-α), with individuals from lowly contaminated site having higher responses than their counterparts from highly polluted site. Animals fed Se-deficient food exhibited lymphocytes decrease, non-significant variation of CORT, and average levels of TNF-α. These results suggest (i) a higher inflammatory response to challenge in free-ranging animals highly exposed to Cd and Pb, (ii) a faster recovery of inflammatory response in animals lowly exposed to pollution when fed non-contaminated standard food than more exposed individuals, and (iii) a functional role of Se in the inflammatory response. The precise role of Se and mechanisms underlying the relationship between glucocorticoid and cytokine remain to be elucidated.

Keywords

Immunomodulation, trace metals, selenium, glucocorticoids, mammals

Graphical abstract



Highlights

- Cadmium, lead and selenium modulate immune response of wild and captive wood mice
- Animals from highly polluted site exhibited high inflammatory response
- Captive animals from lowly polluted site fed standard food showed high response
- Captive animals fed Se-deficient food exhibited low response
- Inflammatory responses were related to Se and might be mediated by corticosterone

Introduction

Severe changes have occurred in many habitats of free ranging mammal populations (*e.g.* fragmentation, habitat loss, pollution, reduction of food availability and/or quality…) turning their living area into a stressful environment. Wild rodents are also constantly exposed to infectious diseases (Strandin *et al.*, 2018; Viney and Riley, 2017a), making the immune system integrity and efficiency crucial to their health and fitness. Pollution and poor nutritional quality have been shown to have severe implication on both immune and endocrine systems (Acevedo-Whitehouse and Duffus, 2009a). The role of immune system is to eliminate pathogens while the endocrine system ensures peripheral organ functioning to maintain homeostasis (Wensveen *et al.*, 2019). However, both maintain an equilibrium between pro- (immune) and anti- (endocrine) inflammatory responses to a stress, in order to avoid autoimmune damages or an increasing susceptibility to infections (Wensveen *et al.*, 2019; Martin, 2009a). In the purpose of the conservation of free ranging mammals, it is therefore of primordial interest to characterise the impact of pollution and nutritional quality on the ability of organisms to respond to an infection, also known as immunocompetence. Wildlife diseases can be affected by pollutants either by increasing pathogen prevalence or by decreasing immune function of the host (Acevedo-Whitehouse and Duffus, 2009a).

Ensuring an efficient immune response against pathogens is central to fitness as it may reduce energetic cost allocated to reproduction for instance (Lochmiller and Deerenberg, 2000). The part of the energy allocated to immunocompetence belongs to the metabolic demand found in diet by organisms, which required a sufficient amount of macro- and micro-nutrients (Downs and Stewart, 2014). Macronutrients (water, fibres, carbohydrates, lipids and proteins) maintain metabolic processes in providing vital elements (carbon, hydrogen, nitrogen…) and releasing energy. Micronutrients are idiosyncratic components of the immune system whose role varies from homeostatic regulators to constitutive elements of immunity (Allen and Ullrey, 2004; Gombart *et al.*, 2020). Among them, dietary selenium (Se) was found to enhance immune cells functioning, proliferation and activity (Huang *et al.*, 2012). Selenium is also a major antioxidant involved in reactive oxygen species (ROS) scavenging *via* selenoproteins and Se-dependent processes (Schweizer *et al.*, 2016). In a previous study, we found that wood mice (*Apodemus sylvaticus*) populations exposed to elevated levels of soil pollution by cadmium (Cd) and lead (Pb) had higher hepatic Se concentrations than populations from less Cd and Pb contaminated areas. We also found several positive relationships between Se concentrations and some haematological parameters, including blood monocyte counting (Powolny *et al.*, 2022), suggesting that Se might play a protective role against Cd and/or Pb toxicity. In laboratory controlled conditions, selenium has been shown to counteract immunosuppressive as well as

hepatic and renal oxidative damage induced by cadmium in rats orally exposed to Cd and supplemented with Se (El-Boshy *et al.*, 2015).

Toxic metals like Pb and Cd are known to be particularly immunotoxic to mammals (Gera *et al.*, 2015). Complete blood cell counts have commonly been used to assess health condition of individuals exposed to various pollution types including toxic metals (Luster *et al.*, 1988). Leukocytes and derived white blood cells (*i.e.* monocytes, neutrophils, granulocytes) can help to interpret infectious status or to give a disease diagnosis, and have also been studied in a metal pollution context in the field (Rogival *et al.*, 2006b) or under controlled-condition exposure in the lab. Laboratory studies on mice and rats exposed to Cd or Pb showed that these metals dampened immune cell functioning, *e.g.* a decrease of lymphocyte proliferation or an increase of inflammatory markers (Gera *et al.*, 2015). Both Cd or Pb can directly impair immune cell functioning by decreasing cell (*e.g.* T cells, NK cells) proliferation and activity (Gera *et al.*, 2015; Wang *et al.*, 2021). Indirectly, Cd and Pb toxicity can result from ROS production, which can affect nuclear factor-κ B pathway in promoting an increase of (pro-)inflammatory cytokines (*i.e.* TNF-α, Il-6) (Hossein-Khannazer *et al.*, 2020; Liu *et al.*, 2012). Toxic metals can enhance cell reactivity to self (autoimmunity) and modulate immune response against pathogenic agents. For instance, Pb can compromise lab mice resistance against *Listeria monocytogenes* by modulating cytokines expressions in decreasing Il-12 and IFN-γ (involved during bacterial infection), and increasing Il-6 (inflammatory cytokine, which was found to correlate with serum corticosterone) levels (Kishikawa *et al.*, 1997).

Both Cd and Pb are also known to enhance endocrine disruption of the adrenal gland, which produces glucocorticoids (GCs) including the main one found in rodents, the corticosterone (CORT) (Spackman and Riley, 1978). As steroid hormones, GC production (in standard conditions) enables to decrease immune response in down-regulating immune system activity once infections are controlled. The production of GCs can reduce circulating lymphocytes, neutrophils, granulocytes and monocytes. Martin (2009) proposed a temporal framework of immune response to a stressor, involving a neuropeptide phase followed by GC production as key regulators of the immune system. Thus, a close interaction exists between hormonal and immune response against stressors to prevent immune self-damages. The production of mice CORT by the adrenal gland can be enhanced by the release of pro-inflammatory cytokines (Il-6, TNF-α and Il-1) from immune cells (Silverman and Sternberg, 2012). Indeed, immune challenge of laboratory mice showed a combined increase of both TNF-α and CORT (Grion *et al.*, 2007). Metabolic CORT disruption could have severe consequences on the regulation of the immune system, as low levels would maintain an up-regulation of the immune response after an episodic infection. A significant decrease of serum CORT was found in male Wistar

rats exposed to chronic injection of Cd chloride, which could thus act as a neuroendocrine disruptor (Nishiyama and Nakamura, 1984).

This study aims at determining how toxic metals can modulate the immune response of wood mouse populations inhabiting polluted (mainly by Cd and Pb) areas in the vicinity of the former smelter of Metaleurop Nord (Noyelles-Godault, Hauts-de-France, France). To test immunocompetence, individuals from highly and lowly polluted sites were subjected to an immune challenge enhanced by bacterial lipopolysaccharides (LPS). This experiment is hereafter referred to as Experiment 1. To test the potential role of nutritional quality, a sub-sample of the animals captured in the contrasted sites were maintained in captivity for 5 days, and fed with a standard or Se-deficient rodent food. This experiment is hereafter referred to as Experiment 2. We hypothesized that the LPS challenge in wood mice from highly polluted sites would promote higher levels of TNF-α due to neuroendocrine disturbances (as assessed by serum and faecal levels of CORT) mediated by Cd and Pb exposure. We also hypothesized that Se-deficient diet would shape discrepancies in wood mice inflammatory response to the challenge among contrasted levels of pollution.

Methods

Study site

The study was conducted in the surroundings of the former smelter Metaleurop Nord (Hauts-de-France, northern France). This smelter, active during more than a century, generated very high levels of pollution, mainly by Cd and Pb in soils, vegetation, and animals (Fritsch *et al.*, 2010b, 2010a). Two sites (of 25 ha), contrasted by their level of soil trace metal concentrations, were studied, and hereafter referred to as High and Low (Table S1). Median soil Cd and Pb concentrations are of 1.4 and 116 µg/g dry mass for Low site, and 59.3 and 1357 for High site, respectively (Douay *et al.*, 2009; Fritsch *et al.*, 2010a). These data come from soil sampling made in 2006. An additional soil sampling performed in 2019 confirmed that total Cd and Pb concentrations did not change significantly between 2006 and 2019, and provided data for other elements like Se (Table S1, Ozaki *et al.*, 2022).

Animal selection

From the 28th of September to the 13th of October 2020, 70 traplines of 10 nonlethal INRA traps (with dormitory boxes) each spaced three meters apart were set for three to five consecutive nights. Dormitory boxes were filled with apple, hay, sunflower seeds, cat food and peanut butter. They ensure survival of the animals for at least 24 h. Traps were checked every morning. The wood mouse (*Apodemus sylvaticus)* was retained as the animal model for this study considering its relevance for bioaccumulation of metals from soil and/or trophic transfer, its ubiquity and its ability to cope with captivity. Among the individuals trapped, individuals from other species than *A. sylvaticus* were immediately released, after checking their health status (no injury, not wet, apparent normal behaviour). Pregnant females and females exhibiting suckling, as well as young individuals (*i.e.* below 15g, (Gelling *et al.*, 2009)) of *A. sylvaticus* species were also released. Adult males and non-pregnant females were kept for the experiment. Finally, 91 wood mice were trapped on which 46 of the 60 males and 13 of the 31 females caught were used for the experiment (Table 1).

Experimental design

The experiment design, sample size and names given to the various groups are summarized in Table 1 and Figure S1.

Table 1 Sampling design, acronyms and number (in parenthesis) of wood mice caught on high and low levels of soil pollution. In bold are the acronyms that are used throughout text, tables and figures to refer to the different experimental groups.

Soil pollution levels	Study design	Type of food	Day 0 (D0)	Day 5 (D5)	Name of the tested groups	Experiment
High (29)	No captivity (6)	No food (6)	Challenged high (6)		**H-LPS** (6)	1
	Captivity (23)	Standard food (12)	Control high (**H**) (23)	Challenged high standard (12)	**H-LPS-Se+** (12)	2
		Se-deficient food (11)		Challenged high Se-deficient (11)	**H-LPS-Se** (11)	2
Low (23)	No captivity (13)	No food (13)	Challenged low (13)		**L-LPS** (13)	1
	Captivity (13)	Standard food (7)	Control low (**L**) (13)	Challenged low standard (7)	**L-LPS-Se+** (7)	2
		Se-deficient food (6)		Challenged low Se-deficient (6)	**L-LPS-Se-** (6)	2

In experiment 1, a first group of 19 individuals (6 from High site, and 13 from Low site) was captured but not maintained in captivity. Animals were immediately challenged (see below) and a blood sample was taken 2h after. Blood was sampled from the retro-orbital sinus (volume of 1% of the body mass corresponding to 0.2 ml for a 20g individual) using a glass Pasteur pipette and was immediately transferred into EDTA-coated tubes (Microvette®, Sarstedt). Corresponding haematological and biochemical data will serve as reference values for non-captive individuals from High (H-LPS) and Low (L-LPS) polluted sites.

In experiment 2, a second group of 36 individuals (23 captured in sites High, 13 from sites Low) was captured and maintained in captivity. Blood was sampled immediately after capture (day 0, D0) using the same method than described above. Corresponding haematological and biochemical data will serve as reference values for High (H) and Low (L) polluted sites, respectively (Table 1). These animals were then maintained in captivity (see below) for 5 days post-capture.

Animals were fed with cornstarch food for rodents under the form of pellets (SAFE® SAS - basis formula AIN93G, see content in Table S2). A first group of animals coming from highly (H-LPS-Se+) and lowly (L-LPS-Se+) polluted sites received the standard food while a second group coming from highly (H-LPS-Se-) and lowly (L-LPS-Se-) polluted sites received a Se deficient food (Table S2). Vitamin E was also deprived from Se-deficient diet as vitamin E can compensate for the antioxidant activity and immune cell protective effect of Se (Noaman *et al.*, 2002). At the end of the captivity period (day 5, D5), animals were sampled again for blood, 2h after the immune challenge (see below), using the same method as previously described.

Housing

Animals used in experiment 2 were housed individually (to avoid inter-individual aggressive interactions) in transparent boxes filled with a solid hemp litter, a shelter containing natural cotton and a paper roll as enrichment (Figure S2). Boxes were placed in shelves in a room with no heating system (*i.e.* day and night temperatures followed roughly the natural ones and was of 18-20°C during days and of 15-18°C at night for all the duration of the experiment). Light also followed nycthemeral regime. Animals had access to water and to food *ad libitum*. Individuals were weighed at day 0, and then at each cleaning of the box (*i.e.* at day 2, 4 and 5 of the period of captivity). Faecal samples and food remains were collected and weighed during each cleaning days. Faecal samples were stored at -20°C until analysis.

Immune challenge

At day 0 for H-LPS and L-LPS, and at day 5 for H-LPS-Se+, L-LPS-Se+, H-LPS-Se-, and L-LPS-Se- individuals were challenged by intraperitoneal injection with a solution of bacterial lipopolysaccharides (LPS O111:B4, Merck) at 500 µg/kg, which is sufficient to enhance TNF-α production as found in several laboratory rodent models (Batista *et al.*, 2019). Two hours after LPS challenge, a blood sampling was performed as described above. The period of 2 hours post-LPS injection corresponds to a sufficient period of time to enhance both TNF-α and CORT release during a systemic inflammation in lab rodents (Batista *et al.*, 2019).

Blood sub-sampling and storage

A small volume of blood (~50µL) was used fresh (*i.e.* stored at +5°C until analysis, which was performed within the 8h following the sampling) for blood counting (see below). The rest of the blood (~150 µL) was centrifuged at 2000g for 15 minutes to collect plasma. Plasma was stored at -20°C during the field session and, when back at the laboratory, at -80°C until analyses.

Tissue sampling

After the immune challenge and blood sampling, whatever was the experimental group (*i.e.* with or without captivity), individuals were sacrificed by cervical dislocation under anaesthesia (see below) following the American Veterinary Medical Association ethical guidelines (Sikes and the Animal Care and Use Committee of the American Society of Mammalogists, 2016). The liver and eyes were extracted to measure trace metal concentrations in the liver and to determine age with crystalline lens mass (Vandorpe and Verhagen, 1979).

Trace metal analysis

Livers were lyophilised until constant mass, and then acid digested with nitric acid (HNO_3 69%, ultra-pure quality for trace analyses) and then diluted with ultra-pure water (Elga, 18.2 MV/cm). The concentration of Cd, Pb and Se in the liver was measured by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Thermo Fisher Scientific X Series 2). The quality of measurement was assessed by regularly measuring calibration solutions and blank (ultra-pure water). Reproducibility was checked by using internal standards of the ICP-MS. Repeatability and accuracy were verified by repeated (6 times) measurements of certified reference materials (National Research Council Canada TORT-3 Lobster hepatopancreas, National Institute of Standards and Technology SRM® 1577c Bovine liver). Values under limits of quantification limits (LoQ) were replaced by the LoQ divided by the square root of two (Helsel, 2008). The concentration of metals was calculated in µg/g dry mass in the liver. Limits of quantification (LoQ), proportion of data < LoQ, certified values and average recoveries of the certified reference materials are provided in Table S3.

Complete blood counting

Complete blood counting was done with a Haematology Analyser automate (Horiba ABX Micros ES60). For the present study, we used the following leukocyte counts (in 10^3 cells/mm^3): white blood cells (WBC), lymphocytes (LYM), monocytes (MON), granulocytes (GRA). Repeatability is automatically assessed by the analyser in measuring 3 times each blood sample. Moreover, each individual sample was measured 2 to 3 times in the cases of non-repeatable measurements (high deviance within sample measurements). Reproducibility and accuracy were determined with an everyday automatic blank control and a measurement of blood quality control “low”, “medium” and “high” (MINOTROL 16, Horiba SAS).

Determination of TNF-αlpha

TNF-α plasma concentration was determined with a high sensitivity commercial Mouse TNF-α quantikine ELISA kit (R&D MH, detection range from 0.781 to 50 pg/ml). Positive controls were used to evaluate the ability of the kit to detect elevated values at 50 and 100 pg/ml

(average recovery 86% in both cases). As low volume of blood did not allow performing linearity or other recovery assays, only 2 samples were used to control inter-assay (recovery of 104% and 105%) and 2 others sample to control intra-assay (recovery of 91% and 80%) quality of measurements. Four samples spiked with known amounts of TNF-α were used to assess if antigen-antibody inhibitors may be present in the sample, giving a recovery rate of 110% for a spike with 100 pg/ml (*n*=1), 92% with 25 pg/ml (*n*=1), and 89 and 73% with 3.13 pg/ml (*n*=2). Average recovery of 91% suggested the absence of such inhibitors.

Faecal and plasma CORT metabolite determination

The determination of faecal CORT metabolite (FCM) was performed according to the method of Touma *et al.* (2003) as detailed in Sánchez-González *et al.* (2018) on the wood mouse. Briefly, faecal samples were oven dried until constant mass (40°C for 10 hours) and homogenized with a mortar and a pestle. An aliquot of 0.056 ± 0.006 g of faeces was then mixed with 500 µL of 80% methanol and 500 µL of phosphate buffer, vortexed for 15 s and left 16 hours in an orbital agitator. Faecal extraction of FCM was then centrifuged for 15 min at 2500 g and the supernatants were collected in microtubes and stored at -20 °C until analyses. FCM determination was done with a commercially available enzyme immunoassay rat/mouse serum/plasma kit from DEMEDITEC Diagnostics (Kiel, Germany). A serial dilution (1:32, 1:16, 1:8, 1:4, 1:2, 1:1) of faecal extract was done to evaluate parallelism of the standard curves and the diluted extracts (73±27%). Recovery assays were performed between plates to evaluate accuracy (63±24%). The CORT concentration expressed in ng/ml was then corrected by the individual faeces mass as it has been shown to have a significant influence on the levels of FCM (Navarro-Castilla and Barja, 2014a). Plasma concentration of CORT was determined using the same DEMEDITEC Diagnostics kit. Assay precision was measured with intra-assay and inter-assay recovery rates, which were respectively of 115±47 % (*n*=5) and 90±5 % (*n*=4). For accuracy, the recovery rate from spiked samples with 185 ng/ml of CORT was 99±23 % (*n*=2) and the one from diluted (1:2) samples was 91±26 % (*n*=2).

Ethics

Blood sampling, immune challenge and euthanasia were performed under gaseous anaesthesia using isoflurane. Experiments were performed under the authorization of the French National Ethical Committee (Project APAFIS N°5340) by skilled and experienced investigators from Chrono-environnement research department (EU0592), following directive 2010/63/EU on the protection of animals used for scientific purposes.

Statistical analysis

A 2-way ANOVA was performed to determine differences in age structure of individuals from both experiments 1 and 2. For experiment 2, individual whole body mass, age and trace metal concentrations were compared with a 2-way ANOVA between soil pollution levels and diet, alone and in interaction. The quantity of food consumed was compared with a 3-way ANOVA between soil pollution levels, diet and the day of the measurement (at day 2, 4 and 5). In experiment 1, Student *t*-tests were used to test mean differences in hepatic metal concentrations, white blood cell counts and CORT between challenged (CH and CL) and unchallenged (H and L) individuals for each level of soil pollution. Linear regressions relating liver Cd, Pb and Se concentrations to soil pollution levels in interaction with age of animals were performed to determine potential age-related patterns in the accumulation of trace metals. In experiment 2 Student paired *t*-tests were used to compare white blood cell counts and CORT between day 0 (D0) and day 5 (D5) for each diet. Mann-Whitney tests were performed to compare TNF-α concentrations among challenged individuals from experiment 1 (H-LPS vs L-LPS) and experiment 2 (H-LPS-Se+ vs L-LPS-Se+, H-LPS-Se- vs L-LPS-Se-, H-LPS-Se+ vs H-LPS-Se-, L-LPS-Se+ vs L-LPS-Se-). In both experiments 1 and 2, CORT concentrations were compared with a 2-way ANOVA between soil pollution levels in interaction with the day of captivity (D0 and D5). Post-hoc analysis of Tukey's Honestly-Significant-Difference was done when interaction between group factors was significant. The 'HSD.test' function (package 'agricolae') was used as it considers unbalanced designs. Pairwise comparisons using paired *t*-tests with the method of Benjamini and Hochberg (1995) was performed when interaction between group variables was not significant and to compare repeated measurement of faecal CORT metabolites of each individual. Linear regressions were performed to determine the effect of LPS challenge on WBC, LYM, MON and GRA in response to plasma TNF-α and CORT concentrations and pollution levels in experiment 1, and in interaction with diet in experiment 2. Spearman rank correlation matrices were run on physiological parameters according to pollution levels and type of diet (Figure S7 and S8). Log transformation was applied to Cd and Pb concentrations, WBC, LYM, MON and GRA counts and CORT to satisfy prerequisite of statistical tests (normality and homoscedasticity), which were verified with Mauchly, Kolmogorov-Smirnoff and Levene's tests. The package 'performance' was used to verify linear models' assumptions (normality of residuals, linear relationship, homogeneity of variance, multicollinearity). All analyses were done with R version 3.6.1. (R Core Team, 2023) and graphics were drawn with 'ggplot2' and 'ggpubr' packages.

Results

Sex-ratio, age structure, body mass and food consumption

The sex-ratio was biased towards males (46 males *vs* 13 females) in the experiment, depending on the trapping success on the field. No difference in age structure (df = 47, F = 0.56, p = 0.57) was found between the tested groups of experiments 1 and 2 (Table S4, H-LPS, L-LPS, H-LPS-Se+, L-LPS-Se+, H-LPS-Se-, and L-LPS-Se-). In experiment 2, neither differences of consumption between standard and Se-deficient food (df = 38, F = 0.32, p = 0.61) nor differences of mass (df = 47, F = 2.8, p = 0.07) were found (Table S4), suggesting satisfactory housing and experimental conditions.

Trace metals

In experiment 1, chronic exposure of wood mice from High and Low polluted sites was significantly different for Pb and Se but not for Cd. Liver Cd concentrations indeed did not differ between H and L (t = 1.2, df = 14.2, p = 0.24, Table 2). Higher hepatic Pb and Se concentrations, however, were found in H-LPS in comparison with L-LPS (Pb: t = 6.1, df = 13.8, p < 0.001, Se: t = 4.4, df = 8.54, p = 0.002, Table 2). The same was found for captive individuals (Table 2).

In experiment 2, five days of captivity did not change significantly liver concentrations of Cd and Pb between experiment 1 and 2, neither from High (Cd: df = 26, F = 1.6, p = 0.21; Pb: df = 26, F = 0.68, p = 0.51) nor from Low (Cd: df = 23, F = 0.09, p = 0.91; Pb: df = 23, F = 2.08, p = 0.15) polluted sites, whatever was the type of food they were fed with (Table 2, Figure S3). Individuals from High level of pollution fed with Se-deficient had lower Se concentration than individuals fed with standard diet (t = 3, df = 16, p = 0.01), while this was not the case for individuals coming from Low polluted sites (t = 0.9, df = 8, p = 0.4).

In both experiments 1 and 2, the concentrations of Cd, Pb and Se in the liver were not influenced by the age of individuals, alone or in interaction with pollution levels (p > 0.05).

Table 2 Mean (and [median, min – max]) Cd, Pb and Se hepatic concentrations in the experimental groups. Different letters stand for significant differences between H-LPS and L-LPS.

Experiment	Group	Cd	Pb	Se
1	H-LPS	4.9[a] [4.5, 0.9 - 11]	0.9[a] [0.9, 0.6 - 1.6]	7.1[a] [6.4, 5.1 - 10]
	L-LPS	4.8[a] [1.3, 0.1 - 26]	0.2[b] [0.04, 0.005 - 1.5]	2.8[b] [2.5, 0.8 - 6]
2	H-LPS-Se+	8.0[a] [4.0, 0.7 - 23]	1.1[a] [0.6, 0.2 - 3.7]	6.8[a] [6.7, 5.2 - 9.4]
	L-LPS-Se+	4.7[a] [4.1, 0.2 - 16]	0.02[b] [0.007, 0.006 - 0.08]	4.1[b] [4.3, 3.4 - 5]
	H-LPS-Se-	5.1[a] [0.9, 0.5 - 25]	1.2[a] [0.5, 0.05 - 5.4]	5.4[c] [5.5, 4.1 - 7.1]
	L-LPS-Se-	3.1[a] [2.9, 0.3 - 5.8]	0.03[b] [0.01, 0.006 - 0.1]	3.7[b] [3.5, 2.6 - 5]

Effect of immune challenge on WBC

In experiment 1, immune challenge induced more changes on WBC in non-captive individuals coming from High than Low polluted sites (Table 3, Figure S4). Significant decreases of 75% of WBC ($t = -5.7$, $df = 8.8$, $p < 0.001$), 77% of LYM ($t = -6.2$, $df = 10$, $p < 0.001$), 76% of MON ($t = -3.8$, $df = 6.7$, $p = 0.007$), and 51% of GRA ($t = -2.7$, $df = 8$, $p = 0.026$) were found in challenged individuals in comparison with non-challenged ones (H *vs* H-LPS). Challenged individuals from Low polluted sites (L *vs* L-LPS) displayed a decrease of 45% of WBC ($t = 2.8$, $df = 23$, $p = 0.009$) and 52% of LYM ($t = 2.9$, $df = 22$, $p = 0.007$) while no significant variations were detected for MON ($t = 1.7$, $df = 18$, $p = 0.09$) and GRA ($t = 2.1$, $df = 19$, $p = 0.05$).

In experiment 2, captive animals fed with standard food showed higher decrease of WBC (H-LPS-Se+: -85% *vs* L-LPS-Se+: -53%), LYM (H-LPS-Se+: -86% *vs* L-LPS-Se+: -56%), MON (H-LPS-Se+:-87% *vs* L-LPS-Se+:-46%) in individuals from Low polluted sites than individuals form High (Table 3, Figure S4). No significant variation of GRA was found in H-LPS-Se+ individuals while a decrease of -71% was found in L-LPS-Se+ ($t = 3$, $df = 5$, $p = 0.02$). Animals fed with Se-deficient diet showed lower variation of leukocytes counts in response to challenge in both individuals from High and Low polluted sites. Only significant decreases of LYM (H-LPS-Se-:-56% *vs* L-LPS-Se-:-75%) and WBC for L-LPS-Se- (that may be attributed to LYM variation) were found in individuals fed with Se-deficient diet.

Pro- and anti-inflammatory responses to LPS challenge

In both experiments 1 and 2, plasma TNF-α concentration was under detection limits in non-challenged individuals (Table 3).

Challenged individuals from experiment 1 had higher plasma TNF-α concentrations in animals coming from High level of pollution than Low pollution level ($W = 53$, $p = 0.047$, Figure 1). A significant increase of plasma CORT of 23% was found between H and H-LPS individuals ($t =$

5, df = 15, $p < 0.001$; Table 3) while no difference was found between L and L-LPS (t = 1, df = 22, $p = 0.3$). Lower levels of CORT were found in non-challenged individuals from High (H) in comparison with H-LPS, L and L-LPS (F = 5.2, df = 51, $p = 0.003$). In experiment 2, challenged individuals fed with standard diet showed lower TNF-α concentrations in animals coming from High polluted sites than individuals from Low (W = 14, $p = 0.03$, Figure 2).

Figure 1 Experiment 1. Concentration of plasma TNF-α (pg/ml) and of CORT (ng/ml) in response to LPS-challenge in wood mice captured in contrasted levels (high and low) of soil pollution. Levels of significance of *t* and *U* (only TNF-α) tests, *, **, *** for p < 0.5, 0.1, 0.01, respectively; <LD: below limits of detection.

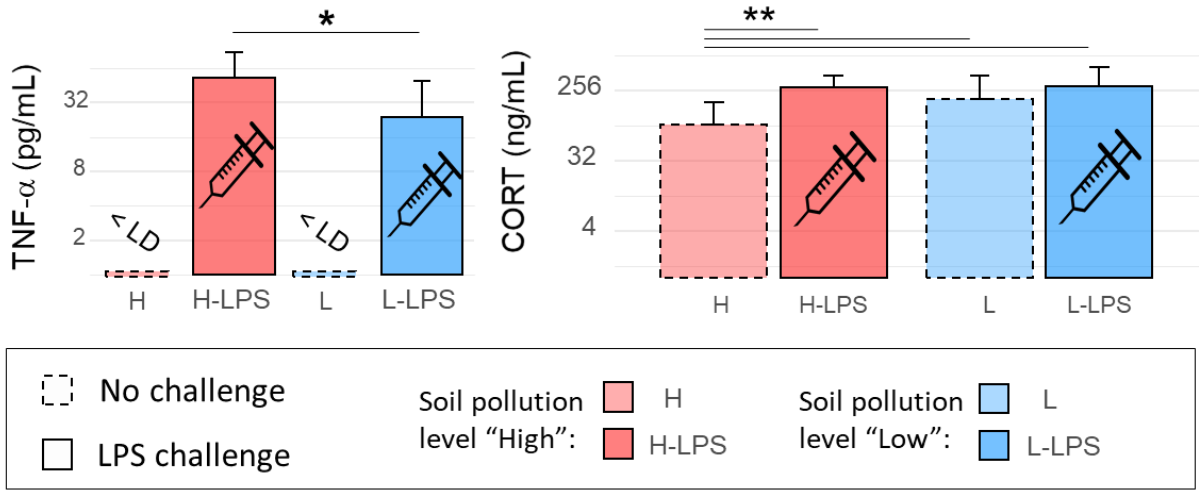
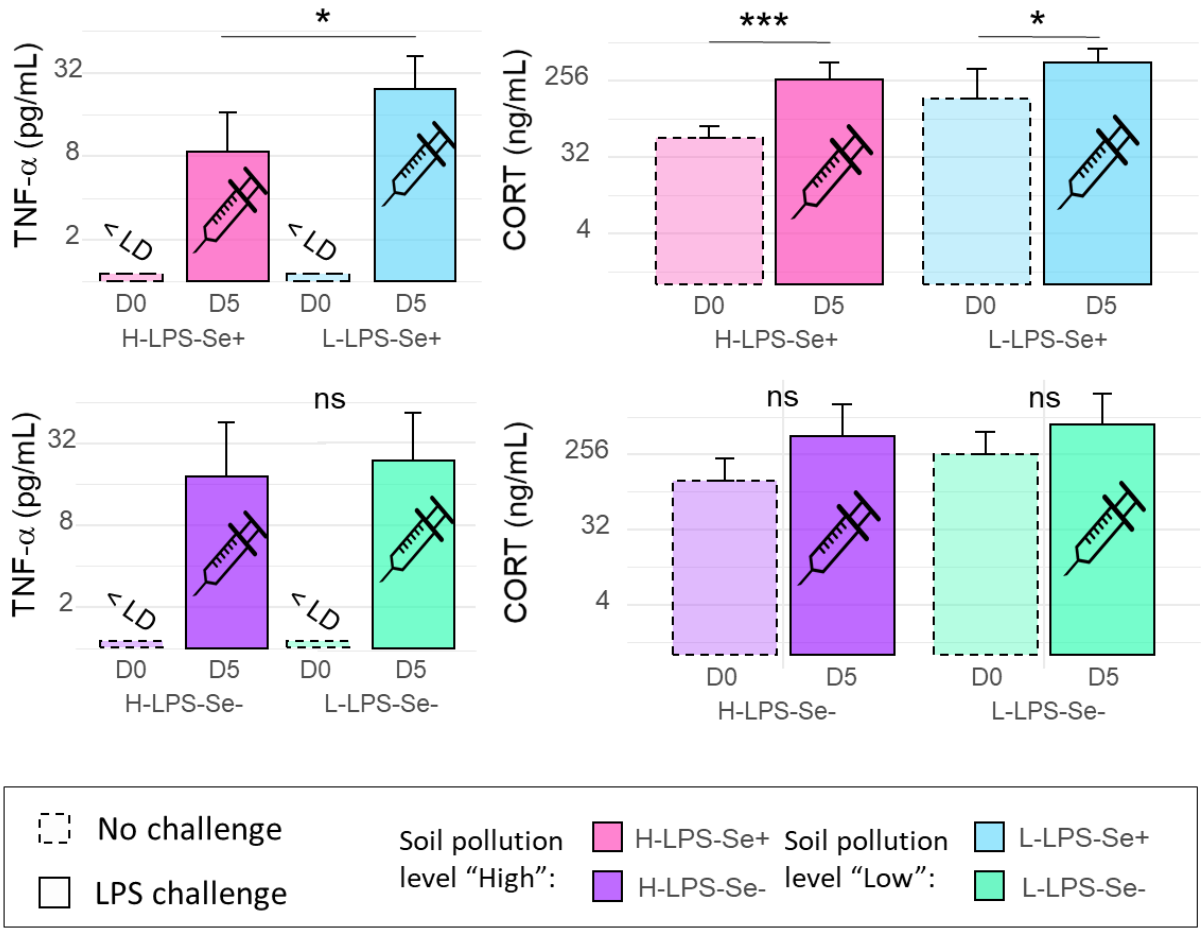


Figure 2 Experiment 2. Concentration of plasma TNF-α (pg/ml) and of CORT (ng/ml) in response to LPS-challenge in wood mice captured in contrasted levels (high and low) of soil pollution and fed for 5 days with standard or Se-deficient diets. Levels of significance of *t* and *U* (only TNF-α) tests: ns: non-significant difference, *, **, *** for p < 0.5, 0.1, 0.01, respectively; <LD: below limits of detection.



Plasma CORT increased by 26% for H-LPS-Se+ and 22% for L-LPS-Se+individuals between D0 and D5 (Table 3, Figure 2). However, no differences neither in TNF-α nor in CORT plasma concentrations were found in challenged individuals fed with Se-deficient diet (Table 3, Figure 2).

Table 3 Variation of total leukocytes counts and corticosterone levels between unchallenged and challenged wood mice (for experiment 1: H *vs* H-LPS, L *vs* L-LPS and for experiment 2: D0 *vs* D5), and median (and [min-max]) concentrations of plasma TNF-α. NS stands for non-significant difference, *, **, *** for p < 0.5, 0.1, 0.01, respectively. Different letters stand for significant differences between H-LPS and L-LPS.

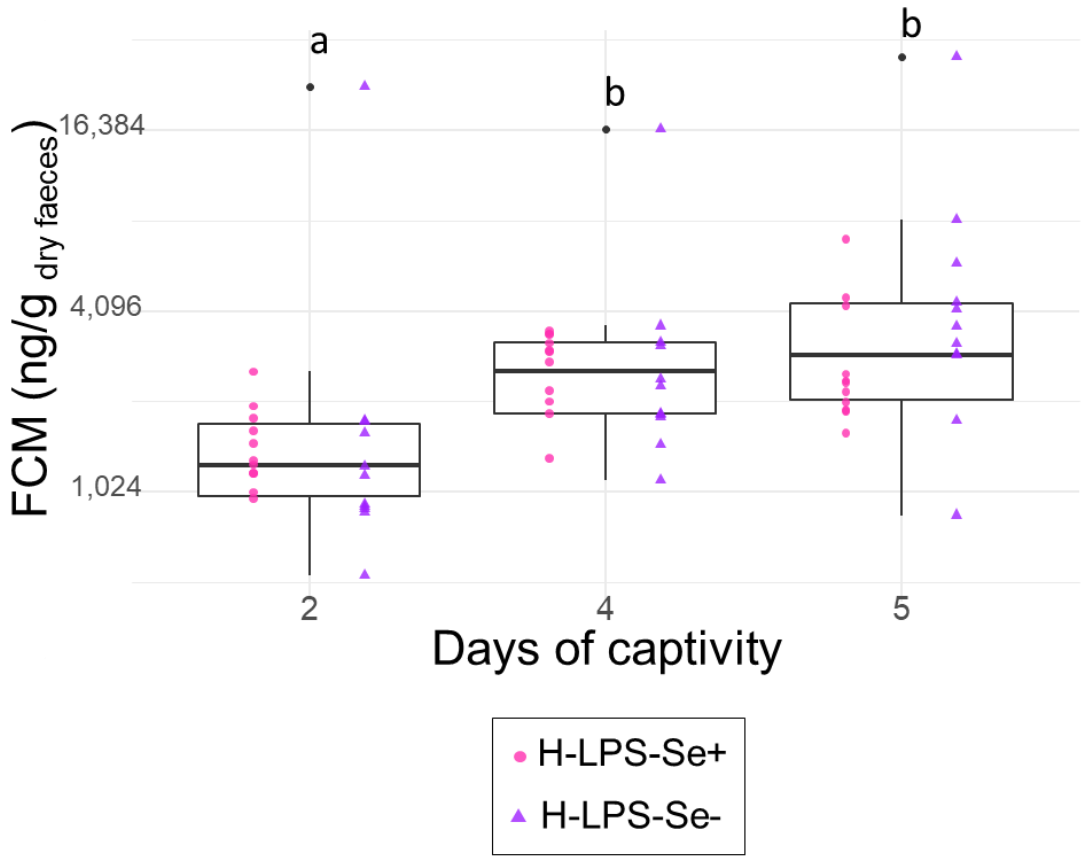
Experiment	Group		Δ[1]WBC	ΔLYM	ΔMON	ΔGRA	ΔCORT	TNF-α (pg/ml)
1	H		-75% ***	-77% ***	-76% **	-51% *	+23% ***	< LD[2]
	H-LPS							49[a] [3 - 94]
	L		-45% *	-52% **	NS	NS	NS	< LD
	L-LPS							24[b] [0.7 - 80]
2	H-LPS-Se+	D0	**-53%** **	**-56%** *	**-46%** **	NS	**+26%** ***	< LD
		D5						4.5 [a] [0.05, 22]
	L-LPS-Se+	D0	**-85%** **	**-86%** **	**-87%** **	**-71%** *	**+22%** *	< LD
		D5						21 [a] [2, 44]
	H-LPS-Se-	D0	NS	**-56%** *	NS	NS	NS	< LD
		D5						11 [a] [1.8, 90]
	L-LPS-Se-	D0	**-71%** *	**-75%** **	NS	NS	NS	< LD
		D5						12 [a] [3.3, 73]

[1] Δ stands for variations of measured parameters (value of *e.g.* H-LPS - value of H)

[2] < LD: under limits of detection

No differences in FCM (Figure 3) were found between Se-deficient and standard diets (F= 1.31, p = 0.26). Lower FCM was found after 2 days of captivity (F= 17.1, p < 0.001) in captive individuals from High polluted sites than from Low polluted sites (H-LPS-Se+ and H-LPS-Se-). However, no differences according to pollution level were detected at day 4 and 5. Post-hoc analyses revealed significant increase of FCM from day 2 to 4 and from day 2 to 5 in individuals from High polluted sites but not between days 4 to 5. No significant variations were found between days 2, 4 and 5 in individuals from Low polluted sites. The mean FCM levels in individuals from High reaches the ones from Low at day 4 (High: 3186 ± 3033 ng/g; Low: 4750 ± 3821 ng/g) and 5 (High: 4390 ± 5506 ng/g; Low: 4770 ± 4539 ng/g).

Figure 3 Experiment 2. Trends in faecal corticosterone metabolite (FCM, ng/$g_{dry\ faeces}$) of wood mice captured in high level of soil pollution during five days of captivity. FCM was measured at day 2, 4 and 5 of the experiment in two groups of individuals fed with standard or Se-deficient diet. Levels of significance of a pairwise paired T-tests between pollution levels: p-value >0.5 (ns), <0.5 (*), <0.01(**), <0.001(***).

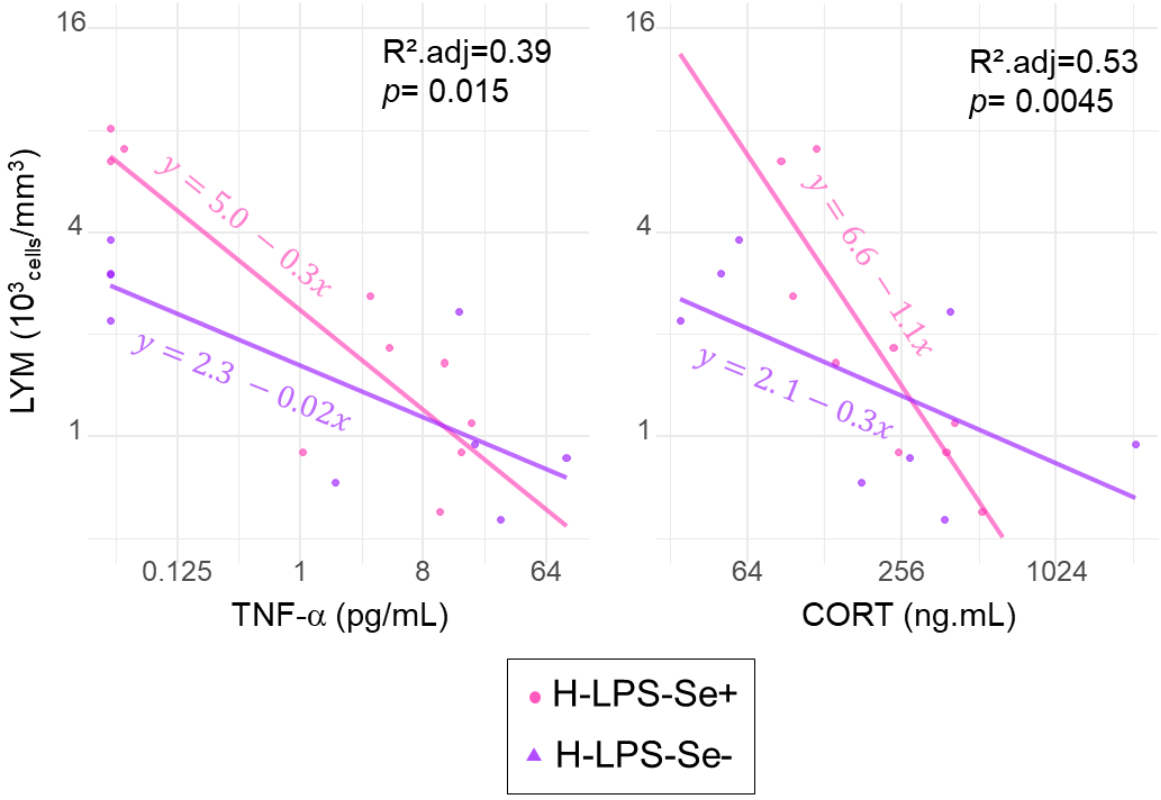


Relationships between WBCs and plasma TNF-α and CORT

In experiment 1, negative relationships between WBC, LYM, MON and GRA were found with TNF-α concentrations whatever the soil pollution level (Figure S5). WBC and LYM were also negatively related to CORT but not MON and GRA.

In experiment 2, WBC and LYM were negatively related to TNF-α and CORT concentrations in individuals from High (Figures 4 and S6), with a significant interaction between standard and deficient food for LYM with TNF ($r^2_{adj.}$ = 0.39, F = 4.8, df = 15, *p* = 0.015) and CORT ($r^2_{adj.}$ = 0.53, F = 7.1, df = 13, *p* = 0.005). The individuals H-LPS-Se+ showed sharper negative relationships between LYM and TNF-α (slope= -0.3 *vs* -0.02) and CORT (slope= -1.1 *vs* -0.3) than in H-LPS-Se-. This was not observed in L-LPS-Se+ and L-LPS-Se-.

Figure 4 Experiment 2. Relationships between lymphocyte counts and plasma TNF-α and CORT after LPS challenge in captive wood mice from "High" levels of soil pollution fed standard (H-LPS-Se+) or Se-deficient (H-LPS-Se-) diet.



The Table 4 summarizes our main results, both in terms of stressors (exposure to environmental pollution, Se-deficient food) and responses measured by variation of WBCs, TNF-α, and CORT)

Discussion

Our data showed that wood mice from the highly polluted site exhibited higher liver concentrations of Pb than their counterparts from the lowly polluted site, confirming a higher chronic exposure to Pb. Concentrations of Pb in the liver from Low and High sites were in the range of those found in the literature for reference or lowly polluted sites and highly polluted sites, respectively (Tête *et al.*, 2014b). Similar levels of hepatic Pb between experiments 1 and 2 showed that five days of captivity with non-contaminated food did not induce a significant decrease of Pb accumulation in the liver, which is in agreement with the half-life of Pb in soft tissues estimated at 40 days in human (Kabata-Pendias and Szteke, 2015). Despite higher concentrations of Cd in soils from High site, concentrations in the liver did not differ significantly from individuals from the Low site. One explanation can be that soil Cd can have been influenced by physio-chemical processes occurring during the aging of metals, which over time have reduced its bioavailability (Lock and Janssen, 2003). Cadmium concentrations in the liver in individuals from both sites were however higher than individuals from "reference" sites in other studies (Tête *et al.* 2014), showing a chronic exposure to Cd. While Cd is known to accumulate with age, this was not found in the present study, maybe because of low sample size. Similarly to Pb, five days of captivity with non-contaminated food did not reduce significantly Cd concentrations in the liver, in agreement with its very long half-life in rat and mouse (200-700 days) and in human (several years or decades) (Nordberg *et al.*, 2015). Our results showed higher levels of hepatic Se in individuals captured on highly polluted site in contrast with individuals from lowly polluted area, which has also been found in a previous study from the same site (Powolny *et al.*, 2022). Mammals' homeostasis as well as reproducing performances have been shown to be connected to individual micronutrient resources (Allen and Ullrey, 2004). Among them, Se is a key antioxidant which deficiency could impact cardiac muscles (*e.g.* mulberry heart disease) and immune cells (*i.e.* leukocytes) functioning (Gombart *et al.*, 2020). Selenium occurs in several mechanisms, including selenoproteins (*e.g.* GPX) and adducts (*e.g.* Cd-Se), which are involved in the detoxification of Cd and Pb notably on ROS (Fu *et al.*, 2019; Vuksic *et al.*, 2018). The critical role of Se in ROS scavenging activity was found to prevent immune cell damages (Avery and Hoffmann, 2018). Given the protective role of Se against the toxicity of metals, one could hypothesise that Se is preferentially taken up and/or accumulated in animals exposed to Cd and Pb (Peraza *et al.*, 1998). However, in the present study site, Se median concentrations in soils from Low and High sites were of 1.8 and 4.1 µg/g, respectively (unpublished data). Our data, therefore, did not allow discriminating between a "passive" higher accumulation of Se in animals from the High site because of higher Se concentrations in soils, and/or a more physiologically "active" uptake or accumulation of Se for its protective function. Contrarily to Cd and Pb, 5 days of captivity with Se-deficient food was sufficient to lead to a decrease of hepatic Se concentrations. This is in line with

the literature, which shows that, in animals, elimination of certain forms of Se through both urine and faeces is at least biphasic (triphasic in human), with one fast elimination phase of approximately 1 day (dose dependent) followed by a slower phase of 30-70 days in most of species (Alexander, 2015). In brief, our data about metal in wood mice suggest a higher exposure to and accumulation of Pb and Se in animals from High site, a higher exposure to but not accumulation of Cd, with a significant decrease of hepatic concentrations during captivity for Se only.

Table 4 Summary of the main trends in the experiments, in terms of stressors encountered by animals and responses as measured by variation of WBCs, CORT, and TNF-α and by Cd, Pb, and Se concentrations. Only statistically significant variations are represented. Decreases and increases are represented by ↘ and ↗, respectively. Variations of 0 to 33%, 34 to 66%, and 67 to 100% are represented by 1, 2 and 3 arrows, respectively. Cd, Pb, and Se concentrations are qualified of high (H) or low (L), and an arrow is used to represent a decrease/increase during captivity.

Group	Stressors	WBCs	LYM	MON	GRA	CORT	TNF-α	Cd	Pb	Se
H-LPS	High exposure Natural conditions	↘↘↘	↘↘↘	↘↘↘	↘↘↘	↗	↗↗↗	H	H	H
L-LPS	Low exposure Natural conditions	↘↘	↘↘				↗↗	H	L	L
H-LPS-Se+	Past high exposure Captivity (no exposure) Standard food	↘↘	↘↘	↘↘		↗↗	↗	H	H	H
L-LPS-Se+	Past low exposure Captivity (no exposure) Standard food	↘↘↘	↘↘↘	↘↘↘	↘↘↘	↗↗	↗↗	H	L	L
H-LPS-Se-	Past high exposure Captivity (no exposure) Se-deficient food		↘↘				↗	H	H	H↘
L-LPS-Se-	Past low exposure Captivity (no exposure) Se-deficient food	↘↘↘	↘↘↘				↗	H	L	L

Overall, our results showed significant decreases of circulating WBCs in individuals exposed to environmental stressors (experiment 1) and also in the ones maintained in captivity (experiment

2) with non-contaminated standard or Se-deficient diet (except H-LPS-Se- where only lymphocytes decreased significantly) (Table 4). The decrease of WBCs was mainly related to lymphocytes decrease and less frequently to decreases of monocytes and granulocytes as well. All groups from both experiments 1 and 2 also exhibited an increase of TNF-α while its level was undetectable in animals before immune challenge. CORT concentrations, on the other hand, increased only in free-ranging animals from High site (experiment 1) and in animals fed standard food for five days in captivity (experiment 2), whatever the site they came from. Brooks *et al.* (2017) found that LPS (15 mg/Kg) induced a decrease of WBCs including lymphocytes, granulocytes and monocytes within 4 hours post-injection of Wistar rats. In line with this, our results suggest an inflammatory response to LPS challenge, characterised by a decrease of WBCs mainly mediated by lymphocytes decrease and a production of TNF-α and CORT. Indeed, CORT can have down-regulating effects on immune parameters in reducing numbers of circulating lymphocytes (T cells > B cells and CD4+ > CD8+) and also antigen presenting cells (*i.e.* macrophages, neutrophils and monocytes) promoting an immune-redistribution (Martin, 2009a). In our study, the stress induced by the LPS challenge always promoted a decrease of circulating lymphocytes, and sometimes of other leukocytes, which may be mediated by elevated CORT enhancing a redistribution of WBCs from the blood to other organs where the infection occurs (*e.g.* peritoneal cavity, lymph node) and clear organisms from pathogens (Dhabhar, 2002). Sharper WBC decrease, involving all types of leukocytes, and higher TNF-α level were found in free-ranging animals coming from the High site, suggesting a higher immune response to LPS challenge than individuals less exposed. Higher levels of TNF-α and a decrease of lymphocyte were also found in cell culture containing 100 ng/mL of LPS and rat peripheral blood polymorphonuclear leukocytes pre-treated with Cd (*i.p.* injection) at 1 mg/kg (Djokic *et al.*, 2014). Lead was also found to enhance a marked increase of TNF-α in rats after co-exposure with different concentrations of Pb and intravenous injection of LPS at 100 µg/kg (Liu *et al.*, 2005). In our study, the difference of TNF-α concentrations and of circulating WBC levels between individuals from High and Low polluted sites could suggest two levels of strength of the immune response to LPS challenge, with a possible peracute immune response in animals highly exposed to pollution. A time sequence measuring TNF-α and WBC count after the challenge would enable to define if the immune reaction (here measured 2 hours after the challenge) is a peracute response or a shortened response to challenge. The absence of significant differences in granulocytes, monocytes and CORT in individuals from Low polluted sites could indicate a differential recruitment of WBCs. One explanation can be that, in High animals, CORT increased to avoid a hyper-inflammatory immune response, which to a certain extent can enhance self-damaged mediated by the activated immune cells. Indeed, in rodents, the main role of CORT is to control inflammation in modulating responses once infections are controlled to avoid self-damage from immune cells, also called autoimmunity (Medzhitov, 2008; Martin, 2009a). However,

lower levels of both plasma CORT and FCM were found in individuals from the highly polluted site before challenge. Cd and Pb were found to inhibit circulating CORT in altering adrenal cortex functions (Nishiyama and Nakamura, 1984; Nishiyama *et al.*, 1985). The adrenal cortex is mainly under the control of the pituitary **adrenocorticotrophic hormone,** which, by a negative feedback mechanism, controls plasmatic CORT levels (Wills and Havard, 2013). Lower circulating CORT could reduce anti-inflammatory levels in wood mice from polluted site. A dampening of anti-inflammatory levels would enable organisms exposed to pollution to maintain at a higher level their basal immune functions leading to an up-regulation of the immune system. We could thus hypothesize that a higher basal immune function in wood mice inhabiting polluted sites allows a shortening of the time of effectiveness to enhance inflammatory immune response and thus quickly enhances CORT release in the blood stream. In experiment 2, FCM levels of individuals from High polluted site were found to increase during the 5 days of captivity towards equivalent levels to the ones from Low polluted site. This variation may provide an explanation of the discrepancies found in TNF-α concentrations, which may be lower in individuals from High polluted sites fed standard diet (H-LPS-Se+), as higher basal CORT could promote a higher anti-inflammatory activity. In contrast, individuals fed Se-deficient diet showed lower immune response to the immune challenge as LYM could probably be less dependent to plasmatic TNF-α and CORT. Lower quality of diet regarding Se would thus disturb both pro- and anti-inflammatory activity of TNF-α and CORT in individuals exposed to High levels of pollution.

Conclusion

Our study provides new insight at the interface of eco-immunology and ecotoxicology to highlight physiological disturbances originating from anthropogenic activities in a terrestrial small mammal. Wood mice exposed to high levels of pollution displayed higher pro-inflammatory immune response to challenge, which was attributed to lower circulating anti-inflammatory CORT. Captivity with low quality (Se-deficient) diet promoted lower immune response of both pro- and anti-inflammatory markers in wood mice populations exposed to Cd and Pb. Altogether, our results highlight an immunomodulation of the inflammatory response indirectly mediated by Cd and/or Pb exposure and diet quality, related to a disturbance in circulating CORT levels. Further experiment should focus on the influence of these two stressors (pollution and nutrition) on both immune and endocrine system of small mammals. A specific focus should be given on the immune system of free-ranging mammals exposed to chronic level of pollution considering the epidemiological disorders it may triggered on host/pathogen interactions. In our study, experiment 1 is in favour of improved immune function of wood mice from polluted sites through neuroendocrine disturbance avoiding a downing of inflammatory mechanism mediated by CORT. The cost of maintaining an elevated immune function may induce an energetic trade-off in the wood mouse, which would allocate lower energy to other components of their fitness (Lochmiller and Deerenberg, 2000). Experiment 2 showed lower immune response to LPS challenge in animals from highly polluted site and fed Se-deficient diet, which is in favour of lower immune function of captive individuals fed low quality diet. In an epidemiological perspective, the consequences of immunomodulation related to pollution and nutrition could have severe impacts in host/pathogen interaction, as lower immune function could increase the susceptibility of host to infections. In an evolutionary perspective, higher immune function in small mammals chronically exposed to (metal) pollution might increase pathogen virulence to reign-in wood mouse immune defences. Further analysis on pathogen prevalence among polluted sites should be done to investigate for discrepancies in prevalence of pathogenic agents in wildlife.

Funding

This work was supported by the Agence De l'Environnement et de la Maîtrise de l'Energie (ADEME, programmes BIOTROPH, grant number N°1172C0030, and DYSPAT, grant number 1572C0309), the Conseil Régional de Franche-Comté (for having financially supported the purchase of the HORIBA ABX Micros ES 60 haematology analyser), the LTER Zone Atelier Arc Jurassien (for the fnancial support to purchase the field anaesthesia device), and the OSU THETA and the University of Franche-Comté (programmes DEMENOL, MEXCO and OREAS, which supported the costs of fieldwork and field and laboratory analyses).

Data availability

Data will be made available on request.

Acknowledgments

The authors gratefully thank Nadia Crini from the PEA2t platform (Chrono-environnement, University Bourgogne Franche-Comté, UMR CNRS 6249, France) for their technical help in metal analyses. Anne-Claude Goydadin is also gratefully thanked for her technical help in sample analyses and Loïc Angonin for his help in the field.

Supplementary Material

Table S1 Characteristics of the study sites (distance from the former smelter, median, minimum and maximum soil concentrations of Cd, Pb and Se) near the former Metaleurop Nord smelter.

		High	Low
Distance from Metaleurop Nord (median, km)		0.6	3.0-11.0
Soil metal concentrations (µg/g dry mass)	Cd	59.3 (15.3 – 236.5)	1.4 (0.7 – 5.3)
	Pb	1357 (755 – 6809)	116 (43 – 200)
	Se	4.1 (3.1 – 10.0)	1.8 (1.5 – 2.3)

Table S2 Composition of standard (AIN93G) and Se-deficient (AIN Deficient) food provided to wood mice for 5 days in captivity (average quantity of 10.1 ± 0.2 g of pellets given every two days).

		AIN93G 18% casein	AIN DEFICIENT Selenium and Vit.E tocophe
mg / kg	Selenium (Se)	0.44	< 0,1
UI / kg	Vit. E (dl-alpha-tocopherol acetate)	87.4	6.9
%	Nitrogen-free extract	63.1	61.6
%	Starch	47.9	48.5
%	Total sugars	13.8	12.2
%	Crude Protein	15.9	16.1
%	Crude Fat	7.4	7.4
%	Crude Ash	2.7	2.7
%	Crude fiber	3.6	3.6
%	Humidity	7.3	8.6
Kcal / kg	ME Atwater Kcal	3,827	3,773
%	% Energy from proteins	16.7	17.1
%	% Energy from lipids	17.4	17.6
%	% Energy from Nitrogen-free extract	65.9	65.3
%	% Energy from sugar	14.5	12.9
%	% Energy from starch	50.1	51.5
mg / kg	Calcium (Ca)	4,973	5,013
mg / kg	Phosphorus (P)	2,621	2,657
mg / kg	Sodium (Na)	1,437	1,582
mg / kg	Zinc (Zn)	42.5	43.8

UI / kg	Vit. A	5,237	4,501
UI / kg	Vit. D3	1,250	1,250

Table S3 Limits of quantification (LoQ) of ICP-MS analysis of metals, proportion of data < LoQ, certified values and average recoveries of the certified reference materials used in this study (the number of reference samples analyzed for each certified reference material is 6).

Parameter	Cd	Pb	Se
LoQ (µg/L)	0.018	0.006	0.056
Proportion of data under LoQ (%)	0%	17.8%	0%
TORT-3 certified values (and uncertainty: 95 confidence limits) in µg/g	42.3(1.8)	0.225(0.018)	10.9(1.0)
Recovery (%)	93±2	71±1	99±3
SRM 1577c certified values (and uncertainty: 95 confidence limits) in µg/g	0.097(0.001)	0.063(0.001)	2.031(0.045)
Recovery (%)	70±5	61±7	105±3

Table S4 Age, whole body mass and food consumption (median, [min - max]) of individuals in function of group and experiment.

Group	Experiment	Age at sacrifice (in days)	Whole body mass (in g)				Daily quantity of food consumed (in g)		
			D0	D2	D4	D5	D0-D2	D2-D4	D4-D5
H-LPS	1	200 [58 - 260]	22.9 [14.9 - 25.7]						
L-LPS	1	105 [47 - 205]	18.5 [14.6 - 21.7]						
H-LPS-Se+	2	130 [56 - 486]	17.6 [14.8 - 23.7]	18.1 [15 - 24.3]	17.5 [14.1 - 25.1]	17.5 [14.4 - 24.8]	2.8 [2.5 - 4.4]	3.2 [2.3 - 4.5]	3.1 [0.4 - 4.9]
L-LPS-Se+	2	108 [53 - 251]	19.3 [15.3 - 23.4]	18.4 [16.6 - 25.2]	19.6 [16.9 - 24.7]	19.1 [15.7 - 23.7]	3.6 [2.6 - 4.1]	3.8 [3.0 - 3.9]	3.4 [0.8 - 4.2]
H-LPS-Se-	2	116 [58 - 502]	16.4 [15.6 - 22.7]	17.5 [15.8 - 21.6]	18.0 [15.9 - 21.3]	17.5 [16.4 - 20.9]	3.2 [2.6 - 3.9]	3.5 [3.1 - 4.5]	3.2 [2.5 - 3.8]
L-LPS-Se-	2	99 [48 - 264]	15.9 [15.1 - 22.6]	16.0 [14.9 - 20.3]	16.5 [14.9 - 20.8]	16.4 [14.6 - 20.8]	3.2 [2.4 - 3.9]	3.6 [3.2 - 4.4]	3.7 [3.1 - 4.7]

Figure S1 Experimental design.

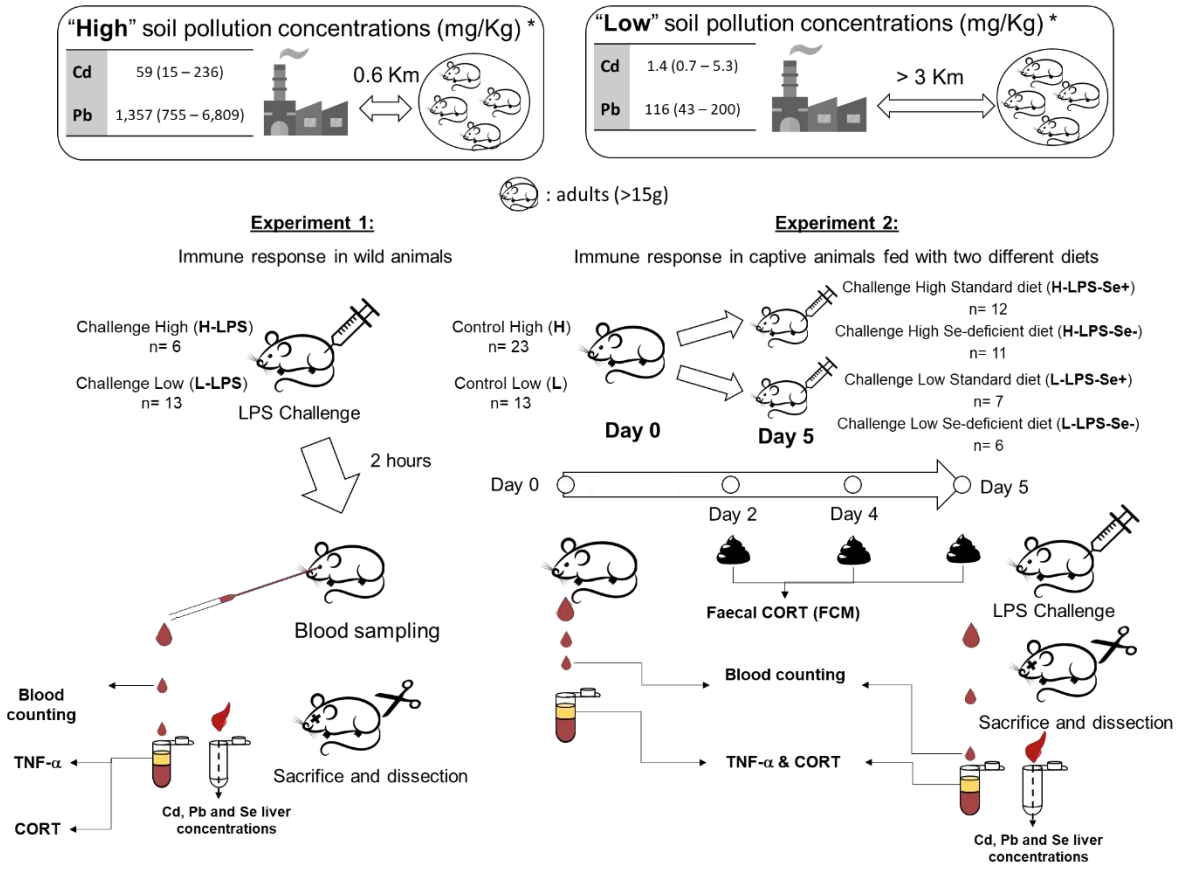


Figure S2 Picture of boxes where animals were maintained in captivity for 5 days.

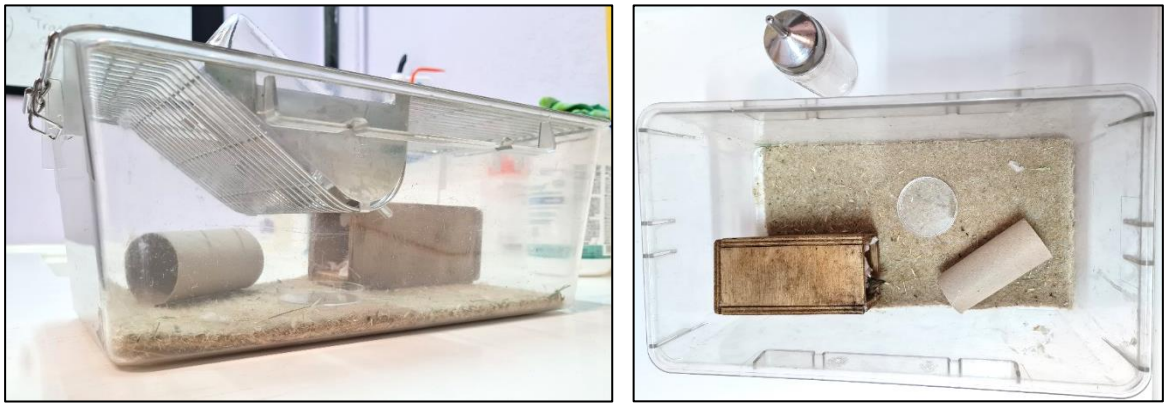


Figure S3 Cadmium (Cd), lead (Pb) and selenium (Se) concentrations (µg/g, log scaled) in the liver of captive and non-captive wood mice among contrasted levels (high and low) of pollution. Levels of significance: $p > 0.5$: ns, $p < 0.5$: *, $p < 0.01$: **, $p < 0.001$: ***.

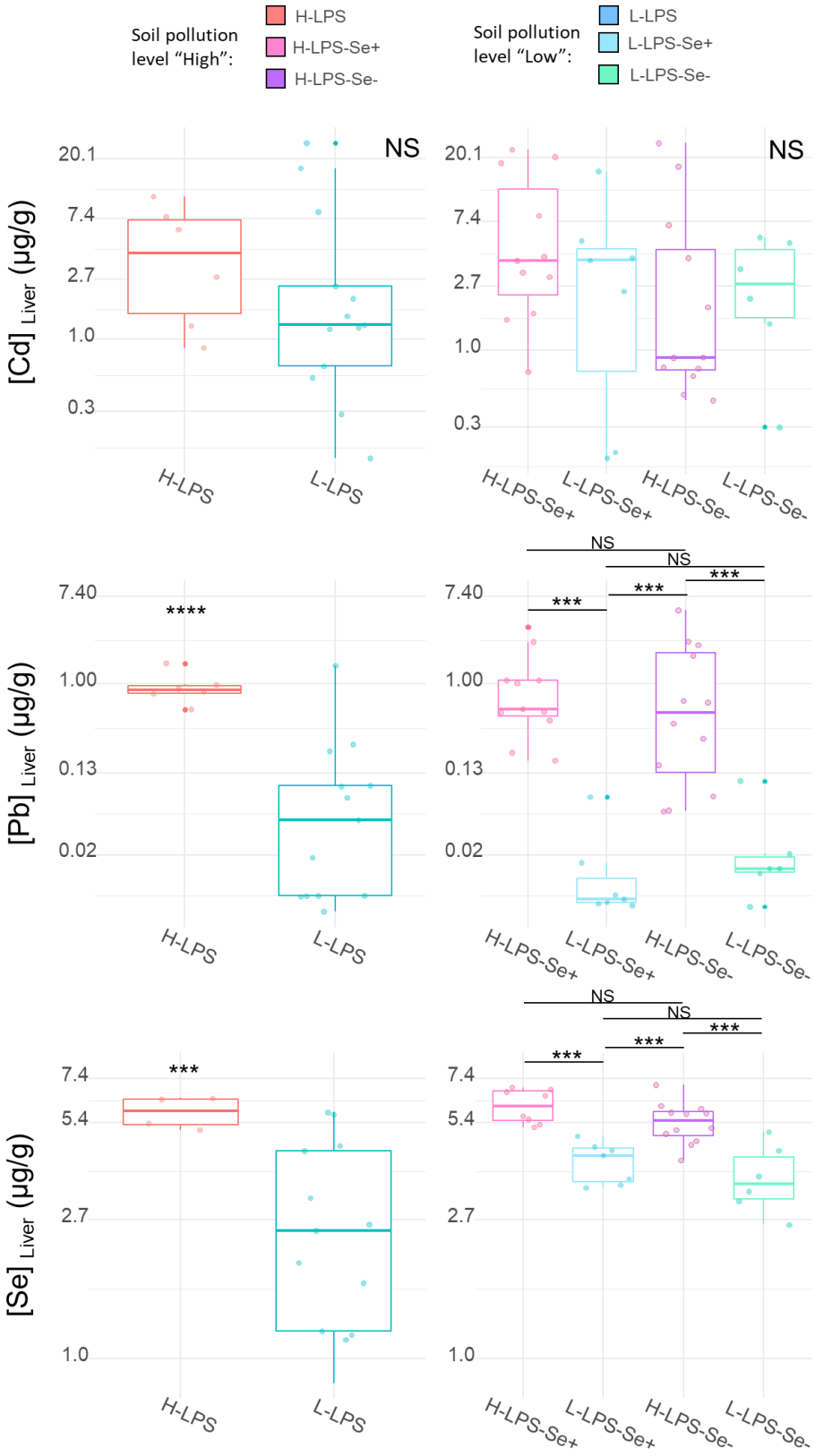


Figure S4 Leukocytes (WBC, log scaled) count between challenged (H- or L-LPS, H- or L-LPS-Se+ D5, H- or L-LPS-Se- D5) and unchallenged (H, L, H- or L-LPS-Se+ D0, H- or L-LPS-Se- D0) wood mice from contrasted levels of soil pollution with Cd and Pb (see Tables 1 and S1 for acronyms). Levels of significance: $p > 0.5$: ns, $p < 0.5$: *, $p < 0.01$: **, $p < 0.001$:***.

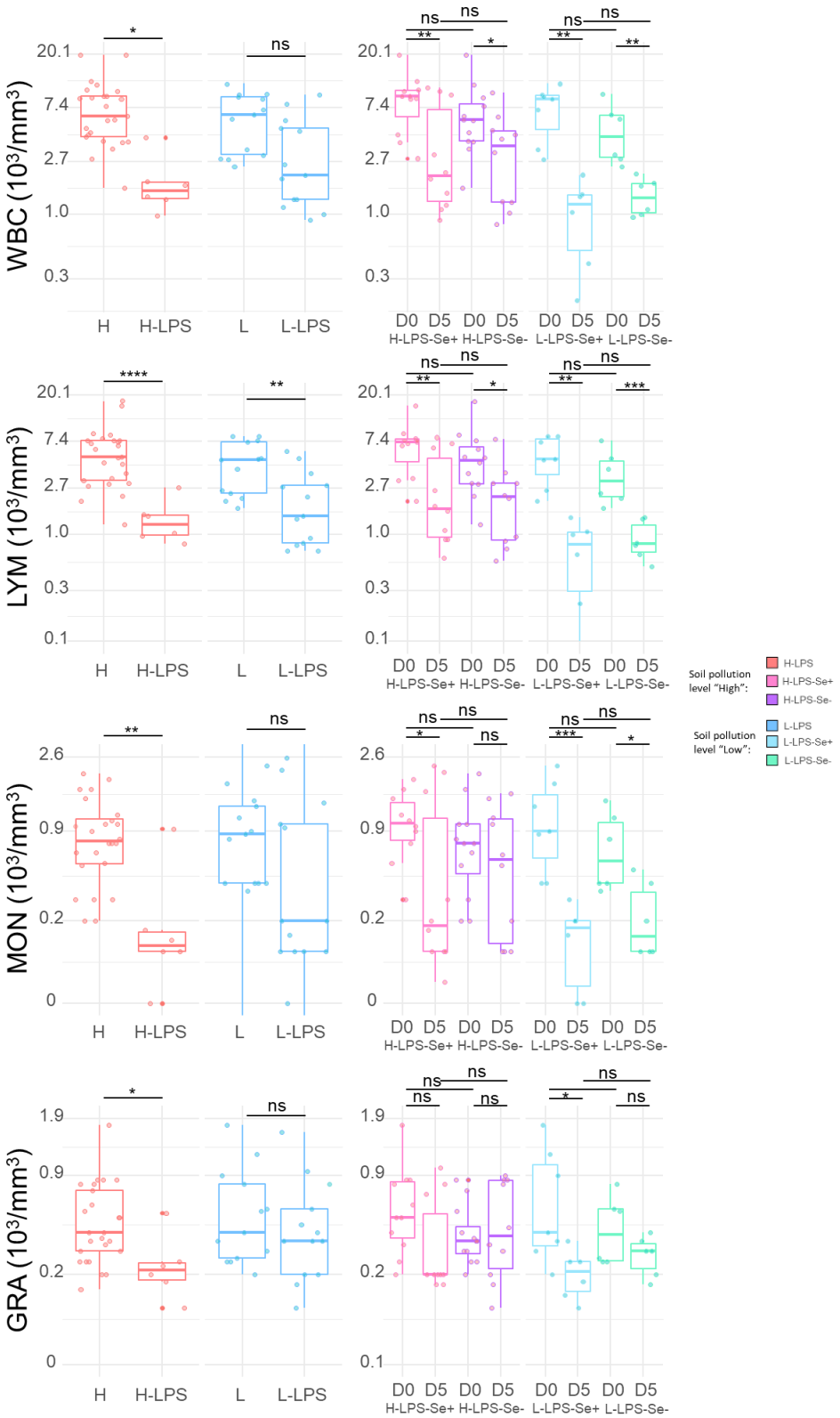


Figure S5 Experiment 1. Variations of white blood cells (WBC), lymphocytes (LYM), monocytes (MON), granulocytes (GRA) counts in response to plasma TNF-α and CORT after LPS challenge in individuals from “High” (red dots) and “Low” (blue dots) levels of soil pollution.

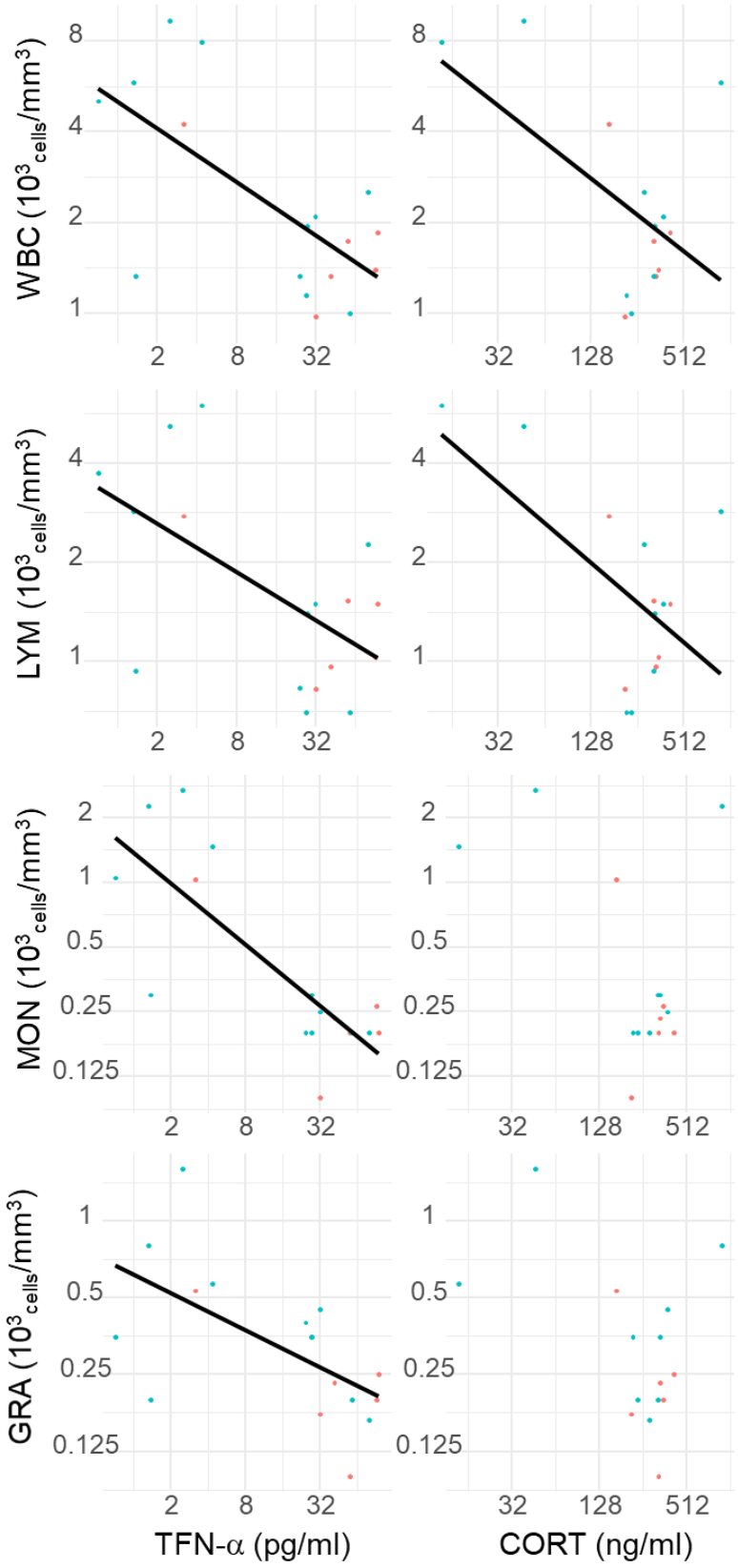


Figure S6 Experiment 2. Variations of white blood cells (WBC), lymphocytes (LYM), monocytes (MON), granulocytes (GRA) counts in response to plasma TNF-α and CORT after LPS challenge in captive wood mice from “High” levels of soil pollution either fed with standard (H-LPS-Se+) and Se-deficient (H-LPS-Se-) diets.

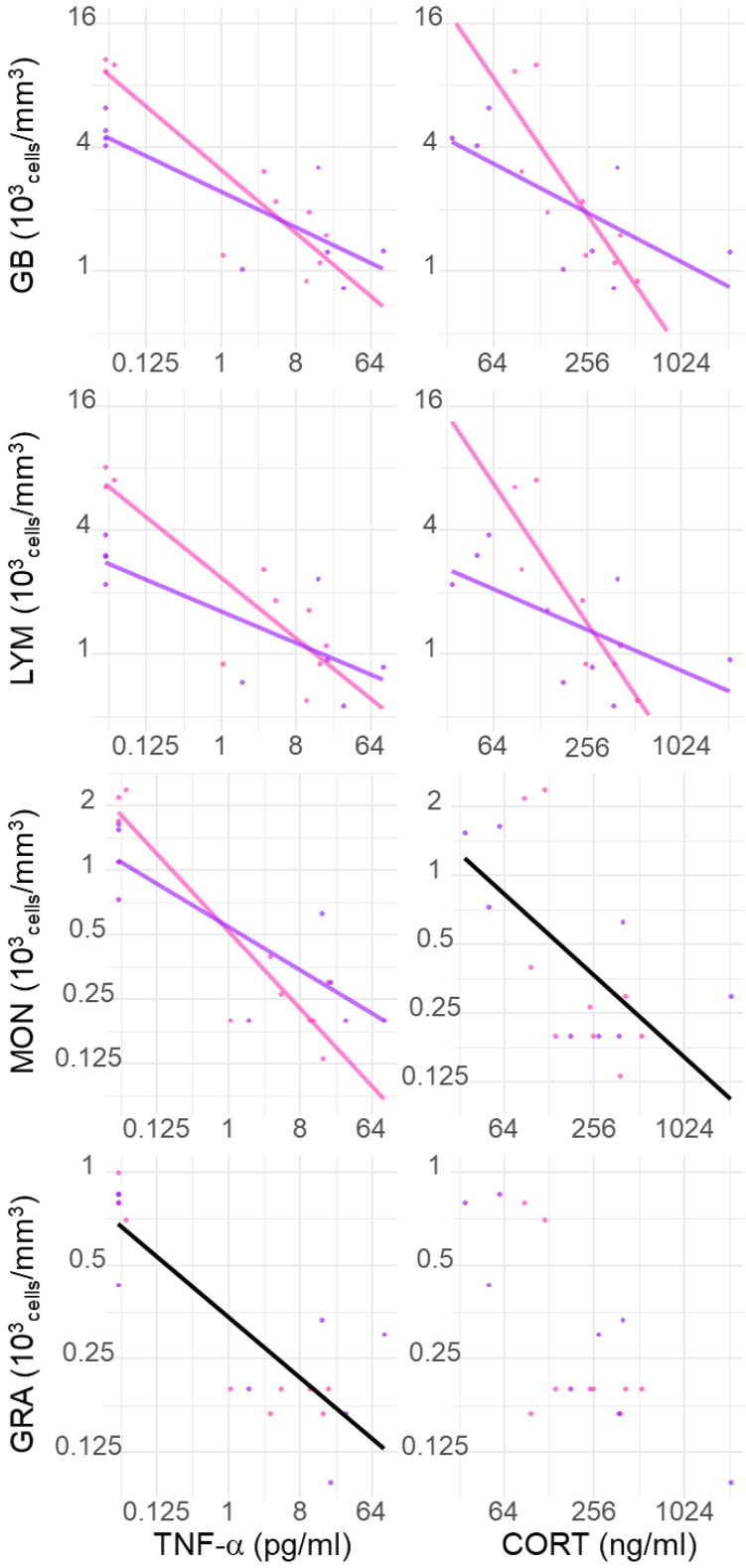


Figure S7 Correlation matrix of Spearman of the physiological variables measured in wood mice captured on contrasted levels of pollution (high and low) measured 2 hours after LPS challenge (i.p. injection).

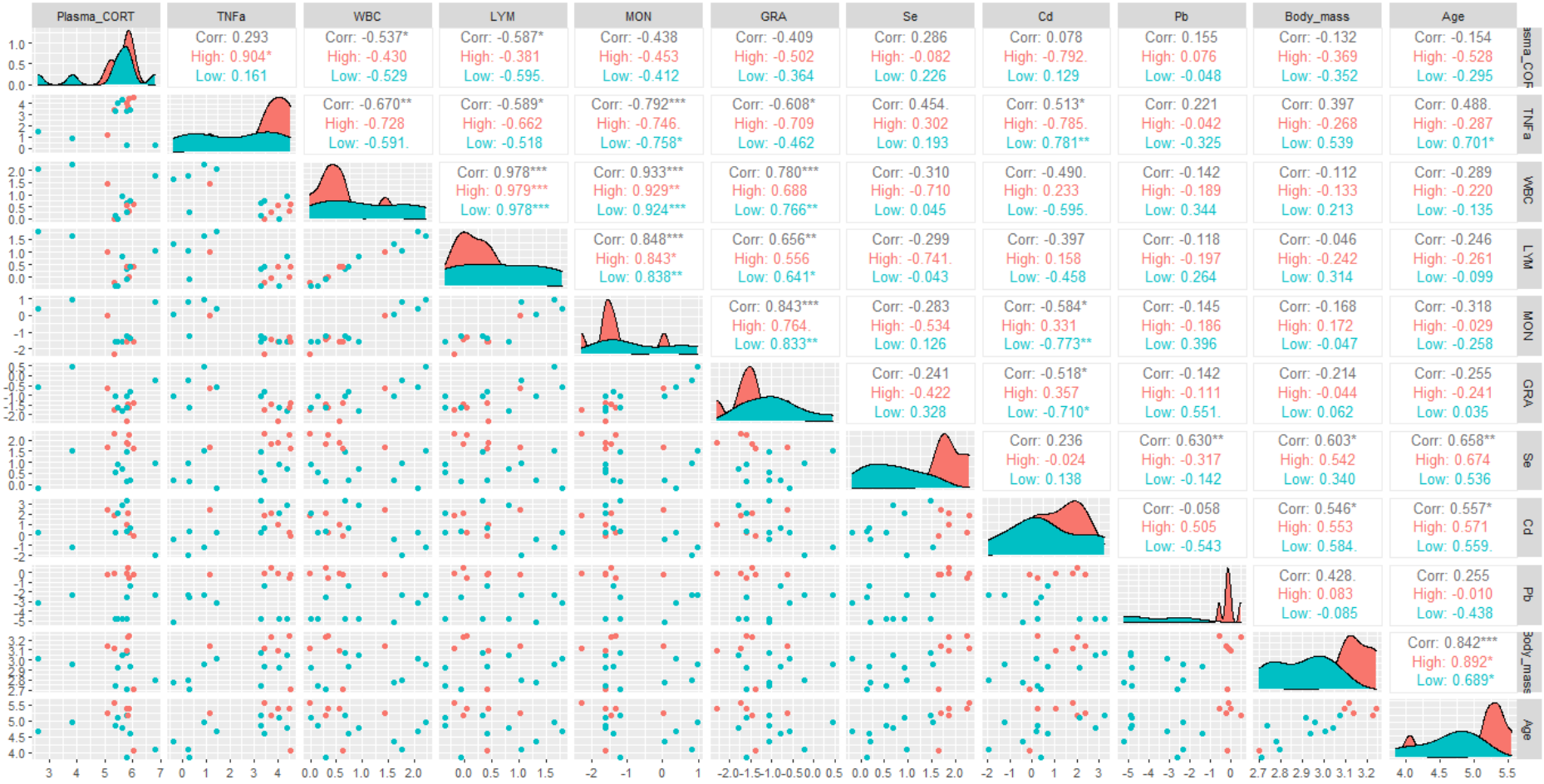
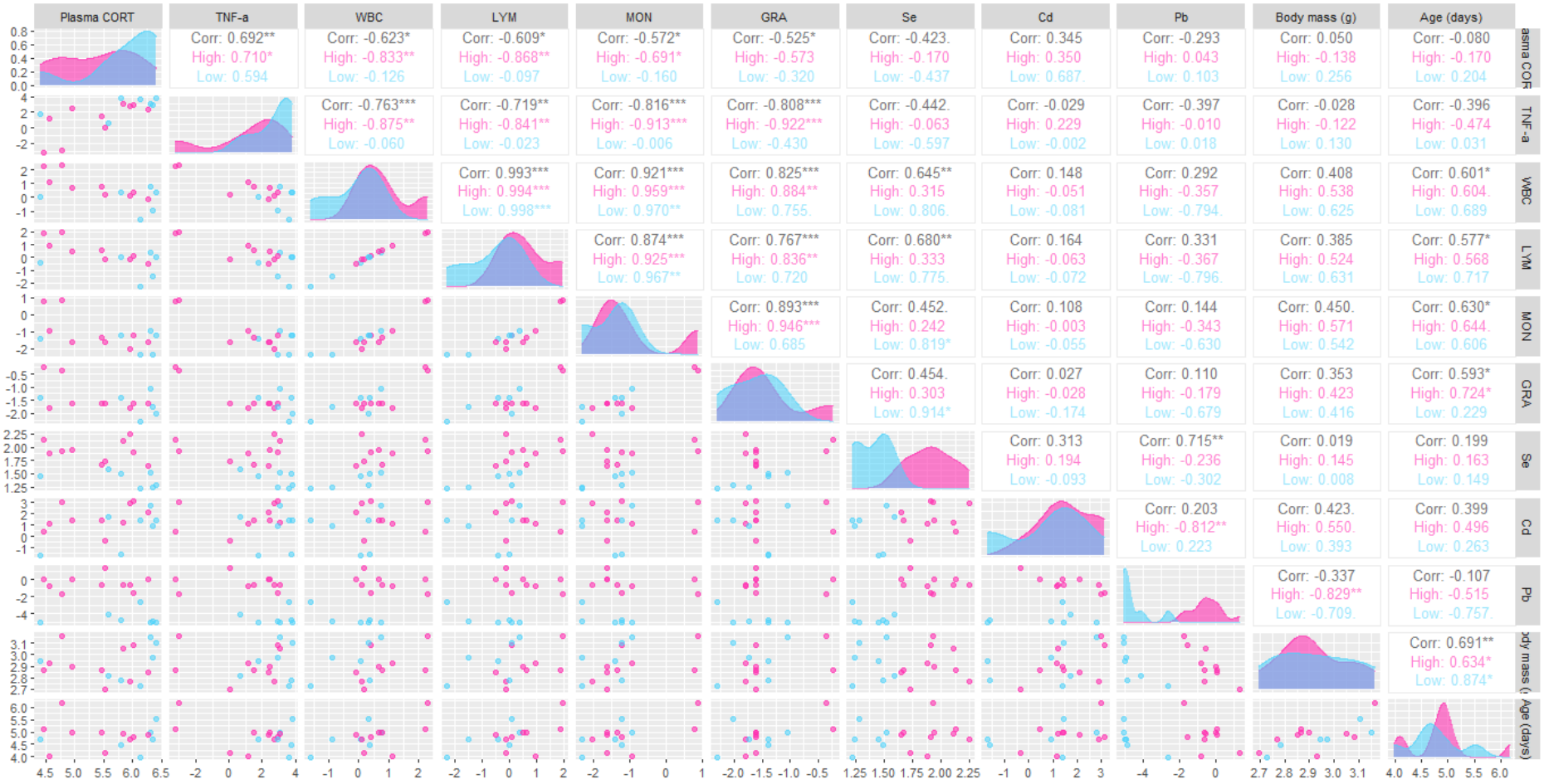
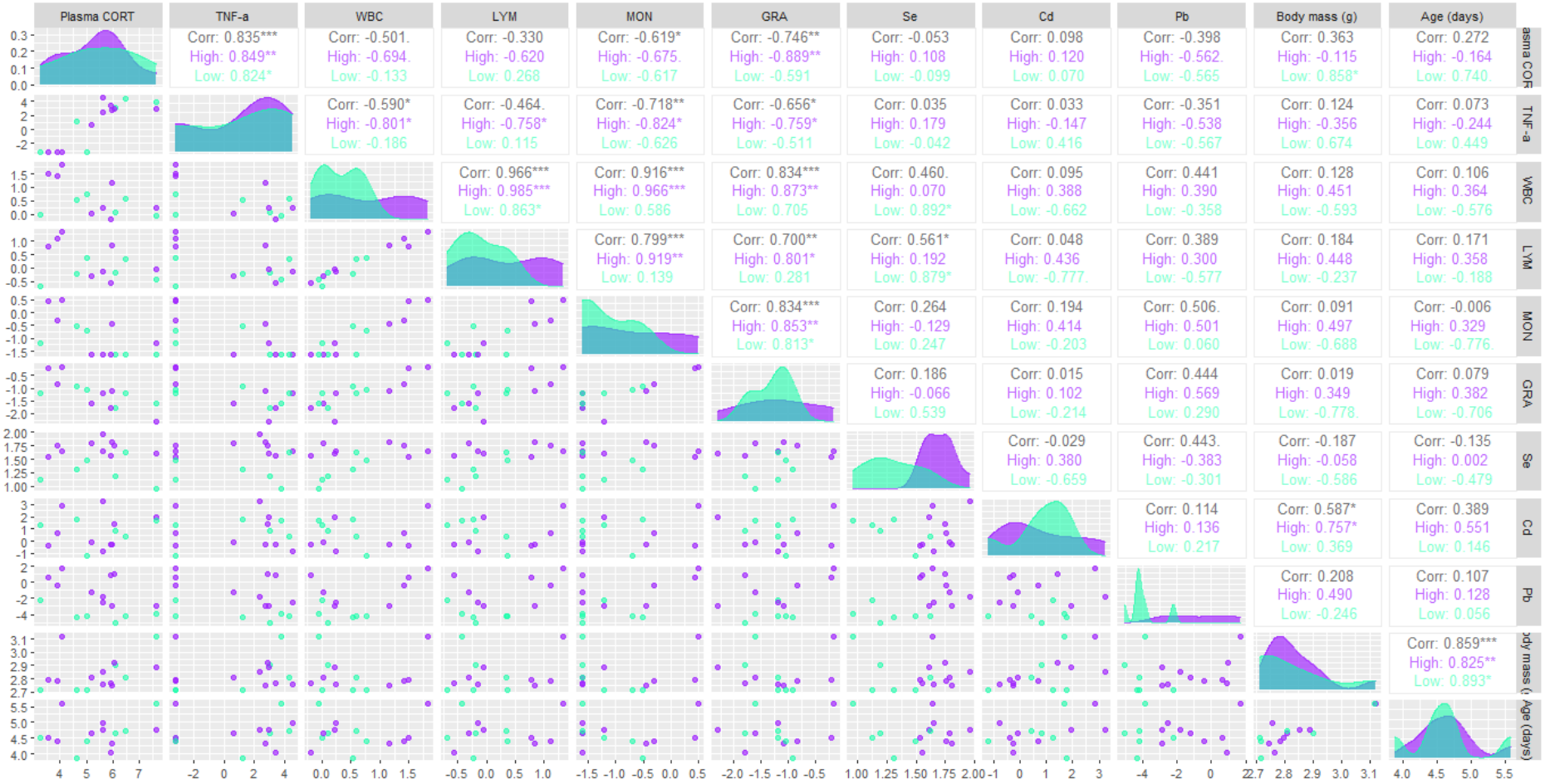


Figure S8 Correlation matrix of Spearman of the physiological variables measured 2 hours after LPS challenge (i.p. injection) in wood mice captured on contrasted levels of pollution (high and low) at 5 days of captivity with standard (A) and selenium deficient laboratory rodent diet.

A/Standard diet



B/Deficient diet



3.3 Chapter 3 (manuscript in preparation)

Moving from haematological parameters analysed at population level to individualised health assessment: reference intervals and blood profiles in small mammals

C. Hadjadji[1,*], Q. Devalloir[1,*], C. Fritsch[1], N. van den Brink[2], V. Driget[1], M. Coeurdassier[1], F. Raoul[1], R. Scheifler[1]

[1]UMR 6249 Chrono-environnement, CNRS / Université Bourgogne Franche-Comté USC INRAe, 16 route de Gray, 25030 Besançon cedex, France

[2]Sub-department of Toxicology, Wageningen University, Box 8000 6700 EA, Wageningen, The Netherlands

*Both authors contributed equally to the paper

Corresponding author: Q. Devalloir (quentin.devalloir@gmail.com)

Abstract:

Haematological parameters remain among the principal proxies to assess health. Haematological reference intervals (RI) represent powerful diagnostic tools to assess health status of human and domestic animals. They are, however, available for a very limited number of wild species, preventing their use to assess health of wildlife at both individual and population levels for *e.g.*, conservation purposes. In rodents, RI exist for lab mouse, rat, and hamster but a growing body of evidence shows that wild species, even close phylogenetically, exhibit different haematological and immunological patterns and responses than lab-reared counterparts. To determine RI, we investigated the effects of environmental and biological variables on 14 haematological parameters using a large dataset of three wild species, the wood mouse *Apodemus sylvaticus* (*n* = 321), the bank vole *Myodes glareolus* (*n* = 71) and the yellow-necked mouse *Apodemus flavicollis* (*n* = 38). Biological variables as gender, age class, body condition and trace metals (TM) concentrations in liver had little effects on blood parameters. In contrast, environmental variables such as region and season were the main drivers of haematological parameters. We determined RI in the wood mouse and provided indicative values for the two other species. We then performed individual health assessment of the individuals that were out of RI values, as it is classically performed in human and veterinary medicine. We argue that this approach brings new insights in the understanding of wildlife health, and that the determination of haematological RI in wild species, particularly associated to other health parameters such as infectious or nutrition status, should be of particular interest to conservation of wildlife.

Keywords: Free-ranging mammals, haematology, reference intervals, wildlife health, immunology

Introduction

Haematological parameters have been used in human health assessment for decades to determine physiological and biological profiles, which give information about probable diseases, infections, or organ deterioration (Etim *et al.*, 2014; Jacob, 2016). These parameters are also used for veterinary purposes, so their variations and particularities have been widely reported in many pets as rabbits, cats, or dogs (Jensen *et al.*, 1998; Özkan *et al.*, 2012; Yaqub, 2013). Furthermore, environmental changes can influence haematological parameters in domestic animals as cattle (Mazzullo *et al.*, 2014). As in human health, blood parameters can be used as an indicator of health status, and thus, be a basis for diagnosis (Otto *et al.*, 2000). In a logical sequence, resolving ecological interrogation using physiological tools represent a growing field of research (Johnstone *et al.*, 2017). The field of wildlife ecology and ecotoxicology has begun to appropriate these tools to assess health status of wild animals. Published data on wildlife health are still limited, although this is an area that tends to expand (Acevedo-Whitehouse and Duffus, 2009b). Using blood and its parameters becomes one of the most common methods to assess wildlife health (Kophamel *et al.*, 2022). Haematological parameters have been often used in population management as indicators to indirectly assess adaptation of animals to their habitat (A.C Nunes *et al.*, 2001). Non-destructive haematological biomarkers could help to reflect an impact on the condition of the studied animals (Rogival *et al.*, 2006a). According to Weeks *et al.* (1992), changes in these parameters could provide early warning signals of physiological variation, possibly due to toxic effect of pollutants or anthropogenic activities in general (Acevedo-Whitehouse and Duffus, 2009b; Martin *et al.*, 2010). In addition, haematology requires relatively low amount of blood and constitutes a lowly invasive approach, which is more and more required for both scientific (long-term surveys of populations) and ethical reasons.

Haematological assessment in wildlife has many interests, advantages, and benefits from the development and the democratisation of haematology analysers and automated instruments. Both white and red blood cells are important factors of fitness and condition (Beldomenico *et al.*, 2008b). Leukocyte profiles inform directly about the relative proportion of each white blood cells (WBC) type (*e.g.* lymphocytes, monocytes, granulocytes…) that are circulating in the blood during the sampling (Davis *et al.*, 2008). Lymphocytes are the direct results of acquired immunity, thus their multiplication indicates a response to a stressor agent or to a pathogen. Because of their long lifespan in the blood, they are mainly eliminated by glucocorticoids (Robert M Sapolsky *et al.*, 2000), during immunosuppressive mechanisms (Feldman *et al.*, 2000; Beldomenico *et al.*, 2008; Scott and Stockham, 2013). Monocytes concentrations increase when subacute and chronic inflammation occur (Feldman *et al.*, 2000; Tizard, 2004).

Granulocytes have a major role in the immune function, they increase rapidly in response to cytokines released during injuries of bacterial infections (Tizard, 2004). Concerning erythrocytes, red blood cells (RBC), haematocrit (HT), haemoglobin (HB) and some other related parameters can easily be measured and calculated such as mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular haemoglobin (MCH) and red blood cell distribution width (RDW). HT and HB are markers for oxygen carrying capacity and oxygen transport. Moreover, RBC are major sites in the blood for reactive oxygen species (Maceda-Veiga *et al.*, 2015a). Low HB concentrations can indicate a possible anaemia and iron deficiencies (Weiss *et al.*, 2010). In addition, low concentration of RBC is mainly due to infection, parasite, or food shortages (Scott and Stockham, 2013). This decrease may also be caused by haemolysis and a diminution of erythrocyte production. Red blood cells in mice have a short lifespan of around 50 days, thus anaemia is to a certain degree quickly visible (Kurata *et al.*, 1993).

These metrics, however, have limitations in terms of health and condition indicators (Davis *et al.*, 2008; Johnstone *et al.*, 2017). Leukocytes profiles are only a rough estimation of immune cell composition and do not really constitute a measurement of a functional immune response (Davis *et al.*, 2008). Contrast between low levels of and very low levels of leukocytes need to be related to the activity of specific WBC to perform efficient immune response (Davis *et al.*, 2008). Immune responses are functional and can be defined as a "complex and interactive framework of specific and non-specific humoral and cell-mediated components" (Acevedo-Whitehouse and Duffus, 2009b; Tizard, 2004). Thus, a sharp decrease of immune cells involved in one or both components of the immune system (*i.e.* humoral and/or cell-mediated) would have severe impact on the ability of host to eliminate pathogens. Concerning erythrocyte parameters, it might also be difficult to interpret them alone, a combination of them being often required to reach a potential diagnosis (Johnstone *et al.*, 2017). Interpretation of such parameters requires caution and needs to be done regarding the ecological context (Beldomenico *et al.*, 2008).

In ecology, haematology has many applications and interests, especially in the understanding of animals cost–benefit decisions, which is the general topic of several studies (McNamara and Houston, 1996; Maceda-Veiga *et al.*, 2015a). Studying blood also represents an ecological interest because it helps to perceive the relationship of blood characteristics regarding the environment (Etim *et al.*, 2014). Leukocytes and erythrocytes profiles are evidence for predictable responses to different types of stressors such as season, life history event, nutrition, disease, parasitism or toxic exposure (Davis *et al.*, 2008; Johnstone *et al.*, 2017). Blood parameters allow studying health and parasite infection dynamics. Significant variations in leukocytes and erythrocytes profiles according to the season have been reported in field

voles (Beldomenico *et al.*, 2008b). Moreover, haematological parameters are increasingly used in ecotoxicology to discriminate populations from unpolluted and polluted sites, or along a pollution gradient (A.C Nunes *et al.*, 2001; Rogival *et al.*, 2006a; Tersago *et al.*, 2004). In the Algerian mouse, significant differences in MCHC and MCH regarding the site, polluted or not, were highlighted (A.C Nunes *et al.*, 2001). Correlation between RBC count and metal exposure along a trace metal (TM) pollution gradient while no differences in WBC among sites were reported (Tersago *et al.*, 2004). Significant differences in HT, MCV and MCHC between different polluted study sites were reported (Rogival *et al.*, 2006a). In this study, however, MCV, HB, RBC and WBC showed no difference among the same sites.

Haematology can thus be a powerful tool to discriminate populations, at least for some blood parameters and particularly when study populations inhabit sufficiently different environments. Interpretation of data, however, is limited by the lack of “normal ranges” (usually called “reference intervals” in the scientific literature) for species other than human and animal models frequently used in animal experimentation such as lab rats and mice (Beldomenico *et al.*, 2008b; Davis *et al.*, 2008; Maceda-Veiga *et al.*, 2015a; Johnstone *et al.*, 2017; Broughton *et al.*, 2017). Determining reference intervals (RI) for haematological parameters therefore constitutes an important issue in wildlife ecology. RI also allow to be more precise in the analysis of haematological profile of any individual sampled (Rostal *et al.*, 2012; Maceda-Veiga *et al.*, 2015a; Johnstone *et al.*, 2017). Having RI could help to discriminate individuals and study their health status more deeply, complementarily to investigation at population level. Reference values of haematology, as in human health or for domestic animals, could represent a useful and an important indicator of the health status of both individuals and population of wild animals (Rostal *et al.*, 2012; Kophamel *et al.*, 2022). One of the objectives of this approach is, at the end, to be able to realise a monitoring focused on the individual level and then provide information in the case of population decline or emerging pathogens (Rostal *et al.*, 2012; Kophamel *et al.*, 2022). This individual-based health assessment, on top of being complementary of an assessment at the scale of the population, might be of interest for elusive species for which few individuals can be captured, and/or for species of particular conservation concern. Determination of RI, however, should follow strict recommendation provided in the literature to ensure their reliability (Broughton *et al.*, 2017; Kophamel *et al.*, 2022). Commonly, RI are defined as range of values comprised between the 2.5^{th} and 97.5^{th} percentiles, which is the confidence interval of 95% (ISO 15189; International Federation of Clinical Chemistry, C28-A3). Sample size must be superior to 120 individuals (Geffré *et al.*, 2009; Friedrichs *et al.*, 2012). Individuals used to define RI should also ideally be rigorously selected regarding predefined criteria like age, sex, body condition or sexual activity (Geffré *et al.*, 2009).

The aim of this study is, first, to explore a large dataset to understand the causes of variation of 14 haematological parameters in small mammal species. Then, we determine RI for a ubiquitous and largely distributed small mammal species, the wood mouse (*Apodemus sylvaticus*) with a large sample size (*n* = 321). For two other species, the bank vole *Myodes glareolus* (*n* = 71) and the yellow-necked mouse *Apodemus flavicollis* (*n* = 38), we provide indicative data, but low sampled size prevents establishing true RI. Altogether, our dataset is made of 430 individuals belonging to three different species, coming from four different habitats (*i.e.* urban, industrial, agricultural, forest) and two regions of France (Bourgogne Franche-Comté and Hauts-de-France). Our hypotheses are that (i) populations can be discriminated upon some specific haematological parameters and/or multivariate analysis of the fourteen study parameters, (ii) using RI and haematological profiles, some individuals deviate from the RI of the population they come from, and (iii) these individuals present some special characteristics explaining why they deviate from the blood profile based on RI and are individuals at potential risk that might be studied further to assess individual-based health.

Materials and methods

1. Study sites

From 2014 to 2021, small mammals were captured in four habitat types (*i.e.* industrial, urban, forest, agricultural) in two regions of France (Bourgogne Franche-Comté and Hauts-de-France) at the pace of successive research programs (Figure S1). Individuals from the industrial habitat type were all captured near the former lead (Pb), zinc (Zn) and cadmium (Cd) smelter site of Metaleurop Nord (Noyelles-Godault, France, 50° 25′ 15″ N, 2° 59′ 41″ E). Soil contamination by TM in this area has been widely documented (Douay *et al.*, 2009b; Fritsch *et al.*, 2010c). TM concentrations vary from 16 to 41,960 µg/g, from 44 to 38,760 µg/g and from 0.1 to 2,402 µg/g for Pb, Zn and Cd, respectively (Fritsch *et al.*, 2010c). Concentrations decreased with the distance from the former smelter site and are related to SW-NE winds. In brief, small mammals were sampled along a gradient with roughly four different levels (high, moderate, low pollution and control). Capture sessions in Metaleurop Nord were performed in September and October in 2014 and 2020. Individuals from the urban habitat type were captured in two different locations of the city of Besançon (France, 47° 14′ 35″ N, 6° 01′ 19″ E), the campus of La Bouloie and “Place Leclerc”. Sampling was done in April 2014 and in August 2021. Individuals from the forest habitat type were captured in the “Forêt de Chaux”, which is the second largest deciduous forest in France located in the region of Bourgogne Franche-Comté (47° 04′ 46″ N, 5° 40′ 58″ E). The dominant species is the Sessile oak (*Quercus petraea)*. Capture sessions in the Forest of Chaux were realised in April 2014. Individuals from the agricultural habitat type were captured in the long-term ecological research (LTER) site

“Jurassian Arc” (https://zaaj.univ-fcomte.fr/). These individuals were captured in April and July 2015, in two organic and three conventional agriculture parcels.

Soil occupancy was characterized in each habitat around all trap lines (see § 2.2). Data of soil occupancy were extracted with the software QuantumGIS v. 3.22 from a classification map provided by Theia laboratory with a resolution equal to 10x10 m. The classification is based on CORINE Landcover code. Centre of each trap line was calculated, and soil occupancy was described in a buffer of 500 m of radius. Once the extraction was completed, we combined some land use classes relatively similar to keep for statistical analysis six classes (crops, forest, industrial, openfield, urban, and water).

2. Trapping and blood sampling

Non-lethal trapping was realised during each field session by using INRA traps. They are coupled with a “dormitory” (*i.e.* small plastic boxes), where hay and food supply (peanuts, sunflower seeds, cat food, apple) are provided to the animals, allowing to keep them alive for at least 24h. All the individuals that were not rodents (*e.g.* shrews) were immediately released. Between 8 and 68 traplines of ten traps spaced of around 5 m were set on the field in the different habitat. Most of animals were measured and weighed. The gender was determined upon the presence of testicles or suckling, and by anogenital distance. For wood mice, individuals weighing up to 15 g were considered as juveniles while animals above 15 g were considered as adults (adapted from Navarro-Castilla and Barja, 2014). Concerning bank voles, individuals weighing up to 17 g were considered as juveniles while animals above 17 g were considered as adults (adapted from Ecke *et al.*, 2020). For yellow-necked mice, the body mass threshold taken for age class determination was 20 g (adapted from Marsh *et al.*, 2008). Animals were anesthetised by inhalation with isoflurane using a Univentor 410 Anaesthesia Unit. Blood was collected from the orbital sinus with a Pasteur pipette and immediately transferred in EDTA coated tubes. Storage of blood samples was done at 4°C until analysis.

3. Measurement of haematological parameters

Haematological parameters were measured within 8 to 10 hours after the blood sampling to guarantee a good quality of measurement. In total, 14 blood parameters were determined using an automated Micros 60 Haematology Analyser (Horiba ABX Micros ES60, guaranteed CV of measurements 0.5-5%). We analysed total WBC counts (10^3/mm^3), total counts and percentage of lymphocytes (LYMn and LYM%, respectively), of monocytes (MONn and MON%), and of granulocytes (GRAn and GRA%), total RBC (10^6/mm^3), HB (g/dL), HT (%), MCV (μm^3), MCH (pg), MCHC (g/dL) and RDW (%). Horiba SAS provided the reagent kits necessary for the blood analysis. Repeatability (within-run precision) is measured

automatically by the Micros device (RSD). Reproducibility (run-to-run precision) of the measurements and quality control (accuracy) is certified by measuring 3 blood controls (provided by Horiba SAS; the 3 different controls referred to low, medium, high levels of each parameter) with known concentrations and blanks (saline solution) at the beginning of each session of analyses and every 30 runs in case of long-lasting measurement session. Each sample was analysed between two and four times and the average of replicates per individual was kept for statistical analyses.

4. Determination of metal concentrations

The concentrations of cadmium (Cd) and lead (Pb) were determined in the liver of 142 wood mice from the area of Metaleurop Nord (Powolny *et al.* Submitted). In brief, livers were dried in an oven at 60°C until constant mass. Liver dry samples were digested in nitric acid (HNO3, 69%, analytical quality, Optima) and diluted by adding ultra-pure water (Elga, 18.2 MV/cm²) prior to analyses. Metals in the liver were analysed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, ThermoFischer Scientific XSeries 2). For the values under quantification limits, values were replaced by the quantification limit divided by square root of two for statistical analysis (Helsel, 2010). Repeatability (within-run precision) and reproducibility of the measurements were surveyed (checking of RSD values and use of internal standards), and quality control (control solutions and blanks to check for the absence of drift) was conducted during measurements. Analysis accuracy was checked using certified reference material (National Research Council Canada TORT-2 Lobster hepatopancreas. Blanks (acid + ultra-pure water) and certified reference material were prepared and analysed using the same methods as the samples. Average (n = 9) recovery was 93% for Cd and 103% for Pb. Concentrations of metals in the liver are expressed as µg/g dry mass.

5. Ethical issues

Sampling authorisation was given by the Direction Régionale de l'Environnement, de l'Aménagement et du Logement (DREAL) of Bourgogne Franche-Comté and Hauts-de-France region. Experiments were performed under the authorization of the French National Ethical Committee by skilled and experienced investigators from Chrono-environnement research department (EU0592), following directive 2010/63/EU on the protection of animals used for scientific purposes.

6. Database management

Before the beginning of the statistical analysis, we grouped and organized all the available data obtained during different research programs between 2014 and 2021. To construct this database, we used SQL request in the software *R* v. 4.1.1 (R Development Core Team, 2022)

with the packages *dplyr* and *sqldf*. At the end, we obtained a full dataset composed of 445 rows, where each row corresponds to one individual. The dataset is composed of individual variables (species, mass, length, body condition, gender, age class, TM concentration in the liver, haematological parameters) and of related environmental variables (region, habitat, soil occupancy, season, geographic coordinates).

7. Statistical analysis

Exploratory analysis

The initial approach was an exploratory analysis where the possible effects of biological and environmental qualitative (gender, age class, region, habitat, season) and quantitative (body condition, TM liver concentrations, soil occupation) variables have been tested on the whole dataset or for a subset of individuals for which the variables tested were available. The effect of categorical variables has been tested using non-parametric Wilcoxon Mann-Whitney (for non-paired variables) or Kruskal Wallis test. Kruskal Wallis tests, when significant, were followed by a post-hoc multiple comparison test. The effect of quantitative data on haematological parameters were studied using non-parametric Spearman rank correlations. We represented these correlations using Spearman correlation matrix, which is a multipanel display of pairwise relationships between variables (Borcard *et al.*, 2018).

Body condition index (BCI) was computed for 265 wood mice out of the 364 sampled, 68 bank voles out of the 71 and 36 yellow-necked mice out of 38, after the suppression of pregnant females and individuals for which we did not have the body mass and/or the body length. For the calculation, we used the method proposed by Peig and Green (2009) based on a standardised regression axis (SMA) and called Scale Mass Index (SMI, Figure 1).

Figure 1. Equation for the calculation of the BCI/SMI where mi and L_i are the body mass (g) and the body length (mm) of an individual i, respectively ; L_0 is the arithmetic mean value of the study population and bSMA is the scaling exponent estimated by the SMA regression (Peig and Green, 2009)

$$\mathrm{SMI} = mi\left(\frac{L_0}{L_i}\right)^{\mathrm{bSMA}}$$

.

Multivariate analysis

For the multivariate approach, we applied canonical ordination using Redundancy Analysis (RDA) on centred data. RDA is a method that combined a linear model based on multiple

regression model and a Principal Component Analysis (Legendre and Legendre, 2012; Borcard *et al.*, 2018). The aim of this method was to find any tendencies between haematological parameters (response variables) selected and the explanatory variables matrix. The selection of explanatory variable was done according to their biological relevance and were trimmed to avoid autocorrelation. In addition, we represented an ellipse of the standard deviation of points on the RDA triplot (scaling 2), representing a 95% confidence limit. RDA scaling type 2 allow to identify the effect of explanatory variables according to the angles of the arrows (Borcard *et al.*, 2018; Legendre and Legendre, 2012). Normality of data (Shapiro-Wilkinson test) of each response variables were checked. We applied log(x+1) transformation for LYMn, MONn and GRAn to reach normal distribution of the data. Significance of the canonical axis was tested using *anova.cca()* and RDA were considered only if at least two axes were significant ($p < 0.05$).

Definition of reference intervals

Reference intervals were determined with non-parametric methods for all the 14 haematological parameters by using 95% confidence intervals (CI 95%) of the 2.5th and 97.5th percentiles (Friedrichs *et al.*, 2012). Ideally, reference intervals need to be calculated with a sample size equal to at least 120 individuals (Geffré *et al.*, 2009; Friedrichs *et al.*, 2012), which is the case for the wood mouse but nor for the yellow-necked mouse and the bank vole. For those two species, results are indicative but cannot be considered as true RI.

All statistical analysis were performed with the software *R* v. 4.1. (R Development Core Team, 2022). Post-hoc multiple comparison test was computed using the package *pgirmess.* Spearman correlation matrix was computed using the package *corrplot*. RDA were performed using the following packages: *ade4, factoextra, FactoMineR, vegan.* Spider charts were processed using *fmsb*.

Results

A total of 364 wood mice, 38 yellow-necked mice and 71 bank voles were captured between 2014 and 2021, at three seasons (spring, summer, autumn), in four different habitat types (agricultural, forest, industrial, urban) within two French region (Bourgogne Franche-Comté and Hauts-de-France, Table S1). Individuals from the industrial habitat (the former smelter of Metaleurop Nord) were captured during a long-term monitoring, at the same period (autumn) of the year. Statistical analyses were performed on 321 wood mice, 38 yellow-necked mice, and 71 bank voles because 43 wood mice over 364 were removed from the dataset. We excluded the individuals that presented abnormalities in the haematological values (*e.g.* LYM equal to 100% or missing data, $n = 15$) and the challenged individuals ($n = 28$).

1. Exploratory analysis

Biological parameters tested did not have a major impact on haematological parameters in the wood mice (Table S2). Males had higher MONn level than females, in contrary to erythrocyte indicators (HT and MCHC), which were higher in females. MONn and RDW were higher in adults, with medians respectively equal to 0.6 $10^3/mm^3$ and 15.9% for the adults, while they were equal to 0.4 $10^3/mm^3$ and 15.4% in juvenile. Positive correlations were observed between BCI and MCH (rho = 0.27), MCV (0.22) and RDW (0.17, Figure S2). Lead concentrations in liver were negatively correlated to RDW (-0.22). Environmental variables had a greater impact on haematological parameters (Table S2, Figure S1). RBC were higher in agricultural habitat compared to the others while HB were only significantly different in the agricultural habitat compared to the industrial one. HT were higher in the agricultural habitat compared to the industrial and the urban habitat. MCH and MCHC were higher in the urban habitat compared to the agricultural and industrial habitat. MCHC were also significantly different between forest, agricultural and industrial habitat. LYMn were higher in the urban habitat compared to others. GRAn significantly differed between industrial and urban habitats. Both MON and GRA numbers and percentages, as well as all erythrocyte indicators but RDW and MCH, were higher in summer compared to spring and autumn. LYM, MON, MONn, GRA, GRAn, RBC, HB, HT, MCH and MCHC differed between regions. Their values were higher in the Bourgogne Franche-Comté *région*, except for LYM, which were lower compared to the Hauts-de-France. Several low (-0.22 ≤ rho ≤ 0.24) positive or negative correlations were found between haematological parameters and soil occupancy.

Concerning bank voles, none of the fourteen haematological parameters differs according to the gender. WBC, MON and RDW were higher in adults. As in wood mice, body condition was positively (rho = 0.31) correlated to RDW (Figure S3). WBC, RDW and MON significantly differed between habitats, seasons, and *region*, while LYM is only different between seasons. Bank voles captured in the agricultural habitat presented higher values of WBC, MON and RDW than individuals from the industrial habitat. WBC values were higher in summer than in autumn (medians equal to 5.0, 7.5 and 2.7 $10^3/mm^3$ for spring, summer, and autumn, respectively). MON values were also higher in summer compared to autumn (medians equal to 10.8, 14.0 and 8.5% for spring, summer, and autumn, respectively). RDW were the lowest in autumn, compared to both others season. Concerning the region, WBC, MON and RDW were higher in Bourgogne Franche-Comté (medians equal to 6.7, 13.3, and 15.4% for WBC, MON and RDW, respectively, in Bourgogne Franche-Comté, while they were 2.7, 8.5, and 14.6% in Hauts-De-France). Several low (-0.39 ≤ rho ≤ 0.27) positive or negative correlations were found between haematological parameters and soil occupancy (Figure S3). These

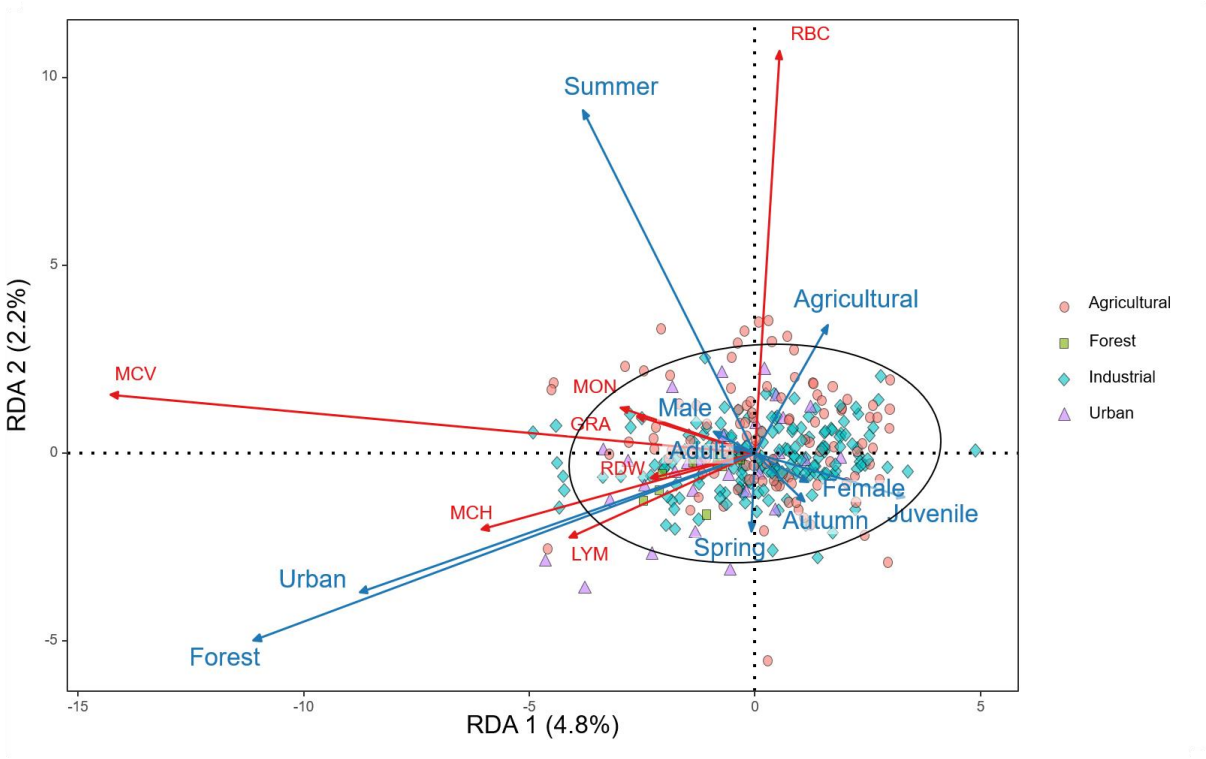
correlations, however, did not concern the same haematological and soil occupancy variables, preventing any generalisation from these correlations.

In yellow-necked mice, none of the fourteen haematological parameters differed according to the gender. Concerning the environmental variables, LYM, LYMn, MON, GRA and MCHC varied according to the habitat. Lymphocytes (count and %) and MCHC were higher in the forest habitat compared to agricultural areas, while the opposite pattern was observed for MON and GRA. LYM, GRA, RBC, HB and HT varied according to the season. LYM was the only parameter presenting significant higher values in spring, while the others were significantly lower during this season. Several ($-0.47 \leq rho \leq 0.47$) positive or negative correlations were found between haematological parameters and soil occupancy (Figure S4), here again between different variables than for both wood mice and bank voles, preventing generalisations.

2. Redundancy analysis

The matrix of explanatory variables was composed of gender, age class (except for *Apodemus flavicollis* for which age class was not included in the analysis because only adults were captured), habitat, and season. We did not include the region because it is a confounding variable, industrial habitats being only represented in Hauts-de-France while the three other habitats were only studied in Bourgogne Franche-Comté. Based on exploratory analysis, only LYM, MON, GRA, RBC, MCV, MCH, and RDW were kept in the matrix of measured variables in the RDA computed for the wood mouse. The explanatory variables significantly explained 7.9% of the variations of the haematological parameters ($R^2 = 0.079$, R^2 adjusted = 0.061, Figure 2). The first axis (RDA 1) explained 4.8% of the variation and significantly constrained the season, while the second axis (RDA 2) explained 2.2% and constrains the habitat type. Higher RBC counts were observed during summer with lower levels were observed in spring and autumn. In contrary to the RBC, increase in RDW and MCH were observed in spring, with lower values in summer and autumn. LYM and MCH were positively correlated to forest and urban habitats. Industrial habitat was negatively correlated to all haematological parameters, while agricultural habitat was positively correlated to RBC. MON and GRA were positively correlated to adult age class and to males. Twenty-seven individuals over 321 (8.5%) were out of the ellipse of the standard deviation (SD) of the global distribution. Over the 27 individuals concerned, 15 came from agricultural habitat, eight from industrial habitat and four from urban habitat. Then, eight, seven and 12 were captured in autumn, spring, and summer, respectively. There were 16 males and 11 females out of the ellipse, two of which were pregnant. In addition, there were 25 adults and two juveniles.

Figure 2. RDA triplot (scaling 2) of the haematological parameters data constrained by biological and environmental variables of *Apodemus sylvaticus* with the ellipse of the standard deviation of points (95% confidence limit). Individuals are discriminated with colours according to the habitat type.



For bank voles, WBC, RBC, MCV and IDR only were kept as measured variables (R^2 = 0.201, R^2 adjusted = 0.153) and LYMn, MONn, GRAn, RBC, HT and MCHC for the yellow-necked mouse (R^2 = 0.230, R^2 adjusted = 0.162). However, RDA was not convincing as the variability was only constrained on a single axis, meaning that structures were not linearly independent (Legendre and Legendre, 2012). Thus, we decided not to consider this in further analysis and interpretations.

3. Determination of the reference intervals

Regarding the exploratory and multivariate analyses, RI were computed for all wood mouse individuals (*n* = 321, Table 1). We decided not to calculate RI according to the significant variables evidenced previously, first because RDA results did not show strong variance in the haematological parameters considered, and second to keep in the RI calculation a large sample size (*i.e.* ≥ 120). Depending on the parameter, from nine to 23 individuals were lower or higher than the RI. Similar tables are provided in Supplementary Material for bank voles and yellow-necked mice (Table S5, Table S6). RI for these two species are only indicative as the

sample size constraint was not respected (71 and 38 individuals, respectively). For bank voles, from two to four individuals were lower or higher than the RI, depending on the parameter (Table S5). Concerning the yellow-necked mouse, only one to two individuals were out of the RI (Table S6).

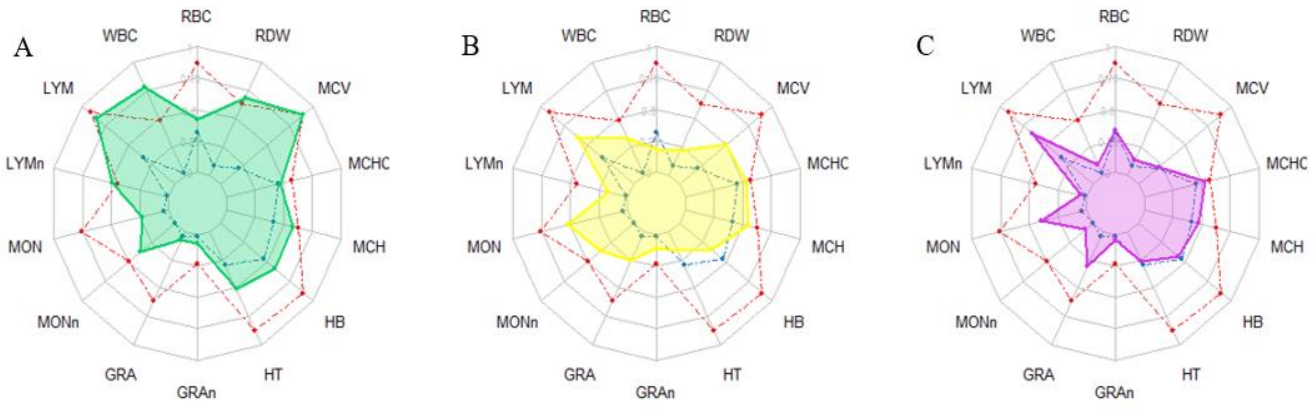
Table 1. *Statistical distribution (confidence intervals based on 2.5th and 97.5th percentiles, number of individuals lower or higher of this range, minimum, median and quartiles, maximum values, mean ± standard deviation (SD) and sample size) of haematological parameters in the wood mouse Apodemus sylvaticus*.

	CI (2.5% – 97.5%)	***n* < 2.5%**	***n* > 97.5%**	**Mean ± SD**	**Min**	**Median [Q1; Q3]**	**Max**
Leukocytes							
WBC (10^3/mm^3)	0.8 – 12.45	10	9	4.99±3.87	0.30	4.2 [2.5; 6.5]	25.50
LYM (10^3/mm^3)	0.65 – 16.05	10	9	4.01±4.35	0.20	3.1 [1.8; 5.1]	35.57
LYM (%)	54.55 – 93.75	9	9	75.88±14.03	31.90	78.8 [72.6; 83.4]	99.85
MON (10^3/mm^3)	0 – 2.2	11	9	0.69±0.65	0	0.5 [0.3; 0.9]	4.45
MON (%)	1.55 – 28.25	9	9	14.77±6.58	0	14.2 [11.5; 17.7]	37.35
GRA (10^3/mm^3)	0.1 – 1.8	23	9	0.50±0.62	0	0.4 [0.2; 0.6]	7.75
GRA (%)	1.3 – 18.7	9	9	7.50±5.04	0.05	6.4 [3.9; 9.9]	30.50
Erythrocyte indicators							
RBC (10^3/mm^6)	6.24 – 12.27	9	9	9.12±1.55	2.87	8.9 [8.2; 10.0]	13.66
HB (g/dL)	10.17 – 18.65	9	9	13.82±2.20	1.13	13.7 [12.5; 14.8]	20.60
HT (%)	30.77 – 62	9	9	44.35±7.39	14.47	43.7 [39.5; 47.9]	68.80
MCV (µm^3)	43.67 – 56	10	10	48.80±3.16	40.00	49.0 [46.5; 5]	58.00
MCH (pg)	13.2 – 17.85	9	9	15.28±1.73	3.93	15.2 [14.3; 16.0]	26.20
MCHC (g/dL)	29.35 – 34.73	9	9	31.34±2.77	7.83	31.0 [30.3; 3]	55.10
RDW (%)	13.85 – 19.75	9	9	16.13±1.45	13.00	15.8 [15.1; 16.6]	23.70

4. Blood profiles

To represent wood mice blood profile based on complete blood count (CBC), spider charts were produced, allowing the comparison of a given individual to the RI (Table 1, Figure 3). We realised a spider-chart for the 27 individuals that were out of the RDA's confidence ellipse. Here, we present three out of the 27 individuals to illustrate this individual health assessment approach. The first individual (Figure 3A) was captured in the highly polluted site of the industrial habitat and its blood profile is characterized by high level of WBC, lymphocytes (count and percentage) and MCV while MCHC is low. The second individual (Figure 3B) came from the agricultural habitat and exhibits a profile different compared to the other individuals coming from the same habitat. Its RBC, HB and HT were lower than the 2.5th confidence limit. The last individual (Figure 3C) came from the urban habitat and had a blood profile characterized by very low erythrocytes indicators, with RBC, HB, and HT below the RI, which makes it quite different from the others caught in this habitat.

Figure 3. Example of spider-chart for three individuals, the first (A) is from Metaleurop Nord, the second (B) is from the agricultural habitat and the third (C) is from the urban habitat. The blue dotted line represents the 2.5th confidence limit and the red dotted line represents the 97.5th confidence limit.



Discussion

Using a large dataset (14 haematological parameters measured on 430 individuals from three species), this study investigated the relationships between biotic (gender, age class, body condition, TM concentrations in the liver) and abiotic (region, season, habitat, soil occupancy) factors, and then determined RI for three free-ranging rodents (with though only indicative values for the bank vole and the yellow-necked mouse because of relatively low sample size).

Health of animals out of RI was then assessed individually using CBC profiles in order to give new insights in wildlife health assessment, complementary to the current approaches that mostly evaluate variations of some parameters among groups (*e.g.* pristine *versus* disturbed habitat) or relationships to explanatory variables (*e.g.* contaminant concentrations in tissues).

Haematological parameters variations according to environmental variables

Our study reveals variations in haematological parameters, in the three species, mainly according to the different environmental variables studied: the habitat type, the region and the season. Habitat type influenced haematological parameters mainly in the wood mouse compared to the two other species. However, it still remains unclear if this significant difference between habitat has any meaningful effect on the organism at the individual level (Rogival *et al.*, 2006a). Individuals that came from an industrial habitat type were not widely different compared to the others. This observation is consistent with the others results already provided in the literature (Gorriz *et al.*, 1996; A.C Nunes *et al.*, 2001; Tersago *et al.*, 2004; Rogival *et al.*, 2006a). TM element seems to not have an important and marked effect on the blood markers, even if elements as Pb are accumulating in blood (Maceda-Veiga *et al.*, 2015a). Most of the time variations seem to be the result of confounding factors rather than of a clear toxic effect (Tersago *et al.*, 2004). More detailed research on the haematological parameters of wood mice and bank voles captured along the pollution gradient of Metaleurop Nord will be soon available (Powolny *et al.* Submitted).

Concerning biogeographical variations of the haematological parameters at the regional level, their values are most of the time higher in the region of Bourgogne Franche-Comté, and this pattern is the same either for the wood mouse or the bank vole. No yellow-necked mice were caught in the region Hauts-De-France. To our knowledge, such disparities in haematological parameters between regions have been barely studied and highlighted in wildlife literature. Apparently, the main geographical influence known in physiology, and most particularly on the immune system, is the latitude (Martin *et al.*, 2008) and it has been demonstrated a few times (Demas and Nelson, 2003; Pyter *et al.*, 2005). The region Hauts-de-France is located at more elevated latitude than the region Bourgogne Franche-Comté. In human health, some researchers started recently to study variations of haematological parameters across different populations coming from several regions around the world or from different ethnicities (Patel *et al.*, 2007; Addai-Mensah *et al.*, 2019). Reference intervals from Caucasian population applied to a sub-Saharan country such as the Ghana could lead to misinterpretation for specific diagnosis (*e.g.* Addai-Mensah *et al.*, 2019). Disparity in anaemia has also been highlighted according to the ethnicity (Patel *et al.*, 2007). Thus, a similar pattern could occur in wild species, with differences among pathologies as anaemia between different population of a

same species. Small mammals coming from the region Hauts-de-France might present different physiological and ecological characteristics that induce long-term modulation of their leukocyte profiles and erythrocyte indicators. In addition, recent studies have focused on nutritional ecology and how the diet could modulate immunity (Birnie-Gauvin *et al.*, 2017b). Changes in environment among different habitat can influence how resources are allocated and available (Martin *et al.*, 2008). We can easily hypothesize that the dissimilarity between both regions is possibly due to the different anthropogenic stressors that create a pressure on wildlife, and thus impact nutritional quality and foraging behaviour. However, presence of confounding factors such as the season could be the reason that explains this pattern.

Seasonal variations in haematological parameters occur mainly because of the variations in metabolic demand through the year and also between years (Nelson *et al.*, 2002; Martin *et al.*, 2008; Viney and Riley, 2017b). Immunological investment and competing metabolic demand are changing, depending on different events as breeding, pathogen infection dynamics, temperature or resource availability (Martin *et al.*, 2008). Changes in haematological parameters among seasons have been barely documented for wood mice. In the present research, both leukocyte profiles and erythrocytes indicators varied depending on the season. Our results showed higher levels of monocytes (count and percentage), granulocytes (count and percentage), RBC, HB, HT, MCV, MCHC during the summer. Results from the literature report only higher HT in autumn and higher HB in spring for wood mice captured in Spain (Pérez-Suárez *et al.*, 1990). In this study, the others haematological variables oscillated only slightly between seasons in the wood mouse, even if correlations were significant (Pérez-Suárez *et al.*, 1990). Concerning the variations in the leukocyte profiles through the season in wood mice, it can be due to changes in their immunological investment. Monocytes are long-lived phagocytic cells involved in phagocytic defence mechanisms, meaning that they ingest and destroy foreign material as bacteria. Granulocytes are non-specific white blood cells of the innate immune system, implicated in several defence mechanisms because they are not specific to a given antigen. Intensification of their proportions during summer could be the sign that wood mice are facing more potential infections (*e.g.* virus or bacteria) during this period (Davis *et al.*, 2008). In addition, the immune investment during summer is generally higher and is probably linked to the end of the breeding season, where the immune investment is “traded-off” (Martin *et al.*, 2008). A trade-off can be defined as “direct or indirect antagonistic interactions between two physiological processes, which can have long-term fitness consequences for organisms” (Martin *et al.*, 2008). Variations of haematological parameters with season were also observed in bank voles, especially on the leukocyte profiles. We found that white blood cells and particularly monocytes are drastically higher in summer and are more than two times lower in autumn. A similar seasonal pattern has already been described before

in the field vole *Microtus agrestis* (Beldomenico *et al.*, 2008b). The lower level of WBC in autumn is a possible consequence of the slow decrease of temperature and of the reduced food availability (Nelson and Demas, 1996; Beldomenico *et al.*, 2008b). In our study, monocyte variations differ from the literature. Beldomenico *et al.* (2008) observed a huge drop of monocyte levels at the end of July while, in our study, monocytes were highest on that period. It could be explained by the fact that climate in July 2008 in United-Kingdom was different compared to the climate in the Bourgogne Franche-Comté region, in July 2015. In the yellow-necked mouse, seasonal variations showed higher LYM during spring while GRA, RBC, HB, and HT were significantly lower. Low erythrocyte parameters in spring have already been reported and are due to the fact that most of the individuals are sexually active (Wołk and Kozłowski, 1989). Interspecific differences are not surprising because it has already been depicted that some rodents do not present a haematic cycle based on season (Pérez-Suárez *et al.*, 1990) while others faced important variations (Beldomenico *et al.*, 2008b). In addition, wood mice from Spain (Pérez-Suárez *et al.*, 1990) and France, field voles from United-Kingdom (Beldomenico *et al.*, 2008b) and bank voles from France were all captured at different period. Environment and climate changed during the last, almost, 30 years. Thus, differences between results of Pérez-Suárez *et al.* (1990), Beldomenico *et al.* (2008) and our results could also be explained by these changes. Seasonal variations in immune activity can occur at intra-annual level and intra-seasonal level, depending on environmental conditions and lifecycle events (Nelson *et al.*, 2002; Martin *et al.*, 2008; Martin, 2009b). Immune function trade-off is species-specific, and even individual-specific (Martin *et al.*, 2008) because all the individuals of a population do not have the same capacity for immune defence. Period of sexual activity and more particularly pregnancy are the more demanding in energy for small mammal species. The increasing of “parental responsibilities” induce a reduction of both cell-mediated and humoral immune activity (Drazen *et al.*, 2003; Martin *et al.*, 2008).

Haematological parameters variations according to biological variables

Modulation of haematological parameters according to the biological variables, such as gender or age class is less marked, if not non-existent. As for the environmental parameters, changes in haematological parameters according to biological variables have been barely documented for wood mouse. Our results show some significant differences, values of leukocytes profiles are higher in females and erythrocytes indicators higher in males. While all the parameters are lower in juvenile individuals. In comparison, few studies reported no significant differences for these variables (Pérez-Suárez *et al.*, 1990; Rogival *et al.*, 2006a). The general effect of gender seems superficial. In the bank voles, the gender has no effect on the haematological parameters. Higher values of WBC, MON and RDW were highlighted in the adults. However, interpreting these variations alone is not easy and does not always depict reality (Maceda-

Veiga *et al.*, 2015a; Johnstone *et al.*, 2017), especially at a population level. Yellow-necked mice presented no variations in haematological parameters according to the gender compared to both other species. As comparison with other studies is impossible, it is difficult to estimate if this result is due to the species physiology itself or simply to the small sample size we had. In another hand, the several multivariate analyses computed showed no strong and clear correlation between haematological parameters, the gender, and the age. It means that these variables do not represent one of the main drivers of the leukocyte and erythrocyte parameters variations. The general effect of gender and age class seems negligible. The gender itself could not have any consistent influence compared to the reproductive status and the specific demand related to breeding season (Martin *et al.*, 2008).

Relevance of using haematological parameters

Using haematological parameters as a health assessment proxy is useful only if we look at a panel of parameters. Precisely attributed variations of such physiological indicators at a population level, with univariate analysis, is difficult because haematological parameters are impacted by many components, as parasitism, infections, sexual activity, season, or anthropogenic activities (Martin *et al.*, 2008; Acevedo-Whitehouse and Duffus, 2009b; Viney and Riley, 2017b). Some of them are also depending on each other's. The leukocyte profile or leukogram approach offers certain advantages, and that mainly when it used lymphocytes, monocytes and granulocytes count and proportion in the blood (Davis *et al.*, 2008; Maceda-Veiga *et al.*, 2015a). For a long time, the main methods to assess stress level in individual was the relative proportion of neutrophils to lymphocytes (N:L ratio), however this approach completely ignore monocytes proportion. In this study, we provide RI and indicator values for the lymphocytes, the monocytes and the granulocytes which better depict the reality (Davis *et al.*, 2008; Maceda-Veiga *et al.*, 2015a). Concerning the erythrocytes indicators, they need to be considered as a whole, and not separately. We saw previously that establish a diagnosis based only on a variation in RBC or in RDW is challenging and not the most relevant approach. Parameters as MCV, MCHC, MCH or RDW inform us more specifically about anaemia and are largely used to establish a diagnosis (Johnstone *et al.*, 2017). Anaemia arises when haemoglobin concentration is decreasing or a diminution of erythrocyte numbers (Bain *et al.*, 2016; Johnstone *et al.*, 2017).

Individual health assessment

The blood profile of an individual or CBC represents, thus, the first basis for a diagnosis in individual health assessment, as it represents the haematological identity of a given individual at a given moment. It is important to keep in mind that each of these parameters can be indicative of a potential disease state or variation in the health status, whether their values are

high or low. As it is a very individual-specific proxy, using blood markers at a population level to assess wildlife health at the beginning is maybe not always the most appropriate. It represents a powerful and non-lethal tool to assess wildlife health (Maceda-Veiga *et al.*, 2015a).

Individuals outside the RDA and generally outside RI do not have particular biological predispositions. Conversely, a major part of these individuals was captured during summer, and 70% were captured in the Bourgogne Franche-Comté region. Our third hypothesis is not fully verified. In addition, we miss some precise information as the reproductive status of all the individuals. If we take the example of the three individuals presented on the Figure 3, we can establish a first diagnosis and provide an individual health assessment. The first one (Figure 3A), captured in the industrial habitat, has a blood profile characterized by high MCV and RDW while MCHC is low which is consistent with the diagnosis of regenerative anaemia (McGrath, 1993; Tyler and Cowell, 1996; Johnstone *et al.*, 2017). This type of anaemia occurs when an individual either releasing immature erythrocyte and/or increasing erythropoiesis (Johnstone *et al.*, 2017), it could be defined as “a generalised emergency state” (Johnstone *et al.*, 2017). Moreover, its individual presented WBC and lymphocytes (count and percentage) out of the RI, which indicates a potential bacterial or viral infection (Davis *et al.*, 2008). To be more precise, we need information about the nature of these lymphocytes, either T or B, belonging to the cell-mediated and humoral immune response, respectively. The second individual (Figure 3B), captured in the agricultural habitat, present RBC, HB, and HT below the RI lower limit. It can indicate microcytic anaemia and a lower oxygen carrying capacity due to low HB and HT (Johnstone *et al.*, 2017). Low RBC, HB and HT also indicates a short-term stress and have also been shown in the black rat *Rattus rattus* (Oishi *et al.*, 1999). In addition, the blood profile pattern of this individual is different from the individuals captured at the same season, in the same environment. The third individual (Figure 3C), captured in the urban habitat, presented low values for all the erythrocyte indicators. As for the second one, it indicates anaemia and low oxygen carrying capacity. It could indicate hypochromic microcytic anaemia which occurs when MCV and MCH are low, and also when the RBC are small (Massey, 1992). Hypochromia of RBC occurs when HB is low in the blood. Deficit in iron is the most common cause of microcytic anaemia (Massey, 1992). Low RDW values also indicates that the RBC are uniform in size (Johnstone *et al.*, 2017). Here, we determined a first and preliminary diagnosis which informs us about the health status of three individuals. This approach can then allow to transpose the results at the population level and then evaluate the global health status of the population.

Limitations and perspectives

RI are still not a widespread diagnosis tool in wildlife health assessment, at a time where it represents an important issue. Determination of RI for a given species requires time and is associated with many constraints. The main requirement is that to establish RI, 120 apparently healthy individuals are needed. At least 74% of the studies that provide RI for wildlife are not following this first guideline that came from veterinary practices and medical research (Kophamel *et al.*, 2022). In conservation biology and physiology, when the studied species are threatened and/or when it is complicated to obtain and sample blood, reach the levels of 120 individuals is not always easy. We had an example here with the bank vole and the yellow-necked mouse. Moreover, when studying wild animals, assess and select which individual of a population can be considered as "healthy" is not always simple to determine. Thus, we could ask ourselves the following question: is it completely relevant to try to follow veterinary recommendations for wildlife species when it concerns health? Drawing on their methods while adapting them to the limitation of the study of wildlife is probably more relevant. Indeed, it has been attested many times that wild animals react differently in term of immune responses compared to lab animals and/or pets (Abolins *et al.*, 2017b; Graham, 2021b). In addition, the haematological approach should be coupled with other diagnostic method, as in human health, if it is needed and conceivable. If only this approach is feasible, the context (*e.g.* season, reproductive status…) should be clear and considered as good as possible in the interpretation (Maceda-Veiga *et al.*, 2015a). The exact method to evaluate wildlife health does not exist and only a mixing of approaches used at different organization level, (*e.g.* community, population, individual) will give a picture representative of the reality (Todgham and Stillman, 2013; Maceda-Veiga *et al.*, 2015a; Kophamel *et al.*, 2022).

Conclusion

Wildlife health assessment is a growing field of research that provide essential baseline in conservation, management and more generally, in understanding impacts of anthropogenic stressors and emerging disease in wildlife populations (Kophamel *et al.*, 2022). Determination of RI for wildlife species is one of the important tools that will help to reach these objectives. Blood and its own markers remain one of the principal proxies to assess health, thus extend RI to wildlife is the logical follow up. In the present study, investigation of the impact of biotic and abiotic parameters on blood parameters in several rodent species revealed that season and region are the main drivers of variations. Thus, they should be considered at the moment of individual health assessment as well as others biological parameters (*e.g.* reproductive status). Following basic diagnosis procedures as veterinarians, but for wildlife is a new challenge for ecologist and ecotoxicologist. Automatic utilisation of blood analyses in wild vertebrates may serve as an early warning signals, and assess if the population and then the

community could be in a potential tipping-point of their health status (Maceda-Veiga *et al.*, 2015a).

Author contributions

R. Scheifler, C. Fritsch, M. Coeurdassier, F. Raoul: Conceptualization; R. Scheifler, C. Fritsch, F. Raoul: Funding acquisition, Methodology, Project administration, Supervision, Validation; C. Hadjadji, Q. Devalloir: Data curation, Formal analysis, Visualization; R. Scheifler, C. Fritsch, V. Driget, F. Raoul, M. Coeurdassier: Investigation; C. Hadjadji, Q. Devalloir, R. Scheifler: Writing - original draft; All: Writing - review & editing.

Funding

This work was supported by the Agence De l'Environnement et de la Maîtrise de l'Energie (ADEME, programme DYSPAT, grant number 1572C0309), the Conseil Régional de Franche-Comté (for having financially supported the purchase of the HORIBA ABX Micros ES 60 haematology analyser), the LTER Zone Atelier Arc Jurassien (for the financial support to purchase the field anaesthesia device), and the OSU THETA and the University of Franche-Comté (programmes DEMENOL, MEXCO and OREAS, which supported the costs of fieldwork and field and laboratory analyses).

Acknowledgements

The authors gratefully thank Nadia Crini and Caroline Amiot from the PEA²t platform (Chrono-environnement, University Bourgogne Franche-Comté, UMR CNRS 6249, France) for their technical help in metal analyses. Virgile Baudrot, Nico van den Brink, Javier Fernandez de Simon, Julie Montaz, Aurore Niechajowicz, Shinji Ozaki, Nicolas Tête, and Núria Vallverdu Coll are also gratefully thanked for their help in the field, and François Gillet for his assistance in data analyses.

Supplementary materials

Figure S1. Map of the different study sites in Bourgogne Franche-Comté and Hauts-de France region.

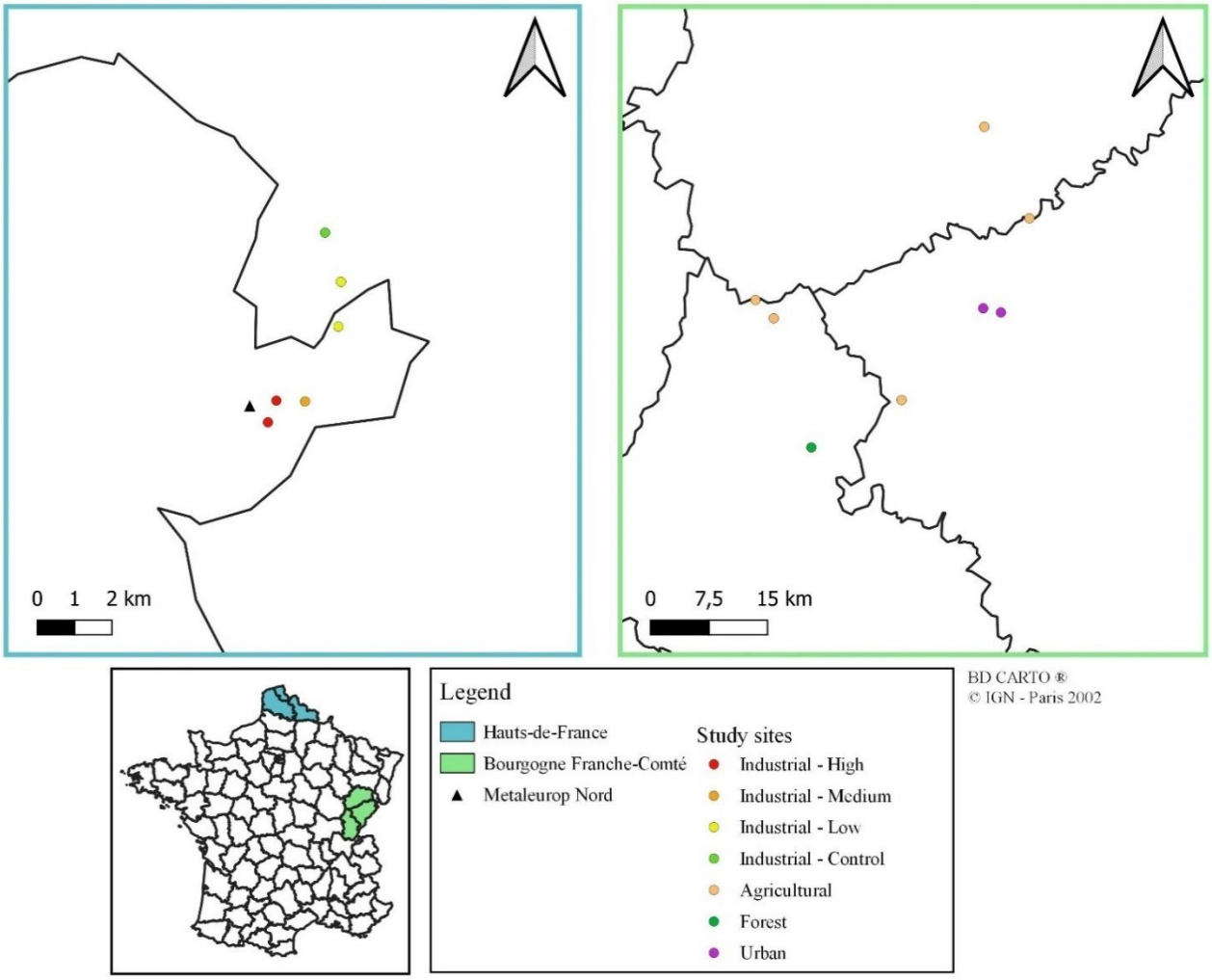


Table S1. Summary of small mammal sample size in the four study sites, according to season, gender, and age class.

	Study sites						
	Industrial (Metaleurop Nord)	Urban (Besancon)		Agricultural (ZAAJ)		Forest (Fôret de chaux)	**Total**
	Autumn 30/09 – 11/10	Spring 24/04 – 26/04	Summer 21/06 – 26/08	Spring 08/04 – 28/04	Summer 01/07 – 24/07	Spring 07/05 – 09/05	
Apodemus flavicollis				**8**	**27**	**3**	**38**
Adult				8	27	3	38
Female				*3*	*12*		*15*
Male				*5*	*15*	*3*	*23*
Apodemus sylvaticus	**199**	**27**	**18**	**72**	**38**	**10**	**364**
Adult	161	22	18	71	36	9	317
Female	*52*	*15*	*6*	*34*	*16*	*6*	*129*
Male	*109*	*7*	*12*	*37*	*20*	*3*	*188*
Juvenile	38	5		1	2	1	47
Female	*25*	*2*		*1*		*1*	*28*
Male	*13*	*3*			*2*		*19*
Myodes glareolus	**27**			**13**	**31**		**71**
Adult	8			13	29		63
Female	*4*			*6*	*14*		*29*
Male	*7*			*7*	*15*		*34*
Juvenile	19				2		8
Female	*8*				*1*		*4*
Male	*11*				*1*		*4*
Total	**226**	**27**	**18**	**93**	**96**	**13**	**473**

Table S2. Effect of biological (gender, age class) and environmental (region, habitat, season) variables on haematological parameters in the wood mouse *Apodemus sylvaticus* (*n* = 321). NS: non-significant, MW: Mann-Whitney test, KW: Kruskal-Wallis test.

	Biological variables		**Environmental variables**		
	sex (MW)	Age class (MW)	Region (MW)	Habitat (KW)	Season (KW)
Leukocytes					
WBC ($10^3/mm^3$)	NS	NS	NS	$Chi^2 = 14.2$ df = 3 $p = 0.003$	NS
LYM ($10^3/mm^3$)	NS	NS	NS	$Chi^2 = 24.3$ df = 3 $p < 0.001$	NS
LYM (%)	NS	NS	$p < 0.001$	$Chi^2 = 12.7$ df = 3 $p = 0.005$	NS
MON ($10^3/mm^3$)	$p = 0.046$	$p = 0.012$	$p = 0.02$	$Chi^2 = 8.3$ df = 3 $p = 0.04$	$Chi^2 = 9.1$ df = 2 $p = 0.01$
MON (%)	NS	$p = 0.017$	$p = 0.003$	$Chi^2 = 16.1$ df = 3 $p = 0.001$	$Chi^2 = 22.3$ df = 2 $p < 0.001$
GRA ($10^3/mm^3$)	NS	NS	$p = 0.005$	$Chi^2 = 17.3$ df = 3 $p < 0.001$	$Chi^2 = 15.5$ df = 2 $p < 0.001$
GRA (%)	NS	NS	$p = 0.003$	$Chi^2 = 10.3$ df = 3 $p = 0.016$	$Chi^2 = 24.0$ df = 2 $p < 0.001$
Erythrocyte indicators					
RBC ($10^3/mm^6$)	NS	NS	$p = 0.008$	$Chi^2 = 20.9$ df = 3 $p < 0.001$	$Chi^2 = 21.8$ df = 2 $p < 0.001$
HB (g/dL)	NS	NS	$p < 0.001$	$Chi^2 = 28.9$ df = 3 $p < 0.001$	$Chi^2 = 35.6$ df = 2 $p < 0.001$
HT (%)	$p = 0.05$	NS	$p = 0.003$	$Chi^2 = 17.8$ df = 3 $p < 0.001$	$Chi^2 = 24.5$ df = 2 $p < 0.001$
MCV (μm^3)	NS	NS	NS	$Chi^2 = 15.8$ df = 3 $p = 0.001$	NS
MCH (pg)	NS	NS	$p < 0.001$	$Chi^2 = 31.0$ df = 3 $p < 0.001$	$Chi^2 = 14.1$ df = 2 $p < 0.001$
MCHC (g/dL)	$p = 0.002$	NS	$p < 0.001$	$Chi^2 = 44.8$ df = 3 $p < 0.001$	$Chi^2 = 60.6$ df = 2 $p < 0.001$
RDW (%)	NS	$p = 0.003$	NS	NS	NS

Table S3. Effect of biological (gender, age class) and environmental (region, habitat, season) variables on haematological parameters in the bank vole *Myodes glareolus* (*n* = 71). NS: non-significant, MW: Mann-Whitney test, KW: Kruskal-Wallis test.

	Biological variables		**Environmental variables**			***n***
	sex (MW)	Age class (MW)	Region (MW)	Habitat (MW)	Season (KW)	
Leukocytes						
WBC ($10^3/mm^3$)	NS	$p < 0.001$	$p < 0.001$	$p < 0.001$	$Chi^2 = 27.1$ df = 2 $p < 0.001$	71
LYM ($10^3/mm^3$)	NS	NS				44
LYM (%)	NS	NS	NS	NS	$Chi^2 = 6.5$ df = 2 $p = 0.04$	71
MON ($10^3/mm^3$)	NS	NS				44
MON (%)	NS	$p = 0.013$	$p = 0.006$	$p = 0.006$	$Chi^2 = 11.2$ df = 2 $p = 0.004$	71
GRA ($10^3/mm^3$)	NS	NS			NS	44
GRA (%)	NS	NS	NS	NS	NS	71
Erythrocyte indicators						
RBC ($10^3/mm^6$)	NS	NS	NS	NS	NS	71
HB (g/dL)	NS	NS	NS	NS	NS	71
HT (%)	NS	NS	NS	NS	NS	71
MCV (μm^3)	NS	NS	NS	NS	NS	71
MCH (pg)	NS	NS	NS	NS	NS	71
MCHC (g/dL)	NS	NS	NS	NS	NS	71
RDW (%)	NS	$p = 0.003$	$p < 0.001$	$p < 0.001$	$Chi^2 = 14.1$ df = 2 $p < 0.001$	71

Table S4. Effect of biological (gender, age class) and environmental (region, habitat, season) variables on haematological parameters in the yellow-necked mouse *Apodemus flavicollis* (*n* = 38). NS: non-significant, MW: Mann-Whitney test.

	Biological variables	**Environmental variables**	
	sex (MW)	Habitat (MW)	Season (MW)
Leukocytes			
WBC (10^3/mm^3)	NS	NS	NS
LYM (10^3/mm^3)	NS	p = 0.007	NS
LYM (%)	NS	p = 0.013	p = 0.03
MON (10^3/mm^3)	NS	NS	NS
MON (%)	NS	p = 0.007	NS
GRA (10^3/mm^3)	NS	NS	NS
GRA (%)	NS	p = 0.01	p = 0.02
Erythrocyte indicators			
RBC (10^3/mm^6)	NS	NS	p = 0.02
HB (g/dL)	NS	NS	p = 0.007
HT (%)	NS	NS	p = 0.003
MCV (µm^3)	NS	NS	NS
MCH (pg)	NS	NS	NS
MCHC (g/dL)	NS	p = 0.03	NS
RDW (%)	NS	NS	NS

Figure S2. Spearman rank correlation plot showing correlations between haematological parameters, soil occupancy (n = 321), body condition index (n = 265) and metal liver concentrations (n = 142) in the wood mouse *Apodemus sylvaticus*. Only significant correlations ($p < 0.05$) are dotted. Colors (blue positive and red negative) and plot size (proportional to correlation coefficient values) indicate the correlation coefficient.

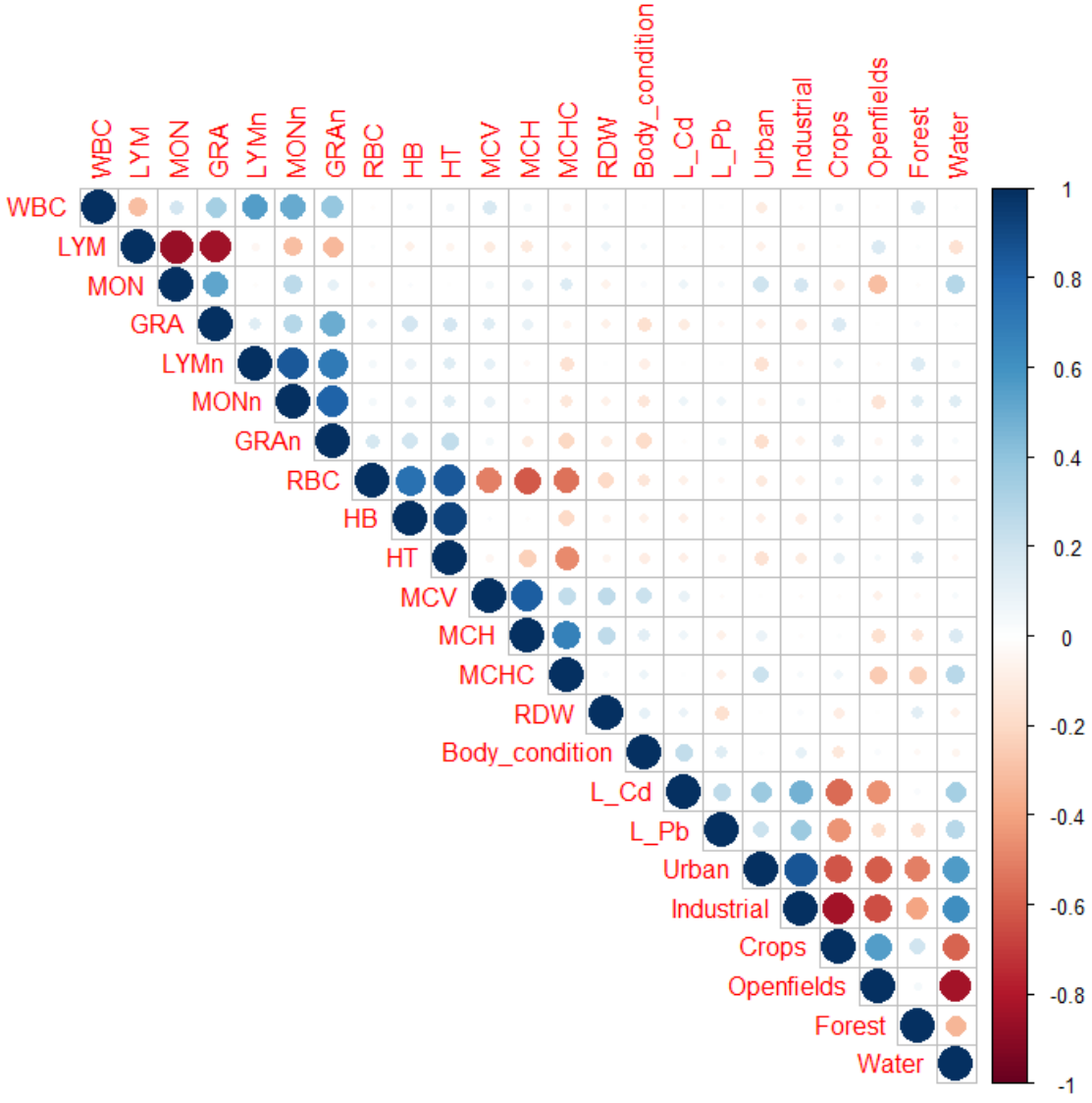


Figure S3. Spearman rank correlation plot showing correlations between haematological parameters, soil occupancy (n = 71) and body condition index (n = 68) in the bank vole *Myodes glareolus*. Only significant correlations (p < 0.05) are dotted. Colors (blue positive and red negative) and plot size (proportional to correlation coefficient values) indicate the correlation coefficient.

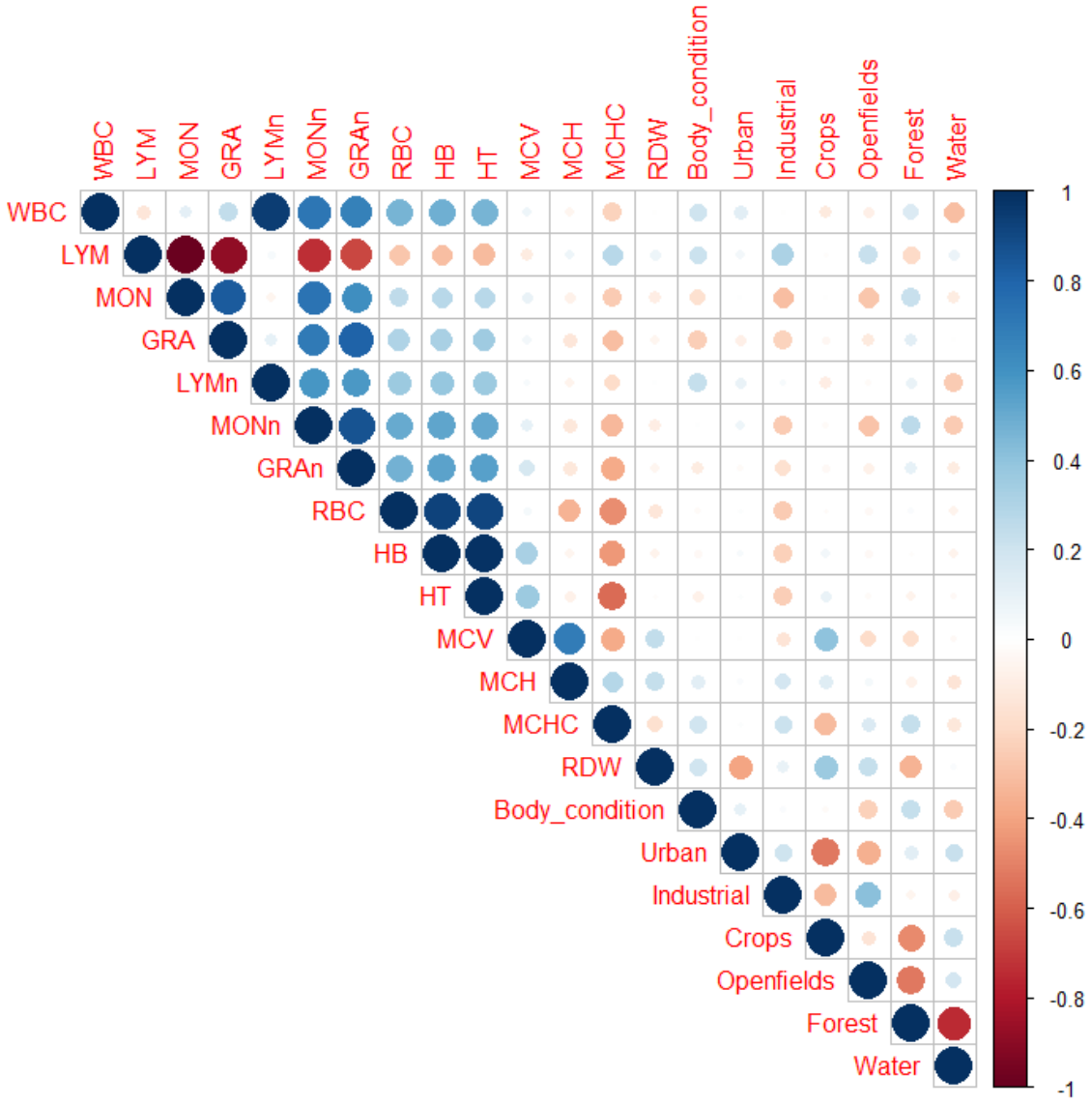


Figure S4. Spearman rank correlation plot showing correlations between haematological parameters, soil occupancy (n = 38) and body condition index (n = 36) in the yellow-necked mouse *Apodemus flavicollis*. Only significant correlations (p < 0.05) are dotted. Colors (blue positive and red negative) and plot size (proportional to correlation coefficient values) indicate the correlation coefficient.

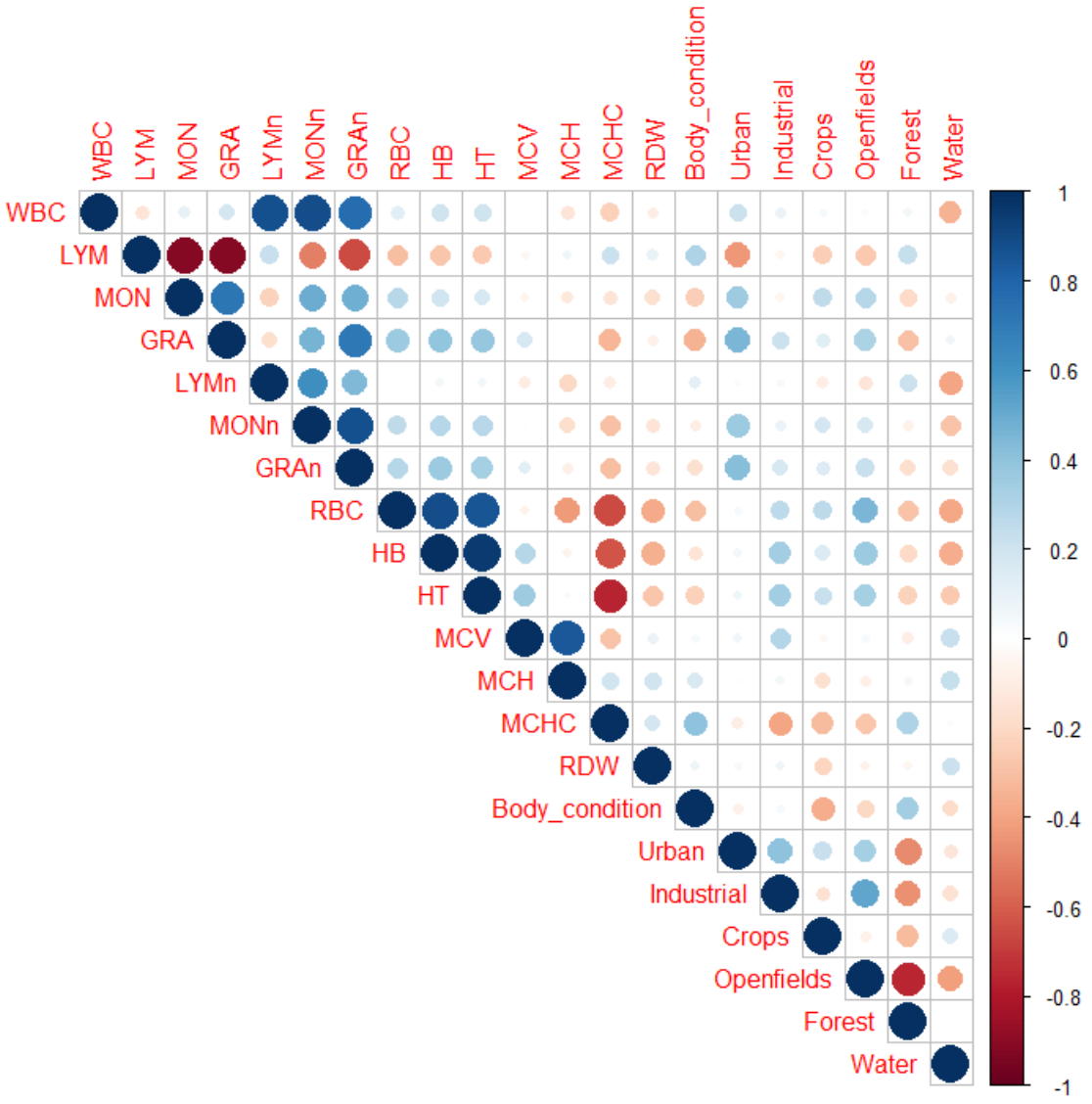


Table S5. Statistical distribution (confidence intervals based on 2.5th and 97.5th percentiles, number of individuals lower or higher of this range, minimum, median and quartiles, maximum values, mean ± standard deviation (SD) and sample size) of haematological parameters in the bank vole *Myodes glareolus*.

	CI (2.5% – 97.5%)	***n* < 2.5%**	***n* > 97.5%**	**Mean ± SD**	**Min**	**Median [Q1; Q3]**	**Max**	***n***
WBC ($10^3/mm^3$)	1.42 – 15.08	2	2	6.08±4.09	1.1	4.74 [3.05; 8.38]	20.3	71
LYM ($10^3/mm^3$)	1.56 – 13.94	2	2	5.98±3.41	1.35	5.5 [3.47; 7.42]	16.65	44
LYM (%)	57.25 – 94.60	2	3	82.94±10.38	50.5	85.60 [77.28; 90.7]	98.25	71
MON ($10^3/mm^3$)	0.10 – 2.89	2	2	1.01±0.82	0*	0.83 [0.42; 1.25]	4.1	44
MON (%)	3.93 – 29.64	2	2	12.50±7.16	1.5	10.83 [7.19; 16.37]	33.05	71
GRA ($10^3/mm^3$)	0.15 – 1.04	4	2	0.43±0.32	0.13	0.35 [0.2; 0.55]	1.83	44
GRA (%)	0.82 – 17.89	2	2	4.55±4.15	0.25	3.15 [2; 5.72]	18.4	71
RBC ($10^3/mm^6$)	4.68 – 13.97	2	2	9.66±1.89	3.36	9.74 [8.96; 10.53]	14.31	71
HB (g/dL)	7.10 – 19.18	2	2	13.55±2.53	5.65	13.53 [12.65; 14.7]	20.03	71
HT (%)	19.92 – 63.86	2	2	43.96±8.94	15.7	44.125 [40.15; 47.63]	68.1	71
MCV (μm^3)	41.27 – 50.17	2	2	45.49±2.42	41	45.165 [44; 47]	52	71
MCH (pg)	12.59 – 16.5	2	3	14.09±0.89	12.55	14.02 [13.42; 14.7]	16.75	71
MCHC (g/dL)	29.26 – 34.15	2	2	30.98±1.28	29.05	30.77 [30.22; 31.45]	36.4	71
RDW (%)	13.55 – 17.87	3	2	15.37±1.18	13.25	15.08 [14.53; 16.15]	18.7	71

Table S6. Statistical distribution (confidence intervals based on 2.5th and 97.5th percentiles, number of individuals lower or higher of this range, minimum, median and quartiles, maximum values, mean ± standard deviation (SD)) of haematological parameters in the yellow-necked mouse *Apodemus flavicollis*.

	CI (2.5% – 97.5%)	***n* < 2.5%**	***n* > 97.5%**	**Mean±SD**	**Min**	**Median [Q1; Q3]**	**Max**
Leukocytes							
WBC (10^3/mm^3)	1.62 – 11.28	1	1	5.49±3.09	0.75	5.00 [2.81; 7.82]	13.5
LYM (10^3/mm^3)	0.99 – 32.03	1	1	5.01±6.95	0.5	3.55 [1.95; 5.02]	34.03
LYM (%)	44.16 – 87.48	1	1	66.78±11.93	36.36	68.12 [57.35; 73.71]	95.93
MON (10^3/mm^3)	0.09 – 3.31	1	1	1.06±0.82	0.03	0.77 [0.46; 1.42]	3.55
MON (%)	7.83 – 29.53	1	1	19.7±6.52	2.73	20.47 [14.91; 24.57]	36.56
GRA (10^3/mm^3)	0.14 – 2.02	1	1	0.85±0.55	0.13	0.75 [0.45; 1.1]	2.3
GRA (%)	3.42 – 26.33	1	1	13.45±6.72	1.33	12.32 [10.02; 17.57]	38.5
Erythrocyte indicators							
RBC (10^3/mm^6)	6.00 – 13.55	1	1	10.02±1.98	5.54	10.35 [8.87; 11.08]	13.72
HB (g/dL)	9.69 – 20.07	1	1	15.49±2.80	9.65	15.85 [14.13; 16.95]	20.35
HT (%)	29.7 – 67.73	2	1	50.01±10.09	29.7	50.9 [45.73; 54.97]	68.2
MCV (µm^3)	44.94 – 56.35	1	1	49.92±3.30	44.33	49 [48; 51.87]	60.66
MCH (pg)	14.06 – 17.33	1	2	15.54±0.92	13.65	15.4 [15.01; 16.08]	17.35
MCHC (g/dL)	29.27 – 32.7	1	2	31.13±1.00	28.33	31.05 [30.61; 31.75]	32.75
RDW (%)	14.4 – 18.04	2	1	15.62±1.10	14.4	15.35 [14.68; 16.13]	19.23

3.4 Chapter 4 (manuscript in preparation)

Developing non-lethal tools to assess immune response to challenge in the wood mouse (*Apodemus sylvaticus*)

Quentin Devalloir[1,*], Clémentine Fritsch[1], Anne-Claude Goydadin[1], Vincent Driget[1], Coralie Barrera[1], Nico van den Brink[3], Renaud Scheifler[1],

[1]UMR 6249 Chrono-environnement, CNRS / Université de Franche-Comté, 25030 Besançon Cedex, France

[3]Division of Toxicology, Wageningen University, 6700 EA Wageningen, The Netherlands

*Corresponding author: quentin.devalloir@gmail.com

Abstract

Environmental and anthropogenic stressors may impact the health status of wild animals. Numerous field studies monitored stress response to these disturbances through physical and physiological changes, but often qualitative and quantitative measurements rely on the use of lethal procedures. This study aims to develop non-lethal methods to measure immune response in free-ranging small mammals. Wood mice captured in two regions of France were used to assess the relevance and compare the results from lethal and non-lethal methods, performing spleen or blood sampling on individuals at capture day (wild) or after a few days of captivity (captive). For lethal procedures, the spleen weight was used as a proxy of the immune status while haematological parameters and cytokines were measured in non-lethal approaches. Blood cell counts were within the range of reference values measured in wood mice. Three commercially available kits designed in lab mice were used to measure inflammatory response among which only a pro-inflammatory cytokine, the TNF-α, cross-reacted with wood mouse samples. Spleen cells showed an increase of TNF-α concentrations in response to LPS medium concentration (*ex vivo*). No significant differences were found between TNF-α concentrations measured in both spleen cell culture and plasma. Plasma TNF-α concentrations were significantly higher in individuals challenge with LPS (*in vivo*) than the control ones. In captive individuals, the blood lymphocyte numbers were found to increase significantly with the spleen weight, suggesting that lymphocyte count can be a good proxy of the spleen weight, however this was not the case in wild animals. In wild individuals, a significant increase was found between the leukocytes (lymphocytes and monocytes only) and plasma TNF-α concentration. Discrepancies in lymphocyte-associated patterns of decrease in response to TNF-α might be attributed to captivity with a sufficient food supply. Further insight may be given into the development of methods able to monitor the immune response of wild animals in their living conditions (*i.e.* exposure to environmental stressors) considering that captivity could modulate these responses.

Keywords

Immunocompetence, immunomarker, wildlife biomonitoring, stress ecology

Highlights

- Lethal and non-lethal methods were compared to assess the immunocompetence of wood mice
- TNF-α concentration was measured in plasma and supernatant of splenocyte culture
- Peripheral lymphocytes and monocytes decrease in response to LPS challenge

- Wild animals showed lymphocyte and monocytes-associated patterns of decrease in response to TNF-α
- Immune response to challenge differed between captive and non-captive individuals

Introduction

Pollution, infectious disease, food scarcity, and habitat loss are among the multiple sources of stress (*i.e.* stressors) occurring in the environment. These stressors can induce several disturbances in animals' behaviour and physiology affecting fitness and evolution (*e.g.* Saaristo *et al.*, 2018; Acevedo-Whitehouse and Duffus, 2009). For instance, wood mice (*Apodemus sylvaticus*) exposed to cadmium (Cd) and lead (Pb) displayed variation in food selection and a decrease in haematocrit in response to pollution (Ozaki *et al.*, 2019; Tête *et al.*, 2015). In field studies, the sensitivity of wild animals to these stressors is monitored with markers associated with behavioural, physical and physiological changes. Physical and physiological changes are closely related as physiological processes ensure the organic structure, the growth and the functioning of physical features of living organisms. Those changes are occurring as a stress response to disturbances in the environment. For instance, the spleen mass is commonly used in immunotoxicity testing to assess the effect of a given compound on the immune system (Luster *et al.*, 1988). Indeed, among vertebrates the spleen acts as a blood filter in replacing senescent erythrocytes, recycling iron and hosting B lymphocytes and monocytes in the white pulp (Murphy and Weaver, 2016). Spleen wet mass was found to be higher (*i.e.* splenomegaly) in rats (Sprague Dawley rats, *Rattus norvegicus*) experimentally exposed to cadmium (Cd) and in Algerian mice (*Mus spretus*) chronically exposed to Cd (Turley *et al.*, 2019; A. C. Nunes *et al.*, 2001). As a lymphoid organ, both splenomegaly and spleen atrophy may have physiological consequences. Spleen atrophy was found to be associated to leukocyte count in juvenile cotton rats (*Sigmodon hispidus*) exposed to cyclophosphamide (used as chemotherapy and to suppress the immune system) and fed with low a protein diet (McMurry *et al.*, 1994). Physiological changes can be measured with bio- or immuno-markers relating disturbances in physiological processes involved in metabolism or immune system functioning, respectively. The measurement of the component of the immune system of wild animals often relies on methods and procedures originally developed in laboratory species (*e.g.* rats and mice). Furthermore, many routines and methodology used in the monitoring of wild animals required invasive (*e.g.* biopsy or blood sampling) or, sometimes, lethal procedures (*e.g.* organ collection) (*e.g.* Espín *et al.*, 2016; Maceda-Veiga *et al.*, 2015). At the same time, ethical considerations on animal use in research tend to improve animal welfare and avoid lethal procedures, with for example the application of the 3 Rs rule (*i.e.* reduce, refine and replace) (Zemanova, 2020; Arck, 2019). In line with this consideration, an emerging concept called New

Approach Methodologies (NAMs) promotes an alternative for chemical hazard and risk assessment through nonlive-animal-based approaches using in silico, in chemico, and *in vitro* methods (Morrissey *et al.*, 2023). Among *in vitro* methods, serum titration (*i.e.* antibody-mediated response) or isolation of lymphoid organs or plasma/serum cells are commonly used to assess the immunocompetence of wild animals. Serum isolation of leukocytes is a non-lethal approach that can be used to evaluate the immunotoxicity of compounds in large mammals but required a substantial volume of blood (*e.g.* Frouin *et al.*, 2010; Dufresne *et al.*, 2010; Kakuschke *et al.*, 2008). However other *in vitro* methods developed in laboratory rodents can be used. Indeed, spleen cell culture allows to harvest a large number of lymphoid cells and evaluate the effect of contaminants (Müller *et al.*, 2005). This technic allows to assess spleen leukocyte sensitivity to chemicals and also immune challenges. In wild mammals, spleen cell cultures of wood mice displayed relevant patterns of inflammatory response, mediated by TNF-α, to different toll-like receptor agonists including lipopolysaccharide (LPS) (Jackson *et al.*, 2009). Among agonist, LPS is a glycolipid found in the external membrane of Gram-negative bacteria. A LPS challenge stimulates toll-like receptor 4 to release inflammatory cytokines (*e.g.* IL-1β, Il-6, TNF…), activate antigen-presenting cells (via major histocompatibility complex of class II) and then recruit T cells (T lymphocytes with clusters of differentiation 4) enhancing a Th1 response (McAleer and Vella, 2008). A peracute injection of LPS produces, within the first hours (0 to 8 hours in rats), an inflammatory response characterised by a significant decrease of lymphocytes and monocytes in the bloodstream corresponding to a relocalisation to the sites of the infection (Brooks *et al.*, 2017). Therefore, haematological measurement such as leukocyte count promotes a non-lethal and potentially less invasive method to estimate animals' health status (Hadjadji *et al.*, in prep; Maceda-Veiga *et al.*, 2015). Nondestructive, non- or less invasive sampling benefit from advances in analytic chemistry and allow to measure body fluids, fat biopsies, feces, and hair or feathers of wild animals (*e.g.* Krug-MacLeod *et al.*, 2022; Zemanova, 2020; Espín *et al.*, 2016). For instance, body fluids can be used to measure inflammatory markers which might berelevant of the underlying mechanism affected by a stressor. This is the case for instance of enzymatic markers of liver injuries (*e.g.* alanine aminotransferase or aspartate aminotransferase), oxidative stress markers (*e.g.* malondialdehyde or glutathione peroxidase) or inflammatory markers (*e.g.* C-reactive protein, cytokines). However, the measurement of bio- or immuno-markers often relies on antigen-antibody recognition to quantify the concentrations of a given protein or peptide of interest (*e.g.* cytokines) which amino acid sequence varies (at least) between species or sub-species (Secombes *et al.*, 2016). Wild immunology is facing a major issue in the cross-compatibility of detection tools as most of the scientific knowledge and commercially available kits rely on captive laboratory model species (Flies and wild comparative immunology consortium, 2020). Even more, a recent paper by Graham (2021)

highlights that laboratory model species (*i.e. Mus musculus*) exposed to natural conditions may display an immune response much more translational of their environmental experience rather than heritable factors. For instance, *in vitro* spleen cell cytokine responses to agonist was lower in wild than in laboratory mice which might suggest that energetic investments of wild animals in immune homeostasis could be unbalanced by environmental stressors not present in captivity.

This study aims to use a lethal and a non-lethal method to determine the health status of a wild small-mammal, the wood mice. Among lethal methods spleen cell cultures are expected to display an increase of TNF-α in response to LPS challenge *in vivo* and/or during *in vitro* assays. Some studies found that a commercially available kit initially developed for mice could cross-react with the wood mice spleen cells and displayed a positive response to *in vitro* LPS challenge (Rynkiewicz *et al.*, 2019; Friberg *et al.*, 2011; Jackson *et al.*, 2009). However, none of those studies used plasma samples to measure circulating levels of TNF-α which can be done in a non-lethal way. We hypothesise that plasma TNF-α could increase while lymphocyte and monocyte decrease in response to *in vivo* LPS challenge promoting an inflammatory response (measured within 2 hours post-injection). Indeed, LPS challenged rats displayed a significant increase in plasma TNF-α and also a significant decrease in leukocytes mainly lymphocytes and monocytes (Brooks *et al.*, 2017). We also hypothesise that haematological parameters could predict a commonly used physical measurement of organ weights in immunotoxicology which could avoid the use of lethal procedures for dissections and organ sampling.

The major role of the spleen as a “blood filter” and lymphocyte host is expected to display positive correlations with haematological parameters in wood mouse populations both in natural conditions and after a short period of captivity.

Methods

Study site

Two sites distant from more than 400 km were selected for this study. The campus of “la Bouloie” (Besançon, Doubs, FRANCE) is an urbanized area characterised by moderate human activities and small patches of woody habitat around the University’s buildings. The animals sampled over this area were chosen to perform spleen cell culture. The “Communauté de commune de Pévèle-Carembault” (CC Pévèle-Carembaut) engulfs several towns among which two locations were selected Thumeries and Tourmignies (Hauts-de-France, FRANCE). The two sites display typical landscape features of the French countryside with alternate

patches of forest, crops and towns. A long-term monitoring of wood mice populations exposed to Cd and Pb is held around the former smelter of Metaleurop Nord SAS which is located at 6.5 and 11.5 km from Thumeries and Tourmignies, respectively. Several studies based on this monitoring used the sites of the CC Pévèle-Carembaut as low polluted sites in their soil contamination gradient (*e.g.* Powolny *et al.*, 2023; Ozaki *et al.*, 2019; Fritsch *et al.*, 2010).

Animal selection

For each field session, traplines of 10 non-lethal INRA traps (with dormitory boxes) each spaced 3 m apart were set for 3 consecutive nights. Dormitory boxes were filled with apples, hay, sunflower seeds, cat food and peanut butter. They ensure the survival of the animals for 24 h. The wood mouse (*Apodemus sylvaticus)* was retained as the animal model for this study considering its relevance in both epidemiological, immuno-ecological and ecotoxicological studies (*e.g.* Friberg *et al.*, 2011; Fritsch *et al.*, 2010a; van den Brink *et al.*, 2010). The wood mouse ubiquity, the intermediate trophic position (*i.e.* prey and primary consumer) and its conservation status in France (status “LC”, www.iucnredlist.org), depicts an efficient model of study to monitor small-mammal health status. Among the individuals trapped, individuals from other species than *A. sylvaticus* were immediately released, after checking their health status (no injury, not wet, apparent normal behaviour). Pregnant females and females exhibiting suckling, as well as young individuals (*i.e.* below 15g, (Gelling *et al.*, 2009)) of *A. sylvaticus* species were also released. Adult males and non-pregnant females were kept for the experiment. Finally, a total of 18 wood mice (14 males and 4 females) were trapped on the campus of la Bouloie and 24 (20 males and 4 females) at the CC Pévèle-Carembault.

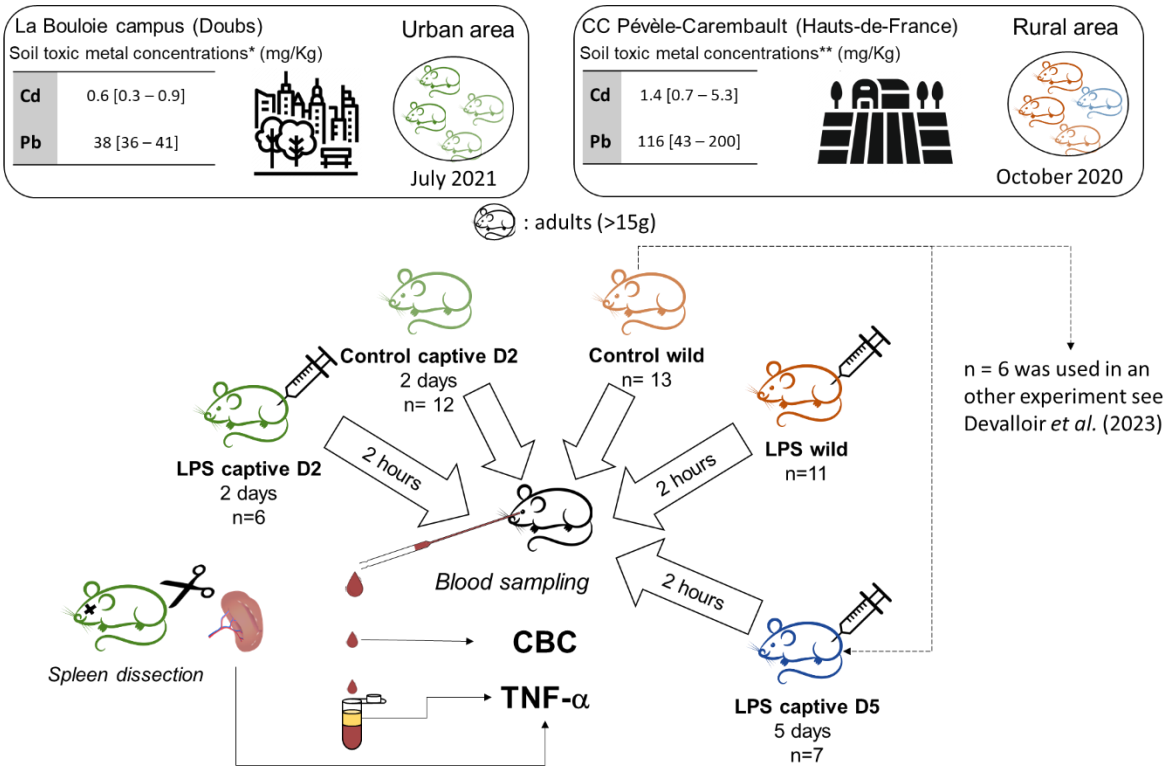
Trace metals soil pollution levels

For the CC Pévèle-Carembault soil trace metal concentrations were presented in Devalloir *et al.* (2023) and details about sampling and methods are detailed in Fritsch *et al.* (2010a) and Douay *et al.* (2009). The concentrations of Cd, Pb and Se in soil at la Bouloie were measured in 2019. Metal analyses were done on soil dried samples (0,25 g) after digestion with 2 ml nitric acid (65% HNO3, ultratrace, Optima) et 5 ml Hydrochloric Acid (32% HCl, Optima). A factor of 10 times was applied for the dilution for ICP-MS analysis (Thermo X2 Series) used for the whole samplings. Precision and accuracy of the analytical measurement were checked using a standard reference material for the soil Loamy Clay 1 (Sigma Aldrich CRM052) displaying acceptable recovery rates for Cd (84%) and Pb (103%). On figure 1, the median concentrations of la Bouloie campus (Cd: 0.6, Pb: 38 mg/kg) and the CC Pévèle-Carembaut (Cd: 1.4, Pb: 116 mg/kg) sites indicate values above the French background values for Cd (0.45 mg/kg) in both soils, but not for Pb (62.3 mg/kg) which is lower at la Bouloie (Villanneau *et al.*, 2008; Baize, 2000).

Experimental design

The study design is presented in figure 1. Wood mice captured during the two field sessions in the two areas were separated into five groups of individuals. For the experiment at la Bouloie, all individuals were maintained in captivity during 2 days. Of 18 individuals, 6 were challenged with LPS (LPS captive D2) and were blood sampled 2h after. The 12 unchallenged individuals (Control captive D2) were blood sampled following the 2 days of captivity as the other group but without LPS challenge. After blood sampling individuals were euthanised for the dissection of the spleen which was directly transferred on ice for the spleen cell culture preparation. For the experiment at CC Pévèle-Carembault, 11 individuals were directly challenged with LPS at capture (LPS wild) and were blood sampled 2h after. The 13 other individuals were blood sampled without challenge directly at capture (Control wild). Among the Control wild, 7 individuals were maintained in captivity for 5 days and then challenged with LPS and then received a blood sampling two hours after (LPS captive D5). The remaining individuals (n=6) were used for another experiment presented in Devalloir *et al.* (2023).

Figure 1. Experimental design. Animals from the la Bouloie (in green): Control and LPS captive D2; animals from CC Pévèle-Carembault (in orange and blue): Control and LPS wild (orange) and the subsection of Control wild set for five days captivity corresponds to LPS captive D5 (in blue).



* measured in 2019

** Douay *et al.*, 2009; Fritsch *et al.*, 2010a

Housing

Animals were housed individually (to avoid inter-individual aggressive interactions) in transparent boxes where animals had access to water and to food *ad libitum.* All the captive individuals were fed with standard rodent pellets composed of cornstarch (SAFE® SAS - basis formula AIN93G). Body weights and food quantity (Table S3) were measured at the day of capture (D0) then during the cleaning of boxes (D2, D4) and at the end (D2 or D5 depending on the field session). Each box was filled with a solid hemp litter, a shelter containing natural cotton and a paper roll as enrichment. Boxes were set up on shelter in a room with no heat and a photoperiod equivalent to the period of daylight. Individuals were weighed at day 0, and then at day 2 or 5 depending on the field session (see above).

Immune challenge

Wood mice were challenged by intraperitoneal injection with a LPS solution (LPS O111:B4, Merck) at 500 µg/kg. The LPS concentration was found to enhance inflammatory immune response and enhance TNF-α production in both the wood mouse and in laboratory rodent models (Devalloir *et al.*, 2023; Batista *et al.*, 2019). A period of two hours post-LPS challenge was chosen to take a blood sampling. The period of 2 hours is sufficient to enhance both TNF-α release during systemic inflammation in lab rodents and in the wood mouse (Devalloir *et al.*, 2023; Batista *et al.*, 2019).

Dissections

After the immune challenge and blood sampling, whatever the experimental group (*i.e.* with or without captivity), individuals were sacrificed by cervical dislocation under anaesthesia (see below) following the American Veterinary Medical Association ethical guidelines (Sikes and the Animal Care and Use Committee of the American Society of Mammalogists, 2016). The eyes were extracted to estimate individuals' age with crystalline lens mass (Vandorpe and Verhagen, 1979).

Blood sampling and storage

Blood was sampled from the retro-orbital sinus (volume of 1% of the body mass corresponding to 0.2 ml for a 20g individual) using a glass Pasteur pipette and was immediately transferred into EDTA-coated tubes (Microvette®, Sarstedt). For the complete blood count 50µL of blood was used fresh and kept stored at +5°C until a maximum period of 8h following the sampling. The rest of the blood (~150 µL) was centrifuged at 2000g for 15 minutes to collect plasma. Plasma was stored at -20°C during the field session and, when back at the laboratory, at -80°C until analyses.

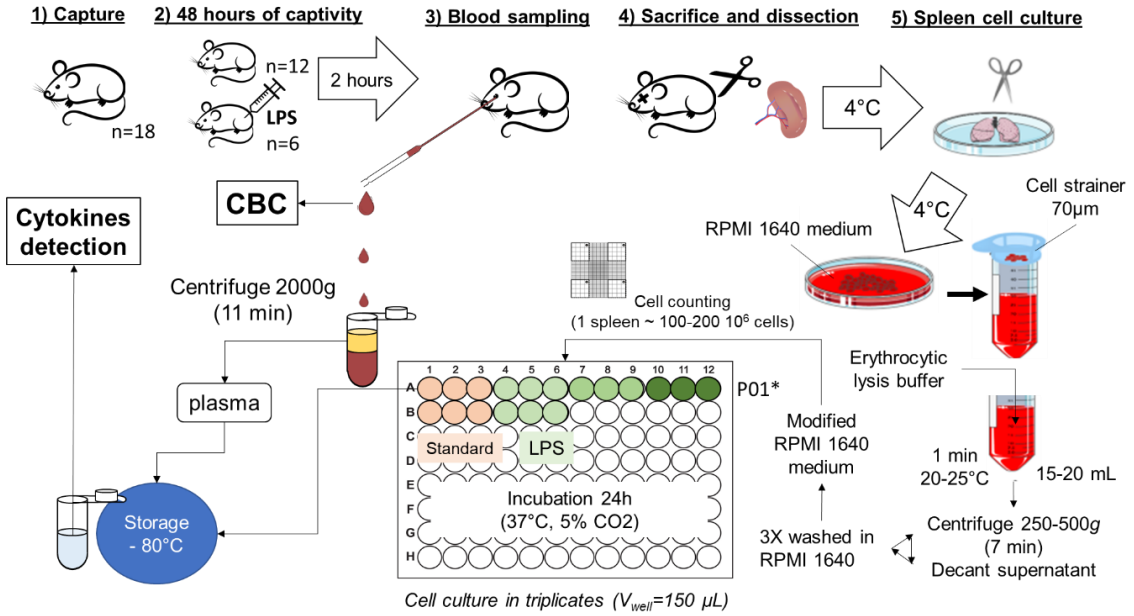
Complete blood counting

Complete blood counting (Table S1 and S2) was done with a haematology analyser automate (Horiba ABX Micros ES60) which gives leukocyte counts (in 10^3 cells/mm^3): white blood cells (WBC), lymphocytes (LYM), monocytes (MON), granulocytes (GRA), and erythrocytic parameters of blood: total red blood cells (RBC in 10^6/mm^3); haemoglobin (HB in g/dL); haematocrit (HT in %), mean corpuscular volume (MCV in μm^3), mean corpuscular haemoglobin (MCH in pg) and mean corpuscular haemoglobin concentration (MCHC in g/dL). The automate ensure repeatability in measuring 3 times each blood sample. Nonetheless, samples were measured between 2 to 3 times in the cases of non-repeatable measurements (high deviance within the sample). Blood quality control “low”, “medium” and “high” (MINOTROL 16, Horiba SAS) and an everyday automatic blank control ensured reproducibility and accuracy during and between each series of measurements. Blood count reference range values computed in the wood mouse including the confidence intervals and quantiles Q1 and Q3 were displayed on Figure 5 (Hadjadji *et al., in prep*).

Spleen cell culture

Spleen cell cultures were performed according to the figure 2 based on the method described in Friberg *et al.* (2011) and in Jackson *et al.* (2009).

Figure 2. Experimental design of the wood mice captured at la Bouloie for the plasma collection and the spleen cells isolation and culture performed for cytokines detection with ELISA and multiplex beads immunoassay. (CBC stands for complete blood counting)



P01* : Individual P01 which gave the highest yield of harvested spleen cells then used to calibrate LPS quantity to add in the cultures (see Fig. 4)

After the sacrifice and the dissection of the wood mice, the spleen was transferred to a sterile tube containing 5 mL of RPMI 1640 medium (Gibco™). (storage at *4°C* within an hour). The spleen was then transferred in a Petri dish placed at 4°C and cut into smaller pieces. Then, pieces of spleen were ground into a cell strainer (mesh 70µm) screwed on the top of a 50mL sterile falcon tube. Clumps were then further dispersed by flushing RPMI1640 with a sterile pipette on the strainer. Falcon tubes were filled with 15 to 20 mL of RPMI1640. The tube was then centrifuged at 250-500 g for 7 minutes and the pellets were resuspended. Then, 1 ml of red blood cell lysis buffer was added and gently mix for 1 min at room temperature. After that, the buffer was diluted with 10 ml of RPMI1640, centrifuged at 300 g for 7 min, the supernatant was discarded and pellets were resuspended. This step was repeated three times to wash spleen leukocytes from the buffer. Then, leukocytes were resuspended in modified RPMI for a concentration of 2 x 10^6 cells/ml. Modified RPMI 1640 was supplemented with 24 mM Na HCO3, 10% heat-inactivated foetal calf serum, 2 mM L-glutamine, 100 u/ml penicillin, 100 mg/ml streptomycin and 60 mM monothioglycerol, according to Jackson *et al.* (2009). Leukocytes were counted with a trypan blue solution on Kova® slides to prepare two triplicate sets of 150 µl volume containing each 2 x10^6 cells/ml in a 96 well-plate. A first triplicate was left unchallenged and the second was supplemented with 30µL of LPS solution at 50µg/mL. The dose of 30µL (*i.e.* final LPS medium concentration of 8.3 µg/ml) was determined with a gradient of LPS concentration (*i.e.* +15, +30, +45 µl) displayed on the figure 4 in the medium

done in one individual (P01). Spleen cells were then cultured (37°C, 5% CO2) for 24 hours and cell viability (~95%) was ensured with another counting. Each well was transferred in tubes and centrifugated for 1 min at 2000 g. The supernatant was stored at – 80°C for further cytokines measurement.

Determination of cytokines concentrations

Cross-reactivity

Only one TNF-α ELISA kit originally developed in lab mouse species was found to cross-react in the literature (Friberg *et al.*, 2011; Jackson *et al.*, 2009). As detailed in Jackson *et al.* (2009) the phylogenetic relationship between *Mus musculus*, *Apodemus sylvaticus* and *Rattus norvegicus* remains unresolved in Murinae. However, some protein-coding sequences including TNF-α amino acid sequences appear to be well conserved between *R. norvegicus* (GenBank NP_036807.1) and *M. musculus* (NP_038721.1) indicating a similarity of 94%. To our knowledge, no studies on TNF superfamily orthologs including the wood mouse were done.

Multiplex beads immunoassay

A large panel of cytokines (IL-17 A, IL-1β, Il-4, Il-9, INF-γ, IL-21, IL-6, IL-12p70, TNF-α) was assayed with an antibody-based multiplex (ProcartaPlex, Invitrogen) using the Luminex technology (*i.e.* magnetic beads for quantification of protein). The analyses were done as follow, 4 individuals from la Bouloie were selected for this experiment: two non-challenged (Control captive D2) and two LPS-challenged *in vivo* including P01 (see above). Plasma and spleen cell cultures (without LPS) were measured in duplicates. The remaining of samples (~25-50µL) was used for a dilution of factor 1:100 to determine if the sample concentration was not too high. Also, two negative controls of plasma taken from laboratory mice (Balb/c, males of 18-19 g) were used to determine if abnormal concentrations of cytokines may be detected on unchallenged laboratory-raised animals. Standard calibration curves gave acceptable recovery rates of 100.4% ± 13.5%. Only two concentrations return as positive in an unchallenged wood mouse with Il-6 (5.74 pg/ml) and another challenged one with TNF-α (8.98 pg/ml). All the other individual concentrations including laboratory mouse samples (*i.e.* good negative control) return below the limit of detection. As these results suggest the absence of cross-compatibility. The results of this experiment were detailed in table S4 and will only be used as basis of discussion in this study.

Measurement of TNF-α

For la Bouloie experiment TNF-α concentration of spleen cell culture (spike and not spiked with LPS) and plasma were measured with a commercially available kit Mouse TNF-α

quantikine ELISA kit (R&D MH, detection range from 10.9 to 700 pg/mL pg/ml, sensitivity 7.21 pg/ml). Two positive controls were used to evaluate the ability of the kit to detect elevated values at 100 pg/ml (average recovery 103%). Linearity was assayed with one previously challenged (*in vivo*) individual (P01) to evaluate TNF-α concentration in response to a gradient of LPS (*ex vivo)* stimulation in spleen cell culture (figure 4). Two replicates of samples spiked with known amounts of TNF-α were used to assess if antigen-antibody inhibitors may be present in the sample, giving an average recovery rate of 94% for a spike with 87.5 pg/ml and 90% with 21.9 pg/ml. Average recovery of 92% suggested the absence of such inhibitors. The ability of the kit to detect TNF-α concentrations below 7.21 pg/ml was estimated with two measurements in replicate of a control sample at 5.45 pg/ml. For plasma, the average recovery rate was of 59% (*i.e.* under-estimated values) and 144% for supernatant of spleen cell culture (*i.e.* over-estimated values). This suggests that samples with values below detection limits should be interpreted separately (Figure 5) and that they may be measured with a more sensitive kit. Indeed, two plasma samples below the detection limit of the Mouse TNF-α quantikine ELISA kit (R&D MH) were measured with a high sensitivity (HS) Mouse TNF-α quantikine ELISA kit (R&D MH, detection range from 0.781 to 50 pg/ml, sensitivity 0.295 pg/ml). One plasma sample gave a value under detection limit and the other one gave a TNF-α concentration 7.7 pg/ml which is at the very limit of the Mouse TNF-α quantikine ELISA kit of 7.21 pg/mL. For this reason, all the samples from CC Pévèle-Carembault were analysed with the HS Mouse TNF-α quantikine ELISA kit results and metrology (briefly, average recoveries of 86% from control samples and of 91% from spiked samples) were presented in Devalloir *et al.* (2023). All Control wild individual were under limits of detection limits (*i.e.* below blank).

Ethics

Blood sampling, immune challenge and euthanasia were performed under gaseous anaesthesia using isoflurane. Experiments were performed under the authorization of the French National Ethical Committee (Project APAFIS N°5340) by skilled and experienced investigators from Chrono-environnement research department (EU0592), following directive 2010/63/EU on the protection of animals used for scientific purposes.

Statistical analysis

Individuals' body wet variation in response to the quantity of food ingested was analysed with linear regressions. For CC Pévèle-Carembault linear regressions of the body mass were tested with the days of the measurement (*i.e.* D2-D0, D4-D2, D5-D4) in interaction with the quantity of food ingested. Polynomial regression was performed on the individual P01 to evaluate the form of the relationship between the TNF-α concentrations and the increasing concentration of LPS in the medium of spleen cell cultures. Pairwise comparisons using *t*-tests with the

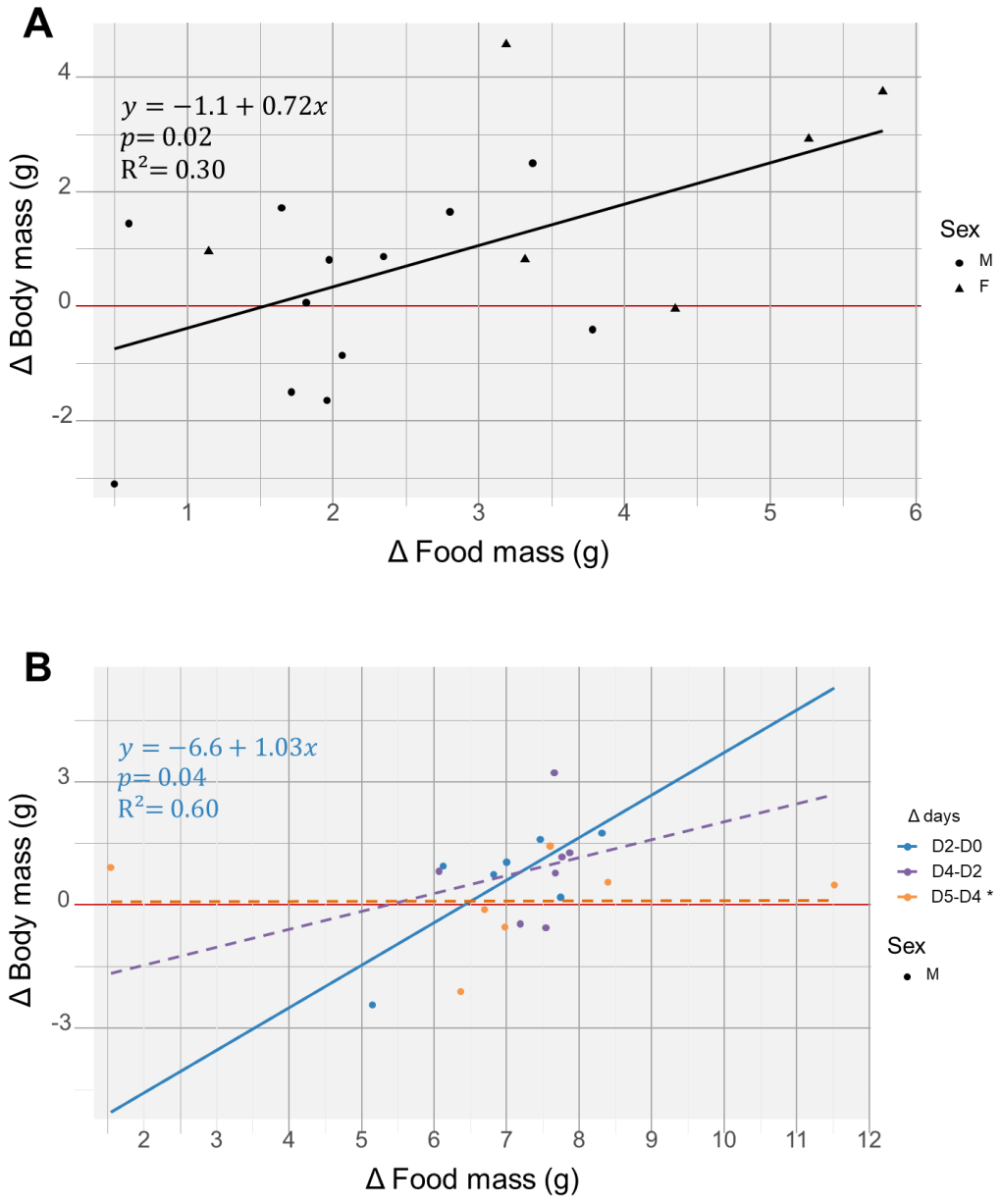
method of Benjamini and Hochberg (1995) were performed to compare TNF-α concentrations in the supernatant of spleen cell culture with and without LPS (*ex vivo*) and plasma of challenged and unchallenged individuals (*in vivo*). The comparison of blood counting parameters between Control and LPS challenged individuals was done with *t*-tests in Captive D2 (Captive D2 *vs* LPS captive D2) and Wild (Control wild *vs* LPS wild) individuals. A Pearson correlations matrix with scatter plots was performed to evaluate the relation between dependent and independent variables which are leukocyte count, TNF-α concentration and morpho-physical features (*i.e.* organs and body weight). Linear regressions were done to test the relationship between the spleen mass or the plasma TNF-α concentrations and lymphocyte or monocyte counts for LPS wild and captive D5 animals. Prerequisites of *t*-tests and Pearson's correlation (normality and homoscedasticity) were verified using Kolmogorov-Smirnoff and Levene's tests. The package 'performance' was used to verify linear models' assumptions (normality of residuals, linear relationship, homogeneity of variance, multicollinearity). All analyses were done with R version 3.6.1. (R Core Team, 2023) and graphics were drawn with 'ggplot2' and 'ggpubr' packages.

Results

Acclimatisation to captivity

The variation of captive individuals' body mass was found to increase with the quantity of food ingested (Figure 3) during the first two days of captivity in both Captive D2 (R^2=0.3, $p<0.05$) and the Captive D5 wood mice (R^2=0.6, p<0.05). However, in the Captive D5 no significant relation was found between day 4 and day 2, and also during the last day of captivity (p>0.05).

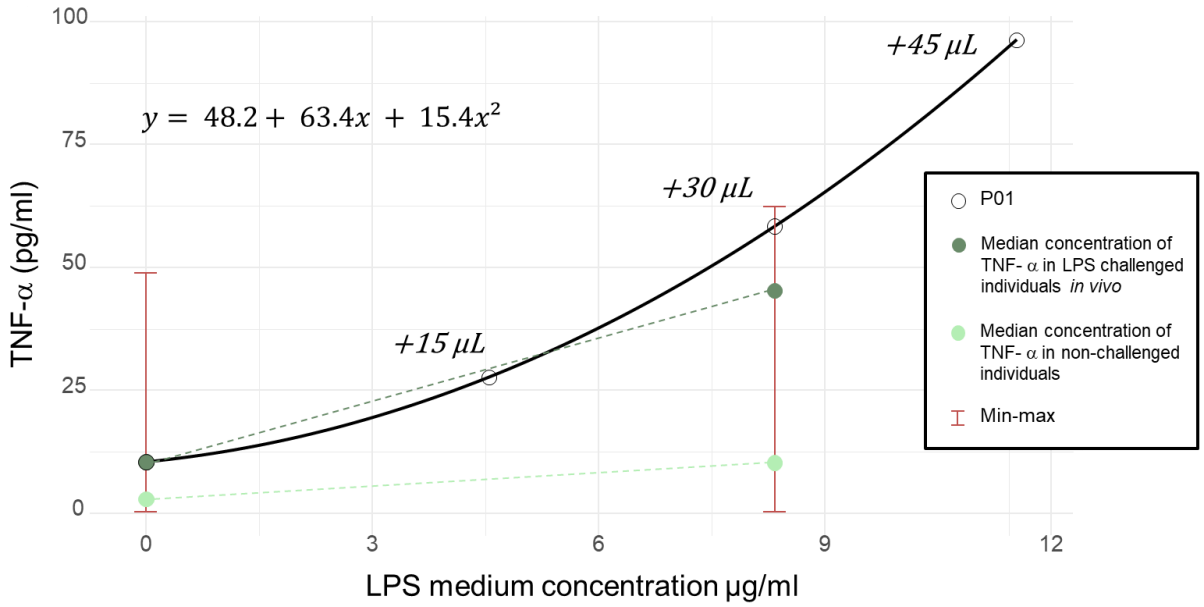
Figure 3. Variation of wood mouse body mass in response to the quantity of food consumed during 48 hours of captivity in la Bouloie (A), and during 5 days of captivity in CC Pévèle-Carembault (B). Δ: differences between the body or the food mass (*e.g.* D2 – D0); in red: Δ Body mass of 0 (allows to discriminate individuals which present weight loss); *: ΔD5-D4 was multiplied by 2 to obtain the same period of two days of D2-D0 and D4-D2; in dotted lines: non-significant trends.



Lethal procedures

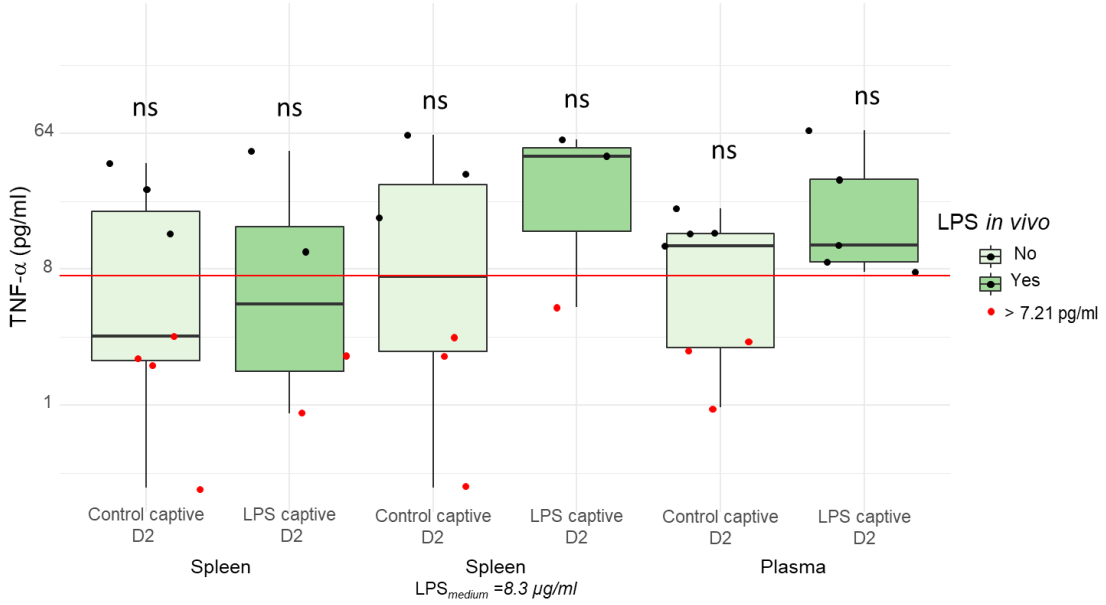
On the figure 4, the spleen cell culture of the individual P01 displayed a significant increase in response to LPS spikes (R^2= 0.37; *p*= 0.0097).

Figure 4. Spleen cell culture TNF-α concentration (pg/ml) of the individual P01 in response to lipopolysaccharide (LPS) concentration in the medium with addition (*i.e. +15, +30, +45 µL*) of LPS solution at 50µg/ml.



The relation displayed a second-order polynom with an exponential shape increasing by 6 times its TNF-α concentration between the unchallenged medium (10 pg/ml) and the LPS-challenged medium at 8.3 µg/ml (60 pg/ml). The median concentrations of TNF-α measured in LPS captive D2 individuals (no LPS to 8.3 µg/ml:10 to 45 pg/ml) displayed higher concentrations and a higher increase than in Control captive D2 (no LPS to 8.3 µg/ml: 2.8 to 10 pg/ml). However, on the figure 5 no significant differences were detected in TNF-α concentrations between Control captive D2 and LPS captive D2 tested with and without values below the detection limit of 7.21 pg/ml (*p>0.1*).

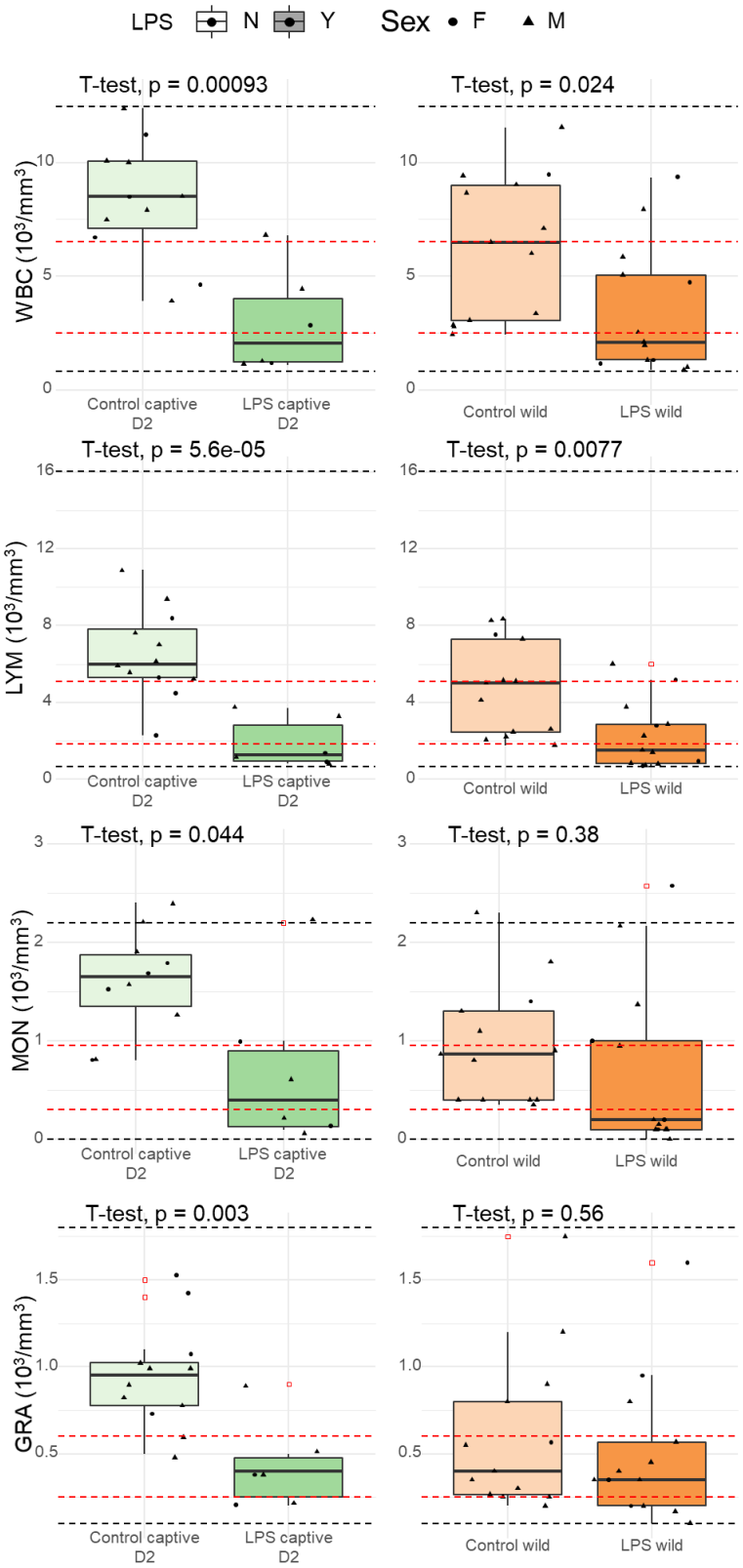
Figure 5. Comparison of TNF-α concentrations (pg/ml) measured in plasma and spleen cells cultured with or without LPS (8.3 µg/ml) of wood mice from la Bouloie (Kit Mouse Quantikine TNF-α, R&D, MH)



Non-lethal procedures

No effect of LPS was detected in erythrocytic parameters (p>0.05, Figure S1). On the figure 6, all leukocyte parameters are included in the reference intervals except for three values on monocytes counting close to the upper interval. However, in Control captive D2 individuals all the inter quartile range are above the Q3 of blood reference intervals while this was not the case in Wild individuals where the median appears as closer to the Q3. Furthermore, the position of the median of leukocytes in LPS captive D2 and in LPS wild is close to the Q1 of blood reference intervals with an inter quartile range below Q3. This statistically translates a significant decrease of leukocyte counts between Control and LPS challenged individuals for Captive D2 and Wild (p<0.001 and p<0.01, respectively). The decrease of leukocytes occurs mainly in lymphocytes of both Captive D2 and Wild (p<0.0001 and p<0.01, respectively). However, a significant decrease of monocytes and granulocytes was found between Control captive D2 and LPS Captive D2 ($p<0.05$) but not in Wild wood mice ($p>0.1$).

Figure 6. Leukocytes parameters of a wood mouse population trapped in la Bouloie (in green, Control and LPS captive D2) and wood mice populations trapped in CC Pévèle-Carembault (in orange, Control wild and LPS wild). Reference intervals leukocyte count (dotted black line) and quartiles Q1 and Q3 (dotted red lines) were obtained from Hadjadji *et al.* (in prep). Boxplot outliers are indicated by red squares.



Lethal vs non-lethal

On figure 7, positive correlations were found among and between the leukocyte total population (*i.e.* white blood cells) and subpopulations (*i.e.* lymphocytes, monocytes, granulocytes). In both LPS Wild and LPS captive D5 white blood cells, lymphocytes and monocytes displayed positive increase in response to challenge ($r>0.87$, $p<0.001$). However, no significant variation of granulocytes depended on the variation of the other leukocytes in LPS captive D5 ($p>0.05$). The body mass displayed positive correlations with the liver ($r=0.74$, $p<0.001$) and the kidneys ($r=0.67$, $p<0.01$) mass and with the spleen mass only when LPS wild and captive D5 were taken together ($r=0.55$, $p<0.05$). The spleen mass of LPS captive D5 displayed positive correlations with white blood cells ($r=0.88$, $p<0.05$), lymphocytes ($r=0.88$, $p<0.05$) and monocytes ($r=0.92$, $p<0.01$). However, no significant correlations were detected between leukocytes and the spleen mass in LPS wild individuals. On the figure 8, both lymphocytes ($R^2=0.78$, *p<0.05*) and monocytes ($R^2=0.85$, *p<0.01*) increased in response to the spleen wet mass of the LPS captive D5 individuals. Nonetheless, no significant increase was found in LPS wild monocytes ($p>0.05$) whilst a positive trend may be observed on lymphocytes ($R^2=0.3$, $p=0.057$). A negative correlation (figure 7) was found between monocytes and plasma TNF-α concentration of LPS Wild ($r=-0.77$, $p<0.01$), but not in LPS captive D5 ($p>0.05$). Indeed, monocyte count was found to decrease in response to plasma TNF-α concentration in LPS Wild ($R^2=0.6$, *p<0.01*), but not in LPS captive D5 ($p>0.05$). A negative trend can be however noticed for lymphocyte count in response to TNF-α concentration of LPS Wild ($R^2=0.3$, $p=0.08$), while no trend was found in LPS captive D5 ($p>0.05$).

Figure 7. Pearson correlation matrix with scatter plots of the relation between immunological (leukocytes and TNF-α) and morphological (body, liver, spleen weights) parameters of wild (LPS wild) and captive (LPS captive D5) wood mice from CC Pévèle-Carembault.

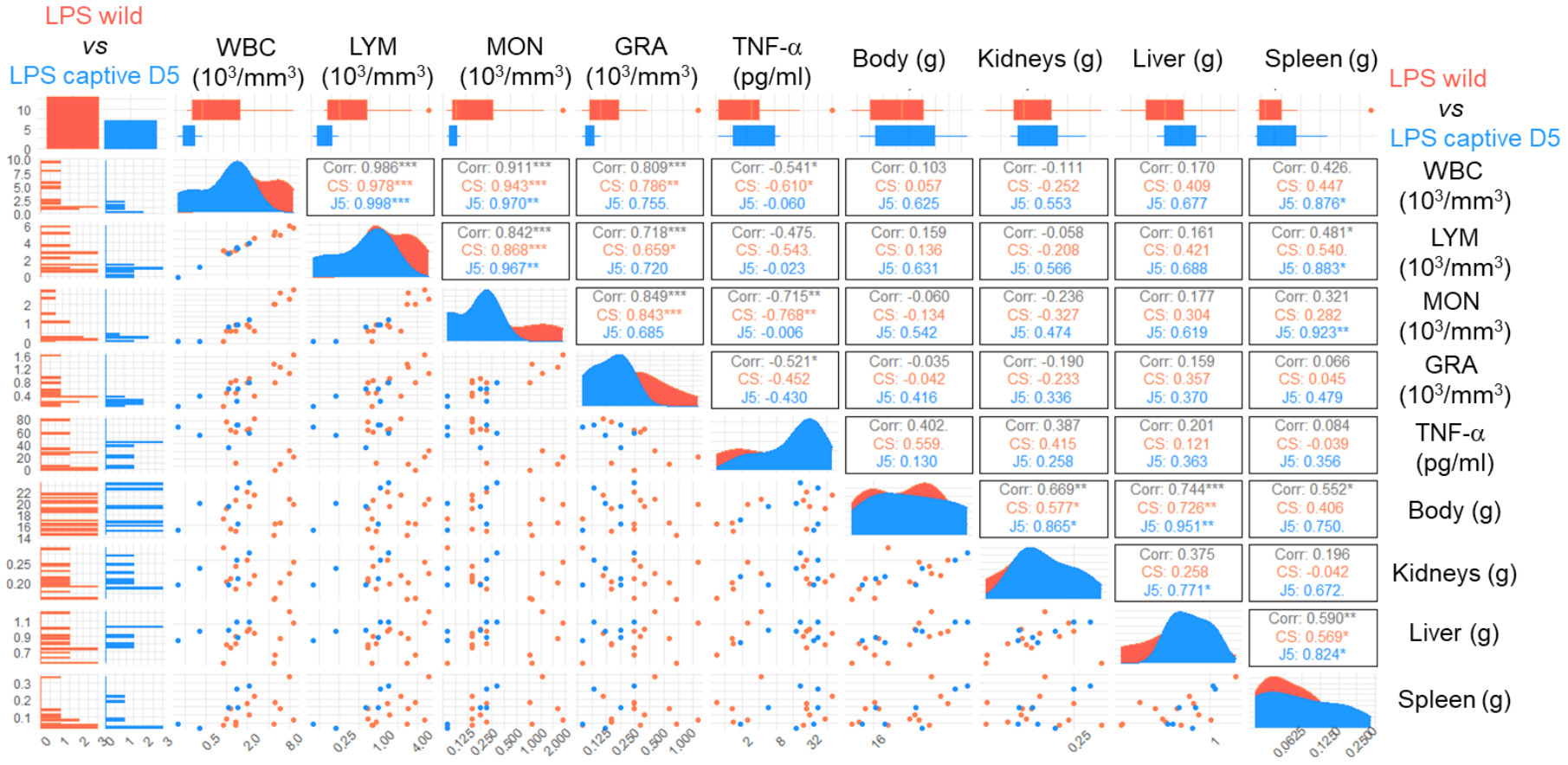
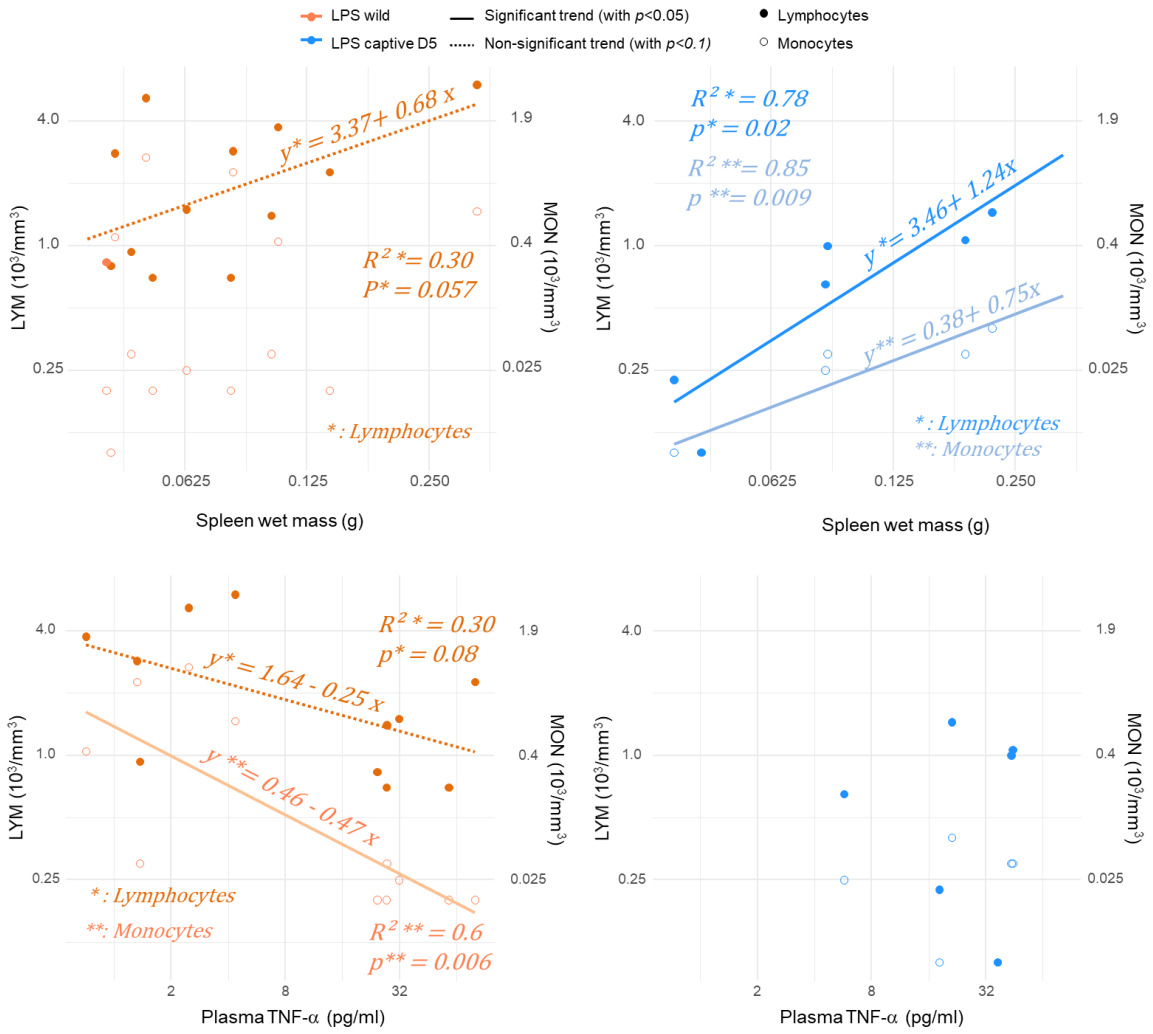


Figure 8. Variation of lymphocytes (LYM) and monocytes (MON) blood counts in response to the spleen wet mass (g) and to plasma TNF-α concentration (pg/ml) in wild (LPS wild) and captive (LPS captive D5) wood mice from CC Pévèle-Carembault.



Discussion

Wood mouse acclimatisation to captivity

No severe mass loss was detected during captivity (<17%) and the positive increase in individuals' mass in response to the food ingested suggested a gain in the body mass within the first two days. However, the absence of significant variation after 2 days of captivity highlighted a stabilisation in the net body mass gained in captivity. The relation between the individual mass variation and the quantity of food ingested might suggest a pattern of acclimatisation to captivity. Further behavioural analysis and hormonal markers of stress (*e.g.* corticosterone) would corroborate any ability of wood mice to cope with a short period of captivity (Kleiman *et al.*, 1996).

Assessment of immunocompetence with the spleen

Among the tested panel of cytokines only TNF-α was found to cross-react with commercially available kits. Though the phylogenetic proximity of the wood mouse and the domestic mouse would indicate that commercially available kits might cross-react with the wood mouse almost all results of the analyses returned below the limit of detection. One measurement gave a positive interpretable value for TNF- α (8.98 pg/ml) as it corresponds to a wood mouse challenged with LPS (*in vivo*). Nonetheless, the antibodies used in Mouse TNF-α quantikine ELISA kit (R&D MH) displayed a significant increase of TNF-α in response to *in vitro* LPS spikes in the medium and to *in vivo* challenge with LPS. Even more, plasma samples displayed better recovery for individuals challenged with LPS from CC Pévèle-Carembault considering that Control wild individuals were all below the detection limit of the high sensitivity mouse TNF-α quantikine ELISA kit (R&D MH) kit. The use of a kit with lower detection range (0.295 *vs* 7.21 pg/ml) suggested that lower circulating levels of cytokines, at least TNF-a, were present in the wood mice. This is in line with Abolins *et al.*, (2017) who found lower levels of cytokines in wild mice in comparison with laboratory mice. The authors attributed lower cytokine response in wild mice to the different costs involved in the maintaining immune homeostasis in a natural environment (*i.e.* environmental stressors).

The use of the spleen cell culture promotes relevant information on the inflammatory response of spleen leukocyte populations. Though 4 individuals were used to determine cross-reactivity and sensitivity of kits (*i.e.* data not shown), our results indicated that both LPS challenge *in vivo* and/or *ex vivo* stimulation enhanced TNF-α secretion from spleen cells. However, the results did not show any differences between TNF-α concentrations with sometimes a large number of values below the limit of detection. For example, spleen cell cultures from Control captive D2 4 on the 7 individuals (>57%) were below the limit of detection of 7.21 pg/ml.

Measurement of TNF-α concentrations in *ex vivo* stimulation of splenocytes by LPS performed by Jackson *et al.* (2009) excluded 53% of samples below the control response (values close to the detection limit of the kit). High sensitivity kit might provide better yields for the measurement of TNF-α in wood mice challenged with 500 µg/kg of LPS. Although, only supernatant was used to quantify cytokines levels, further molecular analysis of mRNA can be done to quantify through real-time Q-PCR the levels of expression of cytokines and transcription involved in the inflammatory cascade (Friberg *et al.*, 2011).

The relationship between spleen mass and lymphocyte or monocyte counts found significant only in LPS captive D5 individuals might be attributed to the supply with an “optimal” food. Indeed, the standard rodent laboratory food provided wood mice in sufficient amount of macro- and micro-nutrients which maintained homeostasis and also immune system functioning. Micronutrients are key modulators of the immune response (*e.g.* Gombart *et al.*, 2020; Smith *et al.*, 2018). For instance, selenium (*i.e.* a major dietary antioxidant) displayed a strong potency to down-regulate immune response to LPS challenge in wood mice captured on sites polluted by Cd and Pb and fed during 5 days with a selenium-deficient diet (Devalloir *et al.*, 2023). Several studies related that environmental stressors including habitat fragmentation or habitat loss may diminish the quantity and the quality of available resources (Bonebrake *et al.*, 2019; Acevedo-Whitehouse and Duffus, 2009a). Further experiments considering the nutritional status of wild animals might validate this interpretation (Strandin *et al.*, 2018).

Using haematological parameters as non-lethal procedures

Haematological parameters displayed relevant capacity to disentangle which compartment of the immune cell populations is particularly affected by the challenge. Here, Captive individuals from la Bouloie (Captive D2) displayed significant decreases of lymphocytes, monocytes and granulocytes while only lymphocytes were found to decrease in wild wood mice from CC Pévèle-Carembault. This dissimilarity might reflect the role of captivity with sufficient amount of macro and micron-nutrients or might be linked to pattern of heterogeneous immunity. Indeed, the upper position (*i.e.* above the Q3) of the inter-quartile ranges indicated higher proportion of leukocytes in Control captive D2 from la Bouloie. This argue in favour with the hypothesis of heterogeneous immunity among wild animals as different areas may host different stressors including pathogens populations and pollution (Jolles *et al.*, 2015). For example, a study on red deer (*Cervus elaphus*) found heterogeneous levels of helminth parasitism and specific antibody levels in response to seasonal and spatial variations on a total study area of 12.7km² (Albery *et al.*, 2019). However, the decrease of lymphocytes and monocytes in response to a per acute injection of LPS was in accordance with other studies on rats which displayed similar patterns of decrease within the 2 hours post injection (Brooks

et al., 2017). The very pattern of lymphocyte-associated decrease in LPS challenged individuals reflected the relocalisation of lymphocytes and monocytes to the site of infection. Nonetheless, a pattern of inflammatory response was observed only in LPS individuals with a decrease of monocytes and lymphocytes in response to plasma TNF-α concentration. The absence of any pattern associated with TNF-α concentrations in captive D5 might may depict a different sequence on the time series of inflammatory processes or the activation of other mechanisms. During an inflammatory response, different components of innate, cellular or humoral immunity can be activated (from recognition to elimination). Thus, the absence of a visible inflammatory response in leukocytes circulating population might indicate that cellular immunity was or is not activated at the time of the measurement (Martin, 2009a). Further analyses on glucocorticoids (*i.e.* involved in anti-inflammatory processes) would confirm this trend. Another possibility linked to the absence of leukocyte relocalisation might be related to a pattern of tolerance. Further analyses on the level of expression of mRNA cytokines (*i.e.* higher expression of TLR4) would validate this hypothesis of tolerance (Jackson *et al.*, 2014).

Blood sampling as substitute for spleen dissection

The spleen displayed several advantages in terms of immunological measurement. For instance, the spleen mass can vary in response to stressors and provides a large number of cells which can be harvested in several replicates (Jackson *et al.*, 2009; A. C. Nunes *et al.*, 2001). Indeed, spleen mass reflected lymphocyte and monocyte variations of captive wood mice but not in wild ones. The differences in spleen mass found between captive and wild populations might be attributed to a delay in the period needed to integrate potential effects of LPS challenge in a lymphoid organ. Haematological parameters on peripheral blood leukocytes' response to stressors can provide an efficient non-lethal alternative to spleen weight. Indeed, stress (*i.e.* during acute response to a stressor) can promote a significant decrease of peripheral lymphocytes and monocytes enhanced by an adrenal gland stimulation mediated by corticosterone (*i.e.* major glucocorticoid in murine species) in rats (Dhabhar *et al.*, 1996). Hormonal mediators might be involved in the absence of lymphocytes and monocytes associated pattern of decrease in response to TNF-α in LPS captive D5 wood mice. Blood lymphocytes and monocytes count showed to be a good proxy of the infectious status of animals as both captive and wild displayed a decreasing count in response to LPS. One particular advantage of blood, out of the non-lethal procedure of collection, is the possibility of splitting whole blood for complete blood counting and the plasma or serum for biochemical analyses. In the wood mouse, a volume of 1% of the body weight (*i.e.* 200µL for a 20g animal) allowed us to perform blood counting (~50µl) and quantification of plasma TNF-α (~50µL x 2 for replicates). Many enzymatic or chemical assays can be performed on low levels of blood

to estimate physiological disturbances mediated by stressors (*e.g.* oxidative stress, hormone quantification…). Furthermore, the development of multiplex analysis, whilst unsuccessfully cross-reactive with the wood mouse, is a promising technology to perform analyses in low volumes of blood. Even leukocyte cultures can be performed in a low amount of peripheral blood as found in *Rhinella arenarum* toads (Agüero *et al.*, 2017). Researches on the development of non-lethal or less invasive methods to evaluate immune response of wild animals, such as NAMs, would meet the needs of further ethical considerations on animal uses in sciences (Zemanova, 2020; Arck, 2019). In addition, developing new and/or cross-reactive analytical methods to evaluate the immunocompetence of wild animals might promote another sight on the effect of anthropogenic stressors on wildlife and the monitoring of the emergence of zoonotic diseases (Acevedo-Whitehouse and Duffus, 2009a).

Conclusion

Our study brought insights into the development of non-lethal procedures to evaluate wild mammals' immunocompetence. Lethal procedures performed to evaluate the performance of commercially available kits (*i.e.* initially developed in laboratory mice) to cross-react with wood mouse specimens ended in the selection of one ELISA kit able to quantify TNF-α concentration in the plasma. Wood mice displayed an interesting capacity to cope rapidly with a short period of captivity within 2 days. Behavioural and hormonal measurements of stress response to captivity may be done for further interpretations. Haematological analyses found heterogeneous proportions of leukocytes between the populations studied. Leukocyte subpopulations displayed different patterns of decrease in response to LPS challenge (*in vivo*) in captive and wild animals. Monocyte recruitment in response to TNF-α was found to be significant in wild animals but not in captive ones. Captive individuals fed with standard laboratory rodent food displayed a potent ability of the spleen mass to be related to lymphocyte and monocyte counts. Further insights might be given into the potential effect of sufficient quantity and good quality diet on the immunocompetence of wild mammals. More non-lethal procedures might estimate the immunological or physiological influence of stressors on wild mammals considering their impact on fitness.

Funding

This work was supported by the Agence De l'Environnement et de la Maîtrise de l'Energie (ADEME, programmes BIOTROPH, grant number N°1172C0030, and DYSPAT, grant number 1572C0309), the Conseil Régional de Franche-Comté (for having financially supported the purchase of the HORIBA ABX Micros ES 60 haematology analyser), the LTER Zone Atelier Arc Jurassien (for the fnancial support to purchase the field anaesthesia device), and the OSU

THETA and the University of Franche-Comté (programmes DEMENOL, MEXCO and OREAS, which supported the costs of fieldwork and field and laboratory analyses).

Authors contributions

C.F., R.S., N.vd.B. and Q.D designed the study. C.F., R.S. and V.D. managed and participated in the long-term survey over the site of Metaleurop Nord since 2006. A-C.G., V.D. and Q.D managed the field session and A-C.G., V.D., C.B. and Q.D the laboratory work. A-C.G., C.F., R.S. and Q.D analysed the data and C.F., R.S., N.vd.B. and Q.D. interpreted the data and wrote, reviewed and edited the manuscript. All co-authors commented on the manuscript and accepted the final version. R.S. and CF acquired the financial support for the projects leading to this publication.

Acknowledgments

The authors thank the university of Franche-Comté and Emmanuelle Uhres from the Communauté de Commune de Pévèle-Carembault for allowing the access to the sites of capture.

Supplementary materials

Table S1 Haematological and TNF-α concentrations in plasma and spleen cell cultures with and without LPS (*in vitro*) measured in non-challenged (control captive) and LPS challenged (*in vivo*) wood mice captured on the la Bouloie campus. (sex: male (M) or female (F); WBC: white blood cells; LYM: Lymphocytes; MON: Monocytes; GRA: Granulocytes)

	Captivity	Age	Sex	WBC	LYM	MON	GRA	TNF-a Spleen[1.]	TNF-a Spleen LPS[1.]	TNF-a Plasma[1,2.]
	Days	*Days*	*M/F*	*10^3/mm^3*	*10^3/mm^3*	*10^3/mm^3*	*10^3/mm^3*	*pg/ml*	*pg/ml*	*pg/ml*
Control captive	2	[5.]	F	4.6	2.3	0.8	1.5	<LD	<LD	<LD [3.]
	2	[5.]	F	8.5	5.3	1.8	1.4	1.88	<LD	<LD
	2	[5.]	M	7.9	5.6	1.3	1	<LD	0.28	20.25
	2	[5.]	M	3.9	5.2	3.3	1	2.88	<LD	0.97
	2	[5.]	M	10.1	7	2.2	0.9	<LD	2.08	<LD
	2	[5.]	F	6.7	4.5	1.5	0.7	2.08	<LD	2.26
	2	[5.]	F	11.2	8.4	1.7	1.1	27.34	62.34	13.87
	2	[5.]	M	15	10.9	3.1	1	<LD	<LD	2.58
	2	[5.]	M	8.5	6.1	1.6	0.8	0.28	2.88	<LD
	2	[5.]	M	7.5	5.9	0.8	0.8	13.84	17.69	11.45
	2	[5.]	M	10	7.6	1.9	0.5	<LD	<LD	<LD
	2	[5.]	M	12.4	9.4	2.4	0.6	40.62	34.24	13.71
LPS captive D2	2	[5.]	M	6.8	3.7	2.2	0.9	0.88	4.48	<LD
	2	[5.]	M	1.1	0.8	0.1	0.2	<LD	<LD	31.5
	2 [4.]	[5.]	M [4.]	4.4 [4.]	3.3 [4.]	0.6 [4.]	0.5 [4.]	10.45 [4.]	58.38 [4.]	8.98 [4.]
	2	[5.]	F	1.2	0.9	0.1	0.2	<LD	<LD	7.71 [2.]
	2	[5.]	M	1.3	1.1	0.2	0.4	2.08	<LD	67.25
	2	[5.]	F	2.8	1.4	1	0.4	48.9	45.28	11.61

[1.] TNF-α concentrations measured with the kit Mouse TNF-α Quantikine (R&D ®)

[2.] TNF-α concentrations measured with the kit High-sensitivity Mouse TNF-α Quantikine (R&D ®)

[3.] Below limits of detection

[4.] Individual P01 which gave the highest yield of harvested spleen cells then used to calibrate LPS quantity to add in the cultures (see Fig. 4)

[5.] Not measured

Table S2 Haematological and TNF-α concentrations in plasma and spleen cell cultures with and without LPS (*in vitro*) measured in wood mice from Burgundy – Franche-Comté with and without LPS challenge *in vivo* (*i.p.* injection).

Group	Localisation	Captivity	Age	Sex	WBC	LYM	MON	GRA	TNF-α plasma[1.]
		Days	*Days*	*M/F*	*$10^3/mm^3$*	*$10^3/mm^3$*	*$10^3/mm^3$*	*$10^3/mm^3$*	*pg/ml*
Control wild	Thumeries	0	[3.]	M	9.4	8.3	0.9	0.3	<LD[2.]
Control wild	Thumeries	0	92	M	2.4	1.8	0.4	0.3	<LD
Control wild	Thumeries	0	107	M	6.0	4.1	1.1	0.8	<LD
Control wild	Thumeries	0	53	M	3.4	2.6	0.4	0.2	<LD
Control wild	Thumeries	0	48	M	3.1	2.5	0.4	0.3	<LD
Control wild	Thumeries	0	145	M	11.6	8.4	2.3	0.9	<LD
Control wild	Thumeries	0	109	M	9.0	7.3	1.3	0.4	<LD
Control wild	Tourmignies	0	112	M	6.5	5.2	0.8	0.6	<LD
Control wild	Tourmignies	0	83	M	2.9	2.2	0.4	0.3	<LD
Control wild	Tourmignies	0	89	M	2.8	2.1	0.4	0.4	<LD
Control wild	Tourmignies	0	265	F	9.5	7.5	1.4	0.6	<LD
Control wild	Tourmignies	0	109	M	7.1	5.0	0.9	1.2	<LD
Control wild	Tourmignies	0	252	M	8.7	5.1	1.8	1.8	<LD
LPS captive D5	Thumeries	5	[3.]	M	[3.]	[3.]	[3.]	[3.]	1.89
LPS captive D5	Thumeries	5	53	M	0.2	0.1	0.0	0.1	36.97
LPS captive D5	Thumeries	5	145	M	2.1	1.5	0.3	0.4	21.17
LPS captive D5	Thumeries	5	109	M	1.1	0.7	0.2	0.3	5.72
LPS captive D5	Tourmignies	5	89	M	1.5	1.0	0.2	0.3	43.51
LPS captive D5	Tourmignies	5	109	M	0.4	0.2	0.0	0.2	18.21
LPS captive D5	Tourmignies	5	252	M	1.4	1.1	0.2	0.1	44.37
LPS wild	Thumeries	0	120	M	2.5	2.3	0.1	0.2	79.85
LPS wild	Thumeries	0	174	M	2.0	1.4	0.2	0.4	27.43
LPS wild	Tourmignies	0	67	F	4.8	2.8	1.0	1.0	<LD
LPS wild	Tourmignies	0	163	M	1.0	0.7	0.1	0.2	58.10
LPS wild	Tourmignies	0	77	M	5.1	3.8	1.0	0.4	0.71
LPS wild	Tourmignies	0	206	M	0.9	0.8	0.0	0.1	<LD
LPS wild	Tourmignies	0	56	M	1.3	0.8	0.1	0.4	24.41
LPS wild	Tourmignies	0	141	F	9.4	5.2	2.6	1.6	2.48
LPS wild	Tourmignies	0	47	F	1.3	0.9	0.2	0.2	1.37
LPS wild	Tourmignies	0	98	M	2.1	1.5	0.2	0.5	31.76
LPS wild	Tourmignies	0	105	M	7.9	6.0	1.4	0.6	4.33
LPS wild	Tourmignies	0	125	F	1.2	0.7	0.1	0.4	27.30
LPS wild	Tourmignies	0	60	M	5.8	2.9	2.2	0.8	1.32

[1.] TNF-α concentrations measured with the kit High-sensitivity Mouse TNF-α Quantikine (R&D ®)

[2.] Below limits of detection

[3.] Missing data

Table S3 Variation of the whole-body wet mass (g) and the quantity of food ingested (g) by captive wood mice from la Bouloie (2 days) and CC Pévèle-Carembault (5 days) between days of captivity (D).

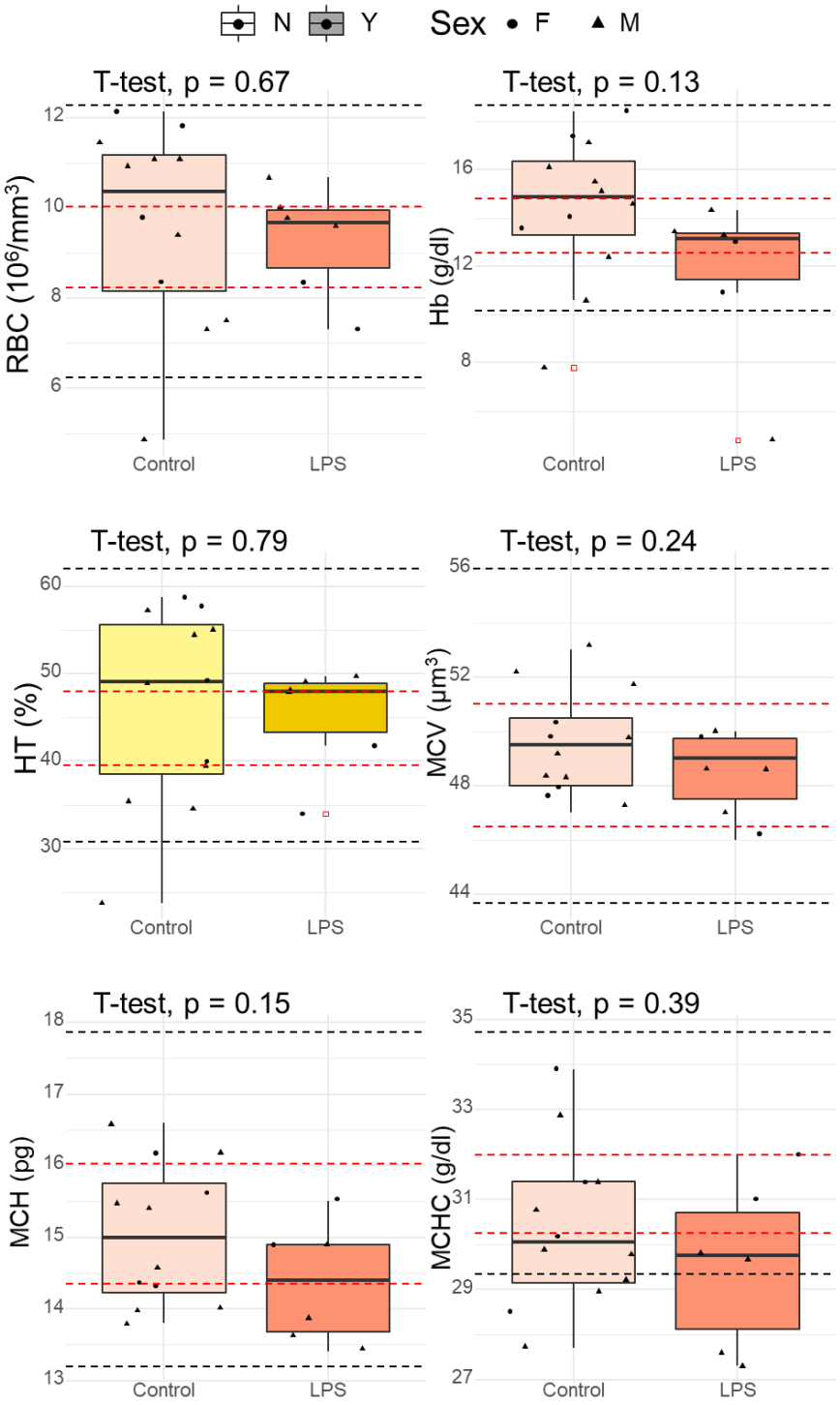
Group	Δ Whole body wet mass (g)			Quantity of food ingested (g)		
	D2-D0	D4-D2	D5-D4	D2-D0	D4-D2	D5-D4
LPS Captive D5	0.736	0.036	-0.533	6.823	7.675	5.76
	1.595	1.618	-2.757	7.463	7.657	0.77
	1.751	-0.482	-0.993	8.315	7.869	4.198
	-2.436	1.969	0.194	5.15	7.188	3.488
	0.943	-0.125	-0.102	6.127	6.065	3.8
	0.181	0.988	-1.224	7.745	7.765	3.348
	1.041	-1.601	-0.499	7.001	7.543	3.185
Control Captive	3.745	-	-	5.774	-	-
	0.951	-	-	1.147	-	-
	0.862	-	-	2.347	-	-
	1.647	-	-	2.802	-	-
	2.496	-	-	3.369	-	-
	2.926	-	-	5.264	-	-
	0.818	-	-	3.316	-	-
	1.444	-	-	0.597	-	-
	-3.108	-	-	0.498	-	-
	0.809	-	-	1.973	-	-
	-0.412	-	-	3.782	-	-
	-1.5	-	-	1.715	-	-
LPS Captive D2	-0.863	-	-	2.062	-	-
	-1.65	-	-	1.959	-	-
	1.713	-	-	1.646	-	-
	4.568	-	-	3.19	-	-
	0.06	-	-	1.815	-	-
	-0.044	-	-	4.346	-	-

Table S4 Results of multiplex beads immune assays on wood mouse plasma and supernatant of spleen cell culture samples (ProcartaPlex, Invitrogen). (Mus01 and 02 are negative controls corresponding to unchallenged laboratory mice).

	LPS *in vivo*	IL-17 A	IL-1 β	Il-4	Il-9	INF-γ	IL-21	IL-6	IL-12p70	TNF-α
P01-plasma	Yes	<LD[1.]	<LD	<LD	<LD	<LD	<LD	<LD	<LD	**8.98**
P01-spleen	Yes	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD
F03-p plasma	Yes	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD
F03-spleen	Yes	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD
Q04-p plasma	No	<LD	<LD	<LD	<LD	<LD	<LD	**5.74**	<LD	<LD
Q04-spleen	No	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD
R01-p plasma	No	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD
R01-spleen	No	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD
P01-plasma 1:100	No	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD
F03- plasma 1:100	No	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD
Q04- plasma 1:100	No	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD
R01- plasma 1:100	No	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD
Mus01-plasma	No	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD
Mus02-plasma	No	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD	<LD

[1.] Below the limit of detection

Figure S1 Erythrocytic indicators measured in wood mouse from la Bouloie given with the reference intervals (dotted black line) and quartiles Q1 and Q3 (dotted red lines) of Hadjadji *et al.* (in prep, see 3.3). Boxplot outliers are indicated by red squares. Boxplot outliers are indicated by red squares.



4 DISCUSSION

4.1 Insights in eco(logy)-immunology

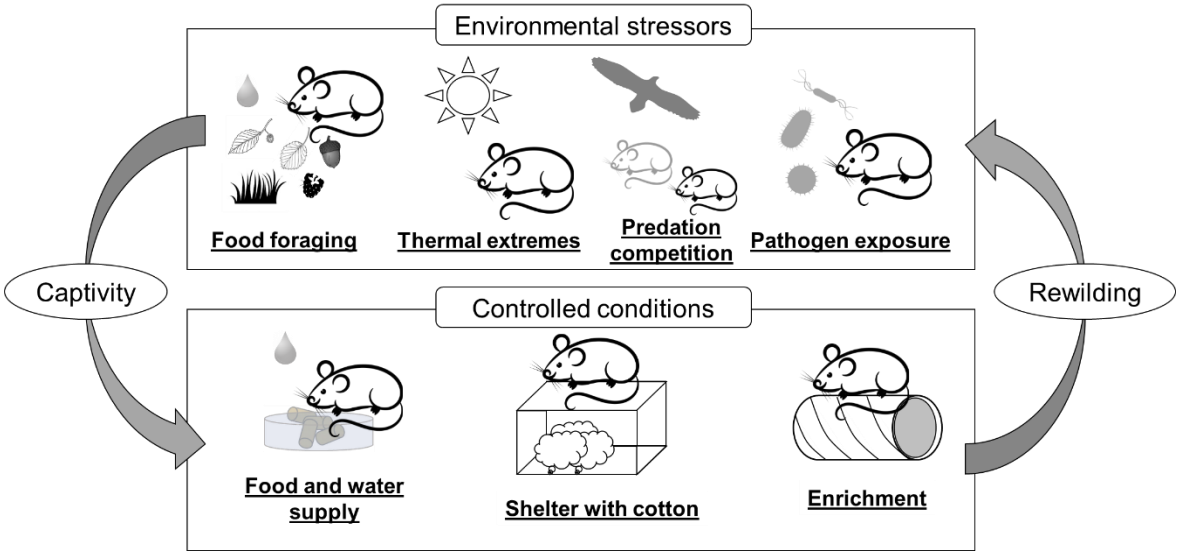
4.1.1 Influence of nutritional quality and captivity on the immune response

The absence of detectable TNF-α in plasma samples of non-challenged wood mice presented in Chapter 3.2 suggests that LPS challenged individuals displayed a general trend of inflammatory response. The results indicate discrepancies in the immune response to LPS challenge between wild and captive individuals. Haematological counting in Chapter 3.4 displayed a higher number of lymphocytes, monocytes and granulocytes in wood mice from the CC Pévèle-Carembault (*i.e.* Control site used in the Hauts-de-France), in comparison with populations from the campus of Besançon (Bourgogne – Franche-Comté). This is in accordance with the hypothesis of heterogeneous immunity that considers that different areas may host different environmental features able to modulate the composition and functioning of the immune system (Jolles et al., 2015). However, Chapter 3.3 shows that biological and environmental variables have a weak influence on haematological parameters (explained 8.5% of the variation with RDA analyses). This suggests that other variables may explain the differences in leukocytes counting between populations. Environmental stressors including pathogens and low diet quality were found to particularly affect mammals immunity (Gombart et al., 2020; Strandin et al., 2018).

In a field experiment, we increased food availability for a portion of the mice in the population by providing sorghum seeds to a set of food stations. We reduced parasite intensity of randomly chosen mice through ivermectin treatment. We determined the number and quality of offspring for the mice using paternity analysis. We quantified seed consumption with stable carbon isotope values of mouse plasma and parasite intensity with faecal egg counts of intestinal nematodes and cestodes (FEC). In a laboratory experiment, we reduced parasite intensity of randomly chosen mice through ivermectin treatment. We quantified their immune functions by total white blood cell count, percent granulocyte count, and percent lymphocyte count through haematological analyses. We measured the FEC and energy intake of the mice. From the field experiment, the number of offsprings in *A. semotus* increased with increasing seed consumption. Due to the trade-off between number and quality of offspring, the offspring quality decreased with increasing seed consumption for the females. The ivermectin treatment did not affect offspring number or quality. However, the FEC was positively correlated with number of offsprings. In the laboratory experiment, the percent lymphocyte/granulocyte count changed with parasite intensity at low energy intake, which was relaxed at high energy intake. This study demonstrated positive effects of food availability and neutral effects of parasitism o

For instance, the study of Shaner et al., (2018) tends to determine if energy intakes could modulate the relationship between parasite intensity and immune responses in captive Taiwan field mice (*Apodemus semotus*) treated with ivermectin and fed with the same diet (natural items). As a result, Taiwan field mouse presenting higher energy intake than their counterparts (estimation of daily calories taken from the diet) displayed a lower decrease of lymphocytes and a lower increase of granulocytes in response to faecal egg load of helminths (Shaner et al., (2018). The authors conclude that food availability may interfere in the relation between parasite intensity and the immune responses displaying “relaxed immuno-defences” with the increase of food availability. In opposition, a study on field vole (*Microtus agrestis*) set in outdoor enclosures (*i.e.* mesocosms) found higher immune responses to nematodes when captive individuals had higher food availability (Forbes et al., 2016). Shaner *et al.* (2018) attributed the difference between the two studies to the locations where field mice from subtropical mountain may suffer less from food scarcity and displayed higher prevalence of nematodes than the field voles captured in boreal Europe. Another explanation provided by Shaner *et al.* (2018) was that the laboratory measurements of the immune response in field mice were done in captivity. Experiments from Forbes *et al.* (2016) on voles were done in outdoor enclosures, where individuals were partly exposed to natural conditions (*i.e.* environmental stressors). In favour of the former explanation, energetic investment in a costly function such as immunocompetence might be less compromised when individuals are maintained in controlled environment without environmental stressors and could thus depict different patterns of immune response to infection (Viney et al., 2005). As illustrated in **Figure 21**, wild animals formerly exposed to environmental stressors have to adjust their metabolic demand to controlled conditions when they are set in captivity. In this context, energetic allocation to biological functions (*i.e.* homeostasis, immune response) may be more likely to occur than costly ecological or behavioural functions involved in survival (*i.e.* food foraging, avoidance of predator…). This would thus allow to evaluate the effect of single (or more) stressor(s) (*e.g*. low-quality diet) under controlled condition on the metabolism of captive individuals. In opposition, the rewilding of laboratory raised mice set in outdoor enclosures with environmental stressors might reflect different strategies of energetic investment (*i.e.* energetic trade-offs). The current experimentations on rewild animals are expected to bring insight in the understanding of how immune response can be shaped by a natural environmental and its stressors (Graham, 2021a; Flies and wild comparative immunology consortium, 2020).

Figure 21. Differences in the living experience of wild animals set in captivity and of laboratory animals exposed to some environmental stressors (rewilding).



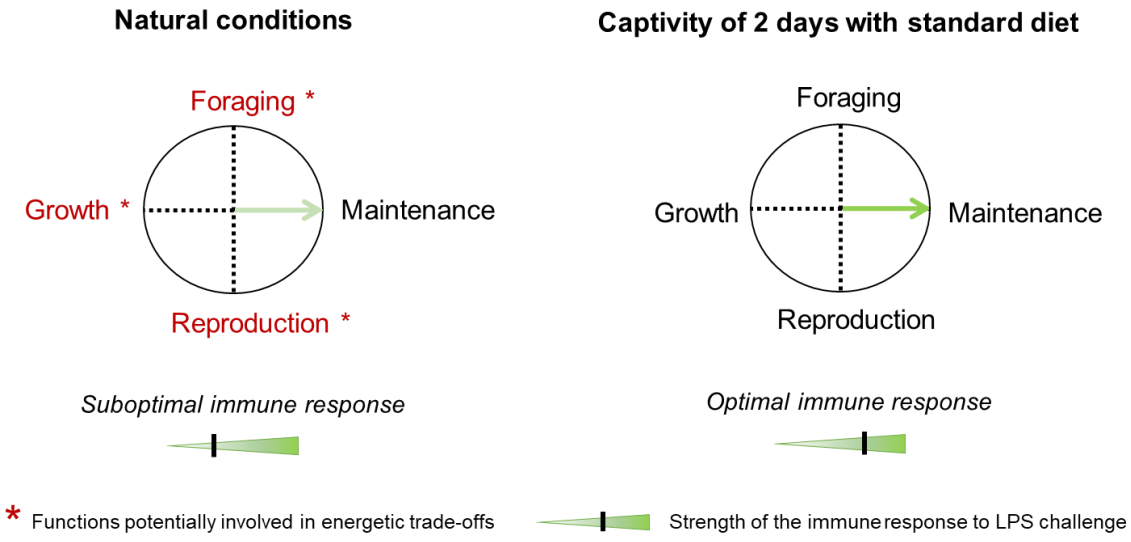
In Chapter 3.4, wild wood mice challenged with LPS showed decreasing trends of monocyte and lymphocyte counts in response to TNF-α, while no relation was found in captive individuals fed with standard laboratory food. This is in line with the observed "relaxation" of the immune defence found in the study of Shaner *et al.* (2018) on captive field mice. As found in Chapter 3.2, wild animals from the Low polluted site (L-LPS) displayed a decrease in lymphocyte count, while captive animals from the same population exhibited a decrease of leukocyte parameters (*i.e.* mainly lymphocytes and monocytes) when fed with standard laboratory food (L-LPS-Se+). This was also the case for captive animals from the La Bouloie campus (LPS captive D2), which also displayed a decrease of all leukocyte parameters (*i.e.* lymphocytes, monocytes, granulocytes) after 2 days of captivity with the same standard food (Chapter 3.4). These results suggest that captivity with standard diet allows a functional immune response to LPS challenge in accordance with the patterns of leukocyte associated decrease found in laboratory rats also challenged with LPS (Brooks et al., 2017). Nonetheless, none of the former studies focused on the stress response to low quality diet (*i.e.* micronutrient deficiency), which was found to directly contribute to immune cell proliferation and protection (see Chapter 3.1). In Chapter 3.2, animals fed Se-deficient diet for Low polluted sites (L-LPS-Se-) displayed similar responses to LPS challenge than wild individuals (L-LPS) from the same population with a decrease only found in lymphocytes. Thus, the quality of diet might modulate the immune response of wood mice to LPS in promoting different patterns of decrease in leukocyte counts suggesting different patterns of immune responses. Further insights should be given to the role of micronutrients in haematopoiesis (*i.e.* the metabolic process by which blood cells including leukocytes are produced). Indeed, Liao *et al.* (2018) found that Se-

deficient mice displayed lower red pulp macrophages in the spleen (*i.e.* impairment in the production of macrophages) associated to a decrease of selenoproteins. It appears that Se not only contributes to the maintenance of leukocytes viability as detailed in Chapter 3.1 (protection against ROS) but also contributes to growth and development of immune cells. Nutritional quality or, at least dietary Se, might be involved in the immune response of wild wood mice to LPS challenge displaying different patterns of leukocytes decrease related to diet content.

4.1.2 Defining optimal immune response in wild and captive animals

The global assessment of circulating leukocytes is probably not sufficient to estimate immunocompetence. The ability of an 'army' of leukocytes to fight pathogens does not only depend on its size but rather on its capacity to eliminate these pathogens. The measurement of a functional response (*i.e.* pro- *vs* anti-inflammatory response) to pathogen like the immune response to LPS challenge may be more relevant to qualify immunocompetence and potential patterns of heterogeneity (Schoenle et al., 2018; Martin et al., 2011). Though immunocompetence would ultimately promotes recovery from an infection, the energetic cost of eliminating pathogens from the organism may outcompete the benefit of tolerating the infection. For instance, over investment in self-maintaining (*i.e.* immune response to infection) rather than valuable functions such as reproduction may be detrimental to the fitness of wild animals. In this view, Viney *et al.* (2005) brought the notion of optimal immune response in opposition with the formal definition of immunocompetence (*i.e.* ability of an organism to resist pathogens or parasites through immune response). In this context, the notion of optimum is context-dependant and considers that the optimal immune response is not necessarily the most efficient. Indeed, 'high' or 'low' strengths of an immune response can be both beneficial or detrimental to fitness. For example, a given individual that invests energy in immunocompetence rather than in visible attractive traits (*e.g.* ornaments supposed to maximize the fitness) will reduce its fitness. For this reason, ecological variables may be integrated to estimate the benefit of an immune response to fitness. In our studies, ecological variables like the reproductive success (*e.g.* number of embryos) cannot be measured because of ethical constraints (*e.g.* all pregnant females were released) and because, more generally, it is very difficult to assess reproduction in small mammals. However, energetic allocation to valuable functions dedicated to fitness can be estimated in considering that an important part of the energy is much likely attributed to immune response to LPS challenge (*i.e.* individual maintenance). In accordance with the energy saving hypotheses, individuals can only invest in one function (*i.e.* growth, forage, reproduction, maintenance) at the time (Sapolsky *et al.,* 2000).

Figure 22. Estimation of the energetic allocation according to the leukocyte-associated pattern of decrease in response to LPS in wild (CC Pévèle-Carembault) and captive (La Bouloie campus) animals from Chapter 4. (in green: color gradient of the strength of the immune response; in italic: the optimal immune response corresponds to similar results found in wild, captive and laboratory small-mammals (Shaner et al., 2018; Brooks et al., 2017; Forbes et al., 2016)).



As detailed above, studies on wild, captive and laboratory small mammals estimate general decrease of leukocytes (*i.e.* mainly lymphocytes and monocytes) as a functional response to infection or challenge (Shaner et al., 2018; Brooks et al., 2017; Forbes et al., 2016). Based on this, the functional measurement of the optimal immune response was proxied with the leukocyte-associated pattern of decrease in response to LPS challenge. We estimated that general decrease of leukocytes translates an optimal immune response to LPS challenge, a single decrease of lymphocytes was estimated to characterise a sub-optimal immune response. The **Figure 22** illustrates the dial of (supposed) energetic allocation for the captive wood mice from la Bouloie campus and the wild ones from the CC Pévèle-Carembault studied in Chapter 4. The captive wood mice fed with standard food would have exhibited an optimal immune response to LPS challenge as leukocyte parameters (*i.e.* mainly lymphocytes and monocytes) were found to decrease. In contrast, wild wood mice only displaying a lymphocyte decrease after LPS challenge showed a suboptimal immune response, in comparison to the response of animals from the campus in captivity. According to the energy saving hypothesis, the captive individuals might allocate sufficient amount of energy to maintain themselves and face a metabolic demand and invest in the immune response. In wild individuals, the single recruitment of lymphocytes might be attributed to another energetic investing strategy, which involved energetic trade-offs between other ecological functions (*i.e.* growth, foraging or

reproduction). However, we cannot determine if the immune system is depressed by an anti-inflammatory response (*e.g.* increase in glucocorticoids) or by a lack of metabolic supplies (*e.g.* micronutrient deficiencies). Further insight in the functional response to challenge (*i.e.* pro- and anti-inflammatory status) including ecological variables and nutritional status of animals might help to determine if wild animals' immune response can be really considered as suboptimal or optimal to their fitness.

Take home messages

1) TNF-α is undetectable in non-challenged individuals while its levels can be detected in all animals challenged with LPS.

2) Leukocytes decrease in response to LPS in captive animals fed standard laboratory diet.

3) Lymphocytes decreased in wild and captive animals fed Se deficient diet.

4) Energetic trade-offs might explain these differences found in the immune response of wild and captive animals.

4.2 Insights in eco(logy)-toxicology

4.2.1 Environmental sources, fate and toxicity of Se

Selenium is essential in the functioning of organisms until a threshold concentration where it becomes toxic to biota. The sources of Se in the environment can be attributed to natural processes (*e.g.* weathering, leaching, volcanic activity…) accounting for 50 to 65% of atmospheric emissions, while anthropogenic activities (*e.g.* mining, smelting…) may release 37 to 40 % of the Se present in the atmosphere (Tan et al., 2016). In soil or water, the inorganic form of Se is mainly found as selenites (Se II), selenids (Se IV) or selenates (Se VI)(Lopes et al., 2017; Tan et al., 2016). The mobility of Se is dependent of its speciation.For instance, elemental Se (0), selenide (Se II) and dimethylselenide (Me_2Se) are the predominant volatile forms of Se (Rosenfeld et al., 2018; Tan et al., 2016). The volatile forms of Se can be transported in ambient air *via*, for example, metal-processing plants, and can be deposited on soils or water in the vicinity of industrial plants (Alexander, 2015). At this stage, environmental Se follows biogeochemical processes to reach biotic or abiotic compartments enabling, or not, its availability to micro-organisms, plants and higher consumers (Natasha et al., 2018). As detailed in Chapter 3.1, the main source of metabolic Se in mammals is supposed to come

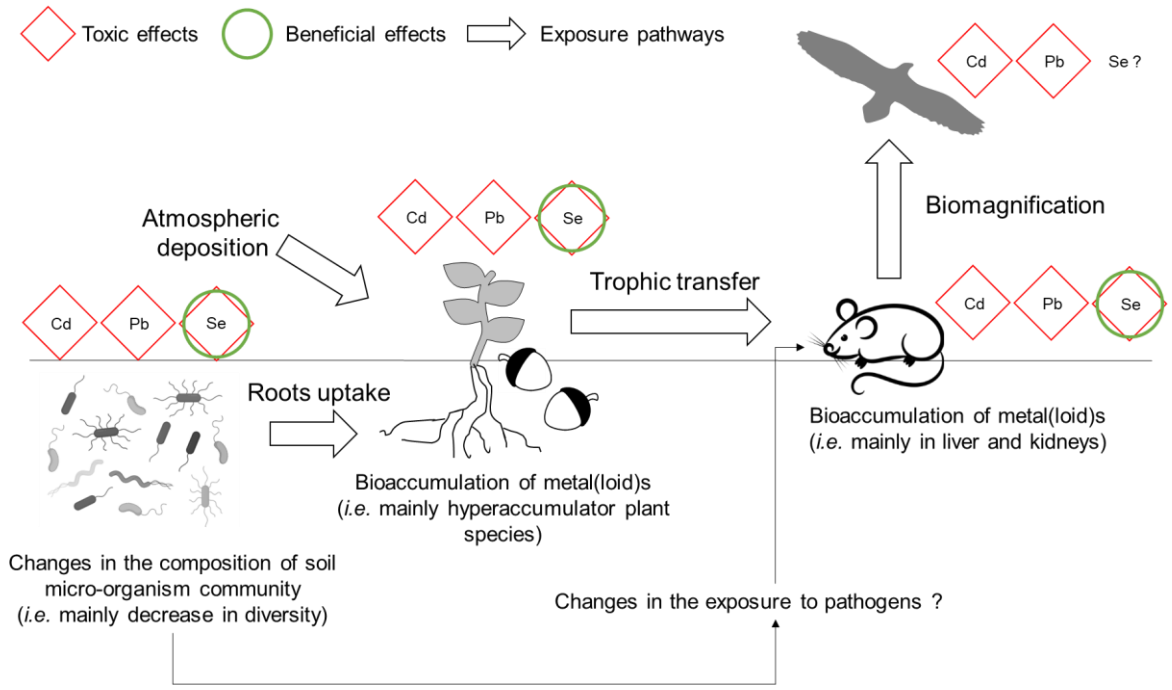
from the diet. However, Se was mainly considered for its beneficial outcomes but not for its toxicity. Chapter 3.2 brought two major issues on environmental Se. One discussed above considered that dietary Se would promote benefit to immunocompetence in improving the immune response. The other issue raised in Chapter 3.2 concerns the elevated hepatic levels of Se found in wood mice from highly polluted sites. Several studies in the vicinity of the former smelter of Metaleurop Nord found elevated concentrations of Cd, Pb and also Se in topsoils (Fritsch et al., 2010; Douay et al., 2009; Sterckeman et al., 2002b). For Cd and Pb, a spatially explicit distribution (*i.e.* gradient) of both exposure in animal and plants and of soil pollution levels was found in response to the distance to the former smelter. Though Sterckeman et al. (2002b) showed similar gradient with Cd and Pb in cultivated areas, the authors did not found significant horizontal pattern of Se distribution but rather a vertical repartition of Se within one meter of depth. The vertical enrichment of Se was attributed to volatile properties of Se, which, when it reaches the soil, undergoes physico-chemical transformations through oxido-reduction processes and biological activities (Sterckeman et al., 2002b). The same study found that the smelter dusts (during its activity) contained higher Se concentrations than those of control soils and that soil concentrations of Se was partially correlated with those of Pb. The authors concluded that the elevated concentrations of Se in the soils (mean concentration in crops next to the smelter 0.55 *vs* 0.031 mg/kg in reference agricultural soils) close to the smelter of Metaleurop can be explained by atmospheric discharge from the plant (Sterckeman et al., 2002a,b). The measurement of trace metal concentrations in soils realised in 2017 (see Chapter 3.2) corroborates higher concentrations of Se in the soil of 'High' site close to the former smelter than in the 'Low' site (median concentration 4.1 *vs* 1.8 mg/kg in woody areas, respectively). However, the measurement of Sterckeman et al. (2002b) were done during the activity of the smelter while measurement of soil concentration in 2017 were done 14 years after the end of Metaleurop Nord activity. Altogether, these results suggest a temporal constancy (*i.e.* persistent soil concentrations) trend of Se contamination in vicinity of the former smelter of Metaleurop Nord. Ozaki et al. (2022) found a constancy in Cd and Pb soil concentrations between 2006 and 2019. Cadmium and Pb are known to persist in the environment while Se persistence is highly dependent of its oxidative status (Tan et al., 2016). As reviewed by Lopes et al., (2017), the mobility of Se rather depends on its speciation and the availability of Se in soils cannot be estimated only by total concentrations (*e.g.* physio-chemical parameters like pH can modulate availability). The Se concentrations found in top soils can help to estimate environmental risk assessment. According to the Canadian guidelines on soil quality (CCME, 2009), Se concentrations in agricultural soils found by Sterckeman et al. (2002b) are below guideline values (0.55 *vs* 1 mg/kg, respectively). On the contrary, Se concentrations found in 'High' site next to the former smelter of Metaleurop Nord were higher than the Canadian guidelines for industrial areas (4.1 *vs* 2.9 mg/kg, respectively)

(CCME, 2009). Therefore, the elevated Se concentrations found in sites close to the former smelter might represent an environmental risk for organisms in this site already polluted with Cd, Pb and Zn.

4.2.2 Influence of Cd, Pb and Se on wood mice environment

As found in Chapter 3.2, elevated Cd, Pb and Se were found in both soils and liver of wood mice captured in the vicinity of the former smelter. The different patterns of exposure to these metals and metalloid are displayed in **Figure 23** and discussed below.

Figure 23. Patterns of exposure to Cd, Pb and Se and their effects in different ecosystem compartments and at different trophic levels.



As reviewed in Abdu et al., (2017), Cd and Pb pollutions can decrease soil micro-organisms diversity and activities. For instance, Cd and Pb can decrease microbial and fungal soil diversity and modulate enzymatic activity in Cd or Pb polluted soils (Sun et al., 2023, 2022; Salam et al., 2020). The same was found in reclaimed phosphorus mine soils where high Se soil concentrations were associated with a decrease of bacterial and fungal diversity (Rosenfeld et al., 2018). Some micro-organisms were found to tolerate and/or facilitate metals and metalloids bioavailability in the rhizosphere of plants (Abdu et al., 2017). Indeed, Rosenfeld et al., (2018) found Se-tolerant bacterial taxa (*Actinobacteria* and *Gemmatimonadetes*) and a fungal taxon (*Ascomycota*) as they were more present in soils polluted with Se (>30mg/kg of Se in soil). The authors suggested that, at least *Actinobacteria,* present high tolerance to Se concentrations and play a major role in the transfer of Se from soil to plant in the rhizosphere (Rosenfeld et al., 2018; Gremion et al., 2003). Another study on the rhizosphere bacterial

community considered that some taxa (including *Gemmatimonadetes*) were involved in Cd bioavailability, promoting Cd uptake and accumulation in wheat (Lu et al., 2023). Though some bacterial and probably fungal taxa might facilitate metals and metalloids transfer at the level of the rhizosphere, the mechanism by which micro-organisms affect bioavailability of metals and metalloids remains unclear. Nevertheless, it is noteworthy that micro-organisms modulate soil physical and biochemical properties (*e.g.* pH, oxidation status…) and thus influence the bioavailability of metals and metalloids promoting a relevant tools for soil remediation (Abdu et al., 2017). Metal hyperaccumulator plants are also particularly interesting in the remediation of polluted soils though they can also represent a major threat to their consumers (Baker et al., 2000). For instance, *Salicacea* are hyperaccumulators of Cd, while Fabaceae, Asteraceae and Brassicaceae represent the three main families of Se hyperaccumulators (Reynolds and Pilon-Smits, 2018; van der Ent et al., 2013). In general, all plants can take up Cd, Pb and Se *via* ionic mimicry in Ca^{2+}, Fe^{2+} and Zn^{2+} transporters (Clemens, 2006). The uptake of metals and metalloids mainly occurs in roots through biochemical processes dependent or not of soil micro-organisms and these elements are then translocated to plant tissues *via* xylem or phloem transportation (Yan et al., 2020). Cadmium and Pb can affect plants through oxidative stress damage and be stored in roots or leaves, favouring transfer to their consumers (Asare et al., 2023). As in mammals, Se can be beneficial or toxic to plant metabolism and can for example enhance the production of ROS (Natasha et al., 2018). In plants, Se (IV) and Se (VI) can be taken up by roots and biochemical processes allow to transform Se in Se-cysteine or methyl-Se (major dietary Se found in herbivores) (Natasha et al., 2018). However, less than 5% of soil Se is utilized by plants (Haug et al., 2007). Elevated concentrations of Cd and Pb were found in plants (grass and nettle) close to the former smelter of Metaleurop Nord (Boshoff et al., 2014). This can be related to results from Ozaki et al., (2019) who found positive correlations between Cd and Pb concentrations in the stomach content of wood mice (*i.e.* principally made of plants) and soil pollution levels. Altogether these results suggest a bioaccumulation of Cd and Pb from soils to plants, and also possibly by atmospheric deposition, which can be then transferred to higher trophic levels (*i.e.* the wood mice). Data analysis in food items of plants consumed by wood mice revealed higher concentrations of Se in 'High' site and positive correlations between Se and Pb concentrations were found in the leaves of these plants (Alchammas et al., unpublished data, publication *in prep*). Regarding the elevated hepatic Se concentrations in wood mice from 'High' site (presented in Chapter 3.2), trophic transfer of Se from plant food items may be considered as a risk to wood mice health. However, Se elimination is biphasic with a rapid phase of excretion (within a day) and a longer one lasting for months (Alexander, 2015). For instance, rats supplemented with 15 mg Se / kg in drinking water for 6 months displayed hepatic damages linked to liver concentrations above 2.0 mg/kg (Crespo et al., 1993). The same study found a highly

significant correlation between dietary Se (II) and liver concentrations after a month of treatment with Se (stable over 6 months). Nonetheless, study on laboratory rats cannot be transposed to wood mice as Se speciation (*i.e.* different toxic concentrations in tissues might be found between Se II and Methyl-Se) and the tolerance of this species are not known. Further biochemical or histological analyses in wood mice might help to determine if higher hepatic Se concentrations can be associated to toxicological outcomes. As reviewed in Alexander (2015), no specific biomarkers exist to evaluate Se toxicity although several studies reported disturbances in Se dependant proteins (*e.g.* variation in the glutathione peroxidase activity) or clinical features of cirrhosis (*e.g.* increase of serum aspartate/alanine aminotransferase). Another possible transfer of Cd, Pb and Se presented in **Figure 23** is the biomagnification of this metalloid to predators feeding on wood mice. The biomagnification of Cd and Pb in top predators feeding on small mammals may be related to ecotoxicological impacts on upper consumers found in Metaleurop Nord (Ali et al., 2019). However, trophic transfer of Se in top predators might be dependent on the metabolic requirements of predators. Moreover, changes in micro-organism community mediated by trace metal pollution might also modulate the community of pathogens present in the environment of the wood mice and thus modulate the pool of pathogens they host. Further insight should be given in the relation between the components of ecosystems regarding their influence on the transfer of trace metals and potentially diseases mediated by this pollution. One hypothesis would be that animals exposed to high concentrations of Cd or Pb may have developed a preferred uptake/accumulation of Se regardless of the source or concentration of Se exposure. This would be attributed to patterns of genetic adaptation or non-heritable tolerance mechanisms to Cd and Pb exposure favouring the maintenance of wild animals in those polluted sites. Further insight on the effect of Se in the wood mice might allow to determine if such adaptation or tolerance can be found in wood mice exposed to Cd and Pb.

Take home messages

1) Selenium is an insidious metalloid whose beneficial or toxicological effects mainly relies on its concentrations and speciation.

2) The sources of Se found in the soils of Metaleurop Nord can be attributed to the smelter dusts.

3) Trophic transfer may be one consequence of elevated Cd, Pb and Se found in the liver of mice.

4) Further insight should be given to the relation between biological levels of organization considering their influence on trace metals transfer and disease transmission.

4.3 Insights in immuno(logy)-toxicology

4.3.1 Endocrine disturbance and immunomodulation by Cd and Pb

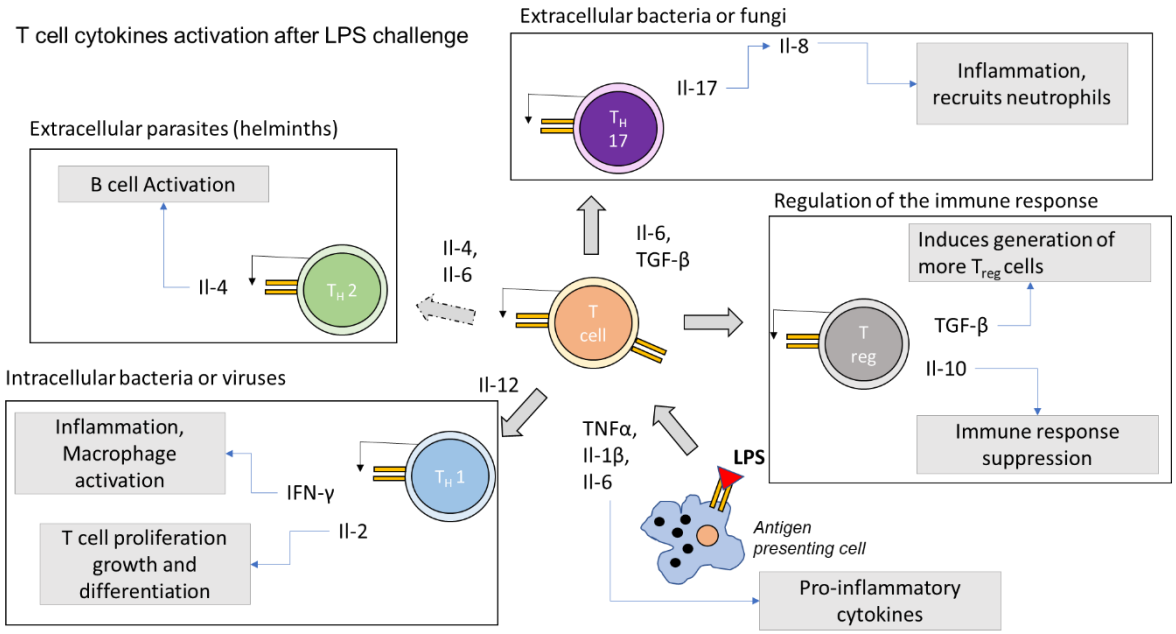
Our results in Chapter 3.2 highlight endocrine disturbance mediated by Cd and Pb exposure. For the immune system, the main role of CORT is to control inflammation in promoting anti-inflammatory responses to avoid autoimmune impacts after an infection (Medzhitov, 2008; Martin, 2009a). Disturbance in the equilibrium between pro-and anti-inflammatory would have severe consequences on health. Nishiyama et al. (1984 and 1985) found that Cd and Pb can inhibit circulating CORT in altering adrenal cortex functions. A mechanism of CORT inhibition mediated by Cd exposure was found in rainbow trout (*Oncorhynchus mykiss*) attributing Cd inhibition in corticosteroidogenesis (Sandhu and Vijayan, 2011). The authors suggest that Cd decrease levels of expression of the MC2R gene involved in the activation of cortocosteroidogenesis by the adrenocorticotrophic hormone (ACTH). Indeed, disturbance in the adrenal cortex might be linked to either an inhibition of ACTH levels or a disturbance of its functioning that controls plasmatic CORT levels (Wills and Havard, 2013). Further analyses on the ACTH circulating concentrations and/or experimentation of the wood mice response to ACTH injection might allow understanding how endocrine disturbance modulates their immune response. It is noteworthy that captivity of wood mice formerly exposed to Cd and Pb might allow recovering basal circulating and excretion levels of CORT (see Chapter 3.2).

This section will enlarge the discussion to components of the immune system not studied in the results but which were found to be affected by Cd and Pb in Chapter 3.1. The influence of Cd, Pb, and LPS injection are summarised in **Table 1** (see end of this section). Laboratory

studies on rats and mice displayed similar decrease of lymphocytes and an increase of white blood cells, monocytes and granulocytes counts in response to Cd and Pb, respectively (Zhang et al., 2017; Li et al., 2017). Brooks et al. (2017) found that LPS (15 mg/Kg) induced a decrease of WBCs including lymphocytes, granulocytes and monocytes within 4 hours post-injection of laboratory rats. Our results found in Chapter 3.2 and 3.4 seem to corroborate rather a response to LPS injection with a decrease of leukocyte parameters (mainly lymphocytes, *i.e.* lymphocyte-associated pattern of decrease described in 3.4) than a response to Cd and Pb exposure. However, no studies testing the effect of Cd and Pb, in similar time laps (*i.e.* 2 hours), on haematological measurements was found in the literature. In line with Chapters 3.3 and 3.4, the use of blood reference intervals might allow trimming healthy *vs* non-healthy individuals in populations with high and low exposure to Cd and Pb. Wood mice that received LPS challenge displayed leukocyte count in the lower range of the reference intervals suggesting an immune response to infection (*i.e.* leukocyte relocalisation). Still at the cellular level, in Chapter 3.1, the population of lymphocytes was found to be modulated by both Cd and Pb in decreasing the CD4+/ CD8 + ratio. Unfortunately, none of the different clusters of differentiation panels tested in flow cytometry cross-reacted with wood mice samples (unpublished data). Another common pattern found in Chapter 3.1 was that MCHII was found to decrease in lymphoid organs (thymus and spleen) and to increase in toxic metal target organs (i.e. kidney and liver) (Kim et al., 2003). Lawrence & McCabe (2002) proposed two possible mechanisms underlying MHCII dysfunction by toxic metal exposure including Hg: (1) adducts with toxic metals can be formed on peptides, formed after the phagocytosis of pathogens by an antigen presenting cell, disturbing the T cell receptor repertoire of peptide recognition towards responsiveness or non-responsiveness; (2) toxic metals may directly alter the structure of MHCII in binding to α or β chains. The first hypothesis can be related to Heo et al. (1996) who postulated that Pb may modulate APC activity *via* antigen density, changes in costimulatory molecule expression and/or changes in membrane fluidity. For the second hypothesis, I propose a mechanism by which toxic metals with affinity for sulphur atoms (i.e. Cd, Hg) may interfere with disulphide bonds that maintain the shape of β1 chains in the peptide-binding domain and/or α2 or β2 chains in the immunoglobulin-like domain (Mak et al., 2014). For instance, the reactivity of Hg with thiol groups and disulphide bonds was related to an inactivation or at least an inhibition of enzyme activity whilst mechanisms are not well known (Rubino, 2015). Thus, the decrease of CD4 T cells with toxic metal exposure may be explained by a loss of viable MCHII recognition. However, the measurement of MHCII in the wood mice would require specific, or at least cross-reactive, tools to estimate disturbances mediated by Cd and Pb exposure. The use of humanized mouse and rat laboratory species for the development of techniques to measure immune response is thus a major constraint in wildlife immunology, which would need further development in non-model species (Flies and wild comparative immunology consortium, 2020;

Jackson, 2015). As detailed in Chapter 3.1, cytokines remain the most promising techniques found to measure or estimate functionally the polarisation of the immune response.

Figure 24. Activation and recruitments of T cells after immune challenge with LPS. Arrows indicate which T cell subpopulation (Th 1,2,17 and Treg) is activated. The arrow in dotted line corresponds to potential co-infection with a helminth.



The **Figure 24** illustrates the immunological recruitment processes of cytokines presented in **Table 1**. In accordance with our results, pro-inflammatory cytokines including TNF-α was found to increase after LPS challenge (within 1.5 to 2 hours) in several species (Williams et al., 2009; Byrne and Reen, 2002; Sadeghi et al., 1999). The same increase of TNF-α (and Il-6 and Il-1β as well) was found in laboratory mice injected with Cd (*i.p*) and laboratory rats exposed to Pb in drinking water (Li et al., 2017; Liu et al., 2012; Kataranovski et al., 1998). In our studies, all of the unchallenged wood mice exhibited TNF-α concentrations below detection limits even if they were captured in 'High' sites (*i.e.* chronically exposed to elevated levels of Cd and Pb). Further investigations might explain the absence of inflammatory immune response in wood mice. For instance, the kit sensitivity (though very low: ~7 pg/ml) or patterns of tolerance to Cd and Pb exposure might be the reason of the difference between findings in laboratory and wild animals' immune response. As found in mouse, macrophage and blood granulocyte cultures showed an increase of pro inflammatory cytokines in response to Cd (*ex vivo* or in medium) alone or to LPS alone (in medium) while other studies showed that the combination of Cd (*ex vivo* or in medium) and LPS challenge (in medium) produced a decrease of TNF-α, Il-6 and Il-1β (García-Mendoza et al., 2019; Djokic et al., 2014). Though spleen cell cultures presented in Chapter 3.4 only focus on TNF-α response to LPS, further experimentation could be done

with spleen cell cultures exposed to Cd, Pb, and LPS. Indeed, further experimentation on spleen cells of non-naïve (i.e. wood mice already exposed to elevated Cd and Pb) and naïve (i.e. wood mice exposed to low levels of pollution) animals might allow to estimate if patterns of tolerance to elevated Cd and Pb concentrations modulate the immune response in wild animals. The measurement of immune cell responses to LPS challenge should be tested with Cd and Pb separately, and in combination. As found in Chiang et al. (2014), both injections of Pb and LPS showed positive influence on pro-inflammatory cytokine secretion, which thus mitigated the patterns found in Cd and LPS interaction. In rat, chronic Cd exposure (drinking water) was found to activate Th1 *via* secretion of IFN-γ and Il2 by splenocytes, promoting an inflammatory response mediated by macrophage activation and T cell proliferation (Turley et al., 2019). The authors also found an increase of Il-10, an anti-inflammatory cytokine that inhibits IFN-γ expression by activated T cells. Moreover, Pathak and Khandelwal (2008) found that cultures of mice thymocytes displayed higher levels of inhibition in IFN-γ, then Il-2 followed by Il-4. Nonetheless, Cd might be involved in cell mediated inflammatory response as found in humans, with Il-17 involved in the recruitment of neutrophils increasing during Cd exposure (Bonaventura et al., 2018). In contrast, Pb may be more associated with Th2 activation normally involved in the clearance of extracellular parasites (Fu et al., 2019; Dobrakowski et al., 2016; Heo et al., 2007; Iavicoli et al., 2006; Heo et al., 1996). Li et al. (2017) found in laboratory rats chronically exposed to Pb (drinking water) that they exhibited an increase of TNF-α and Il-4 and Il-10, which depicts a stimulation of Th2. The increase of anti-inflammatory cytokines may indicate a regulation of IFN-γ increase, which also intervenes after LPS challenge (Sadeghi et al., 1999). Altogether, these results indicate disturbances in the production of pro-inflammatory cytokines, which in some cases affected the activation of Th1, Th2, Th17 and Treg. Further measurements (*i.e.* requiring the development of new techniques to detect cytokines in non-model species) might allow to elucidate which specific compartment of the immune system is affected by Cd and Pb exposure in wood mice population close to the former smelter of Metaleurop Nord.

Table 1. Summary of haematological parameters and cytokine secretions affecting T cell subpopulations in response to Cd and Pb exposure and LPS challenge.

	Leukocyte counts				Pro-inflammatory			Th1		Th2	Th17	Treg
	WBC	**MON**	**LYM**	**NEU**	**TNF-α**	**Il-6**	**IL-1β**	**Il-2**	**IFN-γ**	**Il-4**	**Il-17**	**IL-10**
Cd (*in vivo / in vitro*)	↑ [2]	↑ [2]	↓ [2]	↑ [2]	↑ [1] [5] [6]	↑ [1]	↑ [5]	↓ [3]	↑ [4] ↓ [3]	↓ [3]	↑ [5]	↑ [4]
Pb (*in vivo / in vitro*)	↑ [12]	↑ [12]	↓ [12]	↑ [12]	↑ [9] [12]	↑ [9]	↑ [9]	↑ [7] [10] [11]	↓ [8] [11] [12] [13]	↑ [7] [8] [10] [11] [12]		↑ [12]
LPS (*in vivo*)	↓ [16]	↓ [16]	↓ [16]	↑ [16]	↑ [14] [15] [17]	↑ [14] [15] [17]	↑ [14] [15] [4]					↑ [17]
Cd*LPS (*in vitro*)					↓ [6] [19]	↓ [19]	↓ [19]					
Pb*LPS (*in vivo*)					↑ [18]	↑ [18]	↑ [18]					

[1] Kataranovski *et al.,* 2018 (rat, *acute*); [2] Zhang *et al.,* 2016 (mice, *chronic*); [3] Pathak et Khandelwal, 2007 (*in vitro* mice thymocytes, *gradual*); [4] Turley *et al.,* 2019 (rat, *chronic*); [5] Bonaventura *et al.,* 2018 (human patients *in vitro* cells, *0.1ppm*); [6] García-Mendoza *et al.,* 2019 (mice *in vitro* cells, *gradient*); [7] Dobrakowski *et al.,* 2016 (humans, *chronic*); [8] Heo *et al.,* 1996 (mice, *moderate*); [9] Liu *et al.,* 2012 (rat, *chronic*); [10] Fu *et al.,* 2019 (Chicken, *chronic*); [11] Iavicoli *et al.,* 2006 (mice, *gradient*); [12] Li *et al.,* 2017 (mice, *chronic*); [13] Heo *et al.,* 2007 (mice, *moderate*); [14] Williams *et al.,* 2009 (pig); [15] Byrne *et al.,* 2002; [16] Brooks *et al.,* 2017 (rat, *15mg/Kg*); [17] Sadeghi *et al.,* 1999; [18] Chiang *et al.,* 2014 rat*, 5 mg/kg of Pb and 50 µg/kg of LPS*); [19] Djokic *et al.,* 2014 (*ex vivo* rat cells, *1 mg/kg of Cd + 100 ng/kg of LPS in medium*);.

4.3.1 Immunomodulation by Se

Selenium was found to play an important role in the strength of the immune response to LPS challenge of wood mice exposed to Cd and Pb. Only one study allows to find sufficient elements of comparison with the study present in Chapter 3.2. El-Boshy et al. (2015) study the influence of Se supplies in diet (0.1 mg Se / kg body mass) on health parameters of male albino rats chronically exposed to Cd (40 mg/ml in drinking water) for 30 days. **Table 2** summarises the results found in the study of El-Boshy et al. (2015).

Table 2. Summary of the health parameters measured in male albino rats fed Se-supplemented diet and/or chronically exposed to Cd (40 mg/ml in drinking water) for 30 days.

Health parameters		Treatment		
		Se	**Cd**	**Se + Cd**
Liver concentrations	Cd		↗↗	↗
	Se	↗↗		↗
Haematology	RBC		↘	
	Haematocrit		no effect	
	WBC		no effect	
	Neutrophils		↗↗	↗
	Lymphocytes	↗	↘	
	Monocytes		no effect	
Cytokines	TNF-α		↗	
	Il-1β		↗	↗
	Il-6		↗	
	INF-γ	↗	↘	
	Il-10	↘	↗	
Oxidative stress	GSH	↗	↘	
	GPx	↗	↘	
	CAT	↗	↘	
	SOD	↗	↘	
	MDA		↗↗	↗
Liver biomarkers	ALT		↗	
	AST		↗	
	Total Proteins		↗	
	Albumin		↘↘	↘
	Urea		↗	
	Creatinine		↗	

RBC: Red blood cells, GSH: reduced glutathione, GPx: oxidized glutathione, CAT: catalase, SOD: Super oxide dismutase, MDA: malondialdehyde, ALT: alanine aminotransferases, AST: aspartate aminotransferases, WBC: white blood cells.

As shown in **Table 2**, hepatic Se concentrations were found to increase in rats, which is in accordance with Crespo et al. (1993) who found positive correlations between dietary and liver Se. In El-Boshy et al. (2015), hepatic Se concentrations led to a lower increase when rats exposed to Cd were fed with Se supplement diet. This can be in line with our findings in Chapter 3.2 where Se concentration was found to decrease in wood mice from polluted sites fed with Se-deficient diet. However, no variation of liver Cd concentrations was found in wood mice during the five days of captivity. Further, duration of captivity might allow determining the variation of Cd in response to Se concentrations in the liver. El-Boshy et al. (2015) found positive influence of Se on lymphocyte counts in rats and reactivity of neutrophils in response to Cd. Though erythrocytic parameters were found to be modulated by wood mice hepatic Se in Powolny et al., (2023), only a lower increase of neutrophils is shown in **Table 2**. Cytokine profiles of rats exposed to Cd showed an increase of pro-inflammatory cytokines including TNF-α. Selenium treatment alone seemed to improve Th1 response in rats while Cd and Se treatment might have a single effect on Il-1β. As found in Chapter 3.2, unchallenged wood mice whatever their level of exposure to pollution exhibited TNF-α concentrations below detection limits suggesting no variation mediated by Cd and Pb exposure. El-Boshy et al. (2015) found positive effects of Se on GSH, CAT, GPx and SOD, which suggests patterns of oxidative stress with Cd alone and no variation when Cd and Se are given in combination. These results showed a positive influence of Se on Cd mediated oxidative stress that were concomitant with a decrease of liver and kidney damages. The same was found in liver biomarkers, which showed no variations in ALT, AST, total proteins, urea and creatinine and lower variation in albumin levels. Though neither oxidative stress nor liver biomarkers were measured in this thesis, some promising biomarkers can be measured in wood mice samples. For instance, the measurement of GSH, GPx, SOD, CAT and MDA are not species dependent (*i.e.* do not rely on antibody cross-compatibility) as proposed in Paglia and Valentine (1967). These measurements would provide promising information on the ability of Se to countermeasure the effects of Cd and Pb in wood mice inhabiting polluted sites. Thus, immunotoxicological procedures and assays in wild mammals should have their specific approaches such as Tiers approach considering the conservation status and biologically available resources. A fourth step focusing on the nutritional quality could be added to the Tiers approach when considering natural populations. A new insight is given toward the immunomodulatory role of micronutrients to cope with environmental stressors like exposure to pathogens or pollution (Dietz et al., 2013; O'Brien and Jackson, 2012). Bricknell and Dalmo (2005) proposed a definition of immunostimulant as "a naturally occurring compound that modulates the immune system by increasing the host's resistance against diseases that in most circumstances are caused by pathogens". The energetic requirement for the maintenance of basal immune function can be facilitated by immunostimulants (*e.g.* in fish Ringø et al., 2012). Immunostimulants are usually

given as (pre-/pro- or syn-biotic) feed additives (*e.g.* cattle, fish) and correspond to several types of compounds including minerals and vitamins (micronutrients), amino acids, proteins, organic acids, etc. (Dawood et al., 2018). For example, O'Brien and Jackson (2012) consider vitamin D3 as a key element mediating both innate and adaptive immunities.

Take home messages

1) Haematological response to LPS can be associated to a decrease of, at least, lymphocytes, probably due to their relocalisation.

2) Cd and Pb modulate the immune response in destabilizing the equilibrium between Th1 and Th2.

3) Se probably has ability to countermeasure Cd and/or Pb effects, even if further measurements of *e.g.* oxidative stress parameters would be needed.

4) Further analyses on the role of micronutrients in interaction with toxics might bring pieces of knowledge in the difference between laboratory and wild animal immune response.

4.4 Insights in Eco(logy)-immuno(logy)-toxicology

4.4.1 The sum of stressors is not additive

Stress response is multidimensional as it can affect the metabolism (*e.g.* endocrine or immune response), the behaviour and ultimately the fitness of wild animals (Martin, 2009a; Robert M. Sapolsky et al., 2000). At the metabolic level, both pollution exposure and nutritional quality were found to affect endocrine and immune responses in wood mice. The effect of each stressor measured in this thesis was summarised and related to references when possible in **Table 3**. In this manuscript, endocrine stress response was studied through CORT and immune stress response through lymphocytes counts (*i.e.* lymphocyte-associated pattern of response to stressor) and TNF-α. According to **Table 3**, our results seems to corroborate the lymphocyte-associated pattern of decrease in response to each stressor studied in this manuscript (*i.e.* LPS, Cd and Pb exposure and Se deficiency) and in other studies (Avery and Hoffmann, 2018; Brooks et al., 2017; Gera et al., 2015; Grion et al., 2007; Wang et al., 2021). Similar response were also found in the variation of CORT in response to LPS or Cd and Pb exposure (Brooks et al., 2017; Grion et al., 2007; Nishiyama et al., 1985; Nishiyama and Nakamura, 1984; Spackman and Riley, 1978). However, Cd and Pb exposure did not show a

significant increase of TNF-α in unchallenged individuals but rather a higher inflammatory response in LPS-challenged wood mice from polluted sites (Hossein-Khannazer et al., 2020; Liu et al., 2012). Indeed, the combination of these stressors was found to depict different patterns of immune response to LPS.

Table 3. Respective influence of infection (LPS), pollution exposure (Cd and Pb) and low nutritional quality (Se-deficient diet) on the immune response.

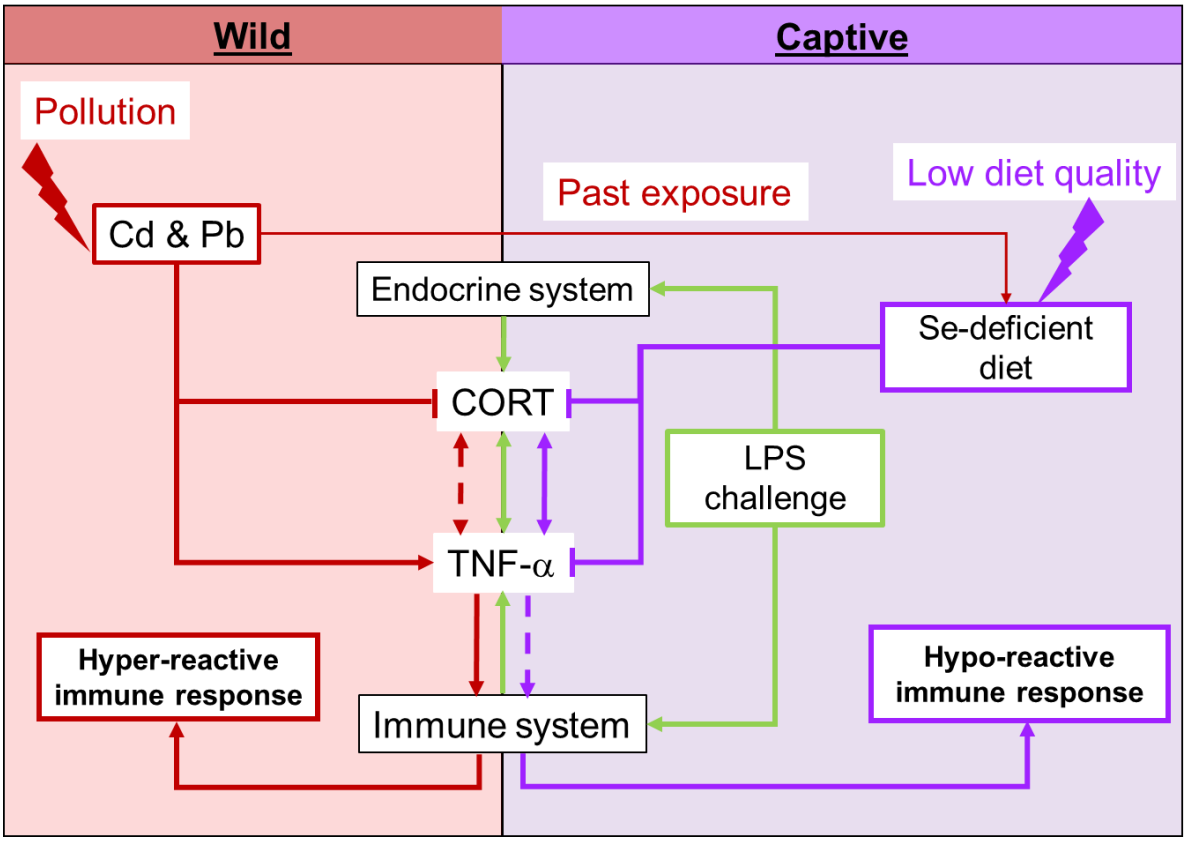
Stressors	Study cases	Response to stressors		
		LYM	**TNF-α**	**CORT**
Infection	Lipopolysaccharide (LPS) injection	↓ [1]	↑ [1]	↑ [1,2]
Pollution exposure	Cadmium (Cd) and lead (Pb)	↓ [3,4]	↑ [5,6]	↓ [7,8,9]
Nutritional quality	Selenium (Se) deficiency	↓ [10]	↓	↓

LYM : Lymphocytes ; CORT : Corticosterone

[1](Brooks et al., 2017); [2](Grion et al., 2007); [3](Gera et al., 2015); [4](Wang et al., 2021); [5](Hossein-Khannazer et al., 2020); [6](Liu et al., 2012); [7](Spackman and Riley, 1978); [8],[9](Nishiyama et al., 1985; Nishiyama and Nakamura, 1984); [10](Avery and Hoffmann, 2018).

Figure 25 illustrates the variation of immune and endocrine responses to LPS in wild and captive individuals fed Se-deficient diet captured in high pollution levels (see details in Chapter 3.2.). In control individuals (i.e. wood mice from low polluted sites, Chapter 3.4), both TNF-α and CORT increased in response to LPS challenge. This is concomitant with laboratory studies presented in **Table 3**, which found a significant increase of both pro-inflammatory (i.e. TNF-α) and anti-inflammatory (i.e. CORT) mediators in rats (Brooks et al., 2017; Grion et al., 2007). Indeed, TNF-α can directly, or *via* cytokine recruitments, activate the HPA axis involved in the secretion of CORT (Bernardini et al., 1990). Conversely, CORT belongs to the glucocorticoids that suppress LPS-stimulated secretion of TNF-α (Mikhaylova et al., 2007). Thus, the close interaction between TNF-α and CORT allows to maintain an immune homeostasis and ultimately facilitates the recovery form the stress of an infection/inflammation (Robert M. Sapolsky et al., 2000). However, a disequilibrium was found in wild wood mice exposed to Cd and Pb exhibiting higher TNF-α levels and lower CORT concentrations.

Figure 25. Effect of pollution exposure (in red: Cd and Pb) and nutritional quality (in purple: Se-deficient diet) on wild and captive wood mice immune response to LPS challenge (in green: 'normal' immune response of control individuals, see details in Chapter 3.4). Dotted lines indicate where disturbances can affect the immune response to LPS.



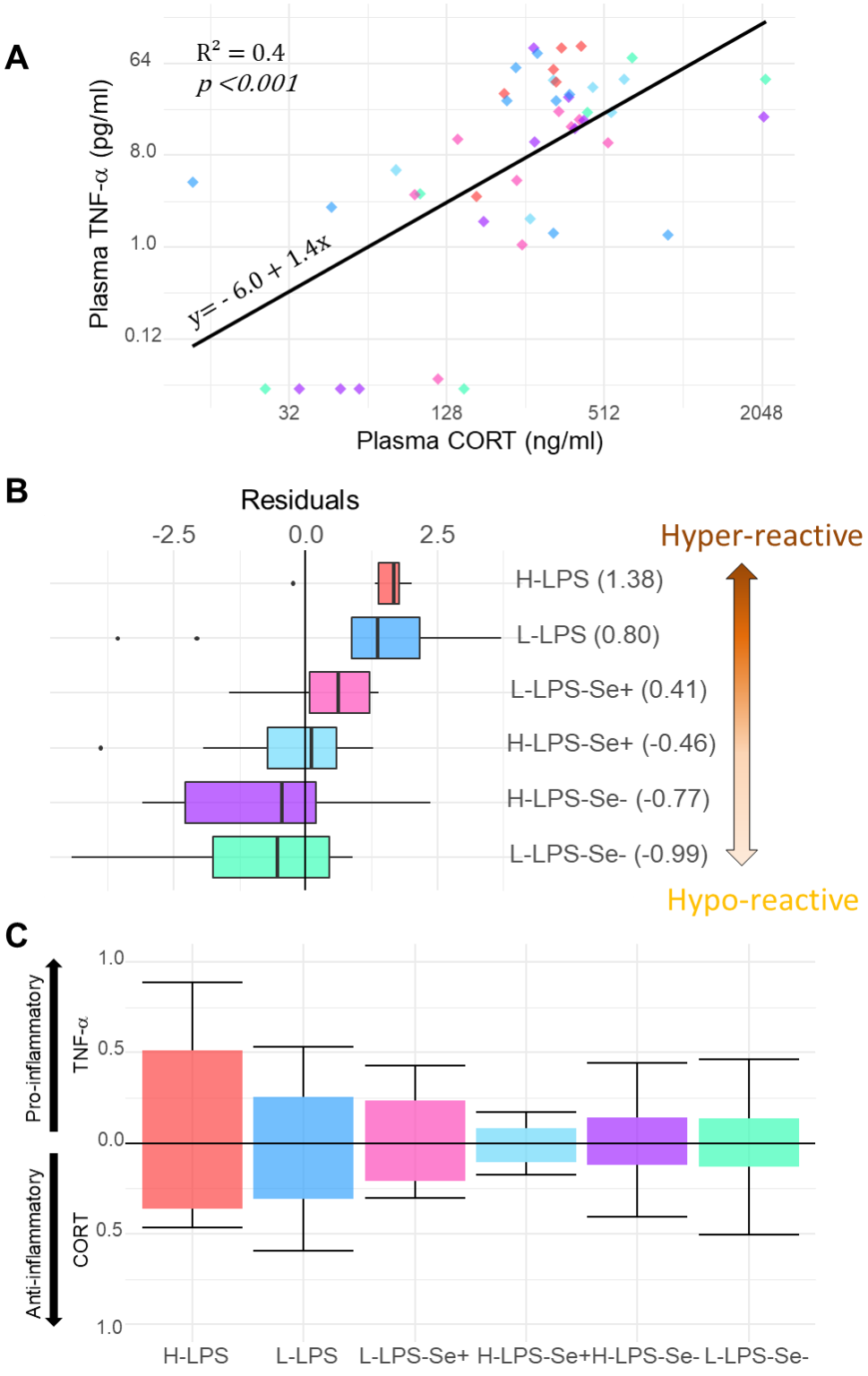
As detailed in **Table 3**, the decrease of CORT levels was attributed to endocrine disorders mediated by Cd and/or Pb exposure (Nishiyama et al., 1985; Nishiyama and Nakamura, 1984). Therefore, the imbalance in favour of proinflammatory response to LPS challenge may be considered as a hyper-reactive immune response. Hyper-reactive immune response might lead to autoimmune damages mediated by the elevated pro-inflammatory response. In contrast, Se-deficient wood mice captured in polluted sites displayed hype-reactive response. As found in Chapter 3.2, lower TNF-α and CORT were measured in wood mice fed Se-deficient food after 5 days of captivity. This pattern of hypo-reactive immune response might be mediated by lower pro-inflammatory response to LPS challenge as a response to quality diet. Selenium has been found to have a positive influence on immune cell protection against ROS (which can be produced by Cd and Pb exposure) and lymphoproliferation (**Table 3**, Avery and Hoffmann, 2018; Schweizer et al., 2016). However, these results did not allow determining if Se-deficient diet is responsible for TNF-α or CORT decrease as both may vary in response to the other. Furthermore, hypo-reactive immune response found in captive animals does not set sufficient basis to estimate the influence of low-quality diet in a natural environment. A mesocosm experimentation with natural food items supplement might allow to, at least,

determine the influence of diet quality (*i.e.* in terms of macronutrient supply) on the immune response to LPS challenge in wild animals. Altogether, these results indicate that complex interactions between stressors might modulate differently immune responses to stressors when considered in combination.

4.4.2 The optimal immune response in a changing world

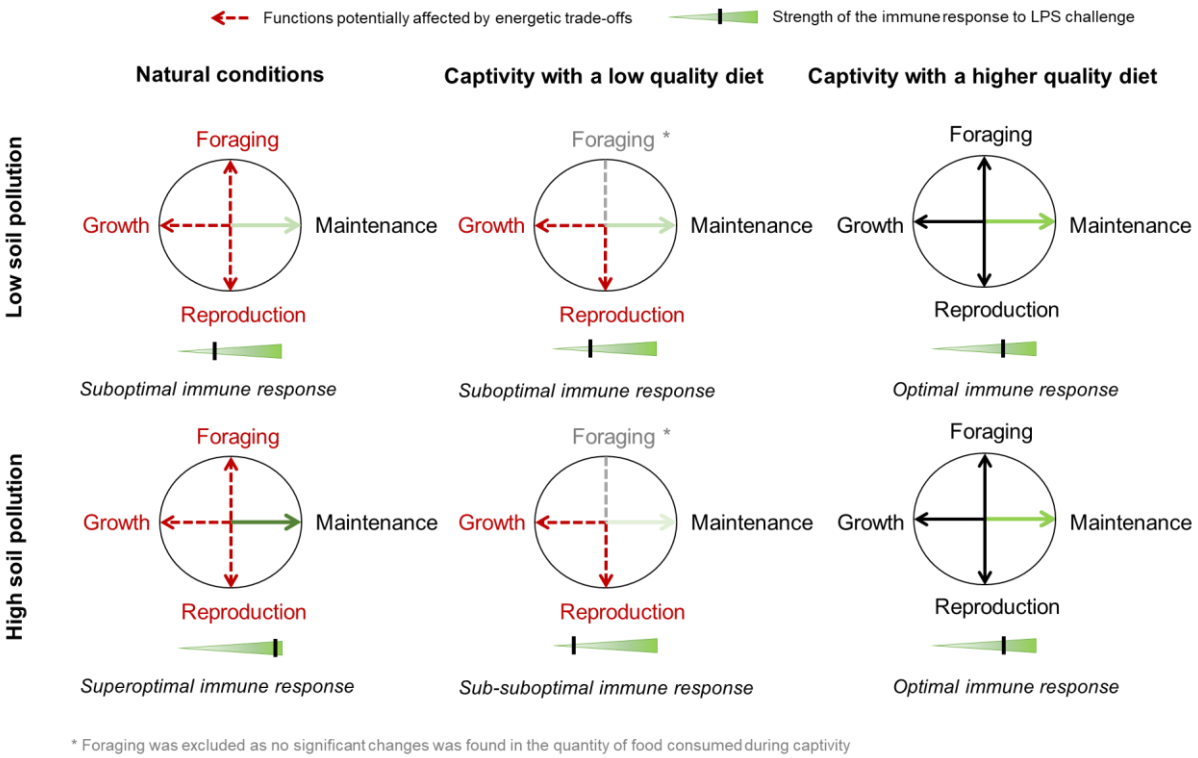
As detailed in § 4.1.2, the optimal immune response considers that the 'high' or 'low' strengths of an immune response can be both beneficial or detrimental to fitness (Viney *et al.* 2005). In this section, we adapted this approach to wood mice exposed to chronic levels of Cd and Pb presented in Chapter 3.2. To this end, the characterization of the strength of the immune response was done in considering the position of the tested groups of wood mice in the equilibrium between pro- (hyper-reactive) and anti- (hypo-reactive) inflammatory response. Studies on wildlife immunology integrated immunomarkers (*e.g.* cytokines) in dimensional analyses, like principal component analyses, to reduce the variability among individuals' response in a single component (*e.g.* Friberg et al., 2011; Jackson et al., 2014). In this view, immune stress response was reduced into two main components or axes that correspond to pro-inflammatory and anti-inflammatory mediator(s), respectively. As only TNF-α and CORT were measured in Chapter 3.2, these two parameters will be used to discriminate hyper- and hypo-reactive immune responses. **Figure 26 A and B** displayed a possible approach used to characterise the strength of the immune response of wood mice *via* a discrimination of hyper- and hypo-reactive immune responses. Min-max normalisation of the TNF-α and CORT plasma concentrations (*i.e.* scale each variable from 0 to 1 by subtracting the minimum and dividing by the maximum of all concentrations) was done to characterise pro- and anti-inflammatory response in each tested group (**Figure 26 C**). Linear regression of TNF-α in response to CORT ($p<0.001$, $R^2= 0.4$) displayed on **Figure 26 A** was used to calculate the mean of the residuals of the regression in each group on **Figure 26 B**. The mean of the residuals aims to estimate the relative distance to the predicted values from the model and ultimately to discriminate groups of individuals (**Figure 26 B**). Therefore, a positive mean of the residuals indicates groups predominantly with hyper-reactive immune response while negative value corresponds rather to hypo-reactive immune response. On **Figure 26 B**, wild individuals from high polluted sites showed higher mean residuals (1.38) suggesting hyper-reactive immune response in comparison with the other groups. This is in accordance with the elevated pro-inflammatory response showed in **Figure 26 C**. Wild individuals from high polluted site would thus describe a super optimal immune response. However, the position of wild individuals from low polluted sites (0.80) also indicated a hyper-reactive immune response, which may be mitigated by the lower pro-inflammatory levels showed in **Figure 26 C**.

Figure 26. A. Characterisation of optimal immune response to LPS challenge in wild and captive wood mice fed standard or Se-deficient diet, through the relation between plasma TNF-α and CORT. B. Discrimination of hyper- and hypo-reactive immune response. C. Mean and standard deviation of pro- (TNF-α) and anti-inflammatory (CORT) mediators min-max normalised.



Furthermore, as found in Chapter 3.2, no significant increase of CORT was found in comparison with their wild unchallenged counterparts. In this view, the immune response of wild individuals from low polluted would rather be considered as suboptimal. No visible disequilibrium between pro- and anti-inflammatory mediators can be seen for captive individuals on **Figure 26 C**, though lower range can be observed in comparison with the wild ones. Captive individuals fed low quality diet showed negative mean of the residuals ("high": -0.77 and "low": -0.99), which can be attributed to hypo-reactive immune response (**Figure 26 B**). Individuals fed Se-deficient diet exhibited a suboptimal immune response, which can be considered as sub-suboptimal in the case of wood mice from polluted sites. Individuals fed standard diet showed mean of their residuals close to zero ("high": -0.46 and "low":0.41) suggesting optimal immune response to LPS (**Figure 26 B**). Though the use of two immune variables constrained the analyses to linear models, the analyses of the mean of the residuals is in accordance with the results found in Chapter 3.2. Further immunomarkers; such as cytokines, of both pro- and anti-inflammatory response might allow using spatial discriminatory analyses (*e.g.* linear discriminant analyses, principal component analyses…), which provided inconclusive results (data not shown). Nonetheless, the classification of the groups of wood mice in Chapter 3.2 detailed in this section was implemented to the dial of energetic allocation presented in **Figure 27**.

Figure 27 Estimation of the energetic allocation according to the discrimination of hyper- from hypo-reactive immune response. (in italic: strength of the immune response).



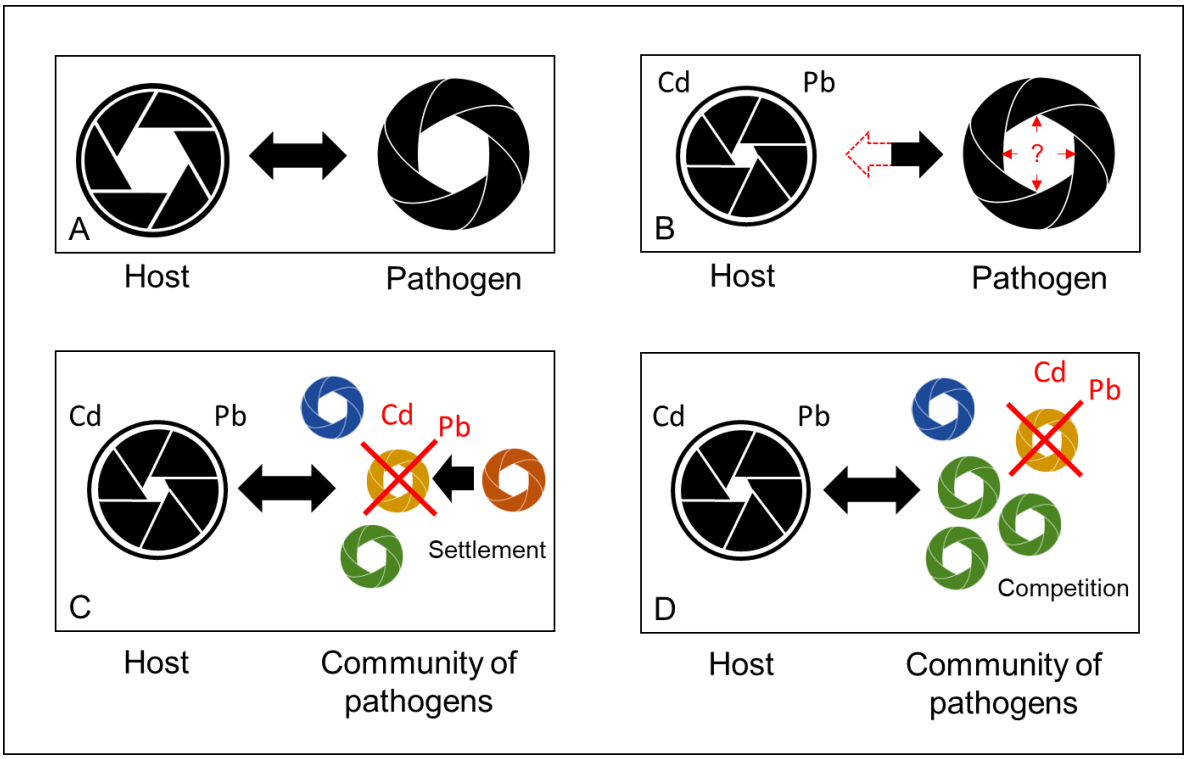
The discrimination of hyper- from hypo-reactive immune response showed similar interpretation of the section 4.1.2. Indeed, individuals from low polluted sites fed standard diet showed optimal immune response, which let suppose that individuals are much likely to invest in their own maintenance in favour of their fitness. Interestingly, the same was found in individuals from polluted site also fed standard diet. The investment of wild animals in functions allocated to their own maintenance might suggest an improvement of immune response mediated by diet quality. In comparison, wild animals from high polluted sites exhibited a super-optimal immune response that may indicate an increase of the cost allocated to maintenance. The main outcome of a super-optimal immune response may be linked to the investment itself in the maintenance rather than in other functions, and also to the energetic investment involved in the repair of autoimmune damages (Martin, 2009a). Such trade-offs might have consequences on the fitness of individuals as less energy could be allocated to reproduction for instance. In opposition, captive individuals fed Se deficient diet showed the lowest strength of the immune response qualified as sub-suboptimal regarding the others. This observation might suggest a potent involvement of diet quality in the energetic allocation to the immune response. Further investigation on the component of the immune system might indicate if this hyporeactive response can be described as an immunosuppression considering that no significant variation of CORT was observed. The immune challenge with LPS also seemed to compromise the immune response of individuals from low polluted sites in wild and in captivity with Se-deficient diet. Hence, immune response was rather characterised as suboptimal in comparison with individuals fed standard diet. In natural conditions and in captivity with low quality diet, individuals invested less in the immune response, which might be explain by potential investment in more valuable function. In captivity, energetic investment in foraging was set outside the possible function where energy might be invested as no differences in food consumption was observed during captivity. Nonetheless, five days captivity might be too short to estimate this trend and further experimentation in mesocosm could shed some light on which valuable function energy may be invest in.

4.4.3 Hosts and pathogens interaction in a changing world

In an evolutionary perception, infections represent a major selective pressure for organisms. Indeed, the relation between hosts and pathogens was described as an ‘arm races’ where host have to take the advantage from pathogens in evolving its resistance (*i.e.* genetic adaptation) while pathogens have to thwart host surveillance to achieve an infection (Sironi et al., 2015). Combes (1997) described two types of ‘arm races’, illustrated as encounter and compatibility filters (or iris), which explained the abundance of parasites in host populations. The first is the encounter filter that determines whether natural selection favours genes of the parasite improving the probability of finding the host, or host genes improving the avoidance of

pathogens. The second is the compatibility filter that considers whether natural selection favours parasite genes improving its survival, or host genes involved in the clearance of pathogens. In this view, we adapted the compatibility filters to host and pathogen interactions (**Figure 28**), where both host and pathogen have to maintain a sufficient aperture to resist or infect the other (**Figure 28 A**). From the side of the host, a wide aperture of the host filter would let a wide variety of pathogens achieving an infection, while a narrow aperture would represent a selective pressure on pathogens (*i.e.* favouring pathogens' evolution). For instance, the emergence of antimicrobial resistance is considered as an evolutionary response to the overuse of antimicrobial drugs in medicine (Holmes et al., 2016). From the side of the pathogen, a wide aperture would imply a low host specificity while a narrow one would indicate a high host specificity.

Figure 28 Illustration of the compatibility filters of Combes (1997) adapted to host-pathogen interaction (A) in a context of soil pollution by Cd and Pb (B), and possible driving force modulating pathogen community (C and D).



In a context of exposure to toxic metals such as Cd and Pb, we can consider that host may have narrow compatibility filter as super-optimal immune response might influence pathogens ability to infect the host (**Figure 28 B**). Furthermore, as detailed above, Cd and Pb are known to decrease soil microbial diversity and might thus have an influence on the pathogen community, at least in bacteria, which could affect the host (Sun et al., 2023, 2022; Salam et al., 2020). As found in Sures, (2008), parasites can aggravate the toxicity of metals like Cd and

Pb, in interfering with host-protection mechanisms (*e.g.* metallothioneins). From this, we can hypothesize that disturbance like Cd and Pb pollution might affect communities of pathogens through different driving forces of selection. As presented in **Figure 28 C**, Cd and Pb exposure might modify pathogen community composition in replacing a given pathogen (*i.e.* with no tolerance to Cd and Pb) with another that could settle in an environment polluted with Cd and Pb (*e.g.* metalliferous bacteria). Another possible mechanism might affect pathogen diversity *via* competition, which would favour only pathogens able to resist to elevated concentrations of Cd and Pb (**Figure 28 D**). However, to our knowledge, no molecular analyses have been done to estimate the community of pathogens that could affect wood mice in the vicinity of Metaleurop Nord.

Take home messages

1) Taken alone, our results confirmed findings of other studies where combination of stressors affected differentially the immune response.

2) Both exposure to pollution and nutritional quality affected wood mice immunocompetence.

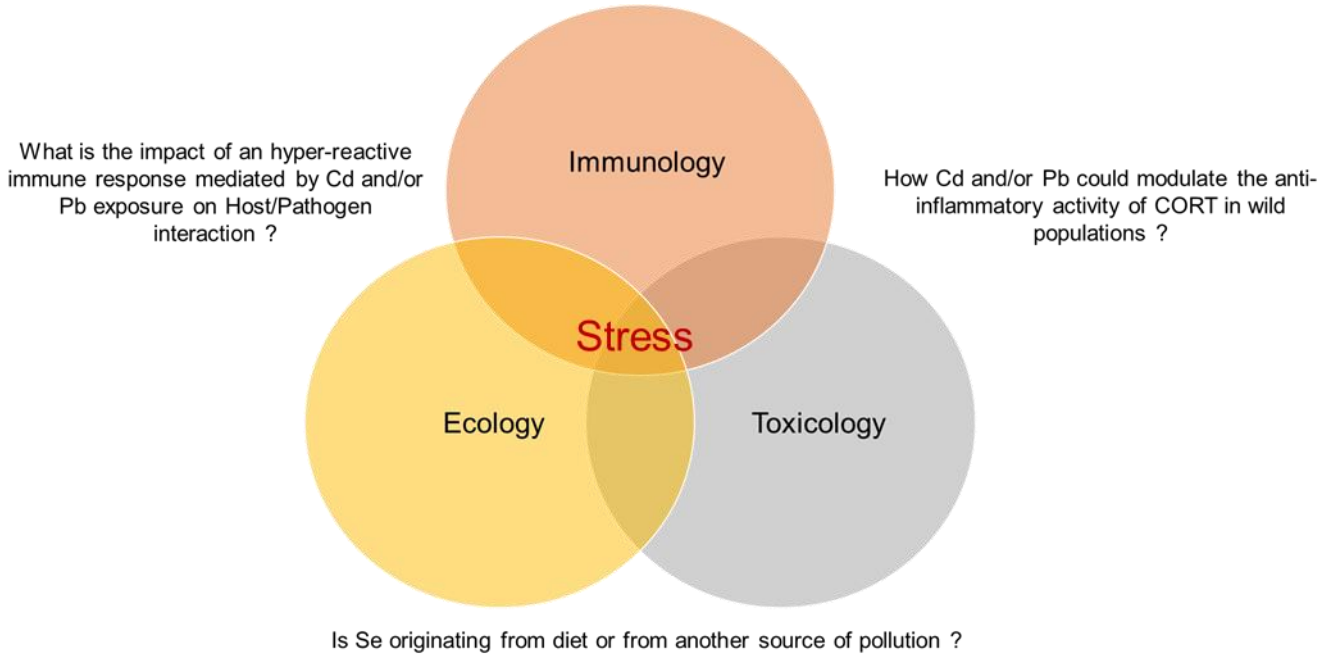
3) Optimal immune response was found in captive animals fed standard diet and not in other groups of animals.

4) Both hosts and pathogens might be influenced by the exposure to Cd and Pb in at least modulating the immune response of the host.

5 CONCLUSION

The present thesis aims to identify the influence of multiple stressors (pollution by toxic metals and low nutritional quality) on the immune response of wood mice. In crossing the borders of three scientific fields of research, our results indicate that both Cd, Pb and Se are potent immunomodulators of the immune response to bacterial challenge. In natural environments, wood mice exhibited higher immune response than their counterparts from low polluted sites, which might compromise their fitness. Energetic trade-offs might be involved regarding the elevated metabolic demand to invest in a very efficient immune response. However, dietary Se was found to alleviate the cost of the immune response in captive animals. At least, haematological parameters served as relevant indicators of the immune response in showing lower strength of the immune response when animals were fed Se-deficient food. Though dietary Se can be found in several natural items (*e.g.* nuts, seeds…), intriguing elevated concentrations of Se were found in the liver of wood mice captured in polluted sites by Cd and Pb. Some authors highlighted that dust from the former smelter of Metaleurop Nord contained elevated levels of Se, which could have been deposited in the surroundings and been mobilised by soil micro-organisms and plants. Nonetheless, the environmental availability and the bioavailability of Se remains unresolved. Trophic transfer at the origin of elevated Cd and Pb can be also a driver of Se accumulation in the liver of wood mice. The accumulation of Cd and Pb in tissues was related to immunological and endocrine impacts which, taken alone, corroborated laboratory studies on small mammal immunocompetence. However, immunomodulation in captive animals was mitigated by the influence of diet quality and this diet quality could be considered as a potent immunomodulator, at least able to modulate the immunotoxicity of Cd and Pb. Variation in the immunocompetence of wild animals may be related to an optimal immune response where the energetic cost of investing in an effective immune response could outweigh the benefits and therefore be at the expense of fitness. This raised an issue on the real efficiency of the immune response, which might be compromised by energetic trade-offs. Immunomarkers and blood reference intervals may provide relevant tools to estimate the trajectory of the immune response toward hyper- or hypo-reactive response and their effective relevance on fitness. Further insight should be gained in studying the relationships between biological levels of organization considering their influence on trace metal transfers and disease transmission. Both host and pathogen might be influenced by the exposure to Cd and Pb in at least modulating the immune response of the host. The results of this manuscript raised three questions, at the border of each scientific fields of research, which might allow elucidating how Cd, Pb and Se interact on wood mice stress (**Figure 29**).

Figure 29. Perspectives on the influence of Cd, Pb, and Se in the immune response of wild animals.



6 BIBLIOGRAPHY

Abdu, N., Abdullahi, A.A., Abdulkadir, A., 2017. Heavy metals and soil microbes. Environ. Chem. Lett. 15, 65–84. https://doi.org/10.1007/s10311-016-0587-x

Abolins, S., King, E.C., Lazarou, L., Weldon, L., Hughes, L., Drescher, P., Raynes, J.G., Hafalla, J.C.R., Viney, M.E., Riley, E.M., 2017a. The comparative immunology of wild and laboratory mice, *Mus musculus domesticus*. Nat. Commun. 8, 14811. https://doi.org/10.1038/ncomms14811

Abolins, S., King, E.C., Lazarou, L., Weldon, L., Hughes, L., Drescher, P., Raynes, J.G., Hafalla, J.C.R., Viney, M.E., Riley, E.M., 2017b. The comparative immunology of wild and laboratory mice, *Mus musculus domesticus*. Nat. Commun. 8, 14811. https://doi.org/10.1038/ncomms14811

Abolins, S., Lazarou, L., Weldon, L., Hughes, L., King, E.C., Drescher, P., Pocock, M.J.O., Hafalla, J.C.R., Riley, E.M., Viney, M., 2018. The ecology of immune state in a wild mammal, *Mus musculus domesticus*. PLOS Biol. 16, e2003538. https://doi.org/10.1371/journal.pbio.2003538

Abolins, S.R., Pocock, M.J.O., Hafalla, J.C.R., Riley, E.M., Viney, M.E., 2011. Measures of immune function of wild mice, *Mus musculus*. Mol. Ecol. 20, 881–892. https://doi.org/10.1111/j.1365-294X.2010.04910.x

Acevedo-Whitehouse, K., Duffus, A.L., 2009a. Effects of environmental change on wildlife health. Philos. Trans. R. Soc. B Biol. Sci. 364, 3429–3438.

Acevedo-Whitehouse, K., Duffus, A.L.J., 2009b. Effects of environmental change on wildlife health. Philos. Trans. R. Soc. B Biol. Sci. 364, 3429–3438. https://doi.org/10.1098/rstb.2009.0128

Addai-Mensah, O., Gyamfi, D., Duneeh, R.V., Danquah, K.O., Annani-Akollor, M.E., Boateng, L., Owiredu, E.-W., Amponsah, F.A., Afriyie, E.Y., Asare, R., Ofosu, D.N., 2019. Determination of Haematological Reference Ranges in Healthy Adults in Three Regions in Ghana. BioMed Res. Int. 2019, 1–6. https://doi.org/10.1155/2019/7467512

Agüero, R., Marsa, S.M., Moreno, L.E., Gomez, M.E.V., 2017. Peripheral Blood Lymphocyte Culture of Rhinella arenarum. Open Access Libr. J. 4, 1–8. https://doi.org/10.4236/oalib.1104212
Al-Attar, A.M., 2011. Vitamin E attenuates liver injury induced by exposure to lead, mercury, cadmium and copper in albino mice. Saudi J. Biol. Sci. 18, 395–401. https://doi.org/10.1016/j.sjbs.2011.07.004
Albalat, R., 2009. The retinoic acid machinery in invertebrates: Ancestral elements and vertebrate innovations. Mol. Cell. Endocrinol. 313, 23–35. https://doi.org/10.1016/j.mce.2009.08.029
Alberdi, A., Aizpurua, O., Bohmann, K., Gopalakrishnan, S., Lynggaard, C., Nielsen, M., Gilbert, M.T.P., 2019. Promises and pitfalls of using high-throughput sequencing for diet analysis. Mol. Ecol. Resour. 19, 327–348. https://doi.org/10.1111/1755-0998.12960
Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P., 2002. Innate Immunity. Mol. Biol. Cell 4th Ed.
Albery, G.F., Becker, D.J., Kenyon, F., Nussey, D.H., Pemberton, J.M., 2019. The fine-scale landscape of immunity and parasitism in a wild ungulate population. Integr. Comp. Biol. 59, 1165–1175.
Albery, G.F., Watt, K.A., Keith, R., Morris, S., Morris, A., Kenyon, F., Nussey, D.H., Pemberton, J.M., 2020. Reproduction has different costs for immunity and parasitism in a wild mammal. Funct. Ecol. 34, 229–239. https://doi.org/10.1111/1365-2435.13475
Alexander, J., 2015. Selenium, in: Handbook on the Toxicology of Metals. Elsevier, pp. 1175–1208. https://doi.org/10.1016/B978-0-444-59453-2.00052-4
Ali, H., Khan, E., Ilahi, I., 2019. Environmental chemistry and ecotoxicology of hazardous heavy metals: environmental persistence, toxicity, and bioaccumulation. J. Chem. 2019.
Allen, M.E., Ullrey, D.E., 2004. Relationships among nutrition and reproduction and relevance for wild animals. Zoo Biol. 23, 475–487. https://doi.org/10.1002/zoo.20029
Alloway, B.J. (Ed.), 2013. Heavy Metals in Soils: Trace Metals and Metalloids in Soils and their Bioavailability, 3rd ed, Environmental Pollution. Springer Netherlands. https://doi.org/10.1007/978-94-007-4470-7
Alpsoy, L., Yildirim, A., Agar, G., 2009. The antioxidant effects of vitamin A, C, and E on aflatoxin B1-induced oxidative stress in human lymphocytes. Toxicol. Ind. Health 25, 121–127. https://doi.org/10.1177/0748233709103413
Amiard-Triquet, C., Amiard, J.-C., Rainbow, P.S., 2012. Ecological Biomarkers: Indicators of Ecotoxicological Effects. CRC Press.
Anderson, S.E., Shane, H.L., 2018. Investigative Immunotoxicology. Methods Mol. Biol. Clifton NJ 1803, 27–46. https://doi.org/10.1007/978-1-4939-8549-4_3
Aoshima, K., 2016. Itai-itai disease: Renal tubular osteomalacia induced by environmental exposure to cadmium—historical review and perspectives. Soil Sci. Plant Nutr. 62, 319–326. https://doi.org/10.1080/00380768.2016.1159116
Apanius, V., 1998. Stress and Immune Defense, in: Møller, A.P., Milinski, M., Slater, P.J.B. (Eds.), Advances in the Study of Behavior, Stress and Behavior. Academic Press, pp. 133–153. https://doi.org/10.1016/S0065-3454(08)60363-0
Arck, P.C., 2019. When 3 Rs meet a forth R: Replacement, reduction and refinement of animals in research on reproduction. J. Reprod. Immunol. 132, 54–59.
Asare, M.O., Száková, J., Tlustoš, P., 2023. The fate of secondary metabolites in plants growing on Cd-, As-, and Pb-contaminated soils—a comprehensive review. Environ. Sci. Pollut. Res. 30, 11378–11398. https://doi.org/10.1007/s11356-022-24776-x
Atkinson, J., Manor, D., Parker, R., 2013. Vitamin E, in: Lennarz, W.J., Lane, M.D. (Eds.), Encyclopedia of Biological Chemistry (Second Edition). Academic Press, Waltham, pp. 545–550. https://doi.org/10.1016/B978-0-12-378630-2.00223-1
Aub, J.C., Fairhall, L.T., Minot, A.S., Reznikoff, P., 1926. Lead Poisoning. Williams & Wilkins.
Avery, J.C., Hoffmann, P.R., 2018. Selenium, Selenoproteins, and Immunity. Nutrients 10, 1203. https://doi.org/10.3390/nu10091203
Bain, B.J., Bates, I., Laffan, M.A., Lewis, S.M., 2016. Dacie and Lewis Practical Haematology: Expert Consult: Online and Print. Elsevier Health Sciences.

Baize, D., 2000. Teneurs totales en «métaux lourds» dans les sols français: résultats généraux du programme ASPITET. Courr. Environ. INRA 39–54.
Baker, A.J.M., McGrath, S.P., Reeves, R.D., Smith, J.A.C., 2000. Metal Hyperaccumulator Plants: A Review of the Ecology and Physiology of a Biological Resource for Phytoremediation of Metal-Polluted Soils, in: Phytoremediation of Contaminated Soil and Water. CRC Press.
Balali-Mood, M., Naseri, K., Tahergorabi, Z., Khazdair, M.R., Sadeghi, M., 2021. Toxic Mechanisms of Five Heavy Metals: Mercury, Lead, Chromium, Cadmium, and Arsenic. Front. Pharmacol. 12, 227. https://doi.org/10.3389/fphar.2021.643972
Bańbura, J., Bańbura, M., Glądalski, M., Kaliński, A., Marciniak, B., Markowski, M., Michalski, M., Nadolski, J., Skwarska, J., Wawrzyniak, J., Zieliński, P., 2013. Consequences of Experimental Changes in the Rearing Conditions of Blue Tit *Cyanistes caeruleus* and Great Tit *Parus major* Nestlings. Acta Ornithol. 48, 129–139. https://doi.org/10.3161/000164513X678784
Barboza, P.S., Parker, K.L., Hume, I.D. (Eds.), 2009. Integrative Wildlife Nutrition. Springer Berlin Heidelberg, Berlin, Heidelberg. https://doi.org/10.1007/978-3-540-87885-8
Batista, C.R.A., Gomes, G.F., Candelario-Jalil, E., Fiebich, B.L., de Oliveira, A.C.P., 2019. Lipopolysaccharide-Induced Neuroinflammation as a Bridge to Understand Neurodegeneration. Int. J. Mol. Sci. 20, 2293. https://doi.org/10.3390/ijms20092293
Beharka, A.A., Wu, D., Serafini, M., Meydani, S.N., 2002. Mechanism of vitamin E inhibition of cyclooxygenase activity in macrophages from old mice: role of peroxynitrite. Free Radic. Biol. Med. 32, 503–511. https://doi.org/10.1016/S0891-5849(01)00817-6
Belcastro, M., Marino, T., Russo, N., Toscano, M., 2005. Interaction of cysteine with Cu2+ and group IIb (Zn2+, Cd2+, Hg2+) metal cations: a theoretical study. J. Mass Spectrom. 40, 300–306.
Beldomenico, P.M., Telfer, S., Gebert, S., Lukomski, L., Bennett, M., Begon, M., 2008a. The dynamics of health in wild field vole populations: a haematological perspective. J. Anim. Ecol. 77, 984–997. https://doi.org/10.1111/j.1365-2656.2008.01413.x
Beldomenico, P.M., Telfer, S., Gebert, S., Lukomski, L., Bennett, M., Begon, M., 2008b. The dynamics of health in wild field vole populations: a haematological perspective. J. Anim. Ecol. 77, 984–997. https://doi.org/10.1111/j.1365-2656.2008.01413.x
Bellamri, N., Morzadec, C., Fardel, O., Vernhet, L., 2018. Arsenic and the immune system. Curr. Opin. Toxicol., Systems Toxicology: Immunotoxicity 10, 60–68. https://doi.org/10.1016/j.cotox.2018.01.003
Bendich, A., 1989. Carotenoids and the Immune Response. J. Nutr. 119, 112–115. https://doi.org/10.1093/jn/119.1.112
Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. B Methodol. 57, 289–300.
Beresford, N.A., Scott, E.M., Copplestone, D., 2020. Field effects studies in the Chernobyl Exclusion Zone: Lessons to be learnt. J. Environ. Radioact. 211, 105893. https://doi.org/10.1016/j.jenvrad.2019.01.005
Bernardini, R., Kamilaris, T.C., Calogero, A.E., Johnson, E.O., Gomez, M.T., Gold, P.W., Chrousos, G.P., 1990. Interactions between tumor necrosis factor-alpha, hypothalamic corticotropin-releasing hormone, and adrenocorticotropin secretion in the rat. Endocrinology 126, 2876–2881. https://doi.org/10.1210/endo-126-6-2876
Besold, A.N., Culbertson, E.M., Culotta, V.C., 2016. The Yin and Yang of copper during infection. JBIC J. Biol. Inorg. Chem. 21, 137–144. https://doi.org/10.1007/s00775-016-1335-1
Birnie-Gauvin, K., Peiman, K.S., Raubenheimer, D., Cooke, S.J., 2017a. Nutritional physiology and ecology of wildlife in a changing world. Conserv. Physiol. 5. https://doi.org/10.1093/conphys/cox030
Birnie-Gauvin, K., Peiman, K.S., Raubenheimer, D., Cooke, S.J., 2017b. Nutritional physiology and ecology of wildlife in a changing world. Conserv. Physiol. 5. https://doi.org/10.1093/conphys/cox030

Biser, J.A., Vogel, L.A., Berger, J., Hjelle, B., Loew, S.S., 2004. Effects of heavy metals on immunocompetence of white-footed mice (*Peromyscus leucopus*). J. Wildl. Dis. 40, 173–184. https://doi.org/10.7589/0090-3558-40.2.173
Bjørklund, G., Dadar, M., Mutter, J., Aaseth, J., 2017. The toxicology of mercury: Current research and emerging trends. Environ. Res. 159, 545–554. https://doi.org/10.1016/j.envres.2017.08.051
Bluhm, B.A., Gradinger, R., 2008. Regional variability in food availability for arctic marine mammals. Ecol. Appl. 18, S77–S96. https://doi.org/10.1890/06-0562.1
Bogdan, C., 2001. Nitric oxide and the immune response. Nat. Immunol. 2, 907–916. https://doi.org/10.1038/ni1001-907
Bonaventura, P., Benedetti, G., Albarede, F., Miossec, P., 2015. Zinc and its role in immunity and inflammation. Autoimmun. Rev. 14, 277–285. https://doi.org/10.1016/j.autrev.2014.11.008
Bonaventura, P., Lamboux, A., Albarède, F., Miossec, P., 2018. Differential effects of TNF-α and IL-1β on the control of metal metabolism and cadmium-induced cell death in chronic inflammation. PloS One 13, e0196285. https://doi.org/10.1371/journal.pone.0196285
Bonebrake, T.C., Guo, F., Dingle, C., Baker, D.M., Kitching, R.L., Ashton, L.A., 2019. Integrating Proximal and Horizon Threats to Biodiversity for Conservation. Trends Ecol. Evol. 34, 781–788. https://doi.org/10.1016/j.tree.2019.04.001
Bonin, M., Dussault, C., Taillon, J., Lecomte, N., Côté, S.D., 2020. Combining stable isotopes, morphological, and molecular analyses to reconstruct the diet of free-ranging consumers. Ecol. Evol. 10, 6664–6676. https://doi.org/10.1002/ece3.6397
Boonstra, R., 2013. Reality as the leading cause of stress: rethinking the impact of chronic stress in nature. Funct. Ecol. 27, 11–23. https://doi.org/10.1111/1365-2435.12008
Borcard, D., Gillet, F., Legendre, P., 2018. Numerical Ecology with R, Use R! Springer International Publishing, Cham. https://doi.org/10.1007/978-3-319-71404-2
Boshoff, M., De Jonge, M., Dardenne, F., Blust, R., Bervoets, L., 2014. The impact of metal pollution on soil faunal and microbial activity in two grassland ecosystems. Environ. Res. 134, 169–180. https://doi.org/10.1016/j.envres.2014.06.024
Brady, P.S., Brady, L.J., Whetter, P.A., Ullrey, D.E., Fay, L.D., 1978. The effect of dietary selenium and vitamin A on biochemical parameters and survival of young among white-tailed deer (*Odocoileus virginianus*). J. Nutr. 108, 1439–1448. https://doi.org/10.1093/jn/108.9.1439
Bricknell, I., Dalmo, R.A., 2005. The use of immunostimulants in fish larval aquaculture. Fish Shellfish Immunol. 19, 457–472.
Bridges, C.C., Zalups, R.K., 2005. Molecular and ionic mimicry and the transport of toxic metals. Toxicol. Appl. Pharmacol. 204, 274–308. https://doi.org/10.1016/j.taap.2004.09.007
Brooks, M., Turk, J., Guerrero, A., Narayanan, P., Nolan, J., Besteman, E., Wilson, D., Thomas, R., Fishman, C., Thompson, K., Ellinger-Ziegelbauer, H., Pierson, J., Paulman, A., Chiang, A., Schultze, A., 2017. Non-Lethal Endotoxin Injection: A Rat Model of Hypercoagulability. PLOS ONE 12, e0169976. https://doi.org/10.1371/journal.pone.0169976
Brophy, M.B., Nolan, E.M., 2015. Manganese and Microbial Pathogenesis: Sequestration by the Mammalian Immune System and Utilization by Microorganisms. ACS Chem. Biol. 10, 641–651. https://doi.org/10.1021/cb500792b
Broughton, H.M., Govender, D., Shikwambana, P., Chappell, P., Jolles, A., 2017.Bridging gaps between zoo and wildlife medicine establishing reference intervals for free ranging African lions (*Panther oleo*). J. Zoo Wildl. Med. 48, 298–311. https://doi.org/10.1638/2016-0021R.1
Brousseau, P., De Guise, S., Voccia, I., Ruby, S., Fournier, M., 2003. 14 Immune status of St. Lawrence estuary beluga whales. Toxicol. Mar. Mamm. 381.
Brousseau, P., Pillet, S., Frouin, H., Auffret, M., Gagné, F., Fournier, M., 2012. Linking Immunotoxicity and Ecotoxicological Effects at Higher Biological Levels. Ecol. Biomark. 24.
Brown, J.H., Marquet, P.A., Taper, M.L., 1993. Evolution of Body Size: Consequences of an Energetic Definition of Fitness. Am. Nat. 142, 573–584.

Budischak, S.A., Hansen, C.B., Caudron, Q., Garnier, R., Kartzinel, T.R., Pelczer, I., Cressler, C.E., van Leeuwen, A., Graham, A.L., 2018. Feeding Immunity: Physiological and Behavioral Responses to Infection and Resource Limitation. Front. Immunol. 8, 1914. https://doi.org/10.3389/fimmu.2017.01914

Buffenstein, R., Pinto, M., 2009. Endocrine function in naturally long-living small mammals. Mol. Cell. Endocrinol., Special Issue: The Endocrinology of Aging 299, 101–111. https://doi.org/10.1016/j.mce.2008.04.021

Bustnes, J.O., Bårdsen, B.-J., Bangjord, G., Lierhagen, S., Yoccoz, N.G., 2013. Temporal trends (1986–2005) of essential and non-essential elements in a terrestrial raptor in northern Europe. Sci. Total Environ. 458–460, 101–106. https://doi.org/10.1016/j.scitotenv.2013.04.027

Butet, A., Paillat, G., 1997. lnsectivores et Rongeurs de France. Le mulot sylvestre - *Apodemus sylvaticus* (Linnée 1758). Arvicola tome IX, 7–23.

Byrne, A., Reen, D.J., 2002. Lipopolysaccharide induces rapid production of IL-10 by monocytes in the presence of apoptotic neutrophils. J. Immunol. 168, 1968–1977.

Cairns, J., 1988. Putting the eco in ecotoxicology. Regul. Toxicol. Pharmacol. 8, 226–238. https://doi.org/10.1016/0273-2300(88)90031-1

Cairns, J., Niederlehner, B.R., 1996. Developing a Field of Landscape Ecotoxicology. Ecol. Appl. 6, 790–796. https://doi.org/10.2307/2269484

Calle, P.P., Dierenfeld, E.S., Robert, M.E., 1989. Serum α-Tocopherol in Raptors Fed Vitamin E-Supplemented Diets. J. Zoo Wildl. Med. 20, 62–67.

Carr, A.C., Maggini, S., 2017. Vitamin C and Immune Function. Nutrients 9. https://doi.org/10.3390/nu9111211

Carson, R., 1962. Silent Spring. Houghton Mifflin Harcourt.

CCME, 2009. Canadian soil quality guidelines for the protection of environmental and human health: Selenium. (Canadian Council of Ministers of the Environment.), Canadian environmental quality guidelines, 1999. Canadian Council of Ministers of the Environment, Winipeg.

Chakraborty, K., Chatterjee, S., Bhattacharyya, A., 2014. Modulation of phenotypic and functional maturation of murine bone-marrow-derived dendritic cells (BMDCs) induced by cadmium chloride. Int. Immunopharmacol. 20, 131–140. https://doi.org/10.1016/j.intimp.2014.02.015

Chaplin, D.D., 2010. Overview of the Immune Response. J. Allergy Clin. Immunol. 125, S3-23. https://doi.org/10.1016/j.jaci.2009.12.980

Charles A Janeway, J., Travers, P., Walport, M., Shlomchik, M.J., 2001. The complement system and innate immunity. Immunobiol. Immune Syst. Health Dis. 5th Ed.

Chi, L., Gao, B., Tu, P., Liu, C.-W., Xue, J., Lai, Y., Ru, H., Lu, K., 2018. Individual susceptibility to arsenic-induced diseases: the role of host genetics, nutritional status and the gut microbiome. Mamm. Genome Off. J. Int. Mamm. Genome Soc. 29, 63–79. https://doi.org/10.1007/s00335-018-9736-9

Chiang, H.-M., Chang, H., Yao, P.-W., Chen, Y.-S., Jeng, K.-C., Wang, J.-S., Hou, C.-W., 2014. Sesamin reduces acute hepatic injury induced by lead coupled with lipopolysaccharide. J. Chin. Med. Assoc. 77, 227–233. https://doi.org/10.1016/j.jcma.2014.02.010

Chmielnicka, J., Sowa, B., 1996. Cadmium Interaction with Essential Metals (Zn, Cu, Fe), Metabolism Metallothionein, and Ceruloplasmin in Pregnant Rats and Fetuses. Ecotoxicol. Environ. Saf. 35, 277–281. https://doi.org/10.1006/eesa.1996.0111

Choby, J.E., Skaar, E.P., 2016. Heme Synthesis and Acquisition in Bacterial Pathogens. J. Mol. Biol. 428, 3408–3428. https://doi.org/10.1016/j.jmb.2016.03.018

Choong, G., Liu, Y., Templeton, D., 2014. Interplay of Calcium and Cadmium in Mediating Cadmium Toxicity. Chem. Biol. Interact. 211. https://doi.org/10.1016/j.cbi.2014.01.007

Clemens, S., 2006. Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants. Biochimie, Facets of Environmental Nuclear Toxicology 88, 1707–1719. https://doi.org/10.1016/j.biochi.2006.07.003

Combes, C., 1997. Fitness of parasites: Pathology and selection. Int. J. Parasitol. 27, 1–10. https://doi.org/10.1016/S0020-7519(96)00168-3
Commissariat général au développement durable, 2019. Les sites et sols pollués [WWW Document]. Notre-Environnementgouvfr. URL https://notre-environnement.gouv.fr/themes/sante/la-pollution-des-sols-ressources/article/les-sites-et-sols-pollues (accessed 11.4.22).
Cook, D.N., Kang, H.S., Jetten, A.M., 2015. Retinoic Acid-Related Orphan Receptors (RORs): Regulatory Functions in Immunity, Development, Circadian Rhythm, and Metabolism. Nucl. Recept. Res. 2. https://doi.org/10.11131/2015/101185
Corp, N., Gorman, M.L., Speakman, J.R., 1997. Ranging behaviour and time budgets of male wood mice Apodemus sylvaticus in different habitats and seasons. Oecologia 109, 242–250. https://doi.org/10.1007/s004420050079
Cousins, R.J., 1979. Metallothionein synthesis and degradation: relationship to cadmium metabolism. Environ. Health Perspect. 28, 131–136.
Crespo, A.M., Neve, J., Pinto, R.E., 1993. Plasma and liver selenium levels in the rat during supplementation with 0.5, 2, 6, and 15 ppm selenium in drinking water. Biol. Trace Elem. Res. 38, 139–147. https://doi.org/10.1007/BF02784050
Cronin, S.J.F., Woolf, C.J., Weiss, G., Penninger, J.M., 2019. The Role of Iron Regulation in Immunometabolism and Immune-Related Disease. Front. Mol. Biosci. 6. https://doi.org/10.3389/fmolb.2019.00116
Culbertson, E.M., Khan, A.A., Muchenditsi, A., Lutsenko, S., Sullivan, D.J., Petris, M.J., Cormack, B.P., Culotta, V.C., 2020. Changes in mammalian copper homeostasis during microbial infection. Met. Integr. Biometal Sci. 12, 416–426. https://doi.org/10.1039/c9mt00294d
Culotta, V.C., Yang, M., O'Halloran, T.V., 2006. Activation of superoxide dismutases: Putting the metal to the pedal. Biochim. Biophys. Acta BBA - Mol. Cell Res., Cell Biology of Metals 1763, 747–758. https://doi.org/10.1016/j.bbamcr.2006.05.003
Dan, G., Lall, S., Rao, D., 2000. Humoral and cell mediated immune response to cadmium in mice. Drug Chem. Toxicol. 23, 349–60. https://doi.org/10.1081/DCT-100100120
Dangleben, N.L., Skibola, C.F., Smith, M.T., 2013. Arsenic immunotoxicity: a review. Environ. Health 12, 73. https://doi.org/10.1186/1476-069X-12-73
Das, K., Dupont, A., De Pauw-Gillet, M.-C., Debier, C., Siebert, U., 2016. Absence of selenium protection against methylmercury toxicity in harbour seal leucocytes in vitro. Mar. Pollut. Bull. 108, 70–76. https://doi.org/10.1016/j.marpolbul.2016.04.060
Das, K., Siebert, U., Gillet, A., Dupont, A., Dipoi, C., Fonfara, S., Mazzucchelli, G., De Pauw, E., De Pauw-Gillet, M.-C., 2008. Mercury immune toxicity in harbour seals: links to in vitro toxicity. Environ. Health 7, 52. https://doi.org/10.1186/1476-069X-7-52
Daum, J.R., Shepherd, D.M., Noelle, R.J., 1993. Immunotoxicology of cadmium and mercury on B-lymphocytes — I. Effects on lymphocyte function. Int. J. Immunopharmacol. 15, 383–394. https://doi.org/10.1016/0192-0561(93)90049-5
Davis, A.K., Maney, D.L., Maerz, J.C., 2008. The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. Funct. Ecol. 22, 760–772. https://doi.org/10.1111/j.1365-2435.2008.01467.x
Dawood, M.A.O., Koshio, S., Esteban, M.Á., 2018. Beneficial roles of feed additives as immunostimulants in aquaculture: a review. Rev. Aquac. 10, 950–974. https://doi.org/10.1111/raq.12209
Dean, J.H., Padarathsingh, M.L., Jerrells, T.R., 1979. Assessment of immunobiological effects induced by chemicals, drugs or food additives. I. Tier testing and screening approach. Drug Chem. Toxicol. 2, 5–17. https://doi.org/10.3109/01480547908993178
Defo, M.A., Spear, P.A., Couture, P., 2014. Consequences of metal exposure on retinoid metabolism in vertebrates: A review. Toxicol. Lett. 225, 1–11. https://doi.org/10.1016/j.toxlet.2013.11.024
Demas, G.E., Nelson, R.J., 2003. Lack of immunological responsiveness to photoperiod in a tropical rodent, Peromyscus aztecus hylocetes. J. Comp. Physiol. [B] 173, 171–176. https://doi.org/10.1007/s00360-002-0325-5

Desforges, J.-P.W., Sonne, C., Levin, M., Siebert, U., De Guise, S., Dietz, R., 2016. Immunotoxic effects of environmental pollutants in marine mammals. Environ. Int. 86, 126–139. https://doi.org/10.1016/j.envint.2015.10.007

Destoumieux-Garzón, D., Mavingui, P., Boetsch, G., Boissier, J., Darriet, F., Duboz, P., Fritsch, C., Giraudoux, P., Le Roux, F., Morand, S., Paillard, C., Pontier, D., Sueur, C., Voituron, Y., 2018a. The One Health Concept: 10 Years Old and a Long Road Ahead. Front. Vet. Sci. 5. https://doi.org/10.3389/fvets.2018.00014

Destoumieux-Garzón, D., Mavingui, P., Boetsch, G., Boissier, J., Darriet, F., Duboz, P., Fritsch, C., Giraudoux, P., Le Roux, F., Morand, S., Paillard, C., Pontier, D., Sueur, C., Voituron, Y., 2018b. The One Health Concept: 10 Years Old and a Long Road Ahead. Front. Vet. Sci. 5.

Devalloir, Q., Fritsch, C., Bangjord, G., Bårdsen, B.-J., Bourgeon, S., Eulaers, I., Bustnes, J.O., 2023. Long-term monitoring of exposure to toxic and essential metals and metalloids in the tawny owl (Strix aluco): Temporal trends and influence of spatial patterns. Sci. Total Environ. 876, 162710. https://doi.org/10.1016/j.scitotenv.2023.162710

Dhabhar, F.S., 2002. Stress-induced augmentation of immune function—The role of stress hormones, leukocyte trafficking, and cytokines. Brain. Behav. Immun. 16, 785–798. https://doi.org/10.1016/S0889-1591(02)00036-3

Dhabhar, F.S., Miller, A.H., McEwen, B.S., Spencer, R.L., 1996. Stress-induced changes in blood leukocyte distribution. Role of adrenal steroid hormones. J. Immunol. 157, 1638–1644.

Dietz, R., Letcher, R.J., Desforges, J.-P., Eulaers, I., Sonne, C., Wilson, S., Andersen-Ranberg, E., Basu, N., Barst, B.D., Bustnes, J.O., Bytingsvik, J., Ciesielski, T.M., Drevnick, P.E., Gabrielsen, G., Haarr, A., Hylland, K., Jenssen, B.M., Levin, M., McKinney, M.A., Norregaard, R.D., Pedersen, K.E., Provencher, J., Styrishave, B., Tartu, S., Aars, J., Ackerman, J.T., Rosing-Asvid, A., Barrett, R., Bignert, A., Borns, E.W., Branigan, M., Braune, B., Bryan, C.E., Dam, M., Eagles-Smith, C.A., Evans, M., Evans, T.J., Fisk, A.T., Gamberg, M., Gustavson, K., Hartman, C.A., Helander, B., Herzog, M.P., Hoekstra, P.F., Houde, M., Hoydal, K., Jackson, A.K., Kucklick, J., Lie, E., Loseto, L., Mallory, M.L., Miljeteig, C., Mosbech, A., Muir, D.C.G., Nielsen, S.T., Peacock, E., Pedro, S., Peterson, S.H., Polder, A., Riget, F.F., Roach, P., Saunes, H., Sinding, M.-H.S., Skaare, J.U., Sondergaard, J., Stenson, G., Stern, G., Treu, G., Schuur, S.S., Vikingsson, G., 2019. Current state of knowledge on biological effects from contaminants on arctic wildlife and fish. Sci. Total Environ. 696, UNSP 133792. https://doi.org/10.1016/j.scitotenv.2019.133792

Dietz, R., Riget, F., Johansen, P., 1996. Lead, cadmium, mercury and selenium in Greenland marine animals. Sci. Total Environ. 186, 67–93. https://doi.org/10.1016/0048-9697(96)05086-3

Dietz, R., Sonne, C., Basu, N., Braune, B., O'Hara, T., Letcher, R.J., Scheuhammer, T., Andersen, M., Andreasen, C., Andriashek, D., Asmund, G., Aubail, A., Baagoe, H., Born, E.W., Chan, H.M., Derocher, A.E., Grandjean, P., Knott, K., Kirkegaard, M., Krey, A., Lunn, N., Messier, F., Obbard, M., Olsen, M.T., Ostertag, S., Peacock, E., Renzoni, A., Riget, F.F., Skaare, J.U., Stern, G., Stirling, I., Taylor, M., Wiig, O., Wilson, S., Aars, J., 2013. What are the toxicological effects of mercury in Arctic biota? Sci. Total Environ. 443, 775–790. https://doi.org/10.1016/j.scitotenv.2012.11.046

Djokic, J., Ninkov, M., Mirkov, I., Popov Aleksandrov, A., Zolotarevski, L., Kataranovski, D., Kataranovski, M., 2014. Differential effects of cadmium administration on peripheral blood granulocytes in rats. Environ. Toxicol. Pharmacol. 37, 210–219. https://doi.org/10.1016/j.etap.2013.11.026

Dobrakowski, M., Boroń, M., Czuba, Z.P., Kasperczyk, A., Machoń-Grecka, A., Kasperczyk, S., 2016. Cytokines related to three major types of cell-mediated immunity in short- and long-term exposures to lead compounds. J. Immunotoxicol. 13, 770–774. https://doi.org/10.1080/1547691X.2016.1184360

Douay, F., Pruvot, C., Roussel, H., Ciesielski, H., Fourrier, H., Proix, N., Waterlot, C., 2008. Contamination of Urban Soils in an Area of Northern France Polluted by Dust Emissions of

Two Smelters. Water. Air. Soil Pollut. 188, 247–260. https://doi.org/10.1007/s11270-007-9541-7
Douay, F., Pruvot, C., Waterlot, C., Fritsch, C., Fourrier, H., Loriette, A., Bidar, G., Grand, C., de Vaufleury, A., Scheifler, R., 2009a. Contamination of woody habitat soils around a former lead smelter in the North of France. Sci. Total Environ. 407, 5564–5577. https://doi.org/10.1016/j.scitotenv.2009.06.015
Douay, F., Pruvot, C., Waterlot, C., Fritsch, C., Fourrier, H., Loriette, A., Bidar, G., Grand, C., de Vaufleury, A., Scheifler, R., 2009b. Contamination of woody habitat soils around a former lead smelter in the North of France. Sci. Total Environ. 407, 5564–5577. https://doi.org/10.1016/j.scitotenv.2009.06.015
Downs, C.J., Stewart, K.M., 2014. A primer in ecoimmunology and immunology for wildlife research and management. Calif. Fish Game 100, 371–395.
Drazen, D.L., Trasy, A., Nelson, R.J., 2003. Photoperiod differentially affects energetics of immunity in pregnant and lactating Siberian hamsters (*Phodopus sungorus*). Can. J. Zool. 81, 1406–1413. https://doi.org/10.1139/z03-120
DRIRE, 2003. L'Industrie au regard de l'environnement en 2002. DRIRE Nord-Pas-de-Calais. Douai.
Dufresne, M.M., Frouin, H., Pillet, S., Lesage, V., De Guise, S., Fournier, M., 2010. Comparative sensitivity of harbour and grey seals to several environmental contaminants using in vitro exposure. Mar. Pollut. Bull. 60, 344–349. https://doi.org/10.1016/j.marpolbul.2009.10.019
Durkalec, M., Nawrocka, A., Krzysiak, M., Larska, M., Kmiecik, M., Posyniak, A., 2018. Trace elements in the liver of captive and free-ranging European bison (*Bison bonasus L*.). Chemosphere 193, 454–463. https://doi.org/10.1016/j.chemosphere.2017.11.050
Duruibe, J.O., Ogwuegbu, M.O.C., Egqurugwu, .N., 2007. Heavy metal pollution and human biotoxic effects. Int. J. Phys. Sci. 2, 112–118.
Ecke, F., Benskin, J.P., Berglund, Å.M.M., de Wit, C.A., Engström, E., Plassmann, M.M., Rodushkin, I., Sörlin, D., Hörnfeldt, B., 2020. Spatio-temporal variation of metals and organic contaminants in bank voles (*Myodes glareolus*). Sci. Total Environ. 713, 136353. https://doi.org/10.1016/j.scitotenv.2019.136353
EEA, 2020. Contamination from local sources — European Environment Agency [WWW Document]. URL https://www.eea.europa.eu/themes/soil/soil-threats (accessed 11.4.22).
El-Boshy, M.E., Risha, E.F., Abdelhamid, F.M., Mubarak, M.S., Hadda, T.B., 2015. Protective effects of selenium against cadmium induced hematological disturbances, immunosuppressive, oxidative stress and hepatorenal damage in rats. J. Trace Elem. Med. Biol. 29, 104–110.
Eroglu, A., Harrison, E.H., 2013. Carotenoid metabolism in mammals, including man: formation, occurrence, and function of apocarotenoids Thematic Review Series: Fat-Soluble Vitamins: Vitamin A. J. Lipid Res. 54, 1719–1730. https://doi.org/10.1194/jlr.R039537
Espín, S., García-Fernández, A.J., Herzke, D., Shore, R.F., van Hattum, B., Martínez-López, E., Coeurdassier, M., Eulaers, I., Fritsch, C., Gómez-Ramírez, P., 2016. Tracking pan-continental trends in environmental contamination using sentinel raptors—what types of samples should we use? Ecotoxicology 25, 777–801.
Espin, S., Sanchez-Virosta, P., 2021. A review of metal-induced effects on vitamins A, E and D3 in birds. Ecotoxicology 30, 1–16. https://doi.org/10.1007/s10646-020-02296-z
Etim, N.N., Williams, M.E., Akpabio, U., Offiong, E.E.A., n.d. Haematological Parameters and Factors Affecting Their Values.
European Commission., 2014. Progress in the management of contaminated sites in Europe. Publications Office, LU.
Exposure to heavy metals and infectious disease mortality in harbour porpoises from England and Wales, 2001. Environ. Pollut. 112, 33–40. https://doi.org/10.1016/S0269-7491(00)00105-6
Feldman, B.V., Zinkl, J.G., Jain, N.C., Schalm, O.W. (Eds.), 2000. Schalm's veterinary hematology, 5th ed. ed. Lippincott Williams & Wilkins, Philadelphia.

Fenga, C., Gangemi, S., Di Salvatore, V., Falzone, L., Libra, M., 2017. Immunological effects of occupational exposure to lead (Review). Mol. Med. Rep. 15, 3355–3360. https://doi.org/10.3892/mmr.2017.6381
Flies, A.S., wild comparative immunology consortium, 2020. Rewilding immunology. Science 369, 37–38. https://doi.org/10.1126/science.abb8664
Flueck, W.T., Smith-Flueck, J.M., Mionczynski, J., Mincher, B.J., 2012. The implications of selenium deficiency for wild herbivore conservation: a review. Eur. J. Wildl. Res. 58, 761–780. https://doi.org/10.1007/s10344-012-0645-z
Folstad, I., Karter, A.J., 1992. Parasites, Bright Males, and the Immunocompetence Handicap. Am. Nat. 139, 603–622. https://doi.org/10.1086/285346
Forbes, K.M., Mappes, T., Sironen, T., Strandin, T., Stuart, P., Meri, S., Vapalahti, O., Henttonen, H., Huitu, O., 2016. Food limitation constrains host immune responses to nematode infections. Biol. Lett. 12, 20160471. https://doi.org/10.1098/rsbl.2016.0471
Forman, R.T., Godron, M., 1981. Patches and structural components for a landscape ecology. BioScience 31, 733–740.
Fossi, M.C., Marsili, L., Neri, G., Natoli, A., Politi, E., Panigada, S., 2003. The use of a non-lethal tool for evaluating toxicological hazard of organochlorine contaminants in Mediterranean cetaceans: new data 10 years after the first paper published in MPB. Mar. Pollut. Bull. 46, 972–982. https://doi.org/10.1016/S0025-326X(03)00113-9
Fournier, M., Cyr, D., Blakley, B., Boermans, H., Brousseau, P., 2000. Phagocytosis as a Biomarker of Immunotoxicity in Wildlife Species Exposed to Environmental Xenobiotics. Integr. Comp. Biol. 40, 412–420. https://doi.org/10.1093/icb/40.3.412
Fox, M.R., Jacobs, R.M., Jones, A.O., Fry Jr, B.E., Stone, C.L., 1980. Effects of vitamin C and iron and cadmium metabolism. Ann. N. Y. Acad. Sci. 355, 249.
Fox, M.R.S., 1979. Nutritional influences on metal toxicity: cadmium as a model toxic element. 10.
França, S.M. de, Breda, M.O., Barbosa, D.R.S., Araujo, A.M.N., Guedes, C.A., França, S.M. de, Breda, M.O., Barbosa, D.R.S., Araujo, A.M.N., Guedes, C.A., 2017. The Sublethal Effects of Insecticides in Insects. IntechOpen. https://doi.org/10.5772/66461
Friberg, I.M., Lowe, A., Ralli, C., Bradley, J.E., Jackson, J.A., 2011. Temporal Anomalies in Immunological Gene Expression in a Time Series of Wild Mice: Signature of an Epidemic? PLOS ONE 6, e20070. https://doi.org/10.1371/journal.pone.0020070
Friedrichs, K., Harr, K., Freeman, K., Szladovits, B., Walton, R., Barnhart, K., Blanco-Chavez, J., 2012. ASVCP reference interval guidelines: determination of de novo reference intervals in veterinary species and other related topics. Vet. Clin. Pathol. https://doi.org/10.1111/vcp.12006
Fritsch, C., Coeurdassier, M., Faivre, B., Baurand, P.-E., Giraudoux, P., van den Brink, N.W., Scheifler, R., 2012. Influence of landscape composition and diversity on contaminant flux in terrestrial food webs: A case study of trace metal transfer to European blackbirds Turdus merula. Sci. Total Environ. 432, 275–287. https://doi.org/10.1016/j.scitotenv.2012.06.004
Fritsch, C., Cœurdassier, M., Giraudoux, P., Raoul, F., Douay, F., Rieffel, D., de Vaufleury, A., Scheifler, R., 2011. Spatially Explicit Analysis of Metal Transfer to Biota: Influence of Soil Contamination and Landscape. PLoS ONE 6, e20682. https://doi.org/10.1371/journal.pone.0020682
Fritsch, C., Cosson, R.P., Cœurdassier, M., Raoul, F., Giraudoux, P., Crini, N., de Vaufleury, A., Scheifler, R., 2010a. Responses of wild small mammals to a pollution gradient: Host factors influence metal and metallothionein levels. Environ. Pollut. 158, 827–840. https://doi.org/10.1016/j.envpol.2009.09.027
Fritsch, C., Giraudoux, P., Cœurdassier, M., Douay, F., Raoul, F., Pruvot, C., Waterlot, C., Vaufleury, A. de, Scheifler, R., 2010b. Spatial distribution of metals in smelter-impacted soils of woody habitats: Influence of landscape and soil properties, and risk for wildlife. Chemosphere 81, 141–155. https://doi.org/10.1016/j.chemosphere.2010.06.075
Fritsch, C., Giraudoux, P., Cœurdassier, M., Douay, F., Raoul, F., Pruvot, C., Waterlot, C., Vaufleury, A. de, Scheifler, R., 2010c. Spatial distribution of metals in smelter-impacted soils

of woody habitats: Influence of landscape and soil properties, and risk for wildlife. Chemosphere 81, 141–155. https://doi.org/10.1016/j.chemosphere.2010.06.075
Fritsch, C., Scheifler, R., Beaugelin-Seiller, K., Hubert, P., Cœurdassier, M., de Vaufleury, A., Badot, P.-M., 2008. Biotic interactions modify the transfer of cesium-137 in a soil-earthworm-plant-snail food web. Environ. Toxicol. Chem. 27, 1698–1707. https://doi.org/10.1897/07-416.1
Fritts, C.A., Erf, G.F., Bersi, T.K., Waldroup, P.W., 2004. Effect of source and level of vitamin D on immune function in growing broilers. J. Appl. Poult. Res. 13, 263–273. https://doi.org/10.1093/japr/13.2.263
Frouin, H., Fortier, M., Fournier, M., 2010a. Toxic effects of various pollutants in 11B7501 lymphoma B cell line from harbour seal (Phoca vitulina). Toxicology 270, 66–76. https://doi.org/10.1016/j.tox.2010.01.014
Frouin, H., Loseto, L.L., Stern, G.A., Haulena, M., Ross, P.S., 2012. Mercury toxicity in beluga whale lymphocytes: Limited effects of selenium protection. Aquat. Toxicol. 109, 185–193. https://doi.org/10.1016/j.aquatox.2011.09.021
Frouin, H., Menard, L., Measures, L., Brousseau, P., Fournier, M., 2010b. T Lymphocyte-Proliferative Responses of a Grey Seal (*Halichoerus grypus*) Exposed to Heavy Metals and PCBs in Vitro. Aquat. Mamm. 36, 365–371. https://doi.org/10.1578/AM.36.4.2010.365
Fu, J., Yang, T., Wang, W., Xu, S., 2019. Effect of selenium antagonist lead-induced damage on Th1/Th2 imbalance in the peripheral blood lymphocytes of chickens. Ecotoxicol. Environ. Saf. 175, 74–82. https://doi.org/10.1016/j.ecoenv.2019.03.036
Fullmer, C.S., 1997. Lead-calcium interactions: involvement of 1,25-dihydroxyvitamin D. Environ. Res. 72, 45–55. https://doi.org/10.1006/enrs.1996.3689
Funk, A.E., Day, F.A., Brady, F.O., 1987. Displacement of zinc and copper from copper-induced metallothionein by cadmium and by mercury: in vivo and ex vivo studies. Comp. Biochem. Physiol. C 86, 1–6. https://doi.org/10.1016/0742-8413(87)90133-2
Galloway, T.S., Depledge, M.H., 2001. Immunotoxicity in Invertebrates: Measurement and Ecotoxicological Relevance. Ecotoxicology 10, 5–23. https://doi.org/10.1023/A:1008939520263
Gallucci, R., Luckett-Chastain, L., Yucesoy, B., 2020. Chapter 18 - Immunotoxicology, in: Pope, C.N., Liu, J. (Eds.), An Introduction to Interdisciplinary Toxicology. Academic Press, pp. 233–244. https://doi.org/10.1016/B978-0-12-813602-7.00018-1
Ganther, 1978. Modification of methylmercury toxicity and metabolism by selenium and vitamin E: possible mechanisms. Environ. Health Perspect. 25, 71–76. https://doi.org/10.1289/ehp.782571
García-Mendoza, D., Han, B., Berg, H.J.H.J. van den, Brink, N.W. van den, 2019. Cell-specific immune-modulation of cadmium on murine macrophages and mast cell lines in vitro. J. Appl. Toxicol. 39, 992–1001. https://doi.org/10.1002/jat.3788
Geffré, A., Friedrichs, K., Harr, K., Concordet, D., Trumel, C., Braun, J.-P., 2009. Reference values: a review. Vet. Clin. Pathol. 38, 288–298. https://doi.org/10.1111/j.1939-165X.2009.00179.x
Gell, P.G.H., Coombs, R.R.A., 1963. Clinical Aspects of Immunology. Clin. Asp. Immunol.
Gelling, M., McLaren, G.W., Mathews, F., Mian, R., Macdonald, D.W., 2009. Impact of trapping and handling on leukocyte coping capacity in bank voles (*Clethrionomys glareolus*) and wood mice (Apodemus sylvaticus). Anim Welf 18, 1–7.
Gera, R., Singh, V., Sharma, A.K., Ghosh, D., 2015. Immunomodulation by Environmental Chemicals: Insights into Mammalian Immune Responses to Arsenic, Cadmium, and Lead, in: Advances in Molecular Toxicology. Elsevier, pp. 109–159.
Germolec, D., Luebke, R., Rooney, A., Shipkowski, K., Vandebriel, R., van Loveren, H., 2017. Immunotoxicology: A brief history, current status and strategies for future immunotoxicity assessment. Curr. Opin. Toxicol., Systems Toxicology: Opportunities and challenges for in vitro assessment of Immunotoxicity 5, 55–59. https://doi.org/10.1016/j.cotox.2017.08.002
Gillet, P., 2018. Histoire de l'écotoxicologie. SMETox J. 1, 1–4.

Gombart, A.F., Pierre, A., Maggini, S., 2020. A Review of Micronutrients and the Immune System–Working in Harmony to Reduce the Risk of Infection. Nutrients 12, 236. https://doi.org/10.3390/nu12010236
Goodwin, T.W., 1986. Metabolism, nutrition, and function of carotenoids. Metab. Nutr. Funct. Carotenoids 6, 273–297.
Gorriz, A., Llacuna, S., Riera, M., Nadal, J., 1996. Effects of air pollution on hematological and plasma parameters in *Apodemus sylvaticus* and *Mus musculus*. Arch. Environ. Contam. Toxicol. 31, 153–158. https://doi.org/10.1007/BF00203920
Goyer, R.A., 1995. Nutrition and metal toxicity. Am. J. Clin. Nutr. 61, 646S–650S.
Graham, A.L., 2021a. Naturalizing mouse models for immunology. Nat. Immunol. 22, 111–117. https://doi.org/10.1038/s41590-020-00857-2
Graham, A.L., 2021b. Naturalizing mouse models for immunology. Nat. Immunol. 22, 111–117. https://doi.org/10.1038/s41590-020-00857-2
Graham, A.L., Shuker, D.M., Pollitt, L.C., Auld, S.K.J.R., Wilson, A.J., Little, T.J., 2011. Fitness consequences of immune responses: strengthening the empirical framework for ecoimmunology. Funct. Ecol. 25, 5–17. https://doi.org/10.1111/j.1365-2435.2010.01777.x
Green, H.N., Mellanby, E., 1928. Vitamin A as an anti-infective agent. Br. Med. J. 2, 691–696. https://doi.org/10.1136/bmj.2.3537.691
Gremion, F., Chatzinotas, A., Harms, H., 2003. Comparative 16S rDNA and 16S rRNA sequence analysis indicates that Actinobacteria might be a dominant part of the metabolically active bacteria in heavy metal-contaminated bulk and rhizosphere soil. Environ. Microbiol. 5, 896–907.
Grion, N., Repetto, E., Pomeraniec, Y., Calejman, C., Astort, F., Sanchez, R., Pignataro, O., Arias, P., Cymeryng, C., 2007. Induction of nitric oxide synthase and heme oxygenase activities by endotoxin in the rat adrenal cortex: Involvement of both signaling systems in the modulation of ACTH-dependent steroid production. J. Endocrinol. 194, 11–20. https://doi.org/10.1677/JOE-06-0199
Gupta, R.S., Gupta, E.S., Dhakal, B.K., Thakur, A.R., Ahnn, J., 2004. Vitamin C and vitamin E protect the rat testes from cadmium-induced reactive oxygen species. Mol Cells 17, 132–139.
Haeckel, E., 1866. Generelle morphologie der organismen. Allgemeine grundzüge der organischen formen-wissenschaft, mechanisch begründet durch die von Charles Darwin reformirte descendenztheorie. G. Reimer, Berlin.
Halbrook, R., Shore, R., Rattner, B., 2003. Ecotoxicology of Wild Mammals. J. Wildl. Manag. 67, 232. https://doi.org/10.2307/3803081
Hallberg, L., Brune, M., Rossander, L., 1989. The role of vitamin C in iron absorption. Int. J. Vitam. Nutr. Res. Suppl. Int. Z. Vitam.- Ernahrungsforschung Suppl. 30, 103–108.
Hamilton, W.D., Zuk, M., 1982. Heritable True Fitness and Bright Birds: A Role for Parasites? Science 218, 384–387. https://doi.org/10.1126/science.7123238
Harrison, X.A., Blount, J.D., Inger, R., Norris, D.R., Bearhop, S., 2011. Carry-over effects as drivers of fitness differences in animals. J. Anim. Ecol. 80, 4–18. https://doi.org/10.1111/j.1365-2656.2010.01740.x
Hart, G.R., Furniss, J.L., Laurie, D., Durham, S.K., 2006. Measurement of vitamin D status: background, clinical use, and methodologies. Clin. Lab. 52, 335–343.
Haug, A., Graham, R.D., Christophersen, O.A., Lyons, G.H., 2007. How to use the world's scarce selenium resources efficiently to increase the selenium concentration in food. Microb. Ecol. Health Dis. 19, 209–228.
Havarinasab, S., Hultman, P., 2005. Organic mercury compounds and autoimmunity. Autoimmun. Rev. 4, 270–275. https://doi.org/10.1016/j.autrev.2004.12.001
Helsel, D.R., 2010. Summing nondetects: Incorporating low-level contaminants in risk assessment. Integr. Environ. Assess. Manag. 6, 361–366. https://doi.org/10.1002/ieam.31
Helsel, D.R., 2008. Detection Limits, in: Encyclopedia of Quantitative Risk Analysis and Assessment. John Wiley & Sons, Ltd. https://doi.org/10.1002/9780470061596.risk0261
Hemilä, H., 2017. Vitamin C and infections. Nutrients 9, 339.

Henderson, D.G., 1970. Some nutritional studies on the-wood mouse apodemus sylvaticus (PhD Thesis). Durham University.
Hennigar, S.R., McClung, J.P., 2016. Nutritional Immunity. Am. J. Lifestyle Med. 10, 170–173. https://doi.org/10.1177/1559827616629117
Heo, Y., Mondal, T.K., Gao, D., Kasten-Jolly, J., Kishikawa, H., Lawrence, D.A., 2007. Posttranscriptional inhibition of interferon-gamma production by lead. Toxicol. Sci. Off. J. Soc. Toxicol. 96, 92–100. https://doi.org/10.1093/toxsci/kfl182
Heo, Y., Parsons, P.J., Lawrence, D.A., 1996. Lead Differentially Modifies Cytokine Productionin Vitroandin Vivo. Toxicol. Appl. Pharmacol. 138, 149–157.
Higdon, J., Drake, V., 2012. An evidence-based approach to vitamins and minerals: health benefits and intake recommendations, 2nd ed. ed. Thieme, Stuttgart ; New York.
Hoffman, D.J., Rattner, B.A., Jr, G.A.B., Jr, J.C., 2002. Handbook of Ecotoxicology. CRC Press.
Holick, M.F., 2004. Sunlight and vitamin D for bone health and prevention of autoimmune diseases, cancers, and cardiovascular disease. Am. J. Clin. Nutr. 80, 1678S–88S. https://doi.org/10.1093/ajcn/80.6.1678S
Holick, M.F., Chen, T.C., Lu, Z., Sauter, E., 2007. Vitamin D and skin physiology: A D-lightful story. J. Bone Miner. Res. 22, V28–V33. https://doi.org/10.1359/JBMR.07S211
Holmes, A.H., Moore, L.S.P., Sundsfjord, A., Steinbakk, M., Regmi, S., Karkey, A., Guerin, P.J., Piddock, L.J.V., 2016. Understanding the mechanisms and drivers of antimicrobial resistance. The Lancet 387, 176–187. https://doi.org/10.1016/S0140-6736(15)00473-0
Hossein-Khannazer, N., Azizi, G., Eslami, S., Alhassan Mohammed, H., Fayyaz, F., Hosseinzadeh, R., Usman, A.B., Kamali, A.N., Mohammadi, H., Jadidi-Niaragh, F., 2020. The effects of cadmium exposure in the induction of inflammation. Immunopharmacol. Immunotoxicol. 42, 1–8.
Houghton, R.A., 1995. Land-use change and the carbon cycle. Glob. Change Biol. 1, 275–287. https://doi.org/10.1111/j.1365-2486.1995.tb00026.x
Hu, H., Möller, G., Abedi-Valugerdi, M., 1997. Major Histocompatibility Complex Class II Antigens are Required for both Cytokine Production and Proliferation Induced by Mercuric Chloridein Vitro. J. Autoimmun. 10, 441–446. https://doi.org/10.1006/jaut.1997.9997
Hua, J., Jones, D.K., Mattes, B.M., Cothran, R.D., Relyea, R.A., Hoverman, J.T., 2015. The contribution of phenotypic plasticity to the evolution of insecticide tolerance in amphibian populations. Evol. Appl. 8, 586–596. https://doi.org/10.1111/eva.12267
Huang, Z., Rose, A.H., Hoffmann, P.R., 2012. The Role of Selenium in Inflammation and Immunity: From Molecular Mechanisms to Therapeutic Opportunities. Antioxid. Redox Signal. 16, 705–743. https://doi.org/10.1089/ars.2011.4145
Hutchinson, G.E., 1965. The ecological theater and the evolutionary play. Yale University Press.
Hutchinson, G.E., 1957. Concluding remarks Cold Spring Harbor Symposia on Quantitative Biology, 22: 415–427. GS SEARCH.
Iavicoli, I., Carelli, G., Stanek, E.J., Castellino, N., Calabrese, E.J., 2006. Below background levels of blood lead impact cytokine levels in male and female mice. Toxicol. Appl. Pharmacol. 210, 94–99. https://doi.org/10.1016/j.taap.2005.09.016
Ilyina, T.N., Baishnikova, I., 2019. Adaptation to Food Deprivation in Mammals: Vitamins A and E. J. Evol. Biochem. Physiol. 55, 184–190. https://doi.org/10.1134/S0022093019030037
Islam, L.N., Nurun Nabi, A.H.M., Rahman, M.M., Zahid, M.S.H., 2007. Association of respiratory complications and elevated serum immunoglobulins with drinking water arsenic toxicity in human. J. Environ. Sci. Health Part A 42, 1807–1814. https://doi.org/10.1080/10934520701566777
Jackson, J.A., 2015. Immunology in wild nonmodel rodents: an ecological context for studies of health and disease. Parasite Immunol. 37, 220–232. https://doi.org/10.1111/pim.12180
Jackson, J.A., Friberg, I.M., Bolch, L., Lowe, A., Ralli, C., Harris, P.D., Behnke, J.M., Bradley, J.E., 2009. Immunomodulatory parasites and toll-like receptor-mediated tumour necrosis factor alpha responsiveness in wild mammals. BMC Biol. 7, 16. https://doi.org/10.1186/1741-7007-7-16

Jackson, J.A., Hall, A.J., Friberg, I.M., Ralli, C., Lowe, A., Zawadzka, M., Turner, A.K., Stewart, A., Birtles, R.J., Paterson, S., Bradley, J.E., Begon, M., 2014. An immunological marker of tolerance to infection in wild rodents. PLoS Biol. 12, e1001901. https://doi.org/10.1371/journal.pbio.1001901

Jacob, E.A., 2016. Complete Blood Cell Count and Peripheral Blood Film, Its Significant in Laboratory Medicine: A Review Study. Am. J. Lab. Med. 1, 34. https://doi.org/10.11648/j.ajlm.20160103.12

Jensen, A.L., Iversen, L., Petersen, T.K., 1998. Study on biological variability of haematological components in dogs. Comp. Haematol. Int. 8, 202–204. https://doi.org/10.1007/BF02752849

Jensen, S.P., 1993. Temporal changes in food preferences of wood mice (Apodemus sylvaticus L.). Oecologia 94, 76–82.

Johnson, M.A., Fischer, J.G., Kays, S.E., 1992. Is copper an antioxidant nutrient? Crit. Rev. Food Sci. Nutr. 32, 1–31. https://doi.org/10.1080/10408399209527583

Johnstone, C.P., Lill, A., Reina, R.D., 2017. Use of erythrocyte indicators of health and condition in vertebrate ecophysiology: a review and appraisal: Erythrocyte indices of vertebrate condition. Biol. Rev. 92, 150–168. https://doi.org/10.1111/brv.12219

Jolles, A.E., Beechler, B.R., Dolan, B.P., 2015. Beyond mice and men: Environmental change, immunity and infections in wild ungulates. Parasite Immunol. 37, 255–266. https://doi.org/10.1111/pim.12153

Kabata-Pendias, A., Szteke, B., 2015. Trace elements in abiotic and biotic environments. Taylor & Francis.

Käkelä, R., Käkelä, A., Hyvärinen, H., Asikainen, J., Dahl, S.-K., 1999. Vitamins A1, A2, and E in minks exposed to polychlorinated biphenyls (Aroclor 1242®) and copper, VIA diet based on freshwater or marine fish. Environ. Toxicol. Chem. 18, 2595–2599. https://doi.org/10.1002/etc.5620181129

Kakuschke, A., Valentine-Thon, E., Fonfara, S., Griesel, S., Siebert, U., Prange, A., 2006. Metal sensitivity of marine mammals: a case study of a gray seal (Halichoerus grypus). Mar. Mammal Sci. 22, 985–996.

Kakuschke, A., Valentine-Thon, E., Griesel, S., Fonfara, S., Siebert, U., Prange, A., 2011. Are metal-induced hypersensitivities in harbor seals associated with liver function? Mar. Pollut. Bull. 62, 1891–1894. https://doi.org/10.1016/j.marpolbul.2011.05.034

Kakuschke, A., Valentine-Thon, E., Griesel, S., Rosenberger, T., Mundry, R., Siebert, U., Prange, A., 2008. Blood metal levels and metal-influenced immune functions of harbour seals in captivity. Mar. Pollut. Bull. 56, 764–769. https://doi.org/10.1016/j.marpolbul.2008.01.015

Kalisinska, E., Lanocha-Arendarczyk, N., Kosik-Bogacka, D., Budis, H., Pilarczyk, B., Tomza-Marciniak, A., Podlasinska, J., Cieslik, L., Popiolek, M., Pirog, A., Jedrzejewska, E., 2017. Muscle mercury and selenium in fishes and semiaquatic mammals from a selenium-deficient area. Ecotoxicol. Environ. Saf. 136, 24–30. https://doi.org/10.1016/j.ecoenv.2016.10.028

Kapustka, L.A., Galbraith, H., Luxon, M., Yocum, J., 2001. Using landscape ecology to focus ecological risk assessment and guide risk management decision-making. Toxicol. Ind. Health 17, 236–246. https://doi.org/10.1191/0748233701th121oa

Karabulut-Bulan, O., Bolkent, S., Yanardag, R., Bilgin-Sokmen, B., 2008. The role of vitamin C, vitamin E, and selenium on cadmium-induced renal toxicity of rats. Drug Chem. Toxicol. 31, 413–426.

Kataranovski, M., Kataranovski, D., Savic, D., Jovcic, G., Bogdanovic, Z., Jovanovic, T., 1998. Granulocyte and plasma cytokine activity in acute cadmium intoxication in rats. Physiol. Res. 47, 453–461.

Kehl-Fie, T.E., Skaar, E.P., 2010. Nutritional immunity beyond iron: a role for manganese and zinc. Curr. Opin. Chem. Biol. 14, 218–224. https://doi.org/10.1016/j.cbpa.2009.11.008

Khan, M.A.K., Wang, F., 2009. Mercury-selenium compounds and their toxicological significance: Toward a molecular understanding of the mercury-selenium antagonism. Environ. Toxicol. Chem. 28, 1567–1577. https://doi.org/10.1897/08-375.1

Kim, B.-E., Nevitt, T., Thiele, D.J., 2008. Mechanisms for copper acquisition, distribution and regulation. Nat. Chem. Biol. 4, 176.
Kim, S.H., Johnson, V.J., Sharma, R.P., 2003. Oral exposure to inorganic mercury alters T lymphocyte phenotypes and cytokine expression in BALB/c mice. Arch. Toxicol. 77, 613–620. https://doi.org/10.1007/s00204-003-0497-0
Kishikawa, H., Song, R., Lawrence, D.A., 1997. Interleukin-12 Promotes Enhanced Resistance to Listeria monocytogenes infection of Lead-Exposed Mice. Toxicol. Appl. Pharmacol. 147, 180–189. https://doi.org/10.1006/taap.1997.8308
Kleiman, D.G., Allen, M.E., Thompson, K.V., Lumpkin, S., 1996. Wild mammals in captivity. Princ. Tech. USA.
Koeman, J.H., Peeters, W.H.M., Koudstaal-Hol, C.H.M., Tjioe, P.S., De Goeij, J.J.M., 1973. Mercury-selenium correlations in marine mammals. Nature 245, 385–386.
Kohlmeier, M., 2015. Chapter 11 - Minerals and Trace Elements, in: Kohlmeier, M. (Ed.), Nutrient Metabolism (Second Edition). Academic Press, San Diego, pp. 673–807. https://doi.org/10.1016/B978-0-12-387784-0.00011-0
Kono, D.H., Balomenos, D., Pearson, D.L., Park, M.S., Hildebrandt, B., Hultman, P., Pollard, K.M., 1998. The prototypic Th2 autoimmunity induced by mercury is dependent on IFN-γ and not Th1/Th2 imbalance. J. Immunol. 161, 234–240.
Kophamel, S., Illing, B., Ariel, E., Difalco, M., Skerratt, L.F., Hamann, M., Ward, L.C., Méndez, D., Munns, S.L., 2022. Importance of health assessments for conservation in noncaptive wildlife. Conserv. Biol. 36. https://doi.org/10.1111/cobi.13724
Kozul, C.D., Ely, K.H., Enelow, R.I., Hamilton, J.W., 2009. Low-Dose Arsenic Compromises the Immune Response to Influenza A Infection in Vivo. Environ. Health Perspect. 117, 1441–1447. https://doi.org/10.1289/ehp.0900911
Krug-MacLeod, A.M., Eng, M.L., Cabezas, S., Marchant, T.A., Morrissey, C.A., 2022. Colour of adult avian integumentary features reveals differences related to in ovo stressors. Ibis.
Kumar, V., Pradheeps, M., Kokkiligadda, A., Niyogi, R., Umapathy, G., 2019. Non-Invasive Assessment of Physiological Stress in Captive Asian Elephants. Animals 9, 553. https://doi.org/10.3390/ani9080553
Kurata, M., Suzuki, M., Agar, N.S., 1993. Antioxidant systems and erythrocyte life-span in mammals. Comp. Biochem. Physiol. Part B Comp. Biochem. 106, 477–487. https://doi.org/10.1016/0305-0491(93)90121-K
Kwong, W., Friello, P., Semba, R., 2004. Interactions between iron deficiency and lead poisoning: Epidemiology and pathogenesis. Sci. Total Environ. 330, 21–37. https://doi.org/10.1016/j.scitotenv.2004.03.017
Lagadic, L., Caquet, T., Amiard, J.C., 1997. Biomarqueurs en écotoxicologie : principes et définitions (introduction), in: Biomarqueurs En Écotoxicologie, Aspects Fondamentaux. Elsevier Mason SAS.
Lall, S.B., Dan, G., 1999. Role of corticosteroids in cadmium induced immunotoxicity. Drug Chem. Toxicol. 22, 401–409. https://doi.org/10.3109/01480549909017843
Landete-Castillejos, T., García, A., Gómez, J.Á., Laborda, J., Gallego, L., 2002. Effects of Nutritional Stress During Lactation on Immunity Costs and Indices of Future Reproduction in Iberian Red Deer (Cervus elaphus hispanicus)1. Biol. Reprod. 67, 1613–1620. https://doi.org/10.1095/biolreprod.102.004507
Lawrence, D.A., McCabe, M.J., 2002. Immunomodulation by metals. Int. Immunopharmacol., Occupational Immunology 2, 293–302. https://doi.org/10.1016/S1567-5769(01)00180-1
Lee, G.Y., Han, S.N., 2018. The Role of Vitamin E in Immunity. Nutrients 10. https://doi.org/10.3390/nu10111614
Legendre, P., Legendre, L., 2012. Numerical Ecology, 3e édition. ed. Elsevier Science Ltd, Amsterdam.
Li, Qian, Zhang, P., Yu, X., Zhao, Y., Li, Qiang, Zhang, Yandong, Yang, Z., Xie, Y., Xue, P., Sun, S., Jia, X., Zhou, Z., He, M., Zhang, Yubin, 2017. Lead Transiently Promotes Granulocyte-Macrophage Progenitor Differentiation and Subsequently Suppresses Common Myeloid Progenitor Differentiation. Toxicol. Sci. 160, 268–283. https://doi.org/10.1093/toxsci/kfx176

Liao, C., Hardison, R.C., Kennett, M.J., Carlson, B.A., Paulson, R.F., Prabhu, K.S., 2018. Selenoproteins regulate stress erythroid progenitors and spleen microenvironment during stress erythropoiesis. Blood 131, 2568–2580. https://doi.org/10.1182/blood-2017-08-800607

Lidicker Jr, W.Z., 2008. Levels of organization in biology: on the nature and nomenclature of ecology's fourth level. Biol. Rev. 83, 71–78.

Liu, C.-M., Sun, Y.-Z., Sun, J.-M., Ma, J.-Q., Cheng, C., 2012. Protective role of quercetin against lead-induced inflammatory response in rat kidney through the ROS-mediated MAPKs and NF-κB pathway. Biochim. Biophys. Acta BBA - Gen. Subj. 1820, 1693–1703. https://doi.org/10.1016/j.bbagen.2012.06.011

Liu, M.-Y., Cheng, Y.-J., Chen, C.-K., Yang, B.-C., 2005. Coexposure of lead-and lipopolysaccharide-induced liver injury in rats: involvement of nitric oxide-initiated oxidative stress and TNF-α. Shock 23, 360–364. https://doi.org/10.1097/01.shk.0000158116.77328.1d

Lochmiller, R.L., 1996. Immunocompetence and Animal Population Regulation. Oikos 76, 594. https://doi.org/10.2307/3546356

Lochmiller, R.L., Deerenberg, C., 2000. Trade-offs in evolutionary immunology: just what is the cost of immunity? Oikos 88, 87–98. https://doi.org/10.1034/j.1600-0706.2000.880110.x

Lochmiller, R.L., Vestey, M.R., Nash, D., 1992. Gut associated lymphoid tissue in the cotton rat (Sigmodon Hipsidus) and its response to proetin restiction. J. Wildl. Dis. 28, 1–9. https://doi.org/10.7589/0090-3558-28.1.1

Lock, K., Janssen, C.R., 2003. Influence of Aging on Metal Availability in Soils, in: Ware, G.W. (Ed.), Reviews of Environmental Contamination and Toxicology, Reviews of Environmental Contamination and Toxicology. Springer, New York, NY, pp. 1–21. https://doi.org/10.1007/0-387-21728-2_1

Lopes, A.C.B.A., Peixe, T.S., Mesas, A.E., Paoliello, M.M., 2016. Lead exposure and oxidative stress: a systematic review, in: Reviews of Environmental Contamination and Toxicology Volume 236. Springer, pp. 193–238.

Lopes, G., Ávila, F., Guilherme, L., 2017. Selenium behavior in the soil environment and its implication for human health. Ciênc. E Agrotecnologia 41, 605–615. https://doi.org/10.1590/1413-70542017416000517

Lopez, C.A., Skaar, E.P., 2018. The Impact of Dietary Transition Metals on Host-Bacterial Interactions. Cell Host Microbe 23, 737–748. https://doi.org/10.1016/j.chom.2018.05.008

Lorentzon, R., Larsson, S.E., 1977. Vitamin D metabolism in adult rats at low and normal calcium intake and the effect of cadmium exposure. Clin. Sci. Mol. Med. 53, 439–446. https://doi.org/10.1042/cs0530439

Lu, M., Huang, L., Wang, Q., Cao, X., Lin, Q., He, Z., Feng, Y., Yang, X., 2023. Soil properties drive the bacterial community to cadmium contamination in the rhizosphere of two contrasting wheat (*Triticum aestivum* L.) genotypes. J. Environ. Sci. 128, 117–128. https://doi.org/10.1016/j.jes.2022.07.028

Luster, M.I., Gerberick, G.F., 2010. Immunotoxicology Testing: Past and Future, in: Dietert, R.R. (Ed.), Immunotoxicity Testing: Methods and Protocols, Methods in Molecular Biology™. Humana Press, Totowa, NJ, pp. 3–13. https://doi.org/10.1007/978-1-60761-401-2_1

Luster, M.I., Munson, A.E., Thomas, P.T., Holsapple, M.P., Fenters, J.D., White, K.L., Lauer, L.D., Germolec, D.R., Rosenthal, G.J., Dean, J.H., 1988. Development of a testing battery to assess chemical-induced immunotoxicity: National Toxicology Program's guidelines for immunotoxicity evaluation in mice. Fundam. Appl. Toxicol. Off. J. Soc. Toxicol. 10, 2–19. https://doi.org/10.1016/0272-0590(88)90247-3

Luster, M.I., Portier, C., Pait, D.G., Rosenthal, G.J., Germolec, D.R., Corsini, E., Blaylock, B.L., Pollock, P., Kouchi, Y., Craig, W., White, K.L., Munson, A.E., Comment, C.E., 1993. Risk Assessment in ImmunotoxicologyII. Relationships between Immune and Host Resistance Tests. Toxicol. Sci. 21, 71–82. https://doi.org/10.1093/toxsci/21.1.71

Maceda-Veiga, A., Figuerola, J., Martínez-Silvestre, A., Viscor, G., Ferrari, N., Pacheco, M., 2015a. Inside the Redbox: Applications of haematology in wildlife monitoring and

ecosystem health assessment. Sci. Total Environ. 514, 322–332. https://doi.org/10.1016/j.scitotenv.2015.02.004
Maceda-Veiga, A., Figuerola, J., Martínez-Silvestre, A., Viscor, G., Ferrari, N., Pacheco, M., 2015b. Inside the Redbox: applications of haematology in wildlife monitoring and ecosystem health assessment. Sci. Total Environ. 514, 322–332.
Macneil, J.D., Patterson, J.R., Salisbury, C.D.C., Tessaro, S.V., 1990. An Investigation of the Trace Element Status of Bison in Wood Buffalo National Park and of Ranch-Raised Bison in Saskatchewan, Canada. Int. J. Environ. Anal. Chem. 41, 99–104. https://doi.org/10.1080/03067319008027353
Mak, T.W., Saunders, M.E., Jett, B.D. (Eds.), 2014. Chapter 6 - The Major Histocompatibility Complex, in: Primer to the Immune Response (Second Edition). Academic Cell, Boston, pp. 143–159. https://doi.org/10.1016/B978-0-12-385245-8.00006-6
Malavia, D., Crawford, A., Wilson, D., 2017. Nutritional Immunity and Fungal Pathogenesis: The Struggle for Micronutrients at the Host-Pathogen Interface, in: Poole, R.K. (Ed.), Microbiology of Metal Ions. Academic Press Ltd-Elsevier Science Ltd, London, pp. 85–103.
Marsh, A.C.W., Poulton, S., Harris, S., 2008. The Yellow-necked Mouse Apodemus flavicollis in Britain: status and analysis of factors affecting distribution: Yellow-necked Mouse in Britain. Mammal Rev. 31, 203–227. https://doi.org/10.1111/j.1365-2907.2001.00089.x
Martin, L.B., 2009a. Stress and immunity in wild vertebrates: Timing is everything. Gen. Comp. Endocrinol., 9th International Symposium on Avian Endocrinology 163, 70–76. https://doi.org/10.1016/j.ygcen.2009.03.008
Martin, L.B., 2009b. Stress and immunity in wild vertebrates: Timing is everything. Gen. Comp. Endocrinol. 163, 70–76. https://doi.org/10.1016/j.ygcen.2009.03.008
Martin, L.B., Hawley, D.M., Ardia, D.R., 2011. An introduction to ecological immunology. Funct. Ecol. 25, 1–4. https://doi.org/10.1111/j.1365-2435.2010.01820.x
Martin, L.B., Hopkins, W.A., Mydlarz, L.D., Rohr, J.R., 2010. The effects of anthropogenic global changes on immune functions and disease resistance: Ecoimmunology and global change. Ann. N. Y. Acad. Sci. 1195, 129–148. https://doi.org/10.1111/j.1749-6632.2010.05454.x
Martin, L.B., Navara, K.J., Weil, Z.M., Nelson, R.J., 2007. Immunological memory is compromised by food restriction in deer mice Peromyscus maniculatus. Am. J. Physiol.-Regul. Integr. Comp. Physiol. 292, R316–R320. https://doi.org/10.1152/ajpregu.00386.2006
Martin, L.B., Weil, Z.M., Nelson, R.J., 2008. Seasonal changes in vertebrate immune activity: mediation by physiological trade-offs. Philos. Trans. R. Soc. B Biol. Sci. 363, 321–339. https://doi.org/10.1098/rstb.2007.2142
Massey, A.C., 1992. Microcytic anemia. Differential diagnosis and management of iron deficiency anemia. Med. Clin. North Am. 76, 549–566. https://doi.org/10.1016/s0025-7125(16)30339-x
Mazzullo, G., Rifici, C., Caccamo, G., Rizzo, M., Piccione, G., 2014. Effect of Different Environmental Conditions on Some Haematological Parameters in Cow. Ann. Anim. Sci. 14, 947–954. https://doi.org/10.2478/aoas-2014-0049
McAleer, J.P., Vella, A.T., 2008. Understanding how lipopolysaccharide impacts CD4 T cell immunity. Crit. Rev. Immunol. 28, 281–299.
McCabe, M.J., Lawrence, D.A., 1991. Lead, a major environmental pollutant, is immunomodulatory by its differential effects on CD4+ T cells subsets. Toxicol. Appl. Pharmacol. 111, 13–23. https://doi.org/10.1016/0041-008x(91)90129-3
McClure, S.J., 2008. How minerals may influence the development and expression of immunity to endoparasites in livestock. Parasite Immunol. 30, 89–100. https://doi.org/10.1111/j.1365-3024.2007.00996.x
McEwen, B.S., Wingfield, J.C., 2010. What is in a name? Integrating homeostasis, allostasis and stress. Horm. Behav. 57, 105–111. https://doi.org/10.1016/j.yhbeh.2009.09.011
McGrath, J.P., 1993. Assessment of hemolytic and hemorrhagic anemias in preclinical safety assessment studies. Toxicol. Pathol. 21, 158–163. https://doi.org/10.1177/019262339302100207

McKee, A.S., Fontenot, A.P., 2016. Interplay of innate and adaptive immunity in metal-induced hypersensitivity. Curr. Opin. Immunol., Allergy and hypersensitivity * Host pathogens 42, 25–30. https://doi.org/10.1016/j.coi.2016.05.001
McKenzie, V.J., Song, S.J., Delsuc, F., Prest, T.L., Oliverio, A.M., Korpita, T.M., Alexiev, A., Amato, K.R., Metcalf, J.L., Kowalewski, M., Avenant, N.L., Link, A., Di Fiore, A., Seguin-Orlando, A., Feh, C., Orlando, L., Mendelson, J.R., Sanders, J., Knight, R., 2017. The Effects of Captivity on the Mammalian Gut Microbiome. Integr. Comp. Biol. 57, 690–704. https://doi.org/10.1093/icb/icx090
McMurry, S.T., Lochmiller, R.L., Vestey, M.R., Qualls, C.W., 1994. Cellular immune responses of nutritionally stressed juvenile cotton rats (*Sigmodon hispidus*) during acute benzene exposure. Arch. Environ. Contam. Toxicol. 27, 14–19. https://doi.org/10.1007/BF00203881
McNamara, J.M., Houston, A.I., 1996. State-dependent life histories. Nature 380, 215–221. https://doi.org/10.1038/380215a0
McNeely, J.A., 1992. The sinking ark: pollution and the worldwide loss of biodiversity. Biodivers. Conserv. 1, 2–18. https://doi.org/10.1007/BF00700247
Medzhitov, R., 2008. Origin and physiological roles of inflammation. Nature 454, 428–435.
Mehri, A., 2020. Trace Elements in Human Nutrition (II) – An Update. Int. J. Prev. Med. 11, 2. https://doi.org/10.4103/ijpvm.IJPVM_48_19
Michaux, J.R., Libois, R., Filippucci, M.-G., 2005. So close and so different: comparative phylogeography of two small mammal species, the Yellow-necked fieldmouse (Apodemus flavicollis) and the Woodmouse (*Apodemus sylvaticus*) in the Western Palearctic region. Heredity 94, 52–63. https://doi.org/10.1038/sj.hdy.6800561
Mikhaylova, I.V., Kuulasmaa, T., Jääskeläinen, J., Voutilainen, R., 2007. Tumor Necrosis Factor-α Regulates Steroidogenesis, Apoptosis, and Cell Viability in the Human Adrenocortical Cell Line NCI-H295R. Endocrinology 148, 386–392. https://doi.org/10.1210/en.2006-0726
Mishra, K.P., 2009. Lead exposure and its impact on immune system: A review. Toxicol. In Vitro 23, 969–972. https://doi.org/10.1016/j.tiv.2009.06.014
Mittal, M., Flora, S.J.S., 2007. Vitamin E Supplementation Protects Oxidative Stress During Arsenic and Fluoride Antagonism in Male Mice. Drug Chem. Toxicol. 30, 263–281. https://doi.org/10.1080/01480540701380075
Mohanta, R.K., Garg, A.K., Dass, R.S., Chaturvedi, V.K., 2014. Effect of vitamin E supplementation on growth, nutrient utilization, mineral balance and immune status of arsenic exposed goats. Anim. Feed Sci. Technol. 194, 26–34. https://doi.org/10.1016/j.anifeedsci.2014.05.003
Moon, J., 1994. The role of vitamin D in toxic metal absorption: a review. J. Am. Coll. Nutr. 13, 559–564.
Moore, H., Dvoráková, K., Jenkins, N., Breed, W., 2002. Exceptional sperm cooperation in the wood mouse. Nature 418, 174–177. https://doi.org/10.1038/nature00832
Mora, J.R., Iwata, M., von Andrian, U.H., 2008. Vitamin effects on the immune system: vitamins A and D take centre stage. Nat. Rev. Immunol. 8, 685–698. https://doi.org/10.1038/nri2378
Morrissey, C., Fritsch, C., Fremlin, K., Adams, W., Borgå, K., Brinkmann, M., Eulaers, I., Gobas, F., Moore, D.R.J., van den Brink, N., Wickwire, T., 2023. Advancing exposure assessment approaches to improve wildlife risk assessment. Integr. Environ. Assess. Manag. n/a. https://doi.org/10.1002/ieam.4743
Morzadec, C., Macoch, M., Robineau, M., Sparfel, L., Fardel, O., Vernhet, L., 2012. Inorganic arsenic represses interleukin-17A expression in human activated Th17 lymphocytes. Toxicol. Appl. Pharmacol. 262, 217–222. https://doi.org/10.1016/j.taap.2012.05.004
Müller, S., Gillert, K., Krause, C., Jautzke, G., Gross, U., Diamantstein, T., 2005. Effects of cadmium on the immune system of mice. Experientia. https://doi.org/10.1007/BF01955143
Murphy, K., Weaver, C., 2016. Janeway's immunobiology. Garland science.
Nairz, M., Dichtl, S., Schroll, A., Haschka, D., Tymoszuk, P., Theurl, I., Weiss, G., 2018. Iron and innate antimicrobial immunity-Depriving the pathogen, defending the host. J. Trace Elem. Med. Biol. 48, 118–133. https://doi.org/10.1016/j.jtemb.2018.03.007

Natasha, Shahid, M., Niazi, N.K., Khalid, S., Murtaza, B., Bibi, I., Rashid, M.I., 2018. A critical review of selenium biogeochemical behavior in soil-plant system with an inference to human health. Environ. Pollut. 234, 915–934. https://doi.org/10.1016/j.envpol.2017.12.019
Navarro-Castilla, Á., Barja, I., 2014a. Does predation risk, through moon phase and predator cues, modulate food intake, antipredatory and physiological responses in wood mice (Apodemus sylvaticus)? Behav. Ecol. Sociobiol. 68, 1505–1512. https://doi.org/10.1007/s00265-014-1759-y
Navarro-Castilla, Á., Barja, I., 2014b. Antipredatory Response and Food Intake in Wood Mice (*Apodemus sylvaticus*) under Simulated Predation Risk by Resident and Novel Carnivorous Predators. Ethology 120, 90–98. https://doi.org/10.1111/eth.12184
Navarro-Castilla, Á., Barja, I., Díaz, M., 2018. Foraging, feeding, and physiological stress responses of wild wood mice to increased illumination and common genet cues. Curr. Zool. 64, 409–417. https://doi.org/10.1093/cz/zox048
Nelson, R.J., Demas, G.E., 1996. Seasonal Changes in Immune Function. Q. Rev. Biol. 71, 38.
Nelson, R.J., Demas, G.E., Klein, S.L., Kriegsfeld, L.J., Bronson, F., 2002. Seasonal Patterns of Stress, Immune Function, and Disease, 1st ed. Cambridge University Press. https://doi.org/10.1017/CBO9780511546341
Newman, M.C., 2015. Fundamentals of Ecotoxicology: The Science of Pollution. 4th. CRC Press Taylor & Francis Group.
Nieboer, E., Richardson, D.H., 1980. The replacement of the nondescript term 'heavy metals' by a biologically and chemically significant classification of metal ions. Environ. Pollut. Ser. B Chem. Phys. 1, 3–26.
Nishiyama, S., Nakamura, K., 1984. Effect of cadmium on plasma aldosterone and serum corticosterone concentrations in male rats. Toxicol. Appl. Pharmacol. 76, 420–425. https://doi.org/10.1016/0041-008X(84)90346-6
Nishiyama, S., Nakamura, K., Ogawa, M., 1985. Effects of heavy metals on corticosteroid production in cultured rat adrenocortical cells. Toxicol. Appl. Pharmacol. 81, 174–176. https://doi.org/10.1016/0041-008X(85)90132-2
Noaman, E., Zahran, A.M., Kamal, A.M., Omran, M.F., 2002. Vitamin E and selenium administration as a modulator of antioxidant defense system. Biol. Trace Elem. Res. 86, 55–64. https://doi.org/10.1385/BTER:86:1:55
Nordberg, G.F., Fowler, B.A., Nordberg, M., 2014. Handbook on the Toxicology of Metals. Academic press.
Nordberg, G.F., Nogawa, K., Nordberg, M., 2015. Cadmium, in: Handbook on the Toxicology of Metals. Elsevier, pp. 667–716. https://doi.org/10.1016/B978-0-444-59453-2.00032-9
Nriagu, J.O., 1996. A History of Global Metal Pollution. Science 272, 223–223. https://doi.org/10.1126/science.272.5259.223
Nunes, A.C, da Luz Mathias, M., Crespo, A.M., 2001. Morphological and haematological parameters in the Algerian mouse (Mus spretus) inhabiting an area contaminated with heavy metals. Environ. Pollut. 113, 87–93. https://doi.org/10.1016/S0269-7491(00)00159-7
Nunes, A. C., Mathias, M.D., Crespo, A.M., 2001. Morphological and haematological parameters in the Algerian mouse (Mus spretus) inhabiting an area contaminated with heavy metals. Environ. Pollut. 113, 87–93. https://doi.org/10.1016/S0269-7491(00)00159-7
Nunez, G., Sakamoto, K., Soares, M.P., 2018. Innate Nutritional Immunity. J. Immunol. 201, 11–18. https://doi.org/10.4049/jimmunol.1800325
O'Brien, M.A., Jackson, M.W., 2012. Vitamin D and the immune system: Beyond rickets. Vet. J. 194, 27–33. https://doi.org/10.1016/j.tvjl.2012.05.022
Odum, E.P., 1977. The Emergence of Ecology as a New Integrative Discipline. Science 195, 1289–1293. https://doi.org/10.1126/science.195.4284.1289
Odum, E.P., Barrett, G.W., 1971. Fundamentals of ecology. Saunders Philadelphia.
Ohnmacht, C., Park, J.-H., Cording, S., Wing, J.B., Atarashi, K., Obata, Y., Gaboriau-Routhiau, V., Marques, R., Dulauroy, S., Fedoseeva, M., Busslinger, M., Cerf-Bensussan, N., Boneca,

I.G., Voehringer, D., Hase, K., Honda, K., Sakaguchi, S., Eberl, G., 2015. The microbiota regulates type 2 immunity through RORγt+ T cells. Science 349, 989–993. https://doi.org/10.1126/science.aac4263
Oishi, K., Yokoi, M., Maekawa, S., Sodeyama, C., Shiraishi, T., Kondo, R., Kuriyama, T., Machida, K., 1999. Oxidative stress and haematological changes in immobilized rats. Acta Physiol. Scand. 165, 65–69. https://doi.org/10.1046/j.1365-201x.1999.00482.x
Otto, F., Vilela, F., Harun, M., Taylor, G., Baggasse, P., Bogin, E., 2000. Biochemical blood profile of Angoni cattle in Mozambique. undefined.
Ozaki, S., Douay, F., Détriché, S., Raoul, F., Scheifler, R., Fritsch, C., 2022. Thirteen years after: dynamics of trace metals in free-ranging small mammals related to soil aging (2006-2019). SETAC Eur. 32nd Annu. Meet., Copenhagen, Denmark.
Ozaki, S., Fritsch, C., Valot, B., Mora, F., Cornier, T., Scheifler, R., Raoul, F., 2019. How Do Richness and Composition of Diet Shape Trace Metal Exposure in a Free-Living Generalist Rodent, Apodemus sylvaticus. Environ. Sci. Technol. 53, 5977–5986. https://doi.org/10.1021/acs.est.8b07194
Ozaki, S., Fritsch, C., Valot, B., Mora, F., Cornier, T., Scheifler, R., Raoul, F., 2018. Does pollution influence small mammal diet in the field? A metabarcoding approach in a generalist consumer. Mol. Ecol. 27, 3700–3713. https://doi.org/10.1111/mec.14823
Özkan, C., Kaya, A., Akgül, Y., 2012. Normal values of haematological and some biochemical parameters in serum and urine of New Zealand White rabbits. World Rabbit Sci. 20, 253–259. https://doi.org/10.4995/wrs.2012.1229
Pacyna, J.M., Pacyna, E.G., 2001. An assessment of global and regional emissions of trace metals to the atmosphere from anthropogenic sources worldwide. Environ. Rev. 9, 269–298. https://doi.org/10.1139/a01-012
Paglia, D.E., Valentine, W.N., 1967. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J. Lab. Clin. Med. 70, 158–169.
Palmer, L.D., Skaar, E.P., 2016. Transition metals and virulence in bacteria. Annu. Rev. Genet. 50, 67–91.
Patel, K.V., Harris, T.B., Faulhaber, M., Angleman, S.B., Connelly, S., Bauer, D.C., Kuller, L.H., Newman, A.B., Guralnik, J.M., 2007. Racial variation in the relationship of anemia with mortality and mobility disability among older adults. Blood 109, 4663–4670. https://doi.org/10.1182/blood-2006-10-055384
Pathak, N., Khandelwal, S., 2008. Impact of cadmium in T lymphocyte subsets and cytokine expression: differential regulation by oxidative stress and apoptosis. BioMetals 21, 179–187. https://doi.org/10.1007/s10534-007-9106-7
Peace, A., Frost, P.C., Wagner, N.D., Danger, M., Accolla, C., Antczak, P., Brooks, B.W., Costello, D.M., Everett, R.A., Flores, K.B., Heggerud, C.M., Karimi, R., Kang, Y., Kuang, Y., Larson, J.H., Mathews, T., Mayer, G.D., Murdock, J.N., Murphy, C.A., Nisbet, R.M., Pecquerie, L., Pollesch, N., Rutter, E.M., Schulz, K.L., Scott, J.T., Stevenson, L., Wang, H., 2021. Stoichiometric Ecotoxicology for a Multisubstance World. BioScience 71, 132–147. https://doi.org/10.1093/biosci/biaa160
Peig, J., Green, A.J., 2009. New perspectives for estimating body condition from mass/length data: the scaled mass index as an alternative method. Oikos 118, 1883–1891. https://doi.org/10.1111/j.1600-0706.2009.17643.x
Pellisso, S.C., Munoz, M.J., Carballo, M., Sanchez-Vizcaino, J.M., 2008. Determination of the immunotoxic potential of heavy metals on the functional activity of bottlenose dolphin leukocytes in vitro. Vet. Immunol. Immunopathol. 121, 189–198. https://doi.org/10.1016/j.vetimm.2007.09.009
Peraza, M.A., Ayala-Fierro, F., Barber, D.S., Casarez, E., Rael, L.T., 1998. Effects of Micronutrients on Metal Toxicity. Environ. Health Perspect. 106, 203–216. https://doi.org/10.2307/3433921
Pérez-Suárez, G., Arévalo, F., López-Caballero, E., López-Luna, P., 1990. Seasonal variations in hematological values and heart weight in two small mammals, a mouse: Apodemus sylvaticus, and a vole: Pitymys duodecimcostatus. Acta Theriol. (Warsz.) 35, 201–208. https://doi.org/10.4098/AT.arch.90-25

Piertney, S.B., Oliver, M.K., 2006. The evolutionary ecology of the major histocompatibility complex. Heredity 96, 7–21.
Pilarczyk, B., Tomza-Marciniak, A., Pilarczyk, R., Marciniak, A., Bąkowska, M., Nowakowska, E., 2019. Selenium, Se, in: Kalisińska, E. (Ed.), Mammals and Birds as Bioindicators of Trace Element Contaminations in Terrestrial Environments: An Ecotoxicological Assessment of the Northern Hemisphere. Springer International Publishing, Cham, pp. 301–362. https://doi.org/10.1007/978-3-030-00121-6_10
Pollard, K.M., Cauvi, D.M., Toomey, C.B., Hultman, P., Kono, D.H., 2019. Mercury-induced inflammation and autoimmunity. Biochim. Biophys. Acta BBA - Gen. Subj., The Biochemistry of Mercury toxicity 1863, 129299. https://doi.org/10.1016/j.bbagen.2019.02.001
Ponomarenko, O., Porte, P.F.L., Singh, S.P., Langan, G., Fleming, D.E.B., Spallholz, J.E., Alauddin, M., Ahsan, H., Ahmed, S., Gailer, J., George, G.N., Pickering, I.J., 2017. Selenium-mediated arsenic excretion in mammals: a synchrotron-based study of whole-body distribution and tissue-specific chemistry. Metallomics 9, 1585–1595. https://doi.org/10.1039/C7MT00201G
Posthuma, L., Suter, G.W., Traas, T.P., 2001. Species Sensitivity Distributions in Ecotoxicology. CRC Press, Boca Raton. https://doi.org/10.1201/9781420032314
Poulsen, A., Escher, B., 2012. Chemically induced immunosuppression and disease susceptibility in marine wildlife: A literature review. Entox Natl. Res. Cent. Environ. Toxicol. Univ. Qld. 110.
Powolny, T., Scheifler, R., Raoul, F., Clémentine Fritsch, M.C., 2022. Effects of chronic exposure to toxic metals on haematological parameters in free-ranging small mammals. Environ. Pollut. 120675. https://doi.org/10.1016/j.envpol.2022.120675
Powolny, T., Scheifler, R., Raoul, F., Coeurdassier, M., Fritsch, C., 2023a. Effects of chronic exposure to toxic metals on haematological parameters in free-ranging small mammals. Environ. Pollut. 317, 120675. https://doi.org/10.1016/j.envpol.2022.120675
Powolny, T., Scheifler, R., Raoul, F., Coeurdassier, M., Fritsch, C., 2023b. Effects of chronic exposure to toxic metals on haematological parameters in free-ranging small mammals. Environ. Pollut. 317, 120675. https://doi.org/10.1016/j.envpol.2022.120675
Powolny, T., Scheifler, R., Raoul, F., Fritsch, C., 2019. Is blood a reliable indicator of trace metal concentrations in organs of small mammals? Chemosphere 217, 320–328. https://doi.org/10.1016/j.chemosphere.2018.10.215
Prasad, A.S., 2000. Effects of Zinc Deficiency on Th1 and Th2 Cytokine Shifts. J. Infect. Dis. 182, S62–S68. https://doi.org/10.1086/315916
Pyter, L.M., Neigh, G.N., Nelson, R.J., 2005. Social environment modulates photoperiodic immune and reproductive responses in adult male white-footed mice (Peromyscus leucopus). Am. J. Physiol. Regul. Integr. Comp. Physiol. 288, R891-896. https://doi.org/10.1152/ajpregu.00680.2004
Qing, H., Desrouleaux, R., Israni-Winger, K., Mineur, Y.S., Fogelman, N., Zhang, C., Rashed, S., Palm, N.W., Sinha, R., Picciotto, M.R., Perry, R.J., Wang, A., 2020. Origin and Function of Stress-Induced IL-6 in Murine Models. Cell 182, 372-387.e14. https://doi.org/10.1016/j.cell.2020.05.054
Qiu, G., Abeysinghe, K.S., Yang, X.-D., Xu, Z., Xu, X., Luo, K., Goodale, E., 2019. Effects of selenium on mercury bioaccumulation in a terrestrial food chain from an abandoned mercury mining region. Bull. Environ. Contam. Toxicol. 102, 329–334.
R Core Team, 2023. R: A Language and Environment for Statistical Computing. 2019.
R Development Core Team, 2022. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. [WWW Document]. URL https://www.r-project.org/
Råberg, L., Grahn, M., Hasselquist, D., Svensson, E., 1998. On the adaptive significance of stress-induced immunosuppression. Proc. R. Soc. Lond. B Biol. Sci. 265, 1637–1641.
Rahman, A., Al-Awadi, A.A., Khan, K.M., 2018. Lead Affects Vitamin D Metabolism in Rats. Nutrients 10. https://doi.org/10.3390/nu10030264

Rahman, Z., Singh, V.P., 2019. The relative impact of toxic heavy metals (THMs) (arsenic (As), cadmium (Cd), chromium (Cr)(VI), mercury (Hg), and lead (Pb)) on the total environment: an overview. Environ. Monit. Assess. 191, 419. https://doi.org/10.1007/s10661-019-7528-7
Ramade, F., 2007. Introduction à l'écotoxicologie : Fondements et applications. Lavoisier.
Reid, W.V., Mooney, H.A., Cropper, A., Capistrano, D., Carpenter, S.R., Chopra, K., Dasgupta, P., Dietz, T., Duraiappah, A.K., Hassan, R., 2005. Ecosystems and human well-being-Synthesis: A report of the Millennium Ecosystem Assessment. Island Press.
Reynolds, R.J.B., Pilon-Smits, E.A.H., 2018. Plant selenium hyperaccumulation- Ecological effects and potential implications for selenium cycling and community structure. Biochim. Biophys. Acta BBA - Gen. Subj., Selenium research in biochemistry and biophysics - 200 year anniversary issue 1862, 2372–2382. https://doi.org/10.1016/j.bbagen.2018.04.018
Ringø, E., Olsen, R.E., Vecino, J.G., Wadsworth, S., Song, S.K., 2012. Use of immunostimulants and nucleotides in aquaculture: a review. J Mar Sci Res Dev 2, 104.
Rodríguez-Estival, J., Álvarez-Lloret, P., Rodríguez-Navarro, A.B., Mateo, R., 2013. Chronic effects of lead (Pb) on bone properties in red deer and wild boar: Relationship with vitamins A and D3. Environ. Pollut. 174, 142–149. https://doi.org/10.1016/j.envpol.2012.11.019
Rodríguez-Estival, J., Martinez-Haro, M., Monsalve-González, L., Mateo, R., 2011a. Interactions between endogenous and dietary antioxidants against Pb-induced oxidative stress in wild ungulates from a Pb polluted mining area. Sci. Total Environ. 409, 2725–2733. https://doi.org/10.1016/j.scitotenv.2011.04.010
Rodríguez-Estival, J., Taggart, M.A., Mateo, R., 2011b. Alterations in vitamin A and E levels in liver and testis of wild ungulates from a lead mining area. Arch. Environ. Contam. Toxicol. 60, 361–371.
Rogival, D., Scheirs, J., Coen, W.D., Verhagen, R., Blust, R., 2006a. METAL BLOOD LEVELS AND HEMATOLOGICAL CHARACTERISTICS IN WOOD MICE (APODEMUS SYLVATICUS L.) ALONG A METAL POLLUTION GRADIENT. Environ. Toxicol. Chem. 25, 149. https://doi.org/10.1897/04-659R1.1
Rogival, D., Scheirs, J., De Coen, W., Verhagen, R., Blust, R., 2006b. Metal blood levels and hematological characteristics in wood mice (Apodemus sylvaticus L.) along a metal pollution gradient. Environ. Toxicol. Chem. 25, 149–157. https://doi.org/10.1897/04-659R1.1
Romagnani, S., 1997. The Th1/Th2 paradigm. Immunol. Today 18, 263–266. https://doi.org/10.1016/S0167-5699(97)80019-9
Ropero, M.P., Fariñas, N.R., Krupp, E., Mateo, R., Nevado, J.B., Martín-Doimeadios, R.R., 2016. Mercury and selenium binding biomolecules in terrestrial mammals (Cervus elaphus and Sus scrofa) from a mercury exposed area. J. Chromatogr. B 1022, 159–166.
Rosenfeld, C.E., James, B.R., Santelli, C.M., 2018. Persistent Bacterial and Fungal Community Shifts Exhibited in Selenium-Contaminated Reclaimed Mine Soils. Appl. Environ. Microbiol. 84, e01394-18. https://doi.org/10.1128/AEM.01394-18
Ross, A.C., 2012. Vitamin A and retinoic acid in T cell–related immunity. Am. J. Clin. Nutr. 96, 1166S-1172S. https://doi.org/10.3945/ajcn.112.034637
Rostal, M.K., Evans, A.L., Solberg, E.J., Arnemo, J.M., 2012. HEMATOLOGY AND SERUM CHEMISTRY REFERENCE RANGES OF FREE-RANGING MOOSE (ALCES ALCES) IN NORWAY. J. Wildl. Dis. 48, 548–559. https://doi.org/10.7589/0090-3558-48.3.548
Rowley, C.A., Kendall, M.M., 2019. To B12 or not to B12: Five questions on the role of cobalamin in host-microbial interactions. PLoS Pathog. 15. https://doi.org/10.1371/journal.ppat.1007479
Rubino, F.M., 2015. Toxicity of Glutathione-Binding Metals: A Review of Targets and Mechanisms. Toxics 3, 20–62. https://doi.org/10.3390/toxics3010020
Ruel-Bergeron, J.C., Stevens, G.A., Sugimoto, J.D., Roos, F.F., Ezzati, M., Black, R.E., Kraemer, K., 2015. Global Update and Trends of Hidden Hunger, 1995-2011: The Hidden Hunger Index. PLoS ONE 10. https://doi.org/10.1371/journal.pone.0143497
Ruuskanen, S., Morosinotto, C., Thomson, R.L., Ratnayake, C.P., Korpimäki, E., 2017. Food supplementation, but not predation risk, alters female antioxidant status during breeding. Behav. Ecol. Sociobiol. 71, 69. https://doi.org/10.1007/s00265-017-2299-z

Rynkiewicz, E.C., Clerc, M., Babayan, S.A., Pedersen, A.B., 2019. Variation in Local and Systemic Pro-Inflammatory Immune Markers of Wild Wood Mice after Anthelmintic Treatment. Integr. Comp. Biol. 59, 1190–1202. https://doi.org/10.1093/icb/icz136
Saaristo, M., Brodin, T., Balshine, S., Bertram, M.G., Brooks, B.W., Ehlman, S.M., McCallum, E.S., Sih, A., Sundin, J., Wong, B.B.M., Arnold, K.E., 2018. Direct and indirect effects of chemical contaminants on the behaviour, ecology and evolution of wildlife. Proc. R. Soc. B Biol. Sci. 285, 20181297. https://doi.org/10.1098/rspb.2018.1297
Sadeghi, S., Wallace, F.A., Calder, P.C., 1999. Dietary lipids modify the cytokine response to bacterial lipopolysaccharide in mice. Immunology 96, 404–410. https://doi.org/10.1046/j.1365-2567.1999.00701.x
Saint-Girons, M.-C., 1967. Etude du genre Apodemus Kauf, 1829 en France.
Sakamoto, K., Kim, Y.-G., Hara, H., Kamada, N., Caballero-Flores, G., Tolosano, E., Soares, M.P., Puente, J.L., Inohara, N., Núñez, G., 2017. IL-22 Controls Iron-Dependent Nutritional Immunity Against Systemic Bacterial Infections. Sci. Immunol. 2. https://doi.org/10.1126/sciimmunol.aai8371
Salam, L.B., Obayori, O.S., Ilori, M.O., Amund, O.O., 2020. Effects of cadmium perturbation on the microbial community structure and heavy metal resistome of a tropical agricultural soil. Bioresour. Bioprocess. 7, 25. https://doi.org/10.1186/s40643-020-00314-w
Sample, B.E., Suter, G.W., 2002. Screening evaluation of the ecological risks to terrestrial wildlife associated with a coal ash disposal site. Hum. Ecol. Risk Assess. 8, 637–656.
Sanchez-Chardi, A., Marques, C.C., Gabriel, S.I., Capela-Silva, F., Cabrita, A.S., Lopez-Fuster, M.J., Nadal, J., Mathias, M.L., 2008. Haematology, genotoxicity, enzymatic activity and histopathology as biomarkers of metal pollution in the shrew Crocidura russula. Environ. Pollut. 156, 1332–1339. https://doi.org/10.1016/j.envpol.2008.02.026
Sánchez-González, B., Planillo, A., Navarro-Castilla, Á., Barja, I., 2018. The concentration of fear: mice's behavioural and physiological stress responses to different degrees of predation risk. Sci. Nat. 105, 16. https://doi.org/10.1007/s00114-018-1540-6
Sandhu, N., Vijayan, M.M., 2011. Cadmium-mediated disruption of cortisol biosynthesis involves suppression of corticosteroidogenic genes in rainbow trout. Aquat. Toxicol. Amst. Neth. 103, 92–100. https://doi.org/10.1016/j.aquatox.2011.02.011
Sapolsky, Robert M., Romero, L.M., Munck, A.U., 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr. Rev. 21, 55–89.
Sapolsky, Robert M, Romero, L.M., Munck, A.U., 2000. How Do Glucocorticoids Influence Stress Responses? Integrating Permissive, Suppressive, Stimulatory, and Preparative Actions 21, 35.
Schoenle, L.A., Downs, C.J., Martin, L.B., 2018. An Introduction to Ecoimmunology. Adv. Comp. Immunol. 901–932. https://doi.org/10.1007/978-3-319-76768-0_26
Schweizer, U., Schomburg, L., Köhrle, J., 2016. Selenoprotein P and Selenium Distribution in Mammals, in: Hatfield, D.L., Schweizer, U., Tsuji, P.A., Gladyshev, V.N. (Eds.), Selenium: Its Molecular Biology and Role in Human Health. Springer International Publishing, Cham, pp. 261–274. https://doi.org/10.1007/978-3-319-41283-2_22
Scott, M.A., Stockham, S.L., 2013. Fundamentals of Veterinary Clinical Pathology. John Wiley & Sons.
Secombes, C.J., Wang, T., Bird, S., 2016. Chapter 5 - Vertebrate Cytokines and Their Evolution, in: Malagoli, D. (Ed.), The Evolution of the Immune System. Academic Press, pp. 87–150. https://doi.org/10.1016/B978-0-12-801975-7.00005-0
Shaik-Dasthagirisaheb, Y.B., Varvara, G., Murmura, G., Saggini, A., Caraffa, A., Antinolfi, P., Tete, S., Tripodi, D., Conti, F., Cianchetti, E., 2013. Role of vitamins D, E and C in immunity and inflammation. J Biol Regul Homeost Agents 27, 291–295.
Shaner, P.-J.L., Yu, A.-Y., Li, S.-H., Hou, C.-H., 2018. The effects of food and parasitism on reproductive performance of a wild rodent. Ecol. Evol. 8, 4162–4172. https://doi.org/10.1002/ece3.3997

Sheldon, B.C., Verhulst, S., 1996. Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. Trends Ecol. Evol. 11, 317–321. https://doi.org/10.1016/0169-5347(96)10039-2
Sheldon, J.R., Skaar, E.P., 2019. Metals as phagocyte antimicrobial effectors. Curr. Opin. Immunol., Allergy and hypersensitivity • Host pathogens 60, 1–9. https://doi.org/10.1016/j.coi.2019.04.002
Shwartz, M., 2007. Robert Sapolsky discusses physiological effects of stress [WWW Document]. Stanf. Univ. URL http://news.stanford.edu/news/2007/march7/sapolskysr-030707.html (accessed 12.9.22).
Sikes, R.S., the Animal Care and Use Committee of the American Society of Mammalogists, 2016. 2016 Guidelines of the American Society of Mammalogists for the use of wild mammals in research and education: J. Mammal. 97, 663–688. https://doi.org/10.1093/jmammal/gyw078
Silverman, M.N., Sternberg, E.M., 2012. Glucocorticoid regulation of inflammation and its behavioral and metabolic correlates: from HPA axis to glucocorticoid receptor dysfunction. Ann. N. Y. Acad. Sci. 1261, 55–63. https://doi.org/10.1111/j.1749-6632.2012.06633.x
Sironi, M., Cagliani, R., Forni, D., Clerici, M., 2015. Evolutionary insights into host–pathogen interactions from mammalian sequence data. Nat. Rev. Genet. 16, 224–236. https://doi.org/10.1038/nrg3905
Siwicki, A.K., Studnicka, M., Morand, M., Pozet, F., Terech-Majewska, E., 1998. Comparative immunotoxicology-a new direction. Acta Vet. Brno 67, 295–301.
Smith, A.D., Panickar, K.S., Urban, J.F., Dawson, H.D., 2018. Impact of Micronutrients on the Immune Response of Animals. Annu. Rev. Anim. Biosci. 6, 227–254. https://doi.org/10.1146/annurev-animal-022516-022914
Sousa, L.L., Silva, S.M., Xavier, R., 2019. DNA metabarcoding in diet studies: Unveiling ecological aspects in aquatic and terrestrial ecosystems. Environ. DNA 1, 199–214. https://doi.org/10.1002/edn3.27
Spackman, D.H., Riley, V., 1978. Corticosterone concentrations in the mouse. Science 200, 87. https://doi.org/10.1126/science.635580
Speakman, J.R., 2008. The physiological costs of reproduction in small mammals. Philos. Trans. R. Soc. B Biol. Sci. 363, 375–398. https://doi.org/10.1098/rstb.2007.2145
Sterckeman, T., Douay, F., Fourrier, H., Proix, N., 2002a. Référentiel pédo-géochimique du Nord-Pas de Calais. Rapp. Final 130 P Annex. 306 P.
Sterckeman, T., Douay, F., Proix, N., Fourrier, H., Perdrix, E., 2002b. Assessment of the Contamination of Cultivated Soils by Eighteen Trace Elements Around Smelters in the North of France. Water. Air. Soil Pollut. 135, 173–194. https://doi.org/10.1023/A:1014758811194
Sternberg, E.M., 2006. Neural regulation of innate immunity: a coordinated nonspecific host response to pathogens. Nat. Rev. Immunol. 6, 318–328. https://doi.org/10.1038/nri1810
Stordeur, P., Poulin, L.F., Craciun, L., Zhou, L., Schandené, L., de Lavareille, A., Goriely, S., Goldman, M., 2002. Cytokine mRNA quantification by real-time PCR. J. Immunol. Methods 259, 55–64. https://doi.org/10.1016/S0022-1759(01)00489-6
Strandin, T., Babayan, S.A., Forbes, K.M., 2018. Reviewing the effects of food provisioning on wildlife immunity. Philos. Trans. R. Soc. B-Biol. Sci. 373, 20170088. https://doi.org/10.1098/rstb.2017.0088
Sugawara, C., Sugawara, N., 1979. The effect of heavy metals on vitamin A metabolism. Toxicol. Lett. 3, 215–218. https://doi.org/10.1016/0378-4274(79)90036-5
Sugawara, C., Sugawara, N., 1978. The effect of cadmium on vitamin A metabolism. Toxicol. Appl. Pharmacol. 46, 19–27. https://doi.org/10.1016/0041-008x(78)90133-3
Sugawara, C., Sugawara, N., Miyake, H., 1981. Decrease of plasma vitamin A, albumin and zinc in cadmium-treated rats. Toxicol. Lett. 8, 323–329. https://doi.org/10.1016/0378-4274(81)90121-1
Sun, H., Shao, C., Jin, Q., Li, M., Zhang, Z., Liang, H., Lei, H., Qian, J., Zhang, Y., 2022. Effects of cadmium contamination on bacterial and fungal communities in Panax ginseng-growing soil. BMC Microbiol. 22, 77. https://doi.org/10.1186/s12866-022-02488-z

Sun, X., Sun, M., Chao, Y., Shang, X., Wang, H., Pan, H., Yang, Q., Lou, Y., Zhuge, Y., 2023. Effects of lead pollution on soil microbial community diversity and biomass and on invertase activity. Soil Ecol. Lett. 5, 118–127. https://doi.org/10.1007/s42832-022-0134-6
Sures, B., 2008. Host–parasite interactions in polluted environments. J. Fish Biol. 73, 2133–2142. https://doi.org/10.1111/j.1095-8649.2008.02057.x
Sweeny, A.R., Clerc, M., Pontifes, P.A., Venkatesan, S., Babayan, S.A., Pedersen, A.B., 2021. Supplemented nutrition decreases helminth burden and increases drug efficacy in a natural host–helminth system. Proc. R. Soc. B Biol. Sci. 288, 20202722. https://doi.org/10.1098/rspb.2020.2722
Tan, L.C., Nancharaiah, Y.V., van Hullebusch, E.D., Lens, P.N., 2016. Selenium: environmental significance, pollution, and biological treatment technologies. Biotechnol. Adv. 34, 886–907.
Tersago, K., De Coen, W., Scheirs, J., Vermeulen, K., Blust, R., Van Bockstaele, D., Verhagen, R., 2004. Immunotoxicology in wood mice along a heavy metal pollution gradient. Environ. Pollut. 132, 385–394. https://doi.org/10.1016/j.envpol.2004.05.029
Tête, N., 2014. Evaluation de l'exposition et des effets des éléments traces métalliques et du parasitisme chez la faune sauvage : contribution au développement d'une approche non létale (These de doctorat). Besançon.
Tête, N., Afonso, E., Bouguerra, G., Scheifler, R., 2015. Blood parameters as biomarkers of cadmium and lead exposure and effects in wild wood mice (*Apodemus sylvaticus*) living along a pollution gradient. Chemosphere 138, 940–946. https://doi.org/10.1016/j.chemosphere.2014.12.031
Tête, N., Afonso, E., Crini, N., Drouhot, S., Prudent, A.-S., Scheifler, R., 2014a. Hair as a noninvasive tool for risk assessment: Do the concentrations of cadmium and lead in the hair of wood mice (Apodemus sylvaticus) reflect internal concentrations? Ecotoxicol. Environ. Saf. 108, 233–241. https://doi.org/10.1016/j.ecoenv.2014.07.010
Tête, N., Durfort, M., Rieffel, D., Scheifler, R., Sánchez-Chardi, A., 2014b. Histopathology related to cadmium and lead bioaccumulation in chronically exposed wood mice, Apodemus sylvaticus, around a former smelter. Sci. Total Environ. 481, 167–177. https://doi.org/10.1016/j.scitotenv.2014.02.029
Tête, N., Fritsch, C., Afonso, E., Coeurdassier, M., Lambert, J.-C., Giraudoux, P., Scheifler, R., 2013. Can Body Condition and Somatic Indices be Used to Evaluate Metal-Induced Stress in Wild Small Mammals? PLOS ONE 8, e66399. https://doi.org/10.1371/journal.pone.0066399
Theodosiou, M., Laudet, V., Schubert, M., 2010. From carrot to clinic: an overview of the retinoic acid signaling pathway. Cell. Mol. Life Sci. 67, 1423–1445. https://doi.org/10.1007/s00018-010-0268-z
Thevenod, F., Lee, W.-K., 2015. Live and Let Die: Roles of Autophagy in Cadmium Nephrotoxicity. Toxics 3, 130–151. https://doi.org/10.3390/toxics3020130
Tizard, I.R., 2004. An Introduction to Veterinary Immunology, Subsequent edition. ed. W B Saunders Co, Philadelphia.
Todgham, A.E., Stillman, J.H., 2013. Physiological Responses to Shifts in Multiple Environmental Stressors: Relevance in a Changing World. Integr. Comp. Biol. 53, 539–544. https://doi.org/10.1093/icb/ict086
Touma, C., Sachser, N., Möstl, E., Palme, R., 2003. Effects of sex and time of day on metabolism and excretion of corticosterone in urine and feces of mice. Gen. Comp. Endocrinol. 130, 267–278. https://doi.org/10.1016/S0016-6480(02)00620-2
Tourkochristou, E., Triantos, C., Mouzaki, A., 2021. The Influence of Nutritional Factors on Immunological Outcomes. Front. Immunol. 12.
Traullé, F., 2022. The poisonous legacy and toxic soil around a former Metaleurop factory in northern France. Le Monde.fr.
Truhaut, R., 1977. Ecotoxicology: Objectives, principles and perspectives. Ecotoxicol. Environ. Saf. 1, 151–173. https://doi.org/10.1016/0147-6513(77)90033-1
Tsuji, P.A., Carlson, B.A., Anderson, C.B., Seifried, H.E., Hatfield, D.L., Howard, M.T., 2015. Dietary Selenium Levels Affect Selenoprotein Expression and Support the Interferon-γ and

IL-6 Immune Response Pathways in Mice. Nutrients 7, 6529–6549. https://doi.org/10.3390/nu7085297
Turley, A.E., Zagorski, J.W., Kennedy, R.C., Freeborn, R.A., Bursley, J.K., Edwards, J.R., Rockwell, C.E., 2019. Chronic low-level cadmium exposure in rats affects cytokine production by activated T cells. Toxicol. Res. 8, 227–237. https://doi.org/10.1039/c8tx00194d
Tyler, R.D., Cowell, R.L., 1996. Classification and diagnosis of anaemia. Comp. Haematol. Int. 6, 1–16. https://doi.org/10.1007/BF00368096
Uchio, R., Hirose, Y., Murosaki, S., Ishigami, A., 2019. High dietary vitamin C intake reduces glucocorticoid-induced immunosuppression and measures of oxidative stress in vitamin C-deficient senescence marker protein 30 knockout mice. Br. J. Nutr. 122, 1120–1129. https://doi.org/10.1017/S0007114519001922
Urban, D.L., O'Neill, R.V., Shugart, H.H., 1987. Landscape Ecology. BioScience 37, 119–127. https://doi.org/10.2307/1310366
US EPA, 1998. Health effects test guidelines, Immunotoxicity (Health Effects Test Guidelines No. OPPTS 870.7800), Immunotoxicity. U.S. Environmental Protection Agency, Washington DC.
Uthus, E.O., 2003. Arsenic essentiality: A role affecting methionine metabolism. J. Trace Elem. Exp. Med. 16, 345–355. https://doi.org/10.1002/jtra.10044
van den Brink, N., Lammertsma, D., Dimmers, W., Boerwinkel, M.-C., van der Hout, A., 2010. Effects of soil properties on food web accumulation of heavy metals to the wood mouse (Apodemus sylvaticus). Environ. Pollut. 158, 245–251. https://doi.org/10.1016/j.envpol.2009.07.013
Van den Brink, P.J., 2008. Ecological Risk Assessment: From Book-Keeping to Chemical Stress Ecology. Environ. Sci. Technol. 42, 8999–9004. https://doi.org/10.1021/es801991c
van der Ent, A., Baker, A.J.M., Reeves, R.D., Pollard, A.J., Schat, H., 2013. Hyperaccumulators of metal and metalloid trace elements: Facts and fiction. Plant Soil 362, 319–334. https://doi.org/10.1007/s11104-012-1287-3
van der Velden, V.H., 1998. Glucocorticoids: mechanisms of action and anti-inflammatory potential in asthma. Mediators Inflamm. 7, 229–237.
Van Straalen, N.M., 2003. Peer Reviewed: Ecotoxicology Becomes Stress Ecology. Environ. Sci. Technol. 37, 324A-330A. https://doi.org/10.1021/es0325720
van Straalen, N.M., van Gestel, C.A.M., 2008. A stress ecology framework for comprehensive risk assessment of diffuse pollution. Sci. Total Environ., Ecological effects of diffuse pollution 406, 479–483. https://doi.org/10.1016/j.scitotenv.2008.06.054
Vandorpe, E., Verhagen, R., 1979. An age reference model for the wood mouse, Apodemus sylvaticus (Linnaeus, 1758), by use of the lens technique. Age Ref. Model Wood Mouse Apodemus Sylvaticus Linnaeus 1758 Use Lens Tech.
Vas, J., Monestier, M., 2008. Immunology of Mercury. Ann. N. Y. Acad. Sci. 1143, 240–267. https://doi.org/10.1196/annals.1443.022
Vestey, M., Mcmurry, S., Lochmiller, R., 1993. Influence of Dietary-Protein on Selected Measures of Humoral and Cellular-Immunity in the Cotton Rat Sigmodon-Hispidus. Can. J. Zool.-Rev. Can. Zool. 71, 579–586. https://doi.org/10.1139/z93-079
Villanneau, E., Perry-Giraud, C., Saby, N., Jolivet, C., Marot, F., Maton, D., Floch-Barneaud, A., Antoni, V., Arrouays, D., 2008. Détection de valeurs anomaliques d'éléments traces métalliques dans les sols à l'aide du Réseau de Mesure de la Qualité des Sols. Etude Gest. Sols 15, 183–200.
Viney, M., Riley, E.M., 2017a. The Immunology of Wild Rodents: Current Status and Future Prospects. Front. Immunol. 8. https://doi.org/10.3389/fimmu.2017.01481
Viney, M., Riley, E.M., 2017b. The Immunology of Wild Rodents: Current Status and Future Prospects. Front. Immunol. 8, 1481. https://doi.org/10.3389/fimmu.2017.01481
Viney, M.E., Riley, E.M., Buchanan, K.L., 2005. Optimal immune responses: immunocompetence revisited. Trends Ecol. Evol. 20, 665–669. https://doi.org/10.1016/j.tree.2005.10.003

von Hurst, P.R., Moorhouse, R.J., Raubenheimer, D., 2016. Preferred natural food of breeding Kakapo is a high value source of calcium and vitamin D. J. Steroid Biochem. Mol. Biol., Proceedings of the 18th Vitamin D Workshop 164, 177–179. https://doi.org/10.1016/j.jsbmb.2015.10.017
Vreugdenhil, E., de Kloet, E.R., Schaaf, M., Datson, N.A., 2001. Genetic dissection of corticosterone receptor function in the rat hippocampus. Eur. Neuropsychopharmacol., The impact of genomic studies on Neuropsychopharmacology 11, 423–430. https://doi.org/10.1016/S0924-977X(01)00119-5
Vuksic, N., Speranda, M., Loncaric, Z., Didara, M., Ludek, E., Budor, I., 2018. The effect of dietary selenium addition on the concentrations of heavy metals in the tissues of fallow deer (Dama dama L.) in Croatia. Environ. Sci. Pollut. Res. 25, 11023–11033. https://doi.org/10.1007/s11356-018-1406-7
Wagner, D., Maser, J., Lai, B., Cai, Z., Barry, C.E., Bentrup, K.H. zu, Russell, D.G., Bermudez, L.E., 2005. Elemental Analysis of Mycobacterium avium-, Mycobacterium tuberculosis-, and Mycobacterium smegmatis-Containing Phagosomes Indicates Pathogen-Induced Microenvironments within the Host Cell's Endosomal System. J. Immunol. 174, 1491–1500. https://doi.org/10.4049/jimmunol.174.3.1491
Waldron, K.J., Rutherford, J.C., Ford, D., Robinson, N.J., 2009. Metalloproteins and metal sensing. Nature 460, 823–830. https://doi.org/10.1038/nature08300
Walker, C H, Sibly, R.M., Hopkin, S.P., 2012. Principles of Ecotoxicology, Fourth Edition 381.
Walker, C.H., Sibly, R.M., Hopkin, S.P., Peakall, D.B., 2012. Principles of ecotoxicology, Fourth edition. ed. CRC Press Taylor & Francis Group.
Wang, C., Zhang, R., Wei, X., Lv, M., Jiang, Z., 2020. Chapter Seven - Metalloimmunology: The metal ion-controlled immunity, in: Dong, C., Jiang, Z. (Eds.), Advances in Immunology, Advances in Immunology in China - Part B. Academic Press, pp. 187–241. https://doi.org/10.1016/bs.ai.2019.11.007
Wang, Z., Sun, Y., Yao, W., Ba, Q., Wang, H., 2021. Effects of Cadmium Exposure on the Immune System and Immunoregulation. Front. Immunol. 12.
Warne, R.W., Baer, S.G., Boyles, J.G., 2019. Community Physiological Ecology. Trends Ecol. Evol.
Warren, S., Patel, S., Kapron, C.M., 2000. The effect of vitamin E exposure on cadmium toxicity in mouse embryo cells in vitro. Toxicology 142, 119–126. https://doi.org/10.1016/S0300-483X(99)00132-8
Wasserman, R.H., Fullmer, C.S., 1995. Vitamin D and intestinal calcium transport: facts, speculations and hypotheses. J. Nutr. 125, 1971S-1979S. https://doi.org/10.1093/jn/125.suppl_7.1971S
Weeks, B.A., Anderson, D.P., DuFour, A.P., Fairbrother, A., Goven, A.J., Lahvis, G.P., Peters, G., 1992. Immunological biomarkers to assess environmental stress, in: R.J. Huggett, R.A. Kimerle, P. M. Mehrle, H.L. Bergman (Eds.), Biomarkers: Biochemical, Physiological and Histological Markers of Anthropogenic Stress. Lewis Publishers, Boca Raton, La, pp. 211–234.
Weiss, D.J., Wardrop, K.J., Schalm, O.W., 2010. Schalm's veterinary hematology. Wiley-Blackwell, Ames, Iowa.
Weiss, G., 2002. Iron and immunity: a double-edged sword. Eur. J. Clin. Invest. 32, 70–78. https://doi.org/10.1046/j.1365-2362.2002.0320s1070.x
Wensveen, F.M., Šestan, M., Turk Wensveen, T., Polić, B., 2019. 'Beauty and the beast' in infection: How immune–endocrine interactions regulate systemic metabolism in the context of infection. Eur. J. Immunol. 49, 982–995. https://doi.org/10.1002/eji.201847895
Wiens, J.A., Moss, M.R., Turner, M.G., Mladenoff, D. (Eds.), 2006. Foundation Papers in Landscape Ecology. Columbia University Press.
Williams, P.N., Collier, C.T., Carroll, J.A., Welsh Jr, T.H., Laurenz, J.C., 2009. Temporal pattern and effect of sex on lipopolysaccharide-induced stress hormone and cytokine response in pigs. Domest. Anim. Endocrinol. 37, 139–147.
Wills, M.R., Havard, B., 2013. Laboratory investigation of endocrine disorders. Butterworth-Heinemann.

Wołk, E., Kozłowski, J., 1989. Changes of body weight and hematological parameters in a fluctuating population of Apodemus flavicollis. Acta Theriol. (Warsz.) 34, 439–464. https://doi.org/10.4098/AT.arch.89-44
Wonderlich, J., Shearer, G., Livingstone, A., Brooks, A., 2006. Induction and Measurement of Cytotoxic T Lymphocyte Activity. Curr. Protoc. Immunol. 72, 3.11.1-3.11.23. https://doi.org/10.1002/0471142735.im0311s72
Woolhiser, M.R., Henry, K.S., Holsapple, M.P., 2005. Ecoimmuntoxicology: state of science. Investig. Immunotoxicol. H Tryphonas M Fournier B Blakley JEG Smits EtP Brousseau CRC Press Book 13Y28.
Wren, C., 1986. A Review of Metal Accumulation and Toxicity in Wild Mammals .1. Mercury. Environ. Res. 40, 210–244. https://doi.org/10.1016/S0013-9351(86)80098-6
Wu, D., Mura, C., Beharka, A.A., Han, S.N., Paulson, K.E., Hwang, D., Meydani, S.N., 1998. Age-associated increase in PGE2 synthesis and COX activity in murine macrophages is reversed by vitamin E. Am. J. Physiol. 275, C661-668. https://doi.org/10.1152/ajpcell.1998.275.3.C661
Yan, A., Wang, Y., Tan, S.N., Mohd Yusof, M.L., Ghosh, S., Chen, Z., 2020. Phytoremediation: A Promising Approach for Revegetation of Heavy Metal-Polluted Land. Front. Plant Sci. 11.
Yang, D.-Y., Chen, Y.-W., Gunn, J.M., Belzile, N., 2008. Selenium and mercury in organisms: Interactions and mechanisms. Environ. Rev. 16, 71–92. https://doi.org/10.1139/A08-001
Yaqub, Y., 2013. Influence of reproductive cycle, sex, age and season on haematologic parameters in domestic animals: A review. J. Cell Anim. Biol. 7, 37–43. https://doi.org/10.5897/JCAB12.063
Yin, N., Han, Z., Jia, W., Fu, Y., Ma, J., Liu, X., Cai, X., Li, Y., Chen, X., Cui, Y., 2022. Effect of vitamin C supplement on lead bioaccessibility in contaminated soils using multiple in vitro gastrointestinal assays: Mechanisms and health risks. Ecotoxicol. Environ. Saf. 243, 113968. https://doi.org/10.1016/j.ecoenv.2022.113968
Young, C., Ganswindt, A., McFarland, R., de Villiers, C., van Heerden, J., Ganswindt, S., Barrett, L., Henzi, S.P., 2017. Faecal glucocorticoid metabolite monitoring as a measure of physiological stress in captive and wild vervet monkeys. Gen. Comp. Endocrinol. 253, 53–59. https://doi.org/10.1016/j.ygcen.2017.08.025
Zelikoff, J.T., Smialowicz, R., Bigazzi, P.E., Goyer, R.A., Lawrence, D.A., Maibach, H.I., Gardner, D., 1994. Immunomodulation by Metals. Toxicol. Sci. 22, 1–7. https://doi.org/10.1093/toxsci/22.1.1
Zemanova, M.A., 2020. Towards more compassionate wildlife research through the 3Rs principles: moving from invasive to non-invasive methods. Wildl. Biol. 2020. https://doi.org/10.2981/wlb.00607
Zeng, H., Uthus, E.O., Combs, G.F., 2005. Mechanistic aspects of the interaction between selenium and arsenic. J. Inorg. Biochem. 99, 1269–1274. https://doi.org/10.1016/j.jinorgbio.2005.03.006
Zhang, X., Lin, W., Yu, R.-Q., Sun, X., Ding, Y., Chen, H., Chen, X., Wu, Y., 2017. Tissue partition and risk assessments of trace elements in Indo-Pacific Finless Porpoises (Neophocaena phocaenoides) from the Pearl River Estuary coast, China. Chemosphere 185, 1197–1207. https://doi.org/10.1016/j.chemosphere.2017.07.080
Zwolak, I., Zaporowska, H., 2012. Selenium interactions and toxicity: a review. Cell Biol. Toxicol. 28, 31–46.

ÉCOLE DOCTORALE
PRES I Bourgogne I Franche-Comté
Environnements - Santé

Titre : Effets de stresseurs multiples (pollution et qualité nutritionnelle) sur l'immunocompétence du mulot sylvestre.

Mots clés : immunotoxicologie, écologie du stress, faune sauvage, qualité nutritionnelle

Résumé :
Dans un contexte de changement rapide de l'environnement, de multiples facteurs de stress peuvent affecter la santé des animaux sauvages. Parmi ces facteurs de stress, l'exposition à des polluants et une mauvaise qualité nutritionnelle peuvent accentuer les troubles physiologiques et moduler la capacité du système immunitaire à répondre à une infection, ce phénomène est appelé l'immunocompétence. L'exposition à des métaux toxiques, comme le cadmium et le plomb, affecte la structure et le fonctionnement des cellules immunitaires tandis que les micronutriments comme le sélénium sont des composés alimentaires qui ont des effets bénéfiques pour la réponse immunitaire lorsqu'ils sont consommés en quantités suffisantes. Cependant, l'influence des micronutriments sur les effets des métaux toxiques a été principalement étudiée dans le cadre d'expériences de laboratoire mais reste obscure chez les animaux sauvages. La présente thèse cherche à déterminer l'influence de l'exposition aux métaux toxiques et de la qualité nutritionnelle sur l'immunocompétence d'un petit mammifère, le mulot sylvestre. Le chapitre 1 traite de la capacité de certains éléments bénéfiques ou vitamines à atténuer les dommages causés sur le système immunitaire liés à l'exposition aux métaux toxiques chez des mammifères sauvages et captifs. Dans le chapitre 2, des mulots sylvestres ont été capturées dans des sites présentant des niveaux élevés et faibles de pollution du sol par le cadmium et le plomb. Elles ont été soumises à un challenge immunitaire (en utilisant des lipopolysaccharides de bactéries, LPS) soit directement ou soit après 5 jours de captivité avec une alimentation standard ou déficiente en sélénium. La réponse immunitaire a été affectée par l'exposition au cadmium et au plomb par le biais de perturbations endocriniennes. Les animaux sauvages exposés à des niveaux élevés de métaux toxiques ont montré des réponses inflammatoires plus élevées à un challenge immunitaire, tandis que les individus maintenus en captivité pendant 5 jours ont montré des réponses immunitaires plus faibles lorsqu'ils ont été nourris avec un régime déficient en sélénium. La caractérisation de la réponse immunitaire des animaux sauvages est généralement limitée par le manque de méthodes appropriées, car la plupart des méthodes et des marqueurs ont été développés sur des animaux de laboratoire. C'est pourquoi le chapitre 3 a été consacré au développement d'intervalles de référence sanguins pour les souris sylvestres et d'autres espèces de rongeurs. Les intervalles de référence sont couramment utilisés en médecine ou en sciences vétérinaires pour les diagnostics de santé, mais ils sont très rares pour les espèces non domestiques en liberté. Il s'est avéré que les variations de la numération des cellules sanguines dépendaient de la saison et de la localisation des populations de mulots sylvestres plutôt que de paramètres biologiques (âge, sexe...). L'utilisation d'intervalles de référence a permis de trier les individus sains et non-sains d'une population donnée en utilisant une approche non létale. Dans le même ordre d'idées, le chapitre 4 a proposé des méthodes non létales pour évaluer la santé et l'état inflammatoire des mulots sylvestres. Une cytokine pro-inflammatoire (TNF-α), un médiateur de l'inflammation, a été mesurée dans le sang et les cellules de la rate de mulots sylvestres capturées dans la nature et mis en captivité. Une augmentation des marqueurs inflammatoires a été constatée chez les mulots exposées au LPS. Dans l'ensemble, ces résultats suggèrent que la qualité nutritionnelle a une influence positive sur l'immunocompétence des animaux sauvages exposés de manière chronique à des métaux toxiques. Les immunomarqueurs et les intervalles de référence sanguins ont fourni des outils pertinents pour évaluer l'immunocompétence des mulots sylvestres.

UBFC
UNIVERSITE BOURGOGNE FRANCHE-COMTE
Université Bourgogne Franche-Comté
32, avenue de l'Observatoire
25000 Besançon

ÉCOLE DOCTORALE
PRES I Bourgogne I Franche-Comté
Environnements - Santé

Title : Effects of multi-stressors (pollution, nutritional quality) on the immunocompetence of wood mouse.

Keywords : immunotoxicology, stress ecology, wildlife, nutritional quality

Abstract :
In a rapidly changing environment, multiple stressors can affect the health of wild animals. Among these stressors, exposure to pollutants and low nutritional quality can enhance physiological disorders and modulate the ability of the immune system to respond efficiently to an infection, a phenomenon called immunocompetence. Exposure to toxic metals, like cadmium and lead, affects the structure and the functioning of immune cells, while micronutrients like selenium are dietary compounds having beneficial effects on the immune response when taken in adequate amounts. However, the influence of micronutrients on the effect of toxic metals has been mainly studied in laboratory experiments but remains obscure in wild animals. The present thesis aimed to disentangle the influence of exposure to toxic metals and of nutritional quality on the immunocompetence of a small mammal, the wood mouse. Chapter 1 reviewed the ability of some beneficial micro-elements or vitamins to alleviate the damage to the immune system caused by exposure to toxic metals in wild and captive mammals. In Chapter 2, free-ranging wood mice were captured in sites exhibiting high and low levels of soil pollution by cadmium and lead and were either immediately challenged (using lipopolysaccharides of bacteria, LPS) or challenged after five days of captivity with standard or selenium-deficient food. Immune response was affected by exposure to cadmium and lead through endocrine disturbances. Wild animals exposed to elevated levels of toxic metals displayed higher inflammatory responses to immune challenge, while individuals maintained in captivity for five days showed lower immune responses when fed a selenium-deficient diet. The characterisation of the immune response of wild animals is, generally speaking, constrained by the lack of appropriate methods since most methods and markers have been developed on laboratory animals. For this reason, Chapter 3 was dedicated to the development of blood reference intervals for wood mice and other rodent species. Reference intervals are commonly used in medicine or veterinary science for health diagnoses but are very scarce for free-ranging non-domestic species. Variations in blood cell counts were found to be affected by season and location of wood mouse populations rather than by biological parameters (age, sex…). The use of reference intervals allowed to sort healthy from unhealthy individuals of a given population by using a non-lethal approach. In line with this, Chapter 4 proposed non-lethal methods to assess health and inflammatory status in wood mice. A pro-inflammatory cytokine (TNF-α), a mediator of the inflammation, was measured in blood and spleen cells of wild-caught captive wood mice. An increase of inflammatory markers was found in wild-caught captive wood mice challenged with LPS. Altogether, these results suggested that nutritional quality has a positive influence on the immunocompetence of wild animals chronically exposed to toxic metals. Immunomarkers and blood reference intervals provided relevant tools for assessing the immunocompetence of wood mice.

UBFC
UNIVERSITE
BOURGOGNE FRANCHE-COMTE

Université Bourgogne Franche-Comté
32, avenue de l'Observatoire
25000 Besançon