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Effects of co-infections on animal health

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Informen:

Que la memòria titulada "**Effects of co-infections on animal health**", presentada per **Cristina Garrido Amaro** per a la obtenció del grau de Doctora en Biodiversitat per la Universitat Autònoma de Barcelona, s'ha realitzat sota la nostra direcció i, un cop considerada satisfactòriament finalitzada, autoritzem la seva presentació per tal que sigui avaluada per la comissió corresponent.

I perquè així consti als efectes oportuns, signem el present informe a Bellaterra, a 13 de novembre de 2024.

Emmanuel Serrano Ferron
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¡Gracias a todos!

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List of abbreviations

- ADA: adenosine deaminase
- AIDS: Acquired Immune Deficiency Syndrome
- AOPP: advanced oxidation protein products
- BC: body condition
- BCS: body condition score
- BD: border disease virus
- BL: bacillary load
- BMI: Body mass index
- CDV: canine distemper virus
- CFU: colony-forming units
- CUPRAC: cupric reducing antioxidant capacity
- CXCL: chemokine
- ExL: exudative lesions
- FRAP: ferric reducing antioxidant power
- GOF: goodness of fit
- HIV: human immunodeficiency virus
- hkMm: heat-killed *M. manresensis*
- IFN-γ: interferon gamma
- IKC: infectious keratoconjunctivitis
- IL: interleukins
- LV: latent variables
- Mtb: *Mycobacterium tuberculosis*
- MV: manifest variables

- N-all: final population size
- p: p-value
- PCA: principal component analysis
- PCV2: porcine circovirus type 2
- PE: probability of extinction
- PLS-PM: Partial Least Squares Path Modelling
- PON1: paraoxonase 1
- PPV: porcine parvovirus
- PRV: pseudorabies virus
- PVA: population viability analysis
- RHD: rabbit hemorrhagic disease
- ROS: reactive oxygen species
- SHV-1: suid herpesvirus 1
- SM: sarcoptic mange
- Stoch-r: growth rate
- TB: tuberculosis
- TC: total citations
- TEAC: trolox equivalent antioxidant capacity
- TNF- α: tumor necrosis factor

1. Summary

1. Summary

Co-infection is a complex phenomenon with significant public health and wildlife conservation implications. While co-infections are common in wildlife populations and have proven epidemiological effects, due to the complexity of natural systems, research has mainly focused on single host and single infectious agent.

Different approaches can include the co-infection study, data obtained from sampling in the field, data obtained experimentally, modelling, or using statistical approaches. In this thesis, we have conducted four studies involving different forms of study co-infection in wildlife. We first executed a literature review with bibliometric analysis to summarise the insights of co-infection research in wildlife during the last century (study I). We then examined the relationship between body condition and infection across various wild vertebrate species, including fish, amphibians, reptiles, birds, and mammals. This analysis uses body condition as a proxy for health and explores its association with infections caused by fungi, viruses, bacteria, protozoa, helminths, and arthropods (study II). Concurrently, the last two studies are study cases. In the first case, we studied the consequences of multiple disease outbreaks with synzootic potential on growth rates and probabilities of extinction of virtual populations exposed to hard winters, density dependence, and co-occurring infectious disease outbreaks, using modelling techniques (study III). Finally, we wanted to understand the impact of helminthiasis and food shortages on tuberculosis progression and explored the cause-effect relationships among these factors with statistical methods (study IV).

Despite the challenges inherent in studying co-infection to understand its mechanisms and epidemiological implications, a combination of experimental tools and theoretical

approaches, including statistical analysis, simulations, and predictive models, offers promising avenues for advancing our understanding in this field.

1.1. Resum

La coinfecció és un fenomen complex amb implicacions significatives per a la salut pública i la conservació de la fauna salvatge. Tot i que les coinfeccions són comunes en poblacions de fauna salvatge i han demostrat efectes epidemiològics, la investigació s'ha centrat principalment en sistemes d'un sol hoste i d'un sol agent infecciós a causa de la complexitat dels sistemes naturals.

L'estudi de la coinfecció es pot fer mitjançant diferents enfocaments, mitjançant l'ús de dades obtingudes a partir del mostreig en camp, dades obtingudes experimentalment, modelització o utilitzant tècniques estadístics. En aquesta tesi, hem realitzat quatre estudis que impliquen diferents formes d'estudi de coinfecció en fauna salvatge. Primer vam realitzar una revisió bibliogràfica amb anàlisi bibliomètrica, per resumir els coneixements de la investigació de coinfecció en fauna salvatge durant el segle passat (estudi I). A continuació, vam examinar la relació entre la condició corporal i la infecció en una sèrie d'espècies de vertebrats salvatges, inclosos peixos, amfibis, rèptils, aus i mamífers. Aquesta anàlisi utilitza la condició corporal com a proxy per a la salut i explora la seva associació amb les infeccions causades per fongs, virus, bacteris, protozous, helmints i artròpodes (estudi II). Paral·lelament, els dos últims estudis són casos d'estudi. En el primer cas, vam estudiar les conseqüències de múltiples brots de malalties amb potencial sinzoòtic sobre les taxes de creixement i probabilitats d'extinció de poblacions virtuals exposades a hiverns durs, la dependència de densitat i els brots de malalties infeccioses coocurrents, utilitzant tècniques de modelització (estudi III). Finalment, vam voler entendre l'impacte de les helmintiasis i l'escassetat d'aliments en la progressió de la

tuberculosi i vam explorar les relacions causa-efecte entre aquests factors amb els mètodes estadístics (estudi IV).

Malgrat els reptes inherents a l'estudi de la coinfecció per entendre els seus mecanismes i implicacions epidemiològiques, tenim a la nostra disposició una combinació d'eines experimentals i enfocaments teòrics, incloent-hi l'anàlisi estadística, les simulacions i els models predictius, que ofereixen vies prometedores per avançar en la nostra comprensió en aquest camp.

1.2. Resumen

La coinfección es un fenómeno complejo con implicaciones tanto para la salud pública como para la conservación de la fauna salvaje. A pesar de que las coinfecciones son comunes en poblaciones de fauna salvaje y han demostrado tener efecto sobre su viabilidad, la investigación sobre el efecto de las enfermedades de la fauna se ha centrado principalmente en sistemas de un solo huésped y de un solo agente infeccioso.

El estudio de la coinfección se puede hacer mediante diferentes enfoques, mediante el uso de datos obtenidos a partir del muestreo en campo, datos obtenidos experimentalmente, modelización o utilizando diferentes técnicas estadísticas. En esta tesis, hemos realizado cuatro estudios que implican diferentes formas de abordar el estudio de coinfección en fauna salvaje. Primero realizamos una revisión bibliográfica con análisis bibliométrico, para resumir los conocimientos de la investigación de coinfección en fauna salvaje durante el siglo pasado (estudio I). A continuación, examinamos la relación entre la condición corporal y la infección en una serie de especies de vertebrados salvajes, incluidos peces, anfibios, reptiles, aves y mamíferos. Este análisis utiliza la condición corporal como proxy para la salud y explora su asociación con las infecciones causadas por hongos, virus, bacterias, protozoos, helmintos y artrópodos (estudio II). Paralelamente, los dos últimos

estudios son casos de estudio. En el primer caso, estudiamos las consecuencias de múltiples brotes de enfermedades con potencial sinzootico sobre las tasas de crecimiento y probabilidades de extinción de poblaciones virtuales expuestas a inviernos duros, la dependencia de densidad y los brotes de enfermedades infecciosas concomitantes, utilizando técnicas de modelización (estudio III). Finalmente, quisimos entender el impacto de las helmintiasis y la escasez de alimento en la progresión de la tuberculosis y exploramos las relaciones causa-efecto entre estos factores con los métodos estadísticos (estudio IV).

A pesar de los retos inherentes en el estudio de la coinfección para entender sus mecanismos e implicaciones epidemiológicas, tenemos a nuestra disposición una combinación de herramientas experimentales y enfoques teóricos, incluyendo el análisis estadístico, las simulaciones y los modelos predictivos, ofreciendo vías prometedoras para avanzar en nuestra comprensión en este campo.

2. Introduction

2.Introduction

2.1. A historical perspective on co-infection

Although co-infections have been known for centuries, the term itself began to be used in the 20th century. Evidence of observations of mixed infections can be traced back to ancient civilisations. For example, the practised medicine of ancient Egypt already knew that several diseases existed in a single individual (Metwaly et al., 2021).

In the Middle Ages, the Black Death was a global epidemic of bubonic plague, a disease caused by the bacterium *Yersinia pestis*, that struck Europe and Asia in the mid-1300s (WHO, 1970). Many of the victims also suffered from secondary infections, such as pneumonia. In many cases, these related infections aggravated the seriousness of the primary infection leading to increased mortality rates (Bennasar-Figueras, 2024; Dennis and Mead, 2009).

In the 19th century, progress in microbiology and pathology led to a deeper understanding of co-infection. Robert Koch made the discoveries, experimenting with anthrax, that led Louis Pasteur to describe how a particular tiny organism (called germ) could invade the body and cause a specific disease (known as 'Koch's Postulates') (National Research Council (US) Committee to Update Science, 2004).

Some examples of co-infections in the nineteenth century: In 1888, in The Medical Journal and Examiner, Doctor Bayard Holmes found a secondary mixed infection in typhoid fever (Holmes, 1888). In 1894, the medical doctors Luther B. Grandy and Miller B. Hutchins in the Medical and Surgical Journal of Atlanta defined the term "mixed infection" as applied in his investigation, *"the combined infection with the virus of chancroid and with that of syphilis. This may occur simultaneously or successively, at the same time or at periods so near*

together as to cause the occurrence of a mixed sore," or one lesion may so closely follow the other as, at any rate, to confuse the diagnosis (Grandy and Miller B. Hutchins, 1894). Later, in 1900 in Calcutta, Dr. Willian C. Hossack found out that two of the patients were suffering not only from the plague, as was thought, but from influenza, which he also referred to as a mixed infection (Hossack, 1900).

The 20th century witnessed a resurgence of interest in co-infections due to new infectious diseases and the development of more sophisticated diagnostic techniques. The HIV/AIDS epidemic, from the first reported cases in 1981, highlighted the importance of co-infections, as individuals infected with HIV were at increased risk for opportunistic infections, such as tuberculosis (Castro, 1995).

Over the past few decades, research on co-infections has grown to include a broader range of pathogens and hosts. Thanks to the advances in genomics, proteomics, and other molecular techniques, researchers can now study the intricate relationships between multiple pathogens and their hosts in depth.

Co-infection is a major public health issue, particularly in developing countries with limited healthcare. Understanding the mechanisms behind co-infections and creating effective prevention and treatment strategies is essential to enhancing global health outcomes.

2.2. Terminology used in co-infection research

According to May and Novak (1995), the term *coinfection indicates a stable coexistence of different parasites (or strains) in the same host*. In 2001, Cox outlined that *The term concomitant infections, alternatively called mixed infections, traditionally refers to a situation in which two or more infectious agents coexist in the same host*. In light of that, we proposed the following definition of co-infection: *the occurrence of more than one*

simultaneous infection by different infectious agents or strains in individual hosts, populations, or communities. In human health, many terms define co-infection (or coinfection), such as mixed infections, concomitant infections, concurrent infections, simultaneous infections, and multiple infections. These terms are frequently used interchangeably even though they describe the same.

Using varying terms to describe the same concept in science can be detrimental. It can cause fragmented searches and increase the risk of missing relevant studies in the same area.

2.3. The study of co-infection in wildlife

Different approaches can be used to study co-infection research: data obtained from sampling in the field, data obtained experimentally or by statistical or mathematical modelling.

The most classical way to study co-infection is to analyse data obtained from natural ecosystems or conduct controlled laboratory experiments. The studies can be either cross-sectional or longitudinal, being longitudinal studies the more common (Vaumourin et al., 2015). Longitudinal studies provide information over time, and cross-sectional studies provide information at the time of sampling.

New approaches like mathematical modelling, simulation analysis and statistical testing offer new opportunities to study co-infection. Tools like the simulation software VORTEX can model many extinction vortices that can threaten the persistence of wildlife populations (deterministic forces as well as demographic, environmental and genetic stochastic events. This software models population dynamics that occur according to probabilities that are random variables following user-specified distributions, this

simulation is iterated many times to generate the probabilities of extinction that the population could experience (Lacy, 2000, 1993; Lacy and J.P. Pollak, 2023).

Parametric and non-parametric tests are two broad classifications of statistical procedures (Walsh, 1962). Parametric tests assume that data are normally (Gaussian) distributed. Non-parametric tests are used when the data don't rely on assumptions about the shape or the population distribution (normally, it is not assumed). In Table 2.3.1.1 we have some examples of parametric and non-parametric test counterpart.

Table 2.3.1.1. Examples of parametric and non-parametric tests.

Parametric test	Non-parametric test	Analysis type
One-way ANOVA	Kruskall-Wallis test	Compare means between multiple distinct/independent groups
Unpaired t-test	Mann-Whitney test	Compare two unpaired groups
Paired t-test	Wilcoxon test	Compare two variables measured in the same sample
Pearson correlation	Spearman correlation	Quantify the association between two variables

2.4. Co-infection interactions

Co-infection has been studied across various pathogen taxonomies, with documented interactions between protozoa, viruses, bacteria, helminths, and other protozoa or helminths (Cox, 2001). Although co-infections involving fungi or arthropods have been less studied, recent years have seen an increase in research on these co-infections in wildlife, livestock, and experimental settings, revealing promising potential for further exploration.

2.4.1. Protozoa

Some co-infections between epidemic and endemic pathogens, which are normally tolerated as a single infection, together result in catastrophic mortality. In 1994 a third of the population of Serengeti lions (*Panthera leo*) died in a canine distemper virus (CDV)

epidemic co-infected with significantly higher levels of *Babesia* (protozoa), which magnified the immunosuppressive effect of CDV (Munson et al., 2008).

In 1995, one of the largest outbreaks of human toxoplasmosis in North America occurred. Years later, a study of co-infection of the protozoans *Toxoplasma gondii* (protozoa) and *Sarcocystis neurona* disease conducted in wild marine mammals showed a higher mortality rate and more severe protozoal encephalitis in co-infected populations, the conclusion of the study was that the waterways was the most likely source of possible contamination by *Toxoplasma gondii* (Gibson et al., 2011).

2.4.2. Helminths

A paper by Reese et al. published in 2014 in the journal Science showed that helminth infection can reactivate latent herpesvirus infection in a murine model. This demonstrates that many mechanisms of co-infection in mammals are far from being totally understood (Reese et al., 2014).

In the tropical and subtropical regions, Schistosomiasis disease continues to inflict significant morbidity and mortality. A study on baboons infected with the helminth *Schistosoma mansonii* showed that co-infection with chronic whipworm (*Trichuris trichura)* intensified schistosome liver damage in the primates (Le et al., 2020).

2.4.3. Bacteria

A model conducted by Risco et al. 2019 showed the importance of wild boar (*Sus scrofa*) as a reservoir of *Mycobacterium tuberculosis*. Helminths of the genera *Metastrongylus* and porcine circovirus type 2 will increase the probability of *M. tuberculosis* in nasal secretions if co-infected with TB-infected animals (Risco et al., 2019).

2.4.4. Viruses

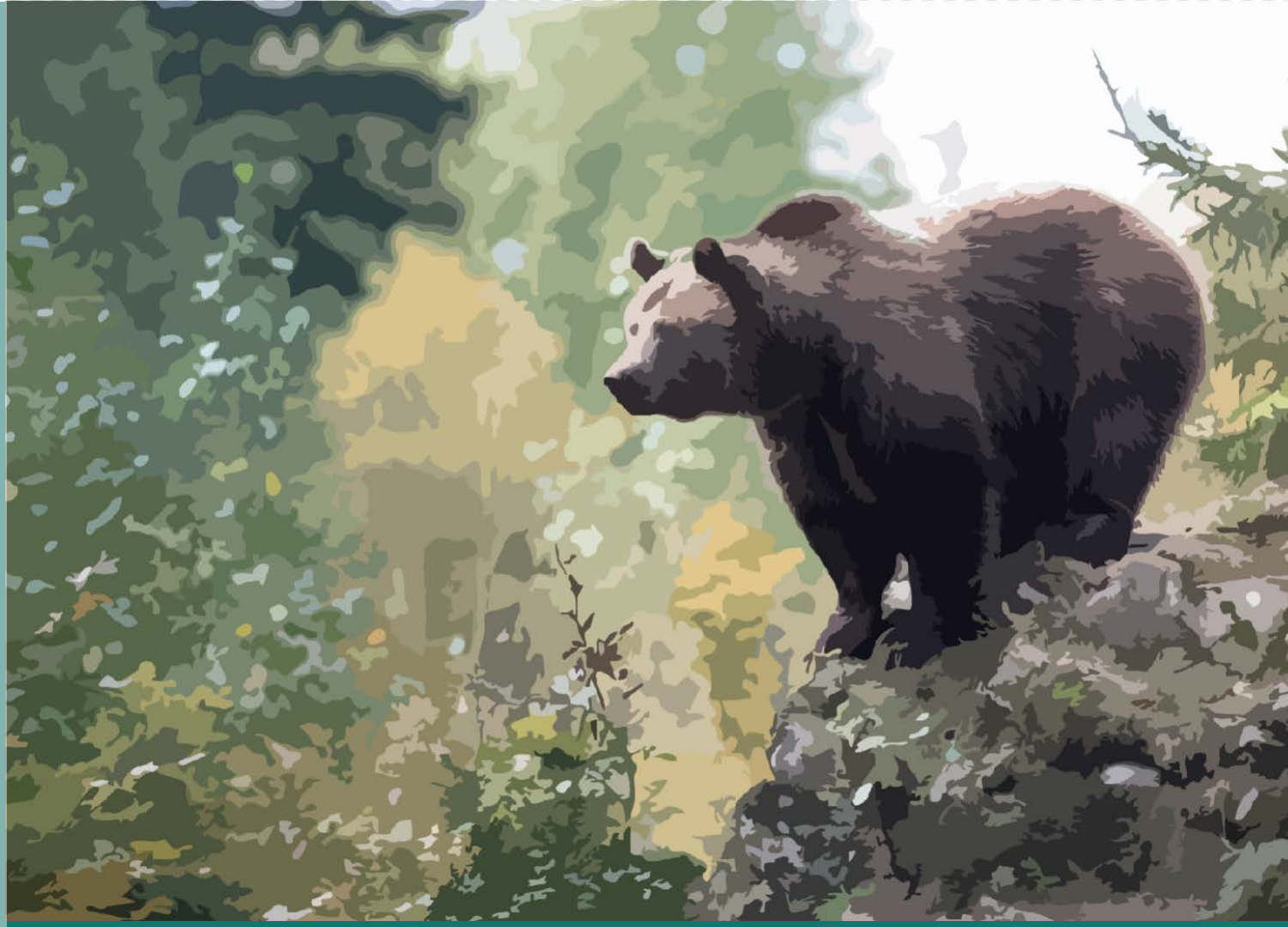
Co-infection can also influence host conditions. In the case of Influenza A virus (IAV) and bacteria *Pseudomonas aeruginosa* co-infection in minks the bacteria played a major role in the progress of the disease contributing to the development of haemorrhagic pneumonia in the host (Bo-shun et al., 2020).

2.4.5. Fungi

The emergence of the *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* co-infection, two fungal pathogens responsible for amphibian declines and extinctions worldwide, led to intensive research focused on understanding the infection mechanisms, the disease progression and the consequences for the host (McDonald et al., 2020; Zamudio et al., 2020).

2.4.6. Arthropods

Some studies show arthropods interacting with viruses or bacteria in the host Atlantic salmon. In the study of Jean-Paul Lhorente et al., they reported a naturally occurring co-infection of the arthropod *Caligus rogercresseyi* and the intracellular bacterial pathogen *Piscirickettsia salmoni* (Lhorente et al., 2014). On the other hand, Barket et al. found that previously infected by sea lice Atlantic salmon are more susceptible to infectious salmon anaemia virus co-infection (Barker et al., 2019).



3. Hypothesis & Objectives

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3.1. Hypothesis

The main hypotheses of this thesis are:

Study I: A century of co-infection research: A bibliometric study

Co-infection research could be dominated by a lack of consensus on using standardised terminology for defining multiple infections and by the lack of a methodological protocol to deal with co-infection data. Along the same lines, I expect to find a bias concerning the pathogen groups included in co-infection research.

Study II: Host condition and infection in wildlife

Because of the energetic requirements of the immune system, I expect to find a relationship between the nutritional status of the host and the susceptibility to infection across host and pathogen types.

Study III: The synzootic potential of common epidemics in chamois populations

I expect to find a relationship between specific epidemic combinations, growth rates, and the probability of extinction of virtual Southern chamois (Rupicapra pyrenaica) populations exposed to hard winters and density dependence.

Study IV: Protective effect of intestinal helminthiasis against tuberculosis progression is abrogated by intermittent food deprivation

I expect to detect a combined effect of intestinal helminthiasis and food deprivation on the tuberculosis progression in tuberculosis susceptible lab mice.

3.2. Objectives

The objectives of the present thesis are:

Study I: A century of co-infection research: A bibliometric study

To summarise the insights of co-infection research in wildlife during the last century after a literature review and bibliometric and data analysis.

Study II: Host condition and infection in wildlife

To examine the relationship between body condition and infection across a range of wild vertebrate species, including fish, amphibians, reptiles, birds, and mammals after a bibliographic review work and statistical analysis.

Study III: The synzootic potential of common epidemics in chamois populations

Case study I: Statistical simulation in the study of co-infection. To evaluate the impact of multiple epidemics and environmental factors on the population viability of Pyrenean chamois (*Rupicapra pyrenaica*).

Study IV: Protective effect of intestinal helminthiasis against tuberculosis progression is abrogated by intermittent food deprivation

Case study II: Statistical analysis of experimental data. To assess the impact of food shortages and *Trichuris muris* and *Heligmosomoides polygyrus* co-infections on the immune response and pathology of *Mycobacterium tuberculosis* infection in mice.

4. Studies

4.1. Study I.

A century of co-infection research: A bibliometric study

4.1.1. Abstract

Co-infection, coinfection, and concomitant infections are all terms used to describe the occurrence of more than one simultaneous infection in individual hosts, populations, or communities. With a growing number of pathogens being discovered and emerging from shifting between different host species, it is perhaps little surprise that co-infection appears to be the rule rather than the exception in nature. Yet, few works have explored the frequency and contribution of specific pathogen groups on disease severity, length, or transmission in wildlife. The broad diversity in methodological approaches and the inconsistency in the terminology used to describe co-infections make the study comparison difficult.

The main aim of this study was to summarise the insights of co-infection research in in animals (wildlife, experimental, modelling, pets, and livestock) during the last century. We conducted a systematic search in the Web of Science (WoS) database and obtained a total of 32,934 articles published between 1920 and 2020. We retrieved 680 validated research articles and extracted valuable information like co-infection terms, population of interest, taxonomic groups, number of species studied, and statistical techniques used.

Despite the wide variety of co-infections, the most studied have been those within the same taxonomic groups (Arthropoda-Arthropoda, Bacteria-Bacteria, Fungi-Fungi, Helminths-Helminths, Protozoan-Protozoan, and Viruses-Viruses), and also between two or three taxonomic groups—like co-infection between Protozoan-Helminths-Bacteria or Protozoan-Viruses-Helminths. Some research involves up to 14 different pathogen species, but the majority only consider two. The study also identifies the range of terms used when referring to co-infection–such as co-infection with and without a dash, concomitant infections, multiple infections, and mixed infections.

To date, the co-infection literature is afflicted by inconsistent terminology, which requires an update in light of the pressing demand to better understand the ecology of co-infection in the context of global change-induced disease spread in wildlife.

4.1.2. Introduction

Co-infection, also referred to as concomitant infection, mixed infection, concurrent infection, or multiple infections, describes the simultaneous occurrence of more than one infection within individual hosts, populations, or communities (Cox, 2001).

The use of different terms to describe the same concept in science presents significant challenges, particularly during literature reviews (Pautasso, 2013). This practice creates inconsistency, making it difficult for researchers to comprehensively locate and synthesise relevant studies. Inconsistent terminology can lead to fragmented searches, where relevant research may be missed if alternative terms are not considered. For example, in infectious disease research, the terms co-infection, concomitant infection, and mixed infection are often used interchangeably despite referring to the same phenomenon. This lack of standardisation complicates database searches, reduces the visibility of related research, and hinders the comparison of findings across studies.

For example, HIV—tuberculosis (TB) and malaria co-infection works have used various terms, making it challenging to track down all relevant literature. Some studies may focus on HIV-TB co-infections, while others use terms like concurrent infection or dual infection, thus leading to fragmented retrieval of data. As a result, researchers must often broaden their searches by including multiple terms to ensure comprehensive coverage.

Bibliometric analysis is a methodology that offers a versatile tool for researchers, policymakers, and funding agencies. By quantitatively analysing the volume, impact, and

development of research within specific fields (Pritchard, 1969), bibliometric methods can identify influential scholars and institutions, track emerging research trends, assess the impact of publications, evaluate research collaboration, and support informed decision-making. These insights can help researchers stay up to date with the latest developments in their field, identify potential collaborators, and allocate resources effectively, enabling a better understanding of the evolution and structure of scientific disciplines (Hood and Wilson, 2001). Furthermore, bibliometric studies also supports decision-making by funding agencies, institutions, and researchers, guiding resource allocation and fostering informed strategies for advancing scientific knowledge. Additionally, it helps assess the global impact and research productivity of different countries and institutions.

For example, in public health, it can summarise the global prevalence and distribution of *Leptospira* infection in rats and add public awareness regarding leptospirosis transmission and prevention (Boey et al., 2019). In contrast, bibliometric analysis can be used in the business and management domains to investigate entrepreneurial universities, shedding light on their distinctive characteristics and contributions to innovation and economic development (Forliano et al., 2021).

Co-infection has been examined across various pathogen groups, with documented interactions between protozoa, viruses, bacteria, helminths, and other protozoa or helminths (Cox, 2001). Although co-infections involving fungi or arthropods are less studied, they offer significant potential for further research.

In humans, approximately 30% of infections may be co-infections, which can rise to 80% in specific populations (Petney and Andrews, 1998). Co-infections often lead to worse health outcomes compared to single infections, exacerbating the severity of disease and increasing mortality. These cases involve various pathogens, with bacterial co-infections

being the most commonly reported (Griffiths et al., 2011). For instance, in Sub-Saharan Africa, the combined effects of co-infections from major diseases like HIV, tuberculosis (TB), and malaria significantly influence disease burden and mortality (Osakunor et al., 2018; Salgame et al., 2013).

While co-infections are common in wildlife populations and have proven epidemiological effects, research has mainly focused on single host and single infectious agent systems due to the complexity of natural systems (Hellard et al., 2015). Co-infections can occur simultaneously, but it is more often sequentially, potentially changing evolutionary and epidemiological outcomes on host-parasite interactions widely across animal ecosystems (Karvonen et al., 2019). Studies in ticks have revealed the presence of high pathogen co-infection rates and the possible implications for human and animal health (Moutailler et al., 2016).

In animal pets, the involvement of co-infecting pathogens in determining the outcome and effectiveness of treatment is also essential, the chosen strategy can determine the effectiveness of the treatment and the course of the disease. For example, immune responses after *Leishmania* infection, result in parasite clearance but also contribute to the pathogenesis increasing the complexity of the course of the disease (Rossi and Fasel, 2018).

Different approaches can be used to study co-infection: data obtained from sampling in the field, data obtained experimentally, modelling, or using statistical approaches (Vaumourin et al., 2015). Simulation models, e.g. population viability modelling have proven the impact of concomitant disease outbreaks and the potential synzootic effects posing an additional threat to the viability in chamois populations previously affected by another disease (Garrido-Amaro et al., 2023). Within-host infectious disease models also provide a

mathematical way forward to study the impact related to the roles of cellular co-infection, collective viral interactions, and viral complementation in within-host viral dynamics and evolution (Koelle et al., 2019).

Statistical approaches can also be used to assess the effects of co-infection. For example, a Partial Least Squares Regression (PLS-R) approach is a statistical tool that works as an extension of multiple regression analysis and has been used e.g. to explore the impact of co-infection on the body condition of feral cats (Serrano and Millán, 2014).

In this study, we conducted a literature review of publications on animals co-infections to explore publication trends, identify the most influential countries and articles in co-infection research, examine the evolution of co-infection terminology, and highlight the most studied hosts. We also investigated the types of co-infections most frequently researched and the number of pathogen species involved in co-infection studies. Finally, we reviewed the statistical techniques most commonly used to analyse co-infection data.

4.1.3. Methods

Literature search

The search protocol applied to this structured literature review was the PRISMA search protocol (O'Dea et al., 2021), and used the *Web of Science (WoS)* search engine to find the literature using the following Boolean search terms: "co-infection*", "coinfection*", "concomitant infection*", "multiple infection*", "simultaneous infection*", and "mixed infection*" were used to search titles, abstracts, and keywords of publication. This search returned a total of 32934 publications from 1920 to 2020.

INCLUSION AND EXCLUSION CRITERIA

Afterwards we conducted an unstructured literature search by reviewing all the reference lists from the obtained structured data. We included studies of livestock (poultry, fishery, and dairy), wildlife, pets, modelling, and experimental (e.g. double co-infections experimentally induced). We excluded records of case reports, research describing a new co-infection in sick patients, description of co-infections in humans or animals without clinical implications, prevalence of missed infections, co-infections reported after transplantation, a single co-infected patient with severed prognosis, co-infections described in less than five individuals. We also excluded co-infections in plant or fungi kingdoms. We did not include references published after December 2020. This method resulted in a 680 manuscripts matching the selection criteria (Figure 4.1.3.1).

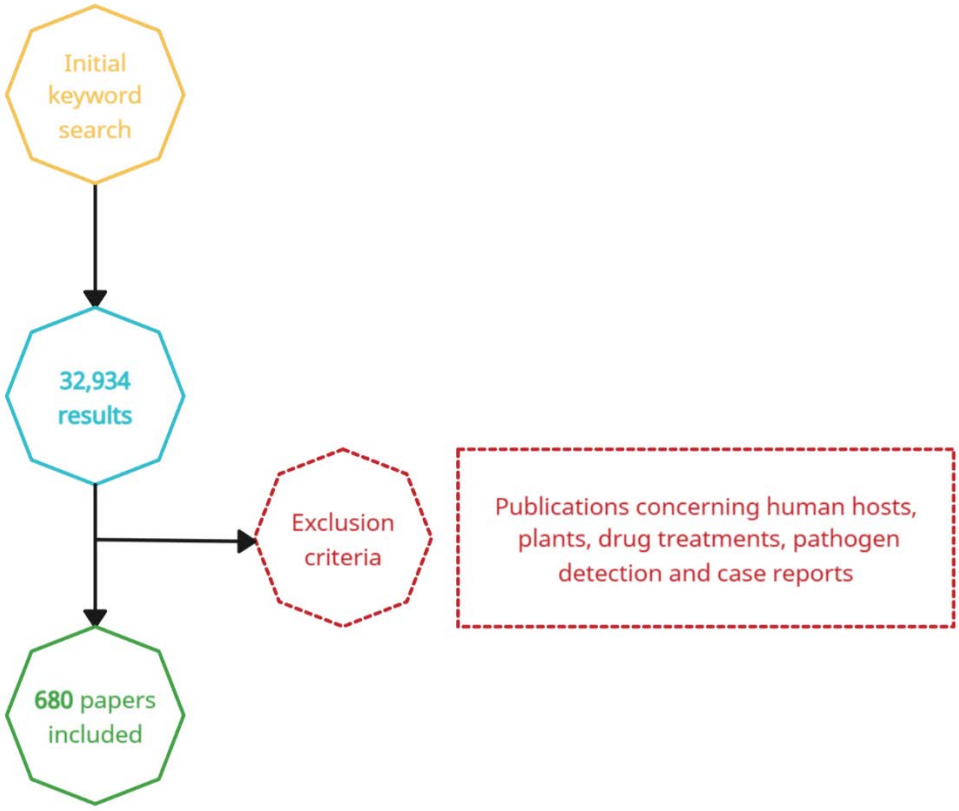


Figure 4.1.3.1. Diagram of the literature review and screening process to review co-infection research in the last century.

Data extraction

We read and extracted data from each applicable reference on all reported co-infections according to the data parameters and descriptions listed in Table 4.1.3.1.

Table 4.1.3.1. Data gathered from the retrieved articles on co-infections.

Parameter	Description	Data options
Category	Classification of the study type	Experimental, Livestock, Wildlife, Pets, Modelling
Co-infection reference	Reference used to address co-infection	Co-infection, Coinfection, Mixed infection, Concurrent infections, Concomitant infections, Multiple infections, Dual infection, Combined infection, Simultaneous infection, Double infection, Subsequent infection, Repeated infection, Synergistic infection, Secondary infections, superinfections, and intercurrent infections, none, N/A.
Population of interest	Species of host	See the database for the full list
Co-infection type		Arthropoda-Arthropoda Arthropoda-Bacteria Arthropoda-Helminths Arthropoda-Protozoan Arthropoda-Viruses Bacteria-Bacteria Bacteria-Fungi Bacteria-Helminths Bacteria-Protozoan Bacteria-Viruses Fungi-Fungi Fungi-Helminths Fungi-Protozoan Fungi-Viruses Helminths-Helminths Helminths-Protozoan Helminths-Viruses Protozoan-Protozoan Protozoan-Viruses Viruses-Viruses See the database for the full list (combinations of three or four types).
Total species studied	Number of species studied	An integer.
Max co-infection sp found	Number of maximum co-infections found	An integer.
N Protozoan species	Number of protozoan species recorded in the study	An integer.
N Helminth species	Number of helminth species recorded in the study	An integer.
N Bacteria species	Number of bacteria recorded in the study	An integer.

N Virus species	Number of virus species recorded in the study	An integer.
N Fungi species	Number of fungi species recorded in the study	An integer.
N Arthropoda species	Number of arthropods recorded in the study	An integer.
Statistical analysis	The study performed a statistical analysis of the data	Yes/No
Notes on statistical analysis	Description of the statistical techniques used	
Post-hoc test	The study performed a posthoc test	Yes/No
Parametric test	The study performed a parametric test	Yes/No
Non-parametric test	The study performed a non-parametric test	Yes/No
Distance-based methods	The study used distance-based methods	Yes/No
Null model tests	The study used null model tests for positive/negative associations between parasites.	Yes/No
Other tests	The study used other tests for positive/negative associations between parasites.	Yes/No
Parasite traits/phylogenetic	The study included traits or phylogenetic information	Yes/No
Regression with environmental data	The study performed a regression with host/immune/environmental data as covariates.	Yes/No

We recorded data about the co-infection reference used in the study, the host species, the co-infection type between the groups (Arthropoda, Bacteria, Fungi, Helminths, Protozoan, and Viruses), the total species studied, the maximum co-infection between species found, and the number of species of each group.

If the statistical analysis was available, we recorded information about the statistical techniques applied in the study. These statistical procedures are sorted by:

- **The post-hoc test**: identifies homogeneous subsets of means that do not differ from each other (Armstrong and Hilton, 2010).
- **Parametric tests**: assume data comes from a normal (Gaussian) distribution, also when from larger datasets. In other words, the data plotted on a frequency histogram will resemble the bell-shaped curve of ‘normal distribution’ (Bernard, 2012).

- **Non-parametric tests**: (or distribution-free) inferential statistical methods are mathematical procedures for statistical hypothesis testing which, unlike parametric statistics, make no assumptions about the probability distributions of the variables being assessed. These tests are typically used where sample sizes are small, or where normality is not assumed (Conover, 1980; Walsh, 1962).
- **Distance-based methods**: The purpose of the distance-based (DB) methods, regression and discrimination, is to properly handle problems with non-real value predictors, including categorical or a mixture of real-valued and categorical explanatory variables (Arenas and Cuadras, 2002).
- **Null model tests**: A null model is a pattern-generating model that is based on randomization of ecological data or random sampling from a known or imagined distribution (Gotelli and Graves, 1996; Gotelli and Ulrich, 2012).

BIBLIOMETRIC ANALYSIS

Bibliometric data was analysed using the web-based data analysis framework Biblioshiny (Aria, M., & Cuccurullo, 2017). This analysis enabled the identification of key authors, keywords, institutions, global collaborations, and the main journals.

4.1.4. Results

BIBLIOMETRIC ANALYSIS

This search returned 32934 publications, a century of co-infection studies from 1920 to 2020 (100 years). The temporal trend in co-infection publications indicates a steady increase in the number of documents published annually, with an average annual growth rate of 3.69%. The global progression of co-infection research can be categorized into three distinct periods: the early twentieth century up to 1925, which experienced a slow increase

with only 42 publications; the second period, spanning 1926 to 2000, characterized by consistent growth; and the last two decades, during which 72% of all research papers in this field were published. (Figure 4.1.4.1).

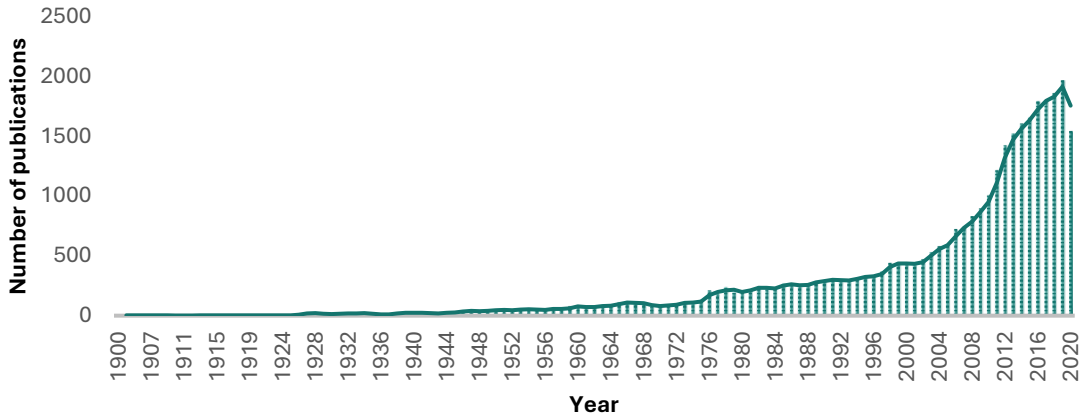
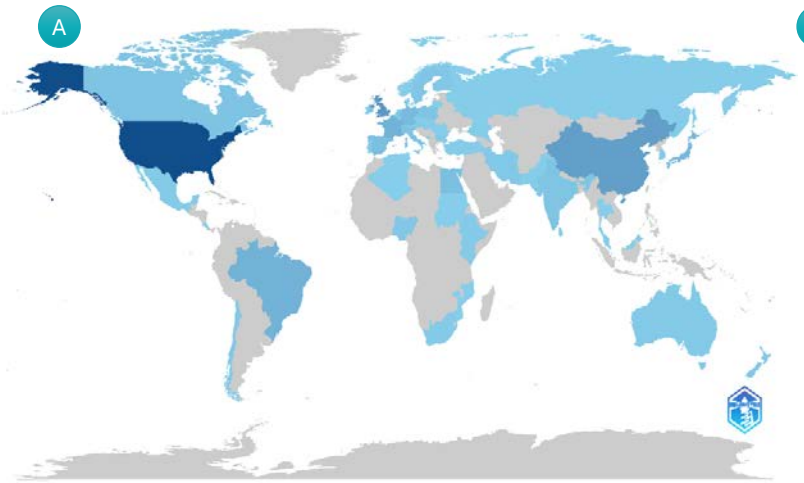


Figure 4.1.4.1. Evolution of co-infection publications production through the last century.

The publications retrieved reported relevant data from 66 geographical locations. The top three countries in terms of number of articles are the USA, China, and the UK (Figure 4.1.4.2).



B

Country	Frequency
USA	424
UK	174
CHINA	144
FRANCE	108
JAPAN	78
BRAZIL	77
SPAIN	69
GERMANY	55
CANADA	47
SWITZERLAND	35

Figure 4.1.4.2. ***(A)*** *Map showing the most active countries in co-infection research. Colour intensity corresponds to scientific production.* ***(B)*** *Table displaying the top ten countries in co-infection scientific output. Source: Biblioshiny, based on the WoS dataset*

The USA also had the highest number of citations (n = 5979), but it was Ireland who scored the highest in the average article citation (61.20) (Table 4.1.4.1).

Table 4.1.4.1. Top ten most cited countries. Source: Biblioshiny, based on the WoS dataset

Country	Total number of citations	The average number of citations/article
USA	5979	38.10
UNITED KINGDOM	2196	41.40
FRANCE	1334	46.00
CHINA	910	17.20
IRELAND	612	61.20
JAPAN	608	20.30
ITALY	607	46.70
SPAIN	548	26.10
AUSTRALIA	405	50.60
BRAZIL	367	11.50

Figure 4.1.4.3 shows the collaboration rates among countries based on the author's affiliations. The rate of collaboration is represented by line thickness, whereas country productivity by color intensity.

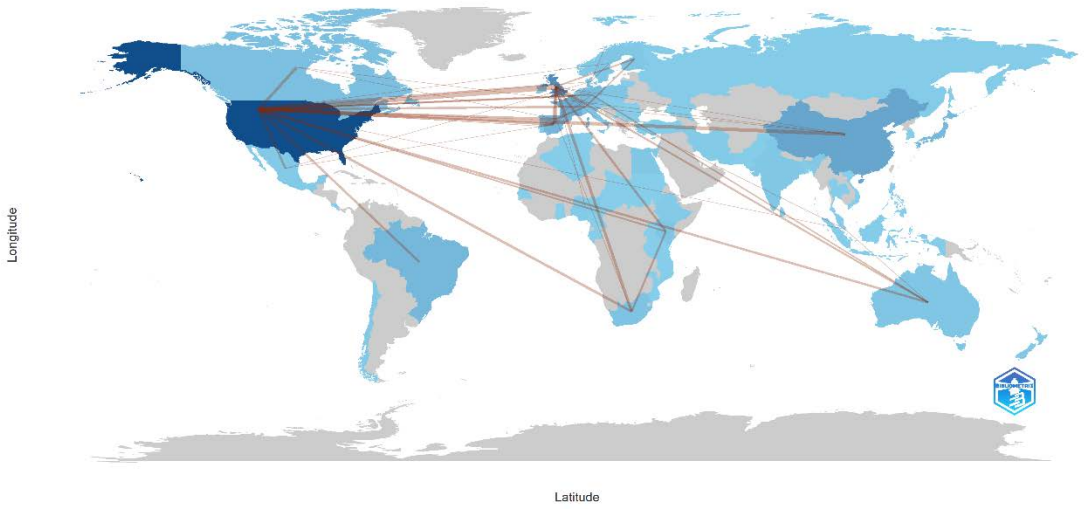


Figure 4.1.4.3. Collaborative network among countries in co-infection research. Source: Biblioshiny, based on the WoS dataset.

The top ten papers per citation related to co-infection research in wildlife are listed in Table 4.1.4.2. The most cited paper, *Concomitant infections, parasites and immune responses,* was published in 2001 by F.E.G. Cox in Parasitology (n = 499). This paper showed that

concomitant infections are common and can include interactions between various organisms. These interactions can either increase or suppress the burden of the infectious agents. They are influenced by the immune system, particularly through immunodepression and cytokine effect, and highlight how understanding these interactions is crucial for clinical disease management and vaccine development. The second paper was published in 2006 by Amy B. Pedersen and Andy Fenton in Trends in Ecology & Evolution (n = 435). This paper emphasizes that in natural systems, individuals often have multiple parasite species co-infecting them, these interactions are not well understood because traditional studies focus on parasite abundance patterns rather than the mechanisms of these interactions. Understanding these within-host interactions is key to predicting the impact of disease control, climate change, and new parasite introductions on health. The third paper (n=392) was published in 1999 in the Journal of Comparative Pathology by G.M. Allan et al. and is an experimental reproduction of severe wasting disease by co-infection of pigs with porcine circovirus (PCV2) and porcine parvovirus (PPV).

Table 4.1.4.2. The ten most cited papers on co-infection. Source: Biblioshiny, based on the WoS dataset

Paper	Total Citations	TC per Year
COX FEG, 2001, PARASITOLOGY	499	20.79
PEDERSEN AB, 2007, TRENDS ECOL EVOL	435	24.17
ALLAN GM, 1999, J COMP PATHOL	392	15.08
NAZZI F, 2012, PLOS PATHOG	344	26.46
MAY RM, 1995, P ROY SOC B-BIOL SCI	293	9.77
ALIZON S, 2013, ECOL LETT	288	24.00
ROVIRA A, 2002, J VIROL	247	10.74
PETNEY TN, 1998, INT J PARASITOL	233	8.63
STACY A, 2016, NAT REV MICROBIOL	205	22.78
MOUTAILLER S, 2016, PLOS NEGLECT TROP D	184	20.44

Author keywords are the terms selected and created by the authors to summarize and represent the content of their papers. The analysis of author keywords showed that the most used were co-infection, coinfection, pigs, and mixed infection (Figure 4.1.4.4).

Figure 4.1.4.4. WordCloud of the top ten author keywords used in co-infection review literature from 1920 to 2020

Data Analysis

Co-infection publications are inconsistent in terminology and have evolved over the years, being the most represented the term co-infection (39.12%), coinfection (20%), mixed infection (10.29%), and concurrent infection (8.68%) (Figure 4.1.4.5).

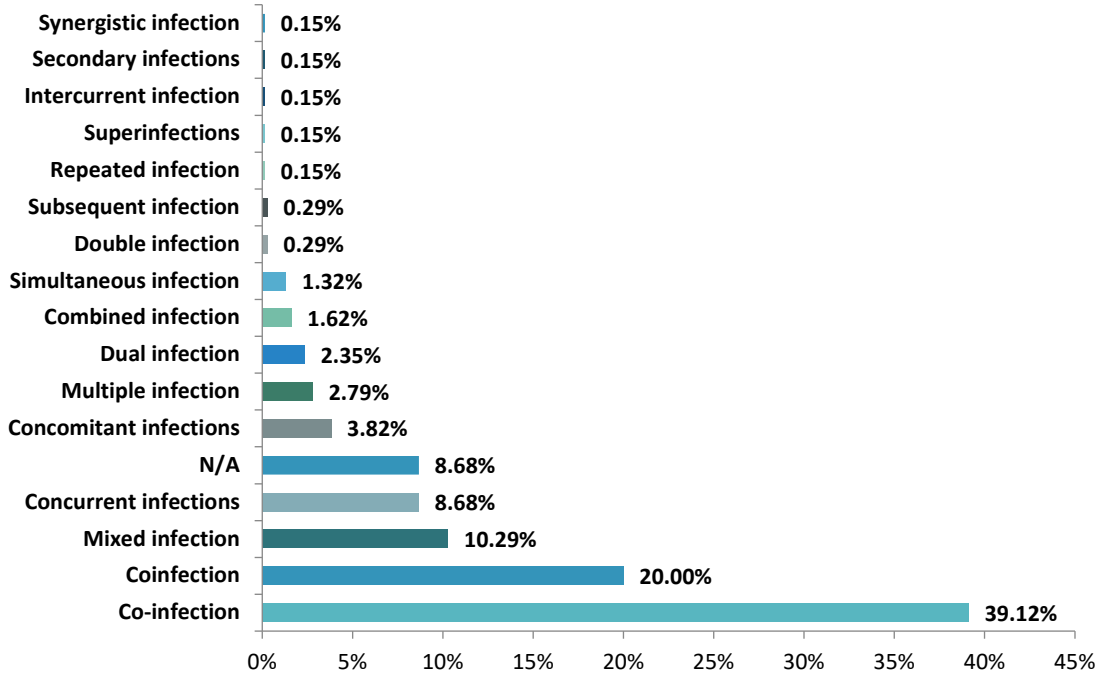


Figure 4.1.4.5. Terms used to describe multiple infections from 1920 to 2020.

The most frequently investigated taxonomic groups were experimental models, primarily murine (47%). Following closely, studies involving livestock, such as poultry and cattle, constituted the second most abundant category (25%). The wildlife taxonomic group,

encompassing a diverse array of host species, ranked third (19%). Pets and other models (theorical research) were less represented, comprising 2% and 7% respectively (Figure 4.1.4.6).

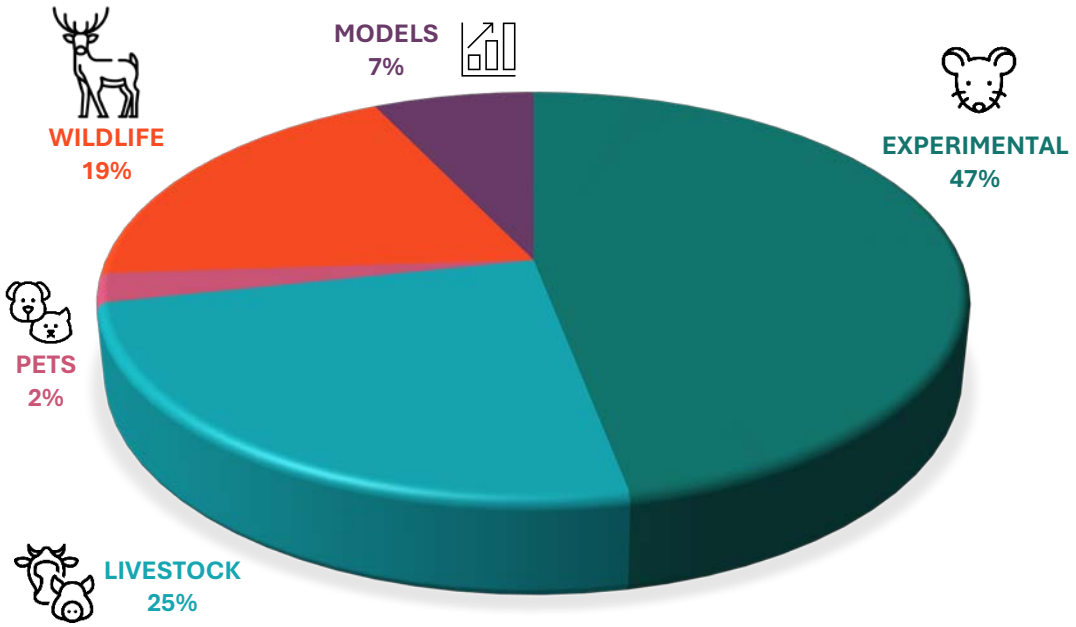


Figure 4.1.4.6. Proportion of co-infection taxonomic groups found in the literature review.

Figure 4.1.4.7 shows the different co-infection types found in the screening process. It is important to note that more than 50% of the publications are related to co-infections between combinations of bacteria-viruses, viruses-viruses, bacteria-bacteria, and helminths-helminths. Less than 3% of the publications relate co-infections between fungi or arthropods and other taxonomic groups like helminths, protozoans, and viruses.

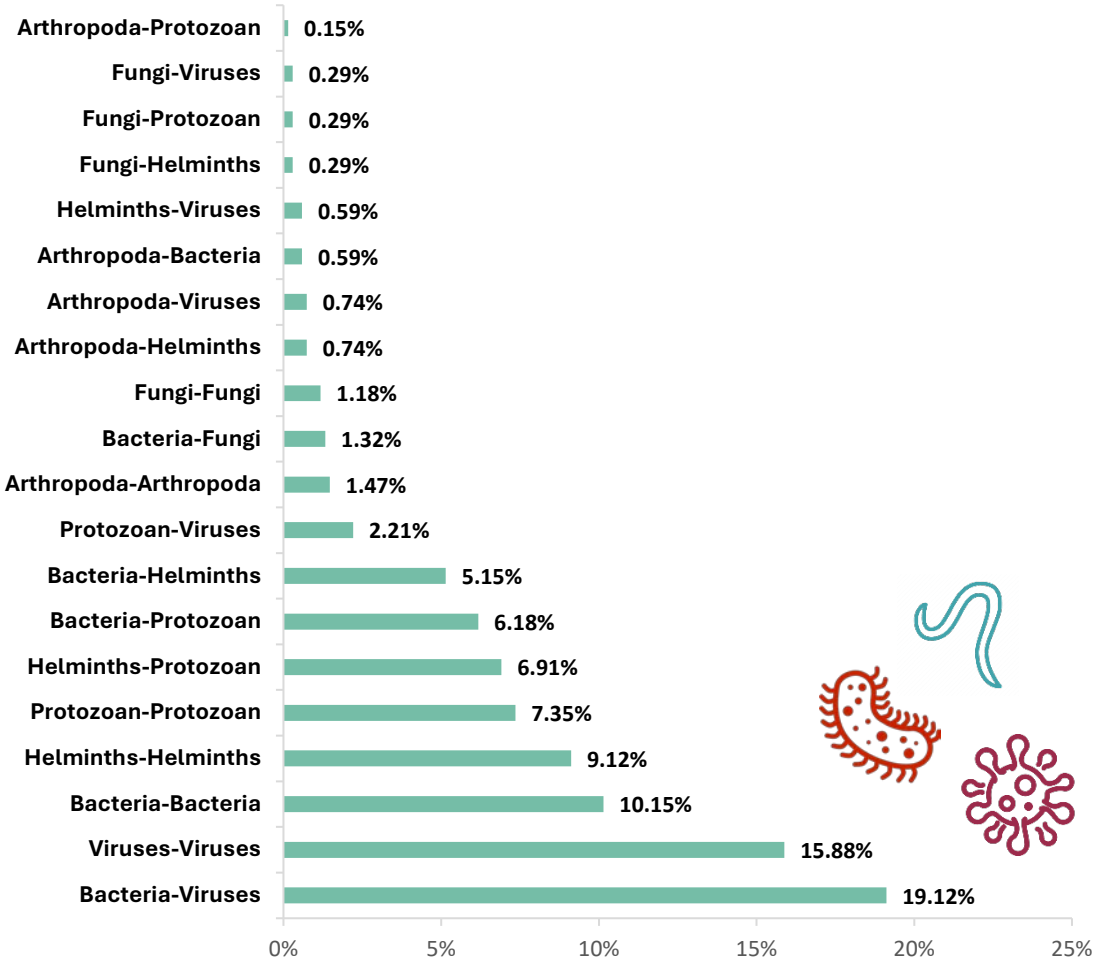


Figure 4.1.4.7. The most extensively studied co-infections in livestock, pets, and wildlife.

If we look at the number of species studied, 86% of publications study the co-infection of two species, and only 14% study more than three co-infected species. Between the publications of 2 co-infected species, we have found that the more studied are between viruses and viruses with 26%, followed by bacteria and viruses with 16%, and bacteria-bacteria with 11%. Within the publications of three co-infected species, bacteria-viruses are the most studied with 35%, and the other studies have in common that they are mostly between co-infected species of the same taxonomic group. Lastly, the studies with more than 4 co-infected species are rare to find, but there are some between 4 species and 5 species. And we only find one publication for 6 and 7 co-infected species (Table 4.1.4.3).

Table 4.1.4.3. Co-infection type and number of species studied.

Co-infection type	2 sp	% 2 sp	3 sp	% 3 sp	4 sp	% 4 sp	5 sp	% 5 sp	6 sp	% 6 sp	7 sp	% 7 sp
Arthropoda-Arthropoda	9	1.66%		0.00%		0.00%		0.00%		0.00%		0.00%
Arthropoda-Bacteria	4	0.74%		0.00%		0.00%		0.00%		0.00%		0.00%
Arthropoda-Bacteria-Helminths		0.00%	1	2.94%		0.00%		0.00%		0.00%		0.00%
Arthropoda-Fungi-Viruses	1	0.18%		0.00%		0.00%		0.00%		0.00%		0.00%
Arthropoda-Helminths	4	0.74%	1	2.94%		0.00%		0.00%		0.00%		0.00%
Arthropoda-Helminths-Bacteria-Viruses		0.00%		0.00%		0.00%		0.00%		0.00%		0.00%
Arthropoda-Helminths-Protozoan	1	0.18%		0.00%		0.00%		0.00%		0.00%		0.00%
Arthropoda-Protozoan	1	0.18%		0.00%		0.00%		0.00%		0.00%		0.00%
Arthropoda-Viruses	5	0.92%		0.00%		0.00%		0.00%		0.00%		0.00%
Arthropods-Helminths-Protozoan-Viruses		0.00%	1	2.94%		0.00%		0.00%		0.00%		0.00%
Bacteria-Bacteria	60	11.07%	7	20.59%	1	16.67%		0.00%		0.00%		0.00%
Bacteria-Fungi	6	1.11%	1	2.94%		0.00%		0.00%		0.00%		0.00%
Bacteria-Fungi-Helminths-Protozoan	1	0.18%		0.00%		0.00%		0.00%		0.00%		0.00%
Bacteria-Helminths	33	6.09%	2	5.88%		0.00%		0.00%		0.00%		0.00%
Bacteria-Helminths-Protozoan		0.00%		0.00%		0.00%		0.00%		0.00%	1	100.00%
Bacteria-Helminths-Viruses		0.00%		0.00%		0.00%		0.00%	1	50.00%		0.00%
Bacteria-Protozoan	37	6.83%	5	14.71%		0.00%		0.00%		0.00%		0.00%
Bacteria-Protozoan-Viruses	1	0.18%	1	2.94%		0.00%		0.00%		0.00%		0.00%
Bacteria-Viruses	110	20.30%	14	41.18%	3	50.00%	1	50.00%		0.00%		0.00%
Fungi-Fungi	6	1.11%	1	2.94%		0.00%		0.00%		0.00%		0.00%
Fungi-Helminths	2	0.37%		0.00%		0.00%		0.00%		0.00%		0.00%
Fungi-Protozoan	2	0.37%		0.00%		0.00%		0.00%		0.00%		0.00%
Fungi-Protozoan-Viruses		0.00%	1	2.94%		0.00%		0.00%		0.00%		0.00%
Fungi-Viruses	2	0.37%		0.00%		0.00%		0.00%		0.00%		0.00%
Helminths-Helminths	56	10.33%	3	8.82%		0.00%		0.00%		0.00%		0.00%
Helminths-Protozoan	43	7.93%	3	8.82%	1	16.67%		0.00%		0.00%		0.00%
Helminths-Protozoan-Viruses	1	0.18%	1	2.94%	1	16.67%		0.00%		0.00%		0.00%
Helminths-Viruses	2	0.37%	2	5.88%		0.00%		0.00%		0.00%		0.00%
Protozoan-Protozoan	44	8.12%	5	14.71%		0.00%		0.00%		0.00%		0.00%
Protozoan-Viruses	12	2.21%	1	2.94%		0.00%		0.00%		0.00%		0.00%
Viruses-Viruses	99	18.27%	5	14.71%		0.00%	1	50.00%	1	50.00%		0.00%

From the screened publications, 439 papers (64.56%) included statistical analysis (Figure 4.1.4.8). 21.89% of the publications incorporated a post-hoc test, 41.54% included parametric tests and 33.79% executed a non-parametric statistical test (Figure 4.1.4.9). Distance-based methods and null models were found at a very low rate, 1.13% and 1.35%, respectively.

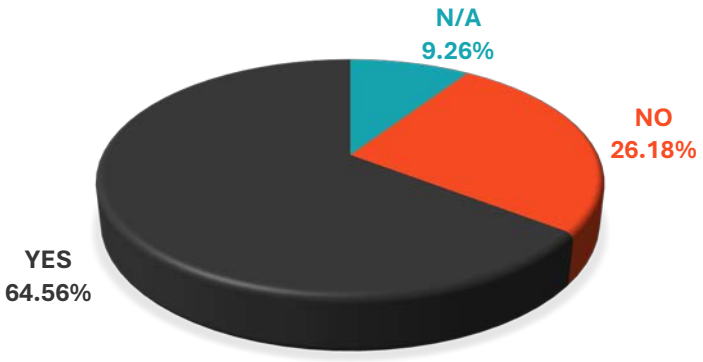


Figure 4.1.4.8. Percentage of publications with statistical analysis

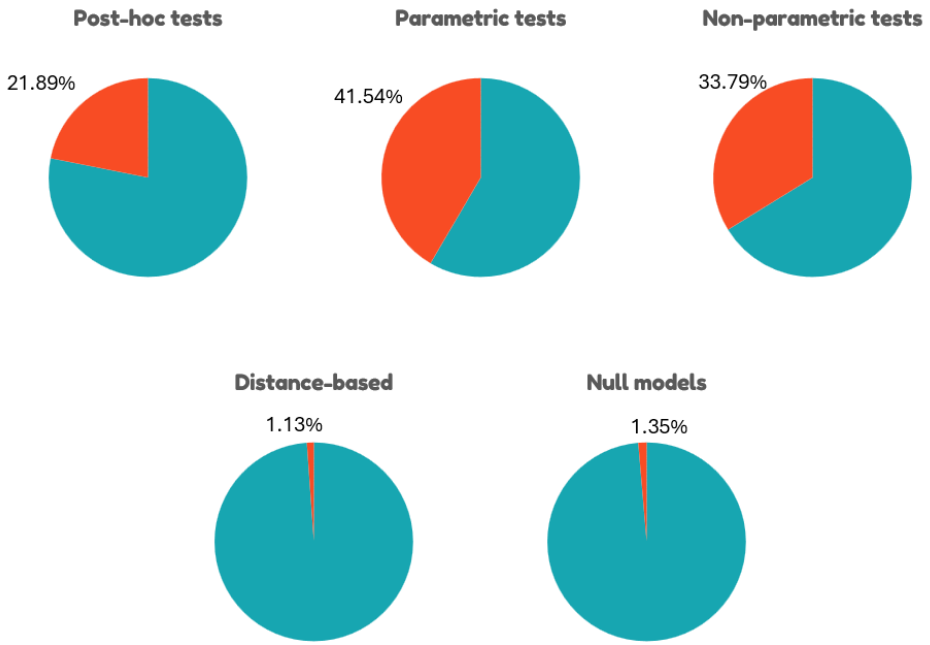


Figure 4.1.4.9. Percentage of each statistical test on publications with statistical analysis

All the statistical tests (post-hoc, parametric, and non-parametric) that were used in the papers are summarized in Table 4.1.4.4.

Table 4.1.4.4. List of the statistical techniques

Post-hoc tests	• Benjamin-Hochberg procedure (BH) • Bonferroni • Duncan new multiple range test (MRT) • Dunnett's correction • Dunn's multiple comparison test • Fisher's least significant difference (LSD) • Holm-Bonferroni Procedure • Newman-Keuls • Rodger's Method • Scheffé's Method • Sidak's • Tukey's Test
Parametric tests	• F-test • GLM • One-way analysis of variance ANOVA • Paired Student t-test • Pearson's correlation • Shapiro-Wilk test • Two-way analysis of variance • Unpaired Student t-test
Non-parametric tests	• Analysis of similarities • Anderson–Darling test • Chi-square test • Cochran's Q • Coefficient gamma • Cohen's kappa • Empirical likelihood • Fisher exact test • Friedman two-way analysis of variance by ranks • Kaplan–Meier • Kappa test • Kendall's tau • Kendall's W • Kolmogorov–Smirnov test • Kruskal–Wallis one-way analysis of variance by ranks • Kuiper's test • Logrank test or Mantel-Cox test • Mann–Whitney U or Wilcoxon rank sum test • McNemar's test • Median test • Odds ratio • PCA • Phi coefficient • Pitman's permutation test • Rank products • Siegel–Tukey test • Sign test • Spearman's rank correlation coefficient • Squared ranks test • Statistical bootstrap methods • Tukey–Duckworth test • Wald–Wolfowitz runs test • Wilcoxon signed-rank test

4.1.5. Discussion

This review aimed to perform a bibliometric analysis of current knowledge on co-infections in animals and to summarize the primary statistical methods used for analyzing available data on animals.

In co-infection research, the terminology used is notably inconsistent. There is no clear consensus on the appropriate terms, with variations such as co-infection, coinfection, concomitant infection, concurrent infection, and others frequently used interchangeably. This lack of standardization has resulted in multiple publications on similar topics using different terminologies over time. This inconsistency can create difficulties for researchers trying to identify relevant literature, as conducting a comprehensive search may require exploring various terms to ensure all pertinent studies are captured.

Co-infection has been investigated across pathogen taxonomies. In this literature review, we found that certain taxonomic combinations are more common to study. The literature includes numerous examples of interactions between protozoa and viruses, protozoa and bacteria, protozoa and other protozoa, protozoa and helminths, helminths and viruses, helminths and bacteria, and helminths and other helminths (Cox, 2001). While less frequently studied, co-infections involving fungi or arthropods present promising avenues for future research.

A comprehensive analysis of the research publications revealed that 64.3% incorporated statistical techniques. Of those statistical methods, 21.89% utilized post-hoc tests to identify specific group differences. Furthermore, 41.54% employed parametric tests, for normally distributed data, while 33.79% opted for non-parametric statistical tests, for data that did not adhere to parametric assumptions. Notably, distance-based methods and null

models were employed at considerably lower rates, with only 1.13% and 1.35% of studies using these approaches, respectively.

Statistical tests are indispensable tools for wildlife researchers, providing a rigorous framework for analyzing data. By understanding the appropriate statistical methods and their limitations, researchers can ensure the reliability and validity of their findings, contributing to a deeper understanding of wildlife populations and their interactions with the environment.

There are inherent methodological limitations in bibliometric analysis of data: the quality of metadata (titles, authors, keywords, etc.) can vary across databases, affecting the accuracy of the results; the publication heterogeneity in terms of methodology, design, and scope, making comparisons challenging; the interpretation of quantitative data must be interpreted carefully, as they do not always reflect the quality or actual impact of the research; the quantitative focus of publications and citations counts, rather than qualitative assessments of research (Belter, 2015; Donthu et al., 2021).

In conclusion, we could say that to date, the co-infection publication is afflicted by inconsistent terminology and definition of concepts and a considerable variation in the statistical approaches. These issues require an update considering the pressing demand to better understand the ecology of co-infection in the time of global change-induced disease spread in all species in general, including wildlife.

4.2. Study II.

Host condition and infection in wildlife

4.2.1. Abstract

The link between nutrition, body condition, and susceptibility to infection has been acknowledged for centuries. Early studies on protein deficiencies in livestock highlighted the critical role of nutrition in supporting immune responses and, thus, infection susceptibility. This study examines the relationship between body condition and infection across a range of wild vertebrate species, including fish, amphibians, reptiles, birds, and mammals. Drawing on data from 1,190 publications, the analysis uses body condition as a proxy for health and explores its association with infections caused by fungi, viruses, bacteria, protozoa, helminths, and arthropods. The findings reveal that, for most taxa, the impact of pathogens on body condition is generally neutral, with positive associations seen particularly in birds and fish infected by fungi and protozoa. Negative effects, notably from helminth and arthropod infections, were more frequent in amphibians, reptiles, and mammals. Overall, there is no consistent link between body condition and susceptibility to infection, as pathogen virulence plays a significant role. Healthy animals may appear thin during specific periods without being sick, challenging the assumption that a lower body condition always indicates a higher infection risk. The principles of tolerance, varying parasite strategies, and the complex top-down and bottom-up regulations between pathogen communities and the host must be considered to fully understand these dynamics.

NOTE. Along the chapter, we will use the term parasite sensu lato, i.e., an organism that lives on or inside another organism, known as the host, and derives nutrients at the host's expense. They often exploit the host for resources necessary for their survival, reproduction, and growth while potentially causing harm to the host by draining nutrients, damaging tissues, or triggering immune responses. Parasites include viruses, bacteria, protozoa, helminths (worms), and ectoparasites like lice and ticks.

4.2.2. Introduction

The connection between nutrition and disease has been acknowledged since early times (Potter, 2022). While the relationship between human nutritional deficiencies and disease was first recognised during sea voyages in the 17th century (Lind, 1757), it wasn't until the 1950s that Professor Nevin Stewart and his colleagues provided substantial evidence showing how malnutrition heightened susceptibility to infections caused by bacteria, viruses, protozoa, and helminths (Scrimshaw et al., 1968, 1959).

While "infection" and "infectious disease" are often used interchangeably, there is an important difference between the two. Infection is the *invasion of an organism's body tissues by a microbial, viral, fungal, or parasitic agent and their multiplication, as well as the reaction by the host to these organisms and/or toxins that the organisms produce* ("NCI Thesaurus," n.d.). On the other hand, an infectious disease is a *disorder resulting from the presence and activity of those agents* ("NCI Thesaurus," n.d.). Clearly and concisely, you can have an infection without having an infectious disease, but you cannot have an infectious disease without an infection.

Infectious diseases can be classified as either acute or chronic. Acute infectious diseases are characterized by short incubation periods and are typically completely cleared from the host within a relatively short time. Examples of acute infectious diseases include chickenpox, scarlet fever, influenza, and pneumonia. In contrast, chronic infectious diseases are defined by longer incubation periods and may persist in the host for extended durations, often more than a month, or may never be entirely cleared. Examples of chronic infectious diseases include chronic hepatitis and HIV, both caused by specific pathogens (Alizon and Van Baalen, 2008; Kuller and Professor, 1987).

The role of nutrition on infection susceptibility and disease progression involves a broad spectrum of nutrients, including macronutrients such as proteins (Chandra, 1992), micronutrients including vitamins and minerals (Wintergerst et al., 2007) and energetic deficiencies (Rytter et al., 2014). From a biological perspective, nutrition influences innate and adaptive immune systems. Macronutrients like protein, essential fatty acids, and carbohydrates, along with micronutrients such as zinc, iron, and vitamins A, D, and E, contribute to immune cell function, including phagocytic activity, antibody production, and cytokine signalling (Calder and Jackson, 2001). In malnourished individuals, these immune functions are impaired, reducing resistance to pathogens and increasing disease severity. For example, vitamin A deficiency weakens mucosal barriers, allowing easier pathogen entry and increasing infection susceptibility (Semba, 1999). Furthermore, studies in malnourished children have shown higher viral shedding and disease severity of diseases like measles and diarrhoea, underscoring the role of nutrition in controlling pathogen spread (Mata, 1992).

During the 20th century, researchers began to explore the interaction between specific nutritional components and immune responses in animals, especially livestock. The relevance of nutrition in livestock gained attention because poor nutrition not only compromised animal health but also negatively impacted productivity (Greer, 2008). Early research on livestock nutrition focused on the protein content of rations (CABI, 2023; Satter and Slyter, 1974). Protein deficiency, in particular, was shown to impair the immune system in ruminants, reducing resistance to gastrointestinal nematodes (Houdijk, 2012). In line with human evidence, protein or energy deficits lead to a weaker response to pathogen infections, allowing for a greater pathogen load and shedding (Van Houtert and Sykes, 1996).

Body condition has been used as a proxy for the nutritional condition and overall health status of farmed animals for decades (Lowman, 1976; Selk et al., 1988). Changes in body condition, often assessed by body weight depletion or body condition scores, have been observed in individuals as a clinical sign of viral (Waldner and Kennedy, 2008), bacterial (Chiodini et al., 1984), protozoa (Heylen et al., 2023), helminth (Fthenakis and Papadopoulos, 2018) and arthropod (Dorchies et al., 1998), disease progression. Although the link between emaciation and disease has promoted the idea that thin animals are infected or at risk of infection, the link between body condition and infection risk has not been proven yet (Bewley and Schutz, 2008).

In recent years, there has been a growing interest in exploring the relationships among host condition, host susceptibility and pathogen intensity of infection in wild populations. Accordingly, host populations in poor average condition might exhibit a higher risk of further infections with subsequent deterioration in condition (see Figure 4.2.2.1, and read (Beldomenico and Begon, 2010)).

In this scenario, hosts in poor condition might have fewer resources for allocation to immune function and are thus more susceptible to infection (Calder and Jackson, 2001). In addition, they may suffer from higher infection intensities because pathogens would encounter weaker opposition to their proliferation (Carrillo et al., 2014). However, poor conditions are not systematically linked to an increase in pathogen susceptibility or intensity of infection, and hosts can often be relatively healthy despite high pathogen burdens. Many examples exist in livestock and wildlife, probably because neither infection susceptibility nor disease progression is strictly linked with body condition. This chapter will systematically review scientific literature evaluating the link between body condition and

infection in wild fish, amphibia, reptiles, birds and mammals infected with fungi, viruses, bacteria, protozoa, helminth and arthropod pathogens.

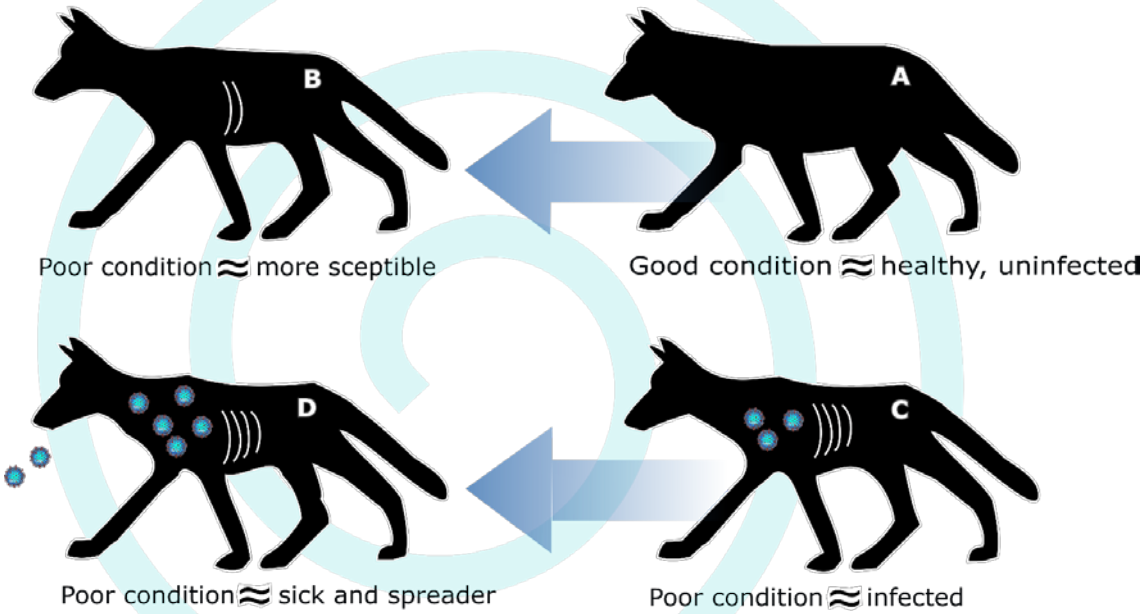


*Figure 4.2.2.1. The prevailing view on the effects of pathogens on animal body reserves is that individuals in better condition **(A)** may have a lower risk of infection due to the connection between body reserves and immune response. However, individuals in poor body condition **(B)** are more susceptible to pathogen infection **(C)** with a subsequent deterioration in condition **(D)**.*

BOX 1 Glossary

Body condition refers to the physical state of an organism, often quantified through fat reserves, organ and muscle mass. Good body condition is often used as a proxy for overall health status including reproductive success, survival, and immune competence.

Body condition score (BCS) is a tool used to assess fat reserves and the physical condition of an animal. BCS is commonly used across livestock species, including cattle, sheep, and horses, to monitor their welfare, particularly in relation to their energy reserves. It typically ranges from 1 to 5 with the lowest number indicating a very thin animal and the highest indicating an excessively fat animal

Dietary restriction refers to the controlled reduction of food intake, typically involving reduced calorie consumption without causing malnutrition.

Fasting is the voluntary abstention from food for a specified period. It impacts physiological processes, particularly immune function, as the body shifts from using glucose to fat reserves for energy. Fasting can alter immune responses by reducing the energy available for immune activities like the production of immune cells and proteins. This metabolic shift influences the immune system, sometimes enhancing certain immune functions while impairing others.

Food restriction refers to a nonspecific restriction of food, often without causing malnutrition, that affects various physiological processes, including immune responses. In some cases, food-restricted hosts exhibit an enhanced tolerance to certain pathogens while showing reduced resistance.

Immunocompetence refers to an organism's ability to mount an appropriate and effective response to pathogens. It represents the overall efficiency of the immune system in recognising, responding to, and neutralising pathogens.

Malnutrition results from an imbalanced diet in which essential nutrients are either lacking, in excess, or poorly absorbed by the body. It can occur due to protein-energy deficiency or specific vitamins and mineral deficiencies.

Starvation occurs when an organism is deprived of food for an extended period. During starvation, the body switches from using readily available glucose and glycogen stores to breaking down fat and muscle protein to sustain essential energy needs.

Undernutrition is the state in which an organism has insufficient intake of essential nutrients, including calories, proteins, vitamins, and minerals, to meet the body's requirements for maintaining normal growth, immune function, and overall health.

Resistance to infection is the host's ability to prevent, control, or eliminate a pathogen, typically through immune system responses. This form of defence involves actively reducing the pathogen burden in the host.

Synzootic is the non-random clustering of two or more diseases within an animal population, with worse health outcomes at the population scale than expected from each condition in isolation.

Tolerance to infection refers to the ability of a host to limit the negative health effects or damage caused by a given burden of infection, without necessarily reducing the pathogen load. Unlike resistance, which aims to reduce or eliminate pathogens, tolerance allows the host to endure infections by minimising the detrimental impacts on health.

4.2.3. Methods

Search protocol

We applied the PRISMA search protocol (O'Dea et al., 2021) and used the online search engine Web of Science. The following terms were utilised to search titles, abstracts, and keywords of publications: "body condition," "wild*," "pathogen*," "parasite*," and "infection*." The search (November 2021) returned 1190 manuscripts (without duplicates).

Eligibility criteria and data extraction

We first read the publications, titles, and abstracts to evaluate the manuscripts' eligibility. We selected only those studies that studied the relationship between body condition and infection in non-human species (amphibians, birds, fish, invertebrates, mammals, and reptiles) using a body condition index as a proxy for body reserves or health status. If a study included more than one pathogen species (e.g., multiple parasites collected in the same host or on different host individuals), each specific body condition-pathogen interaction was listed on a separate row within the database. If a study included more than one parasite or host species (e.g., multiple parasites from one host species or the same parasite on different host species), each specific pathogen was listed on a separate row within the database. We extracted data on all reported interactions between infection and body condition from each applicable reference according to the criteria shown in Table 4.2.3.1. Finally, works were categorised into three categories namely, negative (e.g., infected animals exhibit poor body condition), neutral (no relation between body condition and infection status), and positive (e.g., animals in good body condition are infected).

Table 4.2.3.1. This information was collected in 417 scientific articles exploring the relationship between infection and the body condition of wildlife.

Parameter	Description	Data options
Pathogen	Pathogen systematic	Acantocephala, Actinobacteria, Actinomycetota, Amoebozoa, Annelida, Apicomplexa, Arthropoda, Artverviricota, Ascomycota, Ascomycota, Bivalvia, Caliciviridae, Campylobacterota, Chytridiomycota, Ciliophora, Cossaviricota, Cressdnaviricota, Digenea, Dinoflagelatta, Euglenozoa, Firmicutes, Haemosporidia, Hepeviridae, Iridoviridae, Kitrinoviricota, Metamonada, Microsporidia, Miozoa, Monogenoidea, Negarnaviricota, Nematoda, Nucleocytoviricota, Orthomyxoviridae, Paramyxoviridae, Peploviricota, Pisuviricota, Platyhelminthes, Proteobacteria, Protozoa, Pseudomonadota, Retroviridae, Rhodophyta, Sarcomastigophora, Spirochaetae, Syndermata.
Pathogen type	Functional classification of pathogens	Arthropoda, Bacteria, Fungi, Helminths, Protozoa, Virus.
Pathogen species	Species of parasite	See the database for a complete list
Host type	Functional classification of the host	Invertebrates, Fish, Amphibians, Reptiles, Birds, Mammals
Host order	Host systematic	Accipitriformes, Anguilliformes, Anseriformes, Anura, Artiodactyla, Batrachoidiformes, Carnivora, Characiformes, Chelonia, Chiroptera, Cichliformes, Cingulata, Clupeiformes, Columbiformes, Crocodilia, Cypriniformes, Cyprinodontiformes, Decapoda, Didelphimorphia, Diprotodontia, Falconiformes, Gadiformes, Galliformes, Gobiiformes, Gruiformes, Hymenoptera, Hystricomorpha, Lagomorpha, Mytiloida, Osmeriformes, Passeriformes, Pelecaniformes, Perciformes, Perissodactyla, Primates, Procellariiformes, Rodentia, Salmoniformes, Scombriformes, Scorpaeniformes, Siluriformes, Sphenisciformes, Squamata, Suliformes, Urodela.
Host species	Species of host	See database for complete list
Study type	Type of research conducted	Observational, Experimental.
Study design	Type of study design	Cross-sectional, Longitudinal.
Study type	Type of Research	Observational / Experimental.
Study design	Study design	Cross-sectional / Longitudinal.
Relationship with body condition	Is there a relationship between infection and body condition?	Yes / No.
Methodology	Method to assess body condition	Body Lenth residuals, Body Mass Index, Condition score, Fat score, Fulton index, Liver somatic Index, Mass/length, Kidney Fat Index, Relative condition, Relative fatness, Relative mass, Residual Index, Scaled mass index, Standard weight, other.

Table 4.2.3.1. cont. This information was collected in 417 scientific articles exploring the relationship between infection and the body condition of wildlife.

Parameter	Description	Data options
Relationship with sex	Are there sexual differences in the infection-body condition relationship?	Yes / No.
Relationship with age	Is there a relationship between body condition and age?	Yes / No.
Relationship with environmental factors	Is there a relationship between body condition, infection and environmental factors?	Yes / No.
Co-infection	Is there a relationship between body condition and co-infection?	Yes / No.

DATA ANALYSIS

The G-test was used to compare the differences between the observed and expected frequencies of pathogen effects across vertebrate groups. Bootstrapping was also applied to generate more reliable p-values, especially in small datasets or when parametric assumptions may not hold using the boot package 1.3-31 version (Canty and Ripley, 2022) in the R software 4.4.1 version (R Core Team, 2020).

4.2.4. Results

Our results are summarised in Table 4.2.4.1 and Figure 4.2.4.1.

Concerning Fungi, 50% of fish species experienced an adverse effect from fungal pathogens, 50% showed no effect, and none experienced positive effects. Thus, we did not detect a significant impact of fungal pathogens in fish. Most amphibians showed no effect from fungal pathogens, while only 8.33% of works detected a positive or neutral relationship between fungi infection and amphibian body condition. The G-test indicated a significant link between fungi infection and body condition, but the nature of this impact is primarily neutral or positive. 100% of works on reptiles showed a negative relationship between fungi infection and body condition with no observed neutral or positive effects. The G-test,

however, indicates no significant impact. This may be due to the small sample size (n 0 1), which limits statistical power. 50% of works on bird species found a negative effect of fungi infection on the body condition of birds, while 64.29% showed no effect or positive effect. The G-test is 0.44, which suggests no significant effect of fungal pathogens on bird body condition. 85.71% (G-tests = 5.50, p-value = 0.054) of works about the body condition of mammals showed no effect of fungi infections, suggesting a weak signal of fungal pathogen impact on mammals.

Regarding, virus, 50% of work on fish and amphibians detected a negative link between virus infection and body condition while 50% found no effect from viruses. We found a single work on reptile species and, thus, a minimal sample size to interpret the statistical results. 83.36% of works did not find a relationship between virus infection and body condition of birds (G-test = 21.40, p-value < 0.01). Along the same lines, 88% of works on mammals found no relationship between virus infection and body condition (G-test = 11.56, p-value < 0.001).

For bacterial infections, works on fish, amphibians, and reptiles observed no effect of bacterial infections on the body condition of these vertebrates (G-test = 0.47, p-value = 0.05 for all cases). 55.55% of works on birds observed a positive (44.44%) or neutral (11.11%) effect of bacterial infections and the body condition of birds. Similarly, 66.66% of works observed a neutral (53.33%) or positive (13.33%) relationship between bacterial infections and the body condition of mammals, while 33.33% of works observed a negative effect (G-test = 3.12, p-value = 0.21). In summary, whereas works on fish, amphibians and reptiles did not detect an effect of bacterial infections on their body reserves, works on birds and mammals showed more variation in response to bacterial infections toward a neutral or

positive relationship. Overall, the data suggests that bacterial infections do not significantly affect the body reserves of these vertebrates.

For protozoa, 22.22% of works observed a negative effect of protozoan infections on fish body condition, while 77.78% found no effect (55.56%) or a positive relationship (22.22%). The G-test, however, was not statistically significant (G-test = 4.01, p-value = 0.13) for fish. For amphibians, we only found two works limiting the statistical interpretation of the results. In the case of reptiles, 25% of works observed a negative relationship between protozoal infection and body condition, while the rest had a neutral (62.5%) or positive (12.5%) effect. The G-tests did not detect a significant impact of protozoans on the body condition of amphibians. Similarly, the effect of protozoa infections on reptiles' body condition was not statistically significant (25% of works detected a negative relationship, while 75% was neutral or positive). For birds, 77.12% of works observed no effect of protozoan infections on the body condition. 15.25% observed a negative effect, and 7.63% a positive relationship. The G-test was statistically significant (G-test = 92.23, p-value = < 0.001), suggesting that the proportion for works observing negative effects was lower than their positive or neutral counterparts. 38.71% of works on mammals observed a negative relationship between protozoa infections and body condition, while 58.06% observed a neutral or a positive (3.23%) relationship. The G-test also detected statistically significant differences (G-test = 16.26, $p < 0.01$) towards a neutral effect of protozoal infections.

For helminth infections, 31.58% of fish workers observed a negative relationship with body condition, while 68.42% found a neutral (45.61%) or positive (22.81%) relationship. The G-test did not detect a statistically significant difference among groups. For amphibians (25% negative vs 75% neutral or positive) and reptiles (33.33% negative vs 66.67% neutral or positive), we did not detect a statistically significant effect of helminths on the body

reserves. For birds and mammals, we observed a statistically significant higher proportion of work detecting a neutral impact of helminths on body condition (Table 2).

For the effects of arthropod pathogens on fish, 12.5% of the works found a negative relationship between arthropod infection and body condition, while 87.51% found a positive or neutral relationship. The G-test revealed statistically significant differences (G-test = 24.63, p-value < 0.001) between the proportion of works observing neutral or positive effects and their negative counterparts. 33.33% of works on amphibians observed a negative effect, while 66.67% observed no impact on the body condition of this group (G-test = 0.41, p-value = 0.08). We have not found any work describing the positive effects. In reptiles, 42.86% of works observed a neutral impact of arthropod infection on the body condition of reptiles. 21.43% observed a positive effect, while 35.71% a negative impact. The G-test, however, did not detect statistical differences among effects (G-test = 0.85, p-value = 0.6). In the case of birds, most of the works (88.89%) observed a neutral (83.33%) or positive (5.56%) effect of arthropods on the body condition of birds. These differences differed statistically (G-test = 16.35, p-value < 0.001). Similarly, arthropods' impact on mammals' body condition was mainly neutral (71.58%) or positive (8.24%). The G-tests also revealed statistically significant differences among the proportion of works supporting the negative, neutral and positive effects of arthropod infection in mammals (G-tests = 60.36, p-value < 0.001). Though most works support a neutral effect of pathogens, bootstrapping did not provide statistical support for the neutral or positive relationship between pathogen infection and the body condition in these vertebrate groups.

Table 4.2.4.1. Percentage of works observing a negative, neutral (No effect) and positive relationship between pathogen infection and body condition in fish, amphibians, reptiles and mammals. Wildlife. n = sample size, G-test, and p-value with and without bootstrapping the sample 1000 times.

Fungi	Fish	Amphibians	Reptiles	Birds	Mammals
Negative	50	0	100	50	0
No effect	50	91.67	0.00	50	85.71
Positive	0	8.33	0.00	0	14.29
n	4	12	1	2	7
G-test	1.32	14.13	0.47	0.44	5.94
p-value	0.51	0.00	0.79	0.80	0.051
Bootstrapped p-value	0.66	0.25	0.43	0.65	0.42
Virus	Fish	Amphibians	Reptiles	Birds	Mammals
Negative	50	50	0	13.64	12
No effect	50	50	100	86.36	68
Positive	0	0	0	0	20
n	2	4	1	22	25
G-test	0.43	0.26	0.43	21.40	11.56
p-value	0.84	0.87	0.80	0.00	0.00
Bootstrapped p-value	0.66	0.65	0.66	0.34	0.45
Bacteria	Fish	Amphibians	Reptiles	Birds	Mammals
Negative	0	0	0	44.44	33.33
No effect	100	0	0	11.11	53.33
Positive	0	100	100	44.44	13.33
n	1	1	1	9	15
G-test	0.47	0.47	0.47	1.69	3.13
p-value	0.79	0.79	0.79	0.42	0.21
Bootstrapped p-value	0.47	0.47	0.43	0.65	0.45
Protozoa	Fish	Amphibians	Reptiles	Birds	Mammals
Negative	22.22	100	25	15.25	38.71
No effect	55.56	0	62.50	77.12	58.06
Positive	22.22	0	12.50	7.63	3.23
n	9	2	16	118	31
G-test	4.01	1.48	5.29	95.23	16.26
p-value	0.13	0.47	0.07	0	0
Bootstrapped p-value	0.44	0.44	0.41	0.32	0.46
Helminths	Fish	Amphibians	Reptiles	Birds	Mammals
Negative	31.58	25	33.33	31.25	30.77
No effect	45.61	66.67	50	68.75	63.31
Positive	22.81	8.33	16.67	0	9
n	57	12	12	16	169
G-test	4.03	5.12	1.64	10.99	92.13
p-value	0.13	0.07	0.43	0	0
Bootstrapped p-value	0.45	0.44	0.45	0.44	0.44
Arthropoda	Fish	Amphibians	Reptiles	Birds	Mammals
Negative	12.50	33.33	35.71	11.11	20
No effect	78.13	66.67	42.86	83.33	71.58
Positive	9.38	0	21.43	5.56	8.42
n	32	3	14	18	95
G-test	24.63	0.43	0.85	16.35	6.0.36
p-value	0	0.08	0.60	0	0
Bootstrapped p-value	0.32	0.65	0.47	0.36	0.36

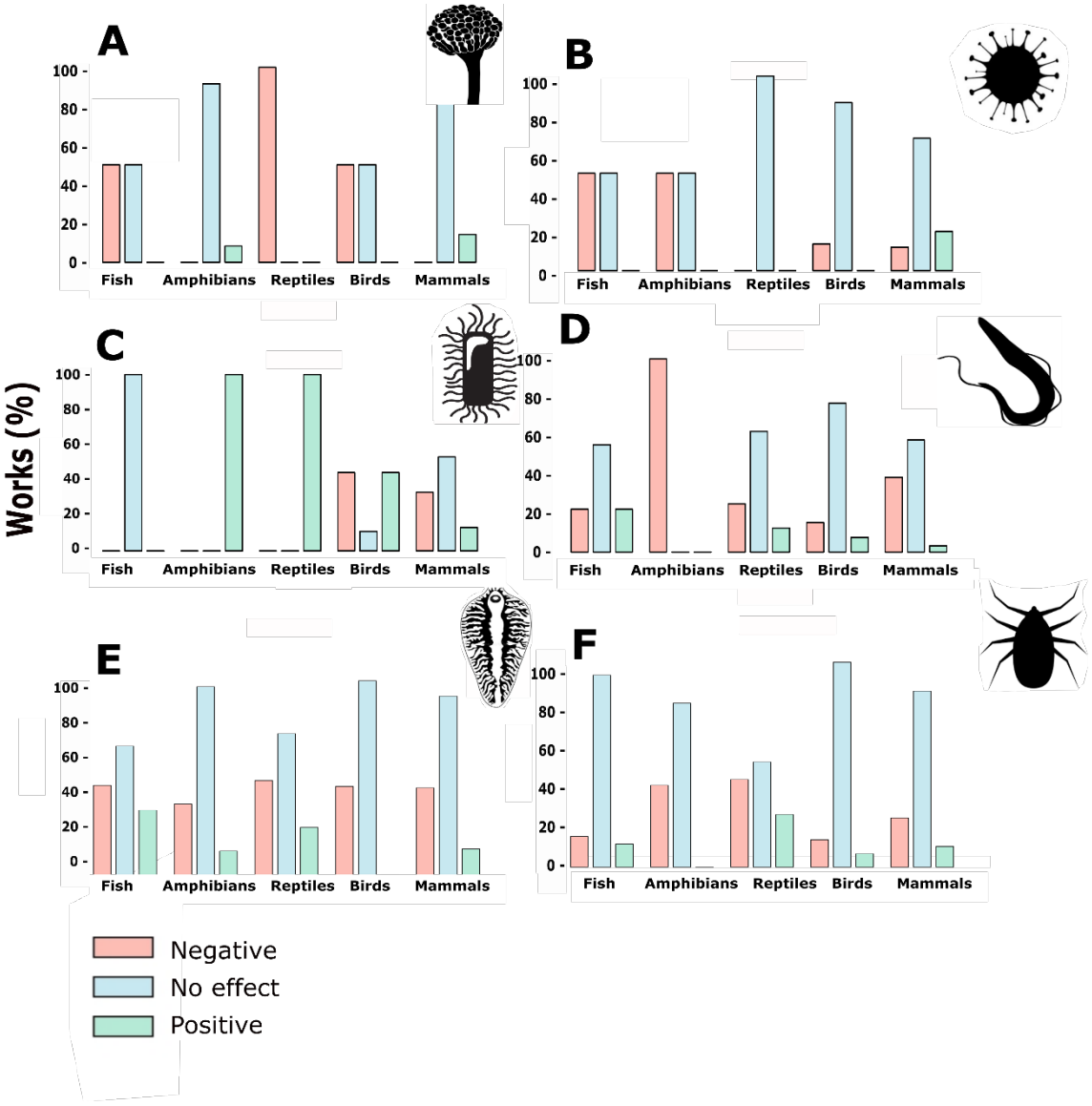


Figure 4.2.4.1. Bar plots showing the proportion of published works on the negative, neutral (No effect) and positive relationship between fungi (A), virus (B), bacteria (C), protozoa (D), helminth (E) and arthropod pathogens on the body condition of wild fish, amphibians, reptiles, birds and mammals.

4.2.5. Discussion

As we have seen, the overall impact of pathogens varies across taxa, with some groups showing more resilience than others. Most works detect a neutral effect of pathogen infections on the body condition of vertebrates, particularly fungi, viruses, protozoa, helminths and arthropods. A positive relationship was considerable in fungi (e.g., birds), bacteria (e.g., birds), and protozoa (e.g., fish) infection. In contrast, the negative effects were mainly observed in helminths and arthropod infections in amphibians, reptiles, birds and mammals.

Poor body condition in infected animals is not the rule in wildlife

For all the wildlife groups, we have observed positive relationships between body condition and infection; in other words, animals infected with microbial or metazoan pathogens show good condition and apparently good health status. A potential explanation is that pathogens can optimise fitness by employing different strategies at various infection stages (Godkin and Smith, 2017). Initially, high transmissibility is favoured to maximise spread, followed by stages prioritising persistence through longer infection duration. This balance helps pathogens adapt to varying host population sizes, with smaller populations favouring longer persistence and larger ones favouring higher transmissibility. Thus, the consequences of a period of food shortage on the disease progression will strongly depend on the pathogen type (Eberhardt et al., 2013). Those agents with low virulence in which survival relies on host resources (e.g., helminths, ectoparasites) will likely achieve greater fitness in hosts in good condition. A synergy between this "pathogen strategy" and the host tolerance strategy may also exist, and two experimental works provided excellent examples of this synergy. Seppälä and colleagues (Seppälä et al., 2008) observed that host starvation limited the amount of resources for helminth parasites, increasing their mortality rate (i.e., decreasing pathogen intensity). (Bize et al., 2008), although hosts in poor condition showed an apparent reduction in immunocompetence, ectoparasites avoided them because individuals in poor condition did not provide adequate food resources. Observational evidence of parasites selecting hosts in a good nutritional state can be found in (Christe et al., 2003). Other interesting examples include the case of trophically transmitted parasites in fish in which individual hosts with a high growth rate before infection are more prone to getting infected by consuming infected prey (Loot et al., 2010).

This latter scenario, however, also occurs in a completely different host-pathogen model, namely influenza A viruses causing systemic infections in mallards. Ducks in normal physiological conditions are more susceptible to infection and shed higher concentrations of the virus than their malnourished counterparts (Arsnoe et al., 2011; Latorre-Margalef et al., 2009). Although the physiological mechanism that regulates this process is still unknown, the host condition appears to not influence antibody production. Moreover, poor condition is associated with changes in the intestinal composition that decrease viral colonisation and propagation, probably due to a decrease in mucin glycoprotein and, consequently, viral receptors.

TRADE-OFFS IN THE ENERGETIC AND SPECIFIC NUTRITIONAL REQUIREMENTS DURING INFECTION

The nutritional regulation of the adaptive immune response appears to be much more complex than the simple link between good food resources, good condition and low pathogen susceptibility. It has recently been proposed that the quantity of nutrients does not limit immune response but rather the variation in the proportion of nutrients consumed by the host (Cotter et al., 2011). In fact, the multiple components of the immune system react differently to nutrient balance (Ponton et al., 2011). More specifically, complex trade-offs in energetic and specific nutritional resources produce cross-regulatory effects on immune system subcomponents, affecting host susceptibility against specific infections (Long and Nanthakumar, 2004). For example, high concentrations of leptin following adequate energy intake (resulting in a good condition state) leads to a more dominant Th1 immune response (protective against intracellular infections by microparasites) but down-regulates the Th2 response (protective against non-invasive infections produced by macroparasites). These cross-regulatory effects become even more interesting in the framework of co-infection since food restriction would influence the success of the entire parasite community in different directions (Pedersen and Fenton, 2007).

4.2.6. Conclusions

We should not forget that pathogen fitness increases with the number of nutritive resources extracted from the host and decreases with the host's immune response. Hence, pathogens must balance these two host components to maximise fitness. However, if infected organisms are ecological systems (Pérez et al., 2006), both the host and their pathogens share a wide range of nutrients required to support normal metabolism and growth. Thus, competition for specific resources can occur between pathogens and the cellular components of the host, so reducing the host's external supply should also lead to a reduction in pathogen growth rates (Smith et al., 2005). It is not surprising that hosts can be healthy despite high pathogen burdens, especially when infected by low virulent strains. The Spanish saying "*A perro flaco todo son pulgas*" (Delibes, 1979), does not always apply to wildlife diseases. Consequently, the principle of tolerance, the existence of different parasite strategies and the complex top-down and bottom-up regulations between pathogen communities and the host in the case of co-infections should be considered to

explain observations of a lack of influence of host condition on susceptibility and intensity of infection.

4.3. Study III.

The synzootic potential of common epidemics in chamois populations

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4.3.1. Abstract

Southern chamois (*Rupicapra pyrenaica*) is a medium-sized and gregarious mountain ungulate with populations affected by periodic outbreaks of border disease virus (BD), infectious keratoconjunctivitis (IKC), and sarcoptic mange (SM). Even though the impact of each disease on chamois populations has been described in detail, there is a lack of information about the potential impact of concomitant epidemics and the synzootic potential (co-occurring enzootic or epizootic processes producing worse health outcomes in wildlife) on chamois populations. Furthermore, whether a specific order of apparition of epidemics is more or less harmful for the host population is practically unknown not only for chamois but also for most mammal populations. Using a population viability analysis (PVA), we studied the consequences of multiple disease outbreaks with synzootic potential on growth rates and probabilities of extinction of virtual populations exposed to hard winters, density dependence, and co-occurring BD, IKC, and SM outbreaks. Such infections are not under cross-immunity nor density-dependent processes and thus are supposed to affect population demography independently. Heavy snowfalls are also likely to occur in our simulated populations. Our simulations showed that a second outbreak, even caused by a low virulent pathogen, causes an increase in the probability of extinction of the host population with regard to the first outbreak. IKC-BD- and SM-BD-affected populations had a higher risk of becoming extinct in 50 years confirming the extra risk of multiple outbreaks on the viability of the affected populations.

4.3.2. Introduction

For more than four decades now, infectious diseases have been recognized as a major demographic driver of wild populations. Virus (Sillero-Zubiri et al., 1996), bacteria (Foreyt and Jessup, 1982), fungi (Berger et al., 1998), or helminth (Goodman and Johnson, 2011)

outbreaks increase the extinction risk of wildlife (Pedersen et al., 2007), threatening global biodiversity (Daszak et al., 2000). Population collapses caused by canine distemper virus in Serengeti lions *(Panthera leo*) (Roelke-Parker ME et al., 1996) or Ebola in African apes (Leroy et al., 2004) are good examples of such deleterious impacts. Pathogens have the potential to affect almost every life-history trait of mammals including energy storage (Carvalho et al., 2015), fecundity and fertility rates (Rhyan et al., 2001; Sarasa et al., 2011), to fetus development (Aleuy et al., 2020), juvenile recruitment (Rossi et al., 2011) or adult survival (Pedersen et al., 2007). Further, the main mechanisms for disease-induced extinctions in wildlife are the pre-epidemic population size and the presence of reservoirs (De Castro and Bolker, 2005).

Even though outbreaks caused by different pathogens are not rare in the wild (Barnett et al., 2018), our knowledge about the impact of synzootics (i.e., co-occurring wildlife diseases) on mammal population demography is scarce. This synzootic concept derives from the term “syndemic”, used in human medicine to assess the consequences of múltiple diseases acting in tandem in a given socio-economic and environmental conditions on human populations (Singer et al., 2017). This syndemic point of view has barely been applied to wildlife (Sweeny et al., 2021), although the risk of suffering from multiple infections in variable environaments is the norm (Bordes and Morand, 2011; Munson et al., 2008) and thus the likelihood of potential synzootic interactions is great. Knowledge about the impact of infectious diseases on wildlife demographics is mainly based on outbreaks by single pathogens. Information about the impact of synzootic on wildlife is limited and often restricted to the impact of comorbidities. Only a few cases such as European wild rabbit (*Oryctolagus cuniculus*) populations affected by rabbit hemorrhagic disease (RHD) but previously exposed to myxoma virus (Mutze et al., 2002) are a good example of the potential of co-occurring epidemics on host population dynamics. With regard to

synzootics, the unprecedented mortalities of African lion populations affected by canine distemper virus (CDV) and *Babesia* spp. in very dry years are an excellent case (Munson et al., 2008).

Southern chamois (*Rupicapra pyrenaica*) is a mèdium-sized mountain ungulate classified as a least concern species by the International Union for the Conservation of Nature, with a global population number of around 50,000 (Herrero et al., 2021). Nevertheless, outbreaks of diseases such as sarcoptic mange (SM), infectious keratoconjunctivitis (IKC), and border disease (BD) affect and have caused dramatic declines in local populations of the Pyrenean (*Rupicapra p. pyrenaica*) and Cantabrian (*Rupicapra p. parva*) subspecies (Fernández-Aguilar et al., 2017; Fernández-Morán et al., 1997; Marco et al., 2007).

SM is caused by the burrowing mite *Sarcoptes scabiei* and is a contagious disease of mammals that induces an allergic-type skin reaction resulting in visible hypersensitive lesions and pruritus (Walton et al., 2004). Although sarcoptic mange epizootics usually do not affect long-term population dynamics, the net effect of mange can have serious conservation consequences in remnant or fragmented populations of threatened or endangered species including mountain ungulates (Pence and Ueckermann, 2002). Apart from the Cantabrian Mountains, SM has been reported to cause mortality in Alpine chamois (*Rupicapra rupicapra rupicapra*) in the Dolomite Alps in Italy (Rossi et al., 2007). Contrary to the typical assumptions of epidemiological models, SM dynamics in carnivores seem to be frequency- rather than density-dependent. In other words, disease transmission is mainly driven by behaviors mediating contact rates (Devenish-Nelson et al., 2014).

In Rupicapra species, social interactions (e.g., contact rates) depend more on social affinities than on any other factors (Crampe et al., 2021). However, in the last work on *Sarcoptes scabiei* transmission (Browne et al., 2021), the authors stated that high

population densities and local population sizes would be key factors for *Sarcoptes* transmission in chamois, but they also argued that the rates of contact within each species are poorly understood. So, this density-dependent transmission issue is unclear, and thus, we have decided to not include the density dependence in mange outbreaks in our viability modelling due to a lack of information for density dependence transmission parameters.

SM outbreaks duration is 5 years on average (Serrano et al., 2015). Mortality rates associated with SM are roughly 10.5% for kids, 14% for yearlings, 52.5% for adult females, and 60% for adult males (Fernández-Morán et al., 1997; Rossi et al., 2007).

IKC, on the other hand, is a highly contagious bacterial disease of the eye characterized by inflammation of the conjunctiva and cornea (Nicholas and Giacometti, 2012). *Mycoplasma conjunctivae* is considered the major cause of IKC in caprine species (Giacometti et al., 2002). IKC outbreaks are characterized by a short duration (1–2 years), high morbidity, low mortality (around 30%), and spontaneous recovery (Loison et al., 1996). After an IKC epizootic episode, the number of kid and adult females typically decreases between 10 and 19% (Arnal et al., 2013), recovering 1 year after the outbreak. Mortality rates associated with IKC are in 6% of kids, 70% of yearlings, 20% of females, and 9% of males (% of kids, 52% of yearlings) (Arnal et al., 2013; Loison et al., 1996).

On the other hand, BD is caused by a pestivirus (Frölich et al., 2012) and in chamois curses emaciation, depression, weakness and difficulties in locomotion (Marco et al., 2007) Pyrenean chamois population in the Pyrenees decreased by 30% due to disease outbreaks (Frölich et al., 2012), which are considered important drivers for chamois population demography (Serrano et al., 2015). Published reports (Fernández-Sirera et al., 2012; Marco et al., 2007) suggest that mortality rates associated with BD outbreaks are 50.5% for kids, 51.8% for yearlings, 45.7% for females, and 47% for males. The consequences of BD are

easily observed 5 years after the first clinical case is detected. The three aforementioned diseases do not induce cross-immunity, and there is no clear evidence for their density-dependent regulation (Fernández-Sirera et al., 2012).

4.3.3. Materials and Methods

In this work, we aimed to simulate the impact of multiple outbreaks on chamois population demography. Our objectives are (I) to explore the consequences of consecutive outbreaks of SM, IKC, and BD on chamois population viability and (II) to determine the specific outbreak pair with the greater demographic impact on the viability of our virtual chamois population. To achieve these objectives, we modelled the consequences of single (SM, IKC and BD) and specific disease outbreak combinations (SM + IKC, SM + BD and IKC + SM) on the viability of a virtual chamois population using a stochastic population viability analysis (PVA, (Lacy, 1993)). Since the impact of chronic BD epidemics is not well understood (Fernández-Sirera et al., 2012), we have decided not to include a secondary outbreak in BD-affected populations. We expect that multiple outbreaks will have greater negative effects than single outbreaks, but more particularly, those combinations involving the more virulent pathogens such as BD or SM. From now on, when we discussed about the extinction of the population in the simulation, we refer to the probability that a chamois population can become extinct locally rather than globally after a potential combination of different disease outbreaks.

Population viability analysis was performed in VORTEX 10.1.6.0 (Lacy, R.C., Pollak, 2015). This computer program simulates the effects of deterministic forces and stochastic events (demographic, environmental, and genetic) to model the growth rate (stoch-r), the final population size (N-all), and the mean probability of extinction (PE). To compare the impact of different simulations, we created a control scenario pristine population), only affected

by winter conditions (Serrano et al., 2015). The effects of heavy winters have also been included in our population viability modelling. This winter effect strongly relies on the local orography, but on average, population reduction due to this natural phenomenon could reach 40% every 10 years (Rughetti et al., 2011). In this scenario, the carrying capacity was fixed at 4000 individuals. We also recreated seven disease scenarios representing single outbreaks (IKC, SM, and BD), and outbreak combinations using the IKC-, SM-, and BD-associated mortalities at specific age classes. In brief, we modelled age and sex-specific mortalities based on the descriptions found in the published reports (Serrano et al., 2015). The likelihood of disease outbreak for each simulation is 0.2 for IKC (the commonest disease in chamois populations) and 0.1 for SM and BD. Two diseases can occur simultaneously or sequentially at the given probabilities. Since there is a likelihood of heavy winters during disease outbreaks, we consider our modelling might reflect the effect of synzootics (comorbidity + adverse environmental conditions, see (Sweeny et al., 2021).

Each model scenario was run for 50 years, 1000 iterations, and 20 initial population sizes proportional to the carrying capacity (from 5 to 100%, with an increment of 5% each time). The Vortex software, however, does not provide population-size-specific outputs (see Table 3.3.4.1 and Figure 3.3.4.1 for a summary). We used non-parametric Mann-Whitney U tests to compare the mean growth rate (stochr), the average population size of a given scenario at the end of the simulation (averaging both surviving and extinct iterations, N-all), and the probability of extinction (PE) between specific outbreak pairs. We performed all the statistical analyses using the statistical software R 4.2.1 (R Core Team, 2022).

The probability of extinction in the pristine scenario was equal to zero, and the final population size was stabilized around the carrying capacity as expected in populations with density dependence regulation (Akçakaya HR, Burgman MA, 1999).

4.3.4. Results

The effect of a single outbreak causes a significant decrease in the growth rate of our virtual chamois population. Compared to pristine populations, the mean probability of extinction after a single disease outbreak increased from 0.22 for SM to 0.53 for BD epidemics that means that 22% and 53% of the simulated populations get extinct, respectively. IKC outbreaks resulted in an intermediate probability of extinction value (0.25, see Table 3.3.4.1).

Table 4.3.4.1. Median, min, and max stochastic growth rates of the population, probabilities of extinction and final population sizes of a hypothetical chamois population of an initial size of 600 individuals and limited by a carrying capacity of 4000 individuals. Simulations were estimated for 50 years and 1000 iterations. The pristine population (control) was only affected by the carrying capacity, the IKC, SM and BD single outbreaks of infectious keratoconjunctivitis (IKC), sarcoptic mange (SM) and border disease (BD), and the IKC+SM, IKC+BD, SM+IKC and SM+BD combined disease outbreaks.

Scenario		**Population growth rate**		**Probability of extinction**		**Final population size**	
		Mean	**Interval (min-max)**	**Mean**	**Interval (min-max)**	**Mean**	**Interval (min-max)**
Pristine population		0.0635	0.0620 - 0.0650	0	-	3723.57	3282.24 - 3795.69
Single disease outbreaks							
	IKC outbreak	0.0463	0.0440 / 0.0540	0.250	0.241–0.263	2782.79	2180.39 / 2857.37
	SM outbreak	-0.0065	-0.0090 / 0.0080	0.229	0.217–0.780	1441.52	597.53 / 1594.47
	BD outbreak	-0.0326	-0.0370 / -0.003	0.534	0.524–0.571	767.86	345.74 / 865.86
Combined disease outbreaks							
	IKC + SM outbreaks	-0.0349	-0.0410 / -0.0090	0.47	0.370–0.549	619.67	245.59–711.92
	IKC + BD outbreaks	-0.0533	-0.0610 / -0.0160	0.634	0.594–0.700	343.22	126.57–430.72
	SM + IKC outbreaks	0.0323	0.0290 / 0.0410	0.253	0.241–0.264	1288.04	1590.07–1398.56
	SM + BD outbreaks	-0.0400	-0.0460 / -0.0100	0.559	0.537–0.617	585.72	213.31–667.66

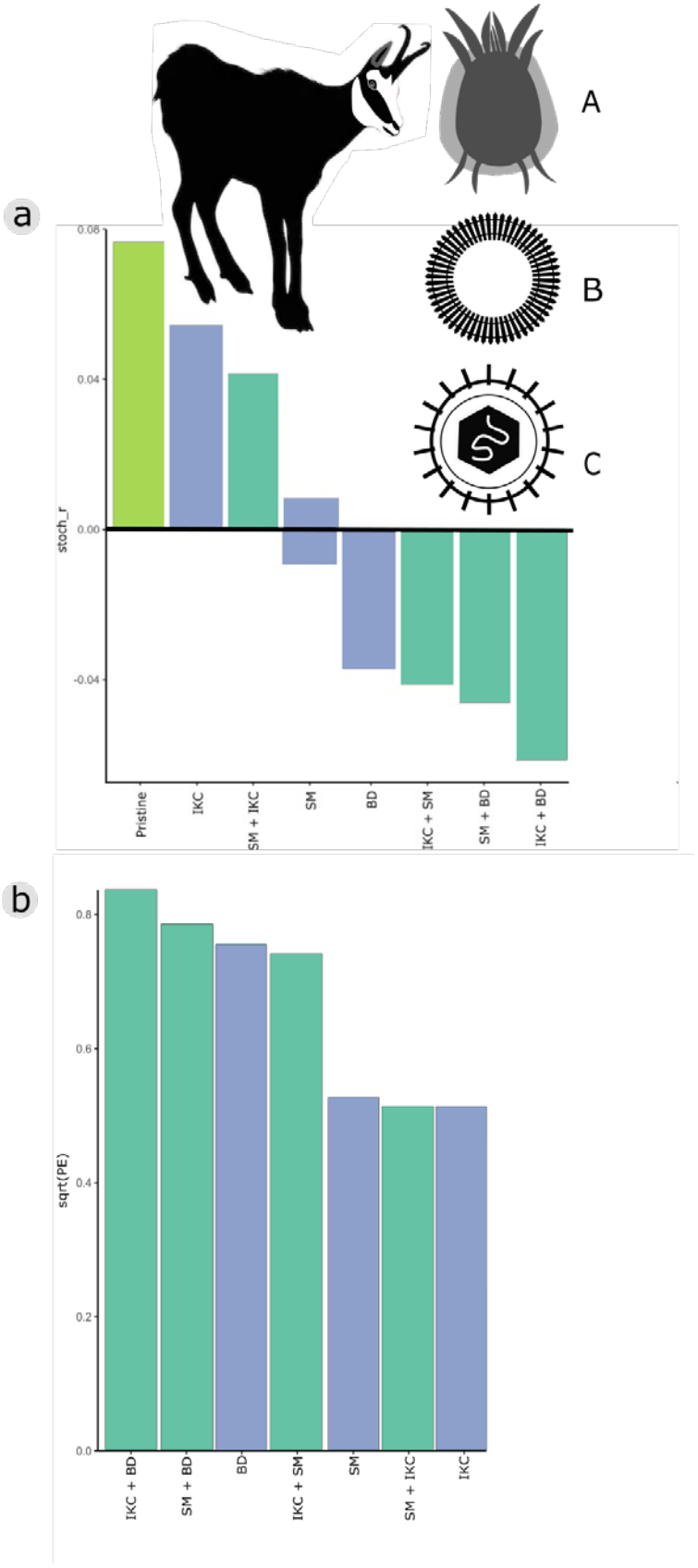


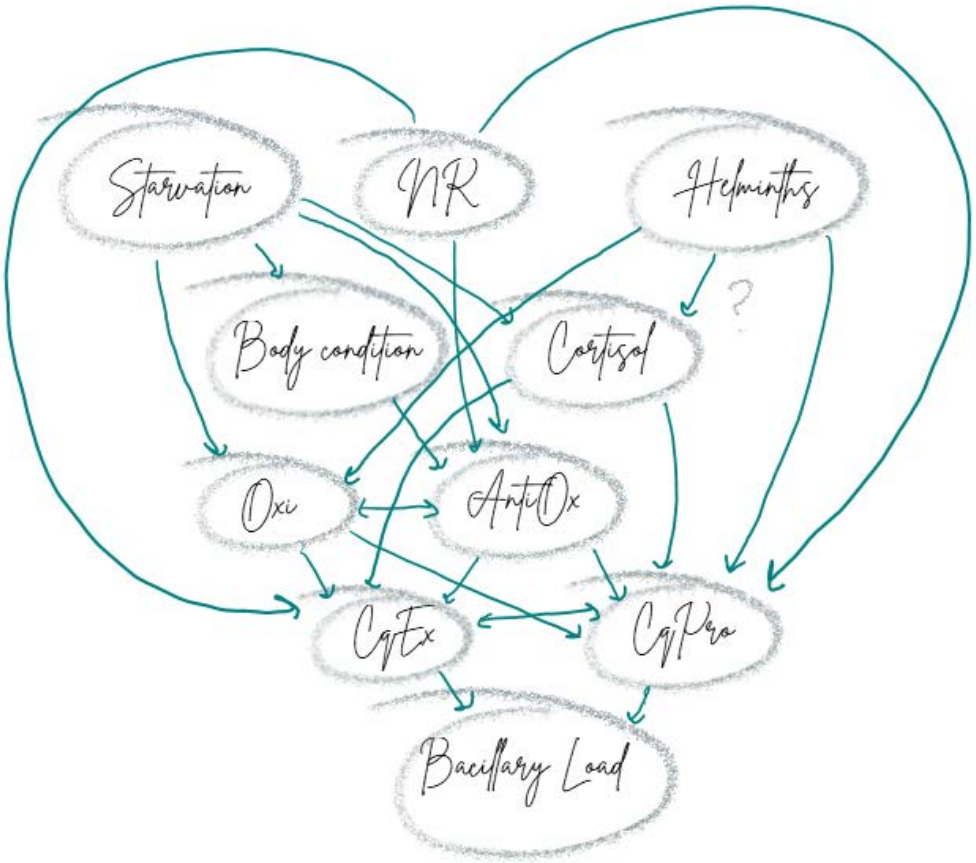
Figure 4.3.4.1. (a) Mean stochastic growth rates (stoch-r) and mean probabilities of extinction (PE, Fig. 1b), of a hypothetical chamois population of an initial size of 600 individuals and limited by a carrying capacity of 4000 individuals. Our modelling scenarios were the following: Pristine population (population only limited by the carrying capacity in light green), the single outbreak scenarios (in purple colour), and combined disease outbreaks (dark green). Infectious keratoconjunctivitis (IKC), sarcoptic mange (SM) and border disease (BD). In b) sqrtPE of our pristine chamois population was equal to zero.

Our population viability modelling clearly shows the negative impacts of a second disease outbreak. PE increased after a second disease outbreak but in particular after BD epidemics. Along the same lines, the growth rate and population size decreased after the second outbreak. For example, SM or BD outbreaks in chamois populations initially affected by IKC resulted in lower growth rate ($W_{IKC\ vs\ IKC\text{-}SM} = 400$, p value = 5.8 e-10, $W_{IKC\ vs\ IKC\text{-}BD} = 400$, p value = 6.02 e-10) and final population size ($W_{IKC\ vs\ IKC\text{-}SM} = 400$, p value = 1.45 e-11, $W_{IKC\ vs\ IKC\text{-}BD} = 400$, p value = 1.5 e-10), but higher PE ($W_{IKC\ vs\ IKC\text{-}SM} = 400$, p value = 6.73 e-08, $W_{IKC\ vs\ IKC\text{-}BD} = 400$, p value = 6.76 e-08) than those only affected by IKC. The same happened in SM-affected populations suffering BD outbreaks, where the growth rate ($W_{SM\ vs\ SM\text{-}BD} = 400$, p value = 5.1 e-08) and final population sizes ($W_{SM\ vs\ SM\text{-}BD} = 336$, p value = 9.2 e-07) are lower than in populations only affected by SM. Final population sizes in mixed epizootics decreased in 59% ($W_{SM\ vs\ SM\text{-}BD} = 400$, p value = 6.7 e-08). For example, the mean probability of extinction for IKC + SM outbreaks is 0.47, whereas = 0.25 or 0.22 for single IKC or SM epizootics.

4.3.5. Discussion

Despite the limitations of our work (e.g., some disease combination outbreaks have not yet been described in natural conditions, and we have only considered demographic consequences, but not transmission or recovery), it seems therefore clear that concomitant outbreaks have potential synzootic effects posing an additional threat to the viability of chamois populations previously affected by one of these three diseases. Interactions among co-infecting pathogens not only alter host pathology and disease spread at different levels of biological organization (Johnson et al., 2015), but also the long-term demography of the affected populations.

Managers in charge of chamois populations chronically affected by infectious diseases should take into account the demographic impacts of synzootics increasing efforts in disease surveillance to avoid new disease epidemics even caused by low virulent pathogens. Our results underline the importance of health surveys to forecast the potential consequences of synzootics on the local extinction risk of wild mammal populations.



4.4. Study IV.

Protective effect of intestinal helminthiasis against tuberculosis progression is abrogated by intermitent food deprivation

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4.4.1. Abstract

Background: Tuberculosis (TB) is still a major challenge for humankind. Because regions with the highest incidence also have a high prevalence of helminthiasis and nutritional scarcity, we wanted to understand the impact of these on TB progression.

Methods: We have developed an experimental murine model for active TB in C3HeB/FeJ, coinfected with *Trichuris muris* and *Heligmosomoides polygyrus* nematodes, and exposed to an environmental mycobacterium (*M. manresensis*) and intermittent fasting. Cause-effect relationships among these factors were explored with Partial Least Squares Path modelling (PLSPM).

Results: Previous parasitization had a major anti-inflammatory effect and reduced systemic levels of ADA, haptoglobin, local pulmonary levels of IL-1β, IL-6, TNF-α, CXCL-1, CXCL-5 and IL-10. Oral administration of heat-killed *M. manresensis* resulted in a similar outcome. Both interventions diminished pulmonary pathology and bacillary load, but intermittent food deprivation reduced this protective effect increasing stress and inflammation. The PLSPM revealed nematodes might have protective effects against TB progression.

Conclusions: Significantly higher cortisol levels in food-deprivation groups showed it is a stressful condition, which might explain its deleterious effect. This highlights the impact of food security on TB eradication policies and the need to prioritize food supply over deworming activities.

4.4.2. Introduction

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (Mtb) and is a major global health problem. In 2018, 10 million people fell ill with TB and it caused 1.4 million deaths worldwide (WHO, 2019). Most TB infection occurs in regions where parasitization by helminths is highly prevalent (Salgame et al., 2013).

Soil-transmitted intestinal parasites are also a global health problem, with *Ascaris lumbricoides*, *Trichuris trichiura* and *Necator americanus* infecting an estimated 804, 477 and 472 million people respectively worldwide (Gazzinelli-Guimaraes and Nutman, 2018). The response to helminth infection can be summarized as causing a bias towards Th2 and an increased Treg response, which modulates both Th1 and Th2, a bias that may be transmitted to infants *in utero* (Blackwell, 2016). Due to the importance of Th1 responses in controlling Mtb infection, it is constantly questioned whether to advocate for a deworming policy or not as a coincidental factor to improve TB vaccination programs. Equally, it is presumed that people in these regions have a high level of contact with environmental mycobacteria, which has a clear impact in the immune response against Mtb. In fact, it is one of the reasons believed to explain the failure of the protection induced by BCG vaccination in several settings (Dockrell and Smith, 2017a), either because exposure to environmental mycobacteria provides some protective immunity to TB (Andersen and Doherty, 2005; Palmer and Long, 1966) or because it blocks the replication of BCG and thus its protective effect (Andersen and Doherty, 2005; Brandt et al., 2002).

Additionally, food insecurity is also common in these regions (FAO et al., 2018). Body mass index (BMI) below 18.5 is a well-known risk factor for TB progression (Leung et al., 2007) due to the reduction of essential nutrients required to build an appropriate immune response. However, little is known about the effect of intermittent food deprivation, as occurs in a

state of food insecurity, and the impact of the stress it causes, even when an overall malnutrition has not been reached.

Surprisingly, our data shows a protective effect of intestinal helminthiasis related to its anti-inflammatory properties, which is abrogated by intermittent food deprivation, thus reinforcing the importance of food security in any TB eradication program.

4.4.3. Materials and Methods

4.4.3.1. Animals

A total of 24 female and 36 male C3HeB/FeJ specific-pathogen-free mice (8–10 weeks old) were obtained from Germans Trias i Pujol Research Institute stock. All procedures were conducted in a BSL-3 facility, according to protocol DMAH6119. This was reviewed by the Animal Experimentation Ethics Committee of the Hospital Universitari Germans Trias i Pujol (registered as B9900005) and approved by the Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural of the Catalan Regional Government, according to current national and European Union legislation regarding the protection of experimental animals. Mice were supervised daily following a strict monitoring protocol to ensure animal welfare and euthanized, if required, by cervical dislocation after anesthesia with isoflurane.

4.4.3.2. Experimental Design

Animals were divided into 6 experimental groups (Figure 3.3.4.1), 10 mice per group evenly matched in sex and weight.

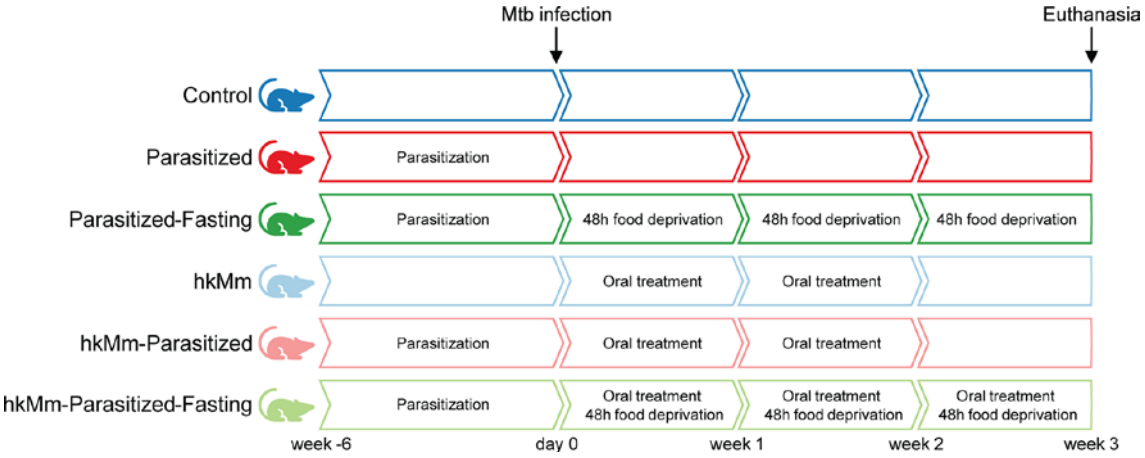

Figure 4.4.3.1. Experimental design. Six groups of 10 animals were used to evaluate the influence of intestine parasitization, intermittent fasting and oral exposure to environmental mycobacteria in an active TB model.

Four groups were orally infected by gavage, twice, with 0.2 mL of a solution containing 30 *Trichuris muris* eggs and 200 *Heligmosomoides polygyrus* larvae as previously done (Johnston et al., 2015; Wakelin, 1967). Once the parasitization had been confirmed by coprologic examination, 6 weeks after parasite infection, all animals were infected with 2×104 CFU (colony-forming units) of *M. tuberculosis* H37Rv Pasteur strain via the caudal vein (Marzo et al., 2014).

Environmental mycobacterial treatment started one day after Mtb infection. Mice received oral treatment with 105 CFU of heat-killed *M. manresensis* (hkMm) (Rech et al., 2015) in a galenic formulation with mannitol. Control groups received mannitol in the same concentration. A total of 7 doses were administered, every other day, for 14 days.

Two groups of animals were submitted to intermittent fasting throughout the experiment, with a weekly regime of 5 days of normal diet and 2 days of food deprivation (Jensen et al., 2013). Otherwise, mice received food and water ad libitum.

Animal weight was recorded every 2-3 days. Fecal samples were obtained from each cage weekly to quantify parasite eggs. After 3 weeks from Mtb infection, mice were anesthetized with isoflurane inhalation and blood samples were obtained through cardiac puncture. They

were then euthanized by cervical dislocation. Lungs, spleen, liver, kidneys and intestines were retrieved. Kidneys and liver were weighed in a precision balance with 0.0001 g accuracy.

4.4.3.3. Bacillary Load (BL)

Spleen and left lobe lung samples from each animal were collected, homogenized and 10-fold serial dilutions were made. 100 µL of these dilutions were plated on nutrient Middlebrook 7H11 agar (BD Diagnostics, Sparks, USA). Visible CFU were counted after incubation for 28 days at 37°C. Data was analyzed as CFU per mL.

4.4.3.4. Lung Pathology

Right caudal lung lobe samples were fixed in 10% buffered formalin, embedded in paraffin and 5-µm sections stained with hematoxylin-eosin for microscopic observation. Two paraffin blocks per group were obtained, each containing samples from 5 animals. Four recuts of every block were used to determine the damaged area as a percentage of total lung area. This analysis was done using the NISElements D version 3.0x software package (Nikon Instruments Inc., Tokyo, Japan).

4.4.3.5. Oxidative Stress, Inflammatory Mediators and Cortisol in Serum

Blood samples were extracted in tubes containing clotting activator (Sarstedt, Nümbrecht, Germany). After centrifugation, sera were obtained and stored at -20°C until analysis.

Total antioxidant capacity was determined by four methods: trolox equivalent antioxidant capacity (TEAC1 and TEAC2), ferric reducing ability of plasma (FRAP) and cupric reducing antioxidant capacity (CUPRAC), as previously reported (Tvarijonaviciute et al., 2017). Thiol was determined using method reported by Jocelyn (Jocelyn, 1987). PON1 activity was analyzed following previously described method (Tvarijonaviciute et al., 2012) and advanced oxidation protein product (AOPP) concentrations as described by Witko-Sarsat

(Witko-Sarsat et al., 1996). All procedures were performed in an automated biochemistry analyzer (Olympus AU600, Beckman Coulter, Brea, USA). Reactive oxygen species (ROS) were estimated by luminol-mediated chemiluminescence assay (Vong et al., 2014) using a microplate reader (Victor 2 1420 Multilabel Counter; PerkinElmer, Finland).

Total adenosine deaminase (ADA), albumin and haptoglobin were measured using commercially available kits (Diazyme Laboratories, Poway, CA, USA; Beckman Coulter, USA and Tridelta Development, Ireland, respectively) in an automated biochemistry analyzer (Olympus AU600, Beckman Coulter, Brea, USA). Cortisol was analyzed following Arantes-Rodrigues et al. (Arantes-Rodrigues et al., 2012) and using a commercially available solid-phase, competitive chemiluminescence enzyme immunoassay (COR Cortisol, Siemens Health Diagnostics, Deerfield, IL, USA) in the automatic analyzer (Immulite 1000, Siemens HealthcareDiagnostic, Deerfiels, IL, USA).

4.4.3.6. Lung Immune Response

A cytokine profile study was performed in lung homogenates from right cranial and middle lobes. The following cytokines were measured by Luminex xMAP® technology: IFN-γ, TNF-α, IL-1β, IL-6, IL-10, IL-12(p40), IL-13, IL-17, CXCL-1 and CXCL-5. Results are expressed as pg per mL of homogenate. The assay was performed with the MILLIPLEX® MAP kit (EMD Millipore Corporation, Billerica, MA, USA) following the manufacturer's instructions and analyzed with xPONENT Software (Luminex Corporation, Austin, TX, USA).

4.4.3.7. Data Analysis

Multiple Comparisons

The scores of the first dimension (PC1) of a principal component analysis (PCA) on liver and kidneys weights were used as proxy for body condition in mice. Likewise, the PC1 of a PCA for the antioxidant (TEAC1, TEAC2, FRAP, CUPRAC, Thiol and PON1) and oxidant (AOPP and

ROS) biomarkers was used as proxy for the oxidative stress profile. The same approach was followed for lungs immune response (analytes detailed in section 2.6). Differences in the PCA scores among experimental groups were explored with ANOVA and a Holm-Sidak post-hoc multiple comparison tests (Blakesley et al., 2009; Olifiers et al., 2015).

On the other hand, a Mann-Whitney test was used to compare damage area and BL among experimental groups. The same statistical test was used to compare differences among treatments in concentration of oxidative stress mediators in serum and cytokines/chemokines in lungs.

PCA was performed using the package "FactomineR" version 2.0 (Lê et al., 2008) of the statistical software R 3.6.2 version (R Core Team, 2022). Graphpad Prism (GraphPad software v7.0, La Jolla, California, USA) was used for graphics and simple statistics, with differences of $p<0.05$ being considered statistically significant. Statistically significant differences are summarized as follows: *$p<0.05$, **$p<0.01$, ***$p<0.001$.

PLS Path Modeling

We used Partial Least Square Path Modelling (PLS-PM) (Aleuy et al., 2020; Sanchez, 2013; Serrano et al., 2014; Tenenhaus et al., 2005) to explore the association of intermittent fasting, helminth infection and hkMm with Mtb load. Briefly, this approach quantifies the network relationship between a set of unobservable latent variables (LV) and a set of parameters directly measured (manifest variables or MV). The LVs are conceptual variables defined by one or several MVs and organized in a network of relationships where the connections among LVs are assumed to represent a cause-effect process called inner model. The links among LV are quantified through path coefficients while the links between LV and MV are quantified through weights (Hair et al., 2013).

Our PLS-PM included 28 MVs organized into 10 LVs, described in Supplementary Table S 3.4.6.1. The initial inner PLS-PM model is shown in Supplementary Figure S 3.4.6.1. The assumed direct effects for the model structure are detailed in Supplementary Table S 3.4.6.2. Even though PLS-PM is distribution free, some variables (IFN-γ, IL-10, IL-12, IL-17, parasite burden and BL) were log-transformed to improve the model fit. After fitting the first model including all the variables, we performed a simplification removing those MVs uncorrelated with their own LVs (Supplementary Table S 3.4.6.3), following Sanchez (Sanchez, 2013). Finally, we also estimated the partial contribution of each LV to the final PLS-PM as well as the goodness of fit [GOF, (Tenenhaus et al., 2004)]. PLS-PM analysis was performed using the package 'plspm' version 0.4.9 (Sanchez, 2013) of the statistical software R 3.6.2 version (R Core Team, 2022).

4.4.4. Results

4.4.4.1. Intermittent Fasting Had a Low Impact on Body Condition

Fasting mice reduced their body weight in a transitory manner (Figure 3.4.4.1A). Nonetheless, the effect of the intermittent fasting was evidenced in liver and kidney weight at the final endpoint. This data was used to perform a PCA, showing that both fasting groups had statistically significant lower scores of PC1 (Figure 3.4.4.1B, C).

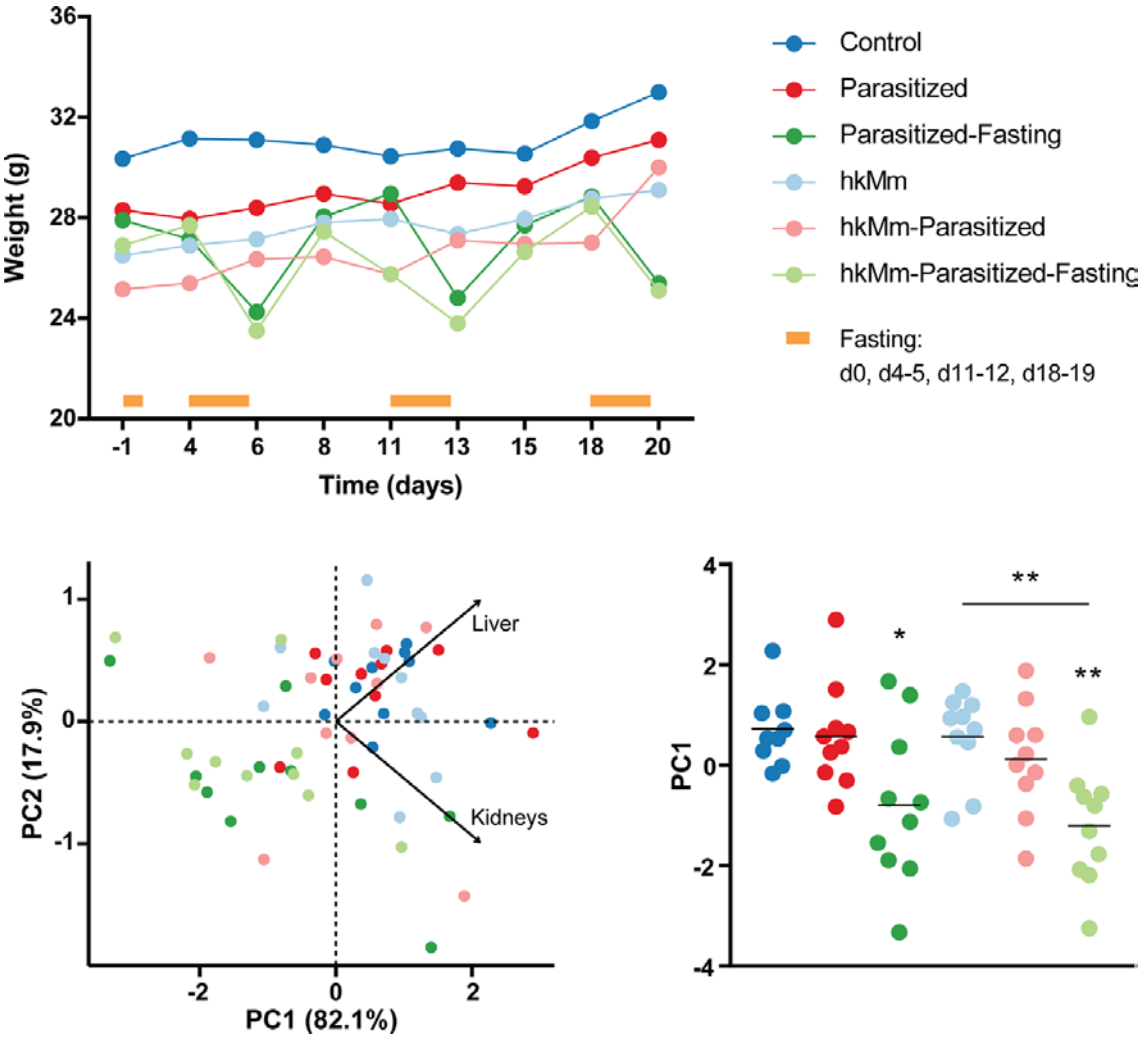

*Figure 4.4.4.1. Change of animals' body condition. (A) Median weight of mice as infection progresses, infection day being time 0. (B) PCA based on liver and kidneys weight at end-point time. (C) PC1 scores, each circle represents an animal and lines are means; ANOVA p-value=0.0004, Holm-Sidak's multiple comparisons test (*p < 0.05, **p < 0.01). hkMm, heat-killed M. manresensis.*

Albumin was also measured in sera, but there were no statistically significant differences between groups (Supplementary Figure S 3.4.6.2).

The number of eggs per gram of feces was determined weekly for samples from each cage. At the endpoint of the experiment, the total number of parasites was determined in the intestines of each animal. Neither measurement showed significant differences between groups (Supplementary Figure S 3.4.6.3).

4.4.4.2. Intestinal Parasites and Oral hkMm Reduced Lung BL and Pathology

The damaged area in lungs was reduced with parasitization and oral hkMm (Figure 3.4.4.2A). Representative lung sections of each group are shown in Supplementary Figure S 3.4.6.4. In agreement with these results, the Mtb load in lungs was significantly reduced in the same groups (Figure 3.4.4.2B). This protective effect against the progression of active TB was not observed in fasting animals. There were no differences between groups for BL in spleen (Supplementary Figure S 3.4.6.5).

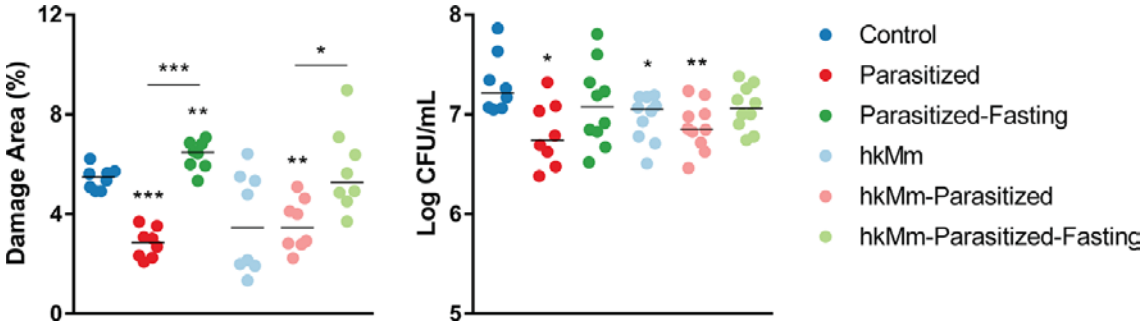

*Figure 4.4.4.2. Damage area (A) and pulmonary bacillary load (B) at week 3 post-infection. Each circle represents a "recut" (A) or an animal (B) and lines show medians. Mann-Whitney test: *p<0.05, **p<0.01, ***p<0.001. hkMm, heat-killed M. manresensis.*

4.4.4.3. Fasting Induced Antioxidant Activity

Oxidative stress was measured in different ways in sera (Supplementary Figure S 3.4.6.6). When compared with control animals, parasitized mice had a significant increase in TEAC2, CUPRAC and FRAP. This was also the case in parasitized-fasting, hkMm and hkMm-parasitized-fasting groups. Fasting animals also showed increased TEAC1 and reduced PON1, regardless of the oral treatment, and increased thiol and reduced ROS when not receiving hkMm. The hkMm group was the only one that presented lower levels of AOPP than control mice. The hkMm-parasitized group had no statistically significant differences from controls.

These results were used to perform a PCA (Figure 3.4.4.3A). The first two components explained 71.1% of the variation in the data set. Fasting induced a significant increase in the PC1 score (Figure 3.4.4.3B), which was mainly explained by TEAC1, CUPRAC, TEAC2, FRAP and thiol variation (Figure 3.4.4.3C).

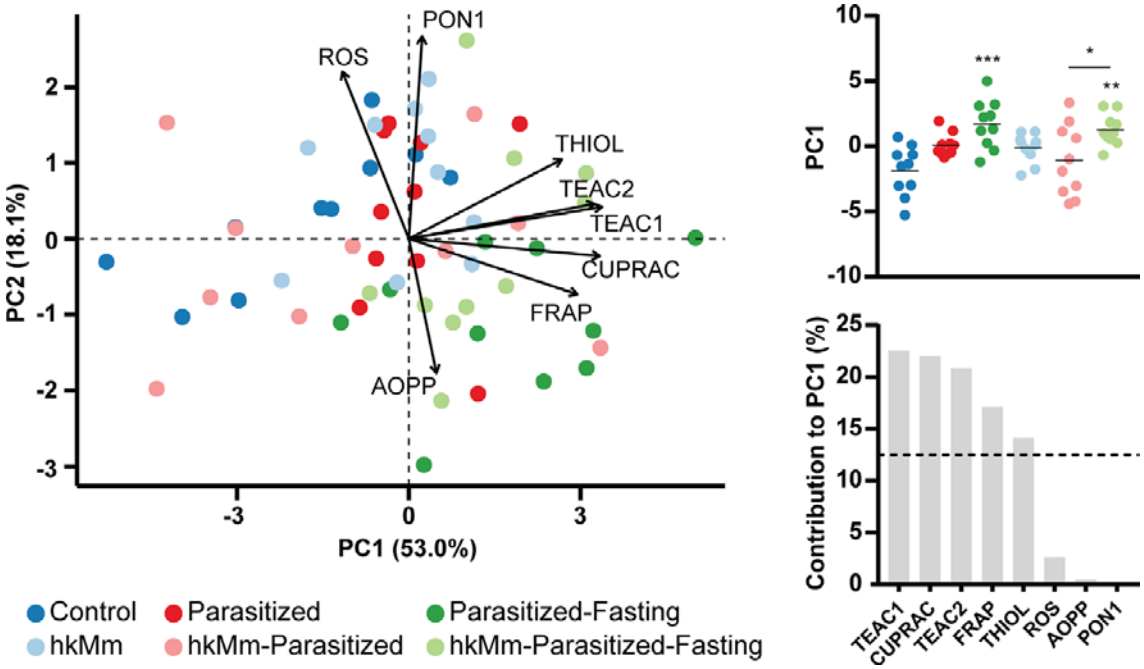

*Figure 4.4.4.3. Oxidative stress analysis at week 3 post-infection. (A) PCA based on mediator concentration in serum. (B) PC1 scores, each circle represents an animal and lines are means; ANOVA p-value=0.0001, Holm-Sidak's multiple comparisons test (*p < 0.05, **p < 0.01, ***p < 0.001). (C) Mediators contribution to PC1. hkMm, heat-killed M. manresensis; TEAC, trolox equivalent antioxidant capacity; CUPRAC, cupric reducing antioxidant capacity; FRAP, ferric reducing antioxidant power; PON1, paraoxonase 1; ROS, reactive oxygen species; AOPP, advanced oxidation protein products.*

4.4.4.4. Intestinal Parasites and Oral hkMm Reduced Inflammation

ADA, haptoglobin and cortisol were measured in sera (Figure 5). ADA was significantly reduced when compared with control animals in all groups except parasitized-fasting. The administration of hkMm in parasitized groups also showed a reduction in ADA levels when compared with their control counterparts. Parasitization induced a reduction of haptoglobin levels, but only under normal feeding conditions. Cortisol was significantly increased in parasitized-fasting animals.

Lung homogenates were used to quantify various cytokines and chemokines (Supplementary Figure S 3.4.6.7). Parasitized animals showed a statistically significant reduction in TNF-α, IL-10, IL-1β, IL-6, CXCL-1 and CXCL-5 levels. Mice receiving oral hkMm had lower levels of TNF-α, IL-1β, IL-6, IL-17, CXCL-1 and CXCL-5. The levels of IL-13 were below the detection limit in all the samples (data not shown).

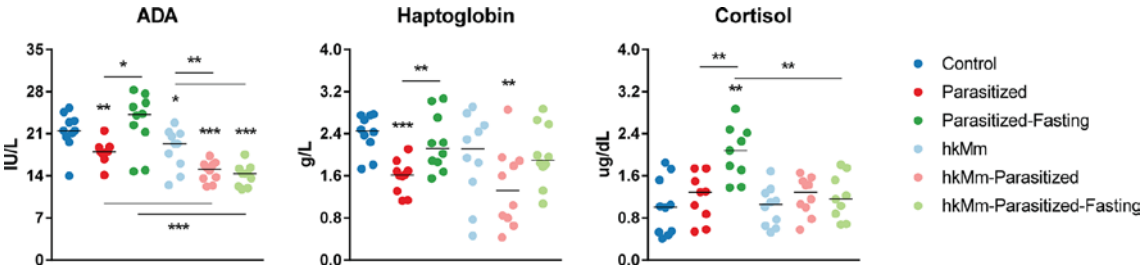

*Figure 4.4.4.4. Adenosine deaminase (ADA) (A), haptoglobin (B) and cortisol (C) levels in serum at week 3 post-infection. Each circle represents an animal and lines are medians, Mann-Whitney test (*p<0.05, **p<0.01, ***p<0.001). hkMm, heat-killed M. manresensis.*

The PCA analysis of lung cytokines and chemokines showed the first two components accounted for 70.9% of the data set variation (Figure 3.4.4.5A). The PC1 score, based mainly on levels of IL-1β, CXCL-1, IL-6 and TNF-α, related to the induction of exudative lesions, was significantly reduced in the parasitized animals (Figure 3.4.4.5B, C).

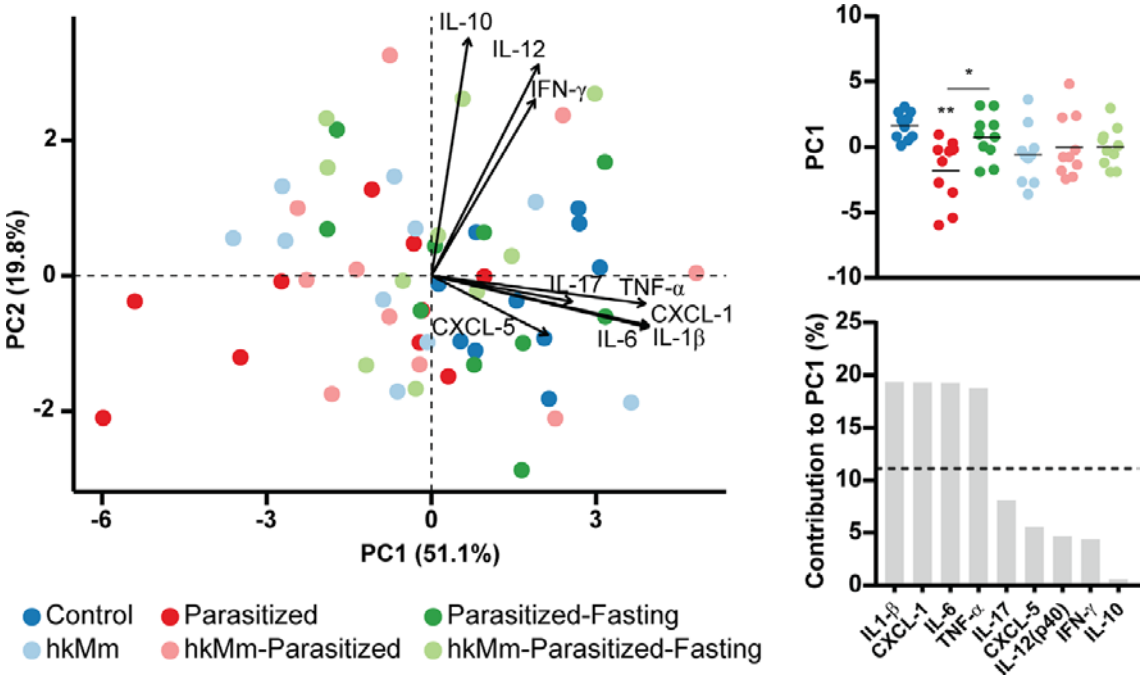

*Figure 4.4.4.5. Immune mediator analysis at week 3 post-infection. (A) PCA based on cytokines concentration in lung homogenates. (B) PC1 scores, each circle represents an animal and lines are means; ANOVA p-value=0.0067, Holm-Sidak's multiple comparisons test (*p < 0.05, **p < 0.01). (C) Mediators contribution to PC1. hkMm, heat-killed M. manresensis.*

4.4.4.5. PLS Path modeling

According to the initial model (Supplementary Table S 3.4.6.4), fasting increases helminth load, both fasting and helminths increase cortisol. Body condition is affected by helminths and fasting. Antioxidants appear increased in fasted mice and oxidation is reduced by the effect of antioxidants. Proliferative lesions were stimulated under food restriction. The bacillary load LV was negatively related to hkMm, helminths and cortisol, but positively related to exudative lesions and antioxidants. However, only exudative lesions were significantly related to BL in the lungs.

After removing the non-significant effects, antioxidant and oxidant LVs were isolated from the path, so these blocks were excluded from the refined model. The LV proliferative lesions was not related to bacillary load and hence also removed from the model. The final refined

model shows both hkMm and helminths reduce exudative lesions that in turn are responsible for higher bacillary loads (Figure 3.4.4.6).

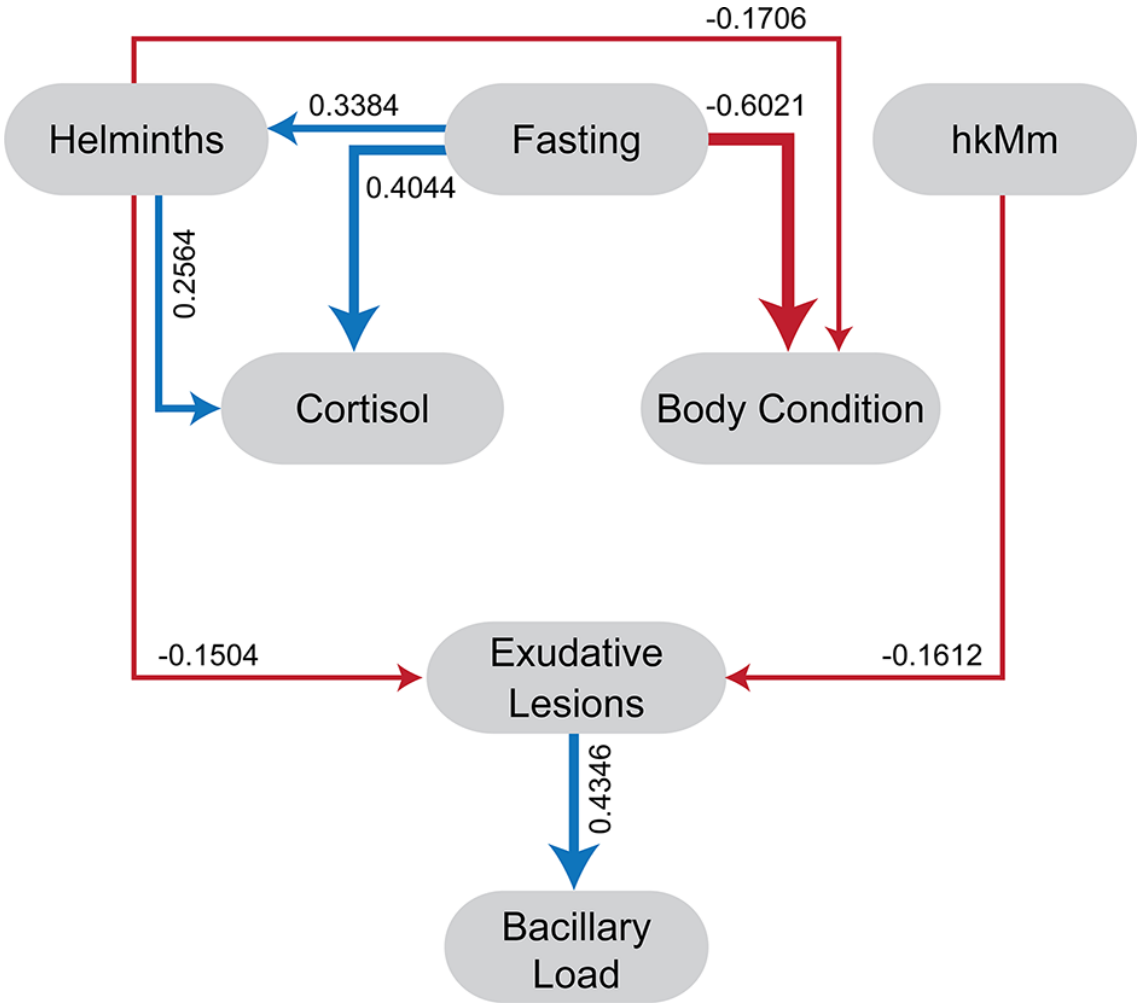

Figure 4.4.4.6. Refined partial least square path model. Red arrows indicate a negative relation among latent variables (path coefficient bellow 0) and blue arrows indicate a positive one (path coefficient over 0).

The goodness of fit for the model was 0.39 (39% of fit). The LV body condition had the greatest impact (46.1%) on the explained variability, followed by cortisol (29.9%) and bacillary load (18.8%).

4.4.5. Discussion

In this work, we aimed to study the relation between three important factors affecting global health: tuberculosis, intestinal helminthiasis and food scarcity. Our data showed a

protective effect of parasitization in the outcome of Mtb infection, which was abrogated by intermittent food deprivation.

Contrary to our findings, most data from cohort studies suggest an increased susceptibility to developing TB when coinfected. Helminthiasis infection has been linked to pulmonary TB in a cohort study that compared infected individuals with a control group (Tristão-Sá et al., 2002). In another study, patients with TB and coinfected also presented a more advanced disease (Resende Co et al., 2007). Lower IFN-γ and higher IL-10 responses were found in coinfected TB patients after stimulation of whole-blood with mycobacterial antigens. Among pediatric household contacts of smear positive TB patients, a positive tuberculin skin test was significantly associated with coinfection (Verhagen et al., 2012). This was interpreted as helminth infection increasing the risk of acquiring latent tuberculosis infection (LTBI). This is in concordance with the improvement of Mtb-specific immune responses in PBMCs after deworming treatment in LTBI persons (Elias et al., 2001). All in all, these results are consistent with the attenuation of Th1 responses caused by helminths. However, even though the host's defense against Mtb requires a Th1 response, it is not a direct correlate of protection (Dockrell and Smith, 2017b). In fact, despite presenting higher levels of Tregs and Th2 cytokine response, helminth coinfection was related with a lower sputum smear-positivity (Abate et al., 2015). Recent data obtained from healthy migrants from Nepal, shows a negative correlation between hookworm infection and LTBI. This has been linked to the increased mycobacterial growth inhibition in the blood of hookworm-infected individuals, leading to a reduced risk of Mtb infection (O'Shea et al., 2018).

A protective effect of *H. polygyrus* infection in air-borne infections, such as respiratory syncytial virus (McFarlane et al., 2017) and *P. aeruginosa* (Long et al., 2019), has been reported in mice. Several authors have tried to understand the impact of helminthiasis on

the progression of TB using experimental models. The design of these studies should be considered to evaluate their relevance. First, most of them have been carried out in mouse strains (mainly BALB/c and C57Bl/6) that develop a sort of chronic infection based on the induction of proliferative lesions, with a strong Th1 response. Thus, they do not develop exudative lesions, characterized by neutrophilic infiltration, which are the key for developing liquefacted lesions with the capacity to become cavitated (Cardona, 2015). So far, the only mouse strain able to develop such lesions is the C3HeB/FeJ, precisely the one used in our study (Marzo et al., 2014). Also, several studies used *M. bovis* BCG as a surrogate of M. tuberculosis infection, thus developing a very attenuated course of infection. Thirdly, it is important to clarify which helminth species have been used. There are several studies where there is a combination of intestinal helminths, with and without a pulmonary phase in their cycle, or even filarial worms (affecting mainly the lymphatic system) thus probably reflecting different scenarios (Budischak et al., 2018; Hübner et al., 2012; Nel et al., 2014; Obieglo et al., 2016). Therefore, by changing the coinfection helminth we may have an alternative immunomodulatory effect, resulting in a new outcome than the one obtained in this experiment. The order of co-infection between nematodes and among nematodes and TB is also a determinant factor (Tompkins et al., 2011).

We have evaluated the parasitization with *T. muris* and *H. polygyrus*, which only affect the intestinal mucosa. We decided to use two nematodes since it is a common situation in nature (Alemu and Mama, 2017; Behnke et al., 2009). Our results within the PLS-PM indicate that, although both species induce similar immune response (Filbey et al., 2019), there was a greater effect of *T. muris* with respect to the *H. polygyrus* counterpart, evidenced in the higher weight on the helminth LV. A recent study with *M. bovis* murine coinfection showed that even when *T. muris* parasitization led to a Th2 immune background, it did not influence *M. bovis* BCG proliferation and dissemination. Coinfection increased the percentage of

CD4+, CD4+IL4+ and CD8+IL4+ cells and decreased TNF-α secretion after BCG stimulation in the spleen when compared with the BCG infection alone. Detection of mRNA in the lung showed that coinfection decreased the expression of TGF-β, Foxp3 and IFN-γ, when compared with BCG infection alone (Nel et al., 2014). In our study, we have not been able to detect the parasitization effect on the Th2 response. However, matching the lower levels of Mtb load, these mice did present a reduction in the pro-inflammatory response in lungs. This was also observed with the oral administration of hkMm, which additionally reduced IL-17 levels, as already described (Cardona et al., 2016). PLS pathway modeling supported the role of helminths and hkMm in the reduction of exudative lesions, and thus reduction of the BL. The levels of IL-10 were also lower in parasitized animals, suggesting a reduction in the regulatory response, as in Nel et al. (Nel et al., 2014). Furthermore, levels of IFN-γ did not experience significant differences between experimental groups, thus dismantling the idea of a Th2-based response in parasitization competing for the Th1 immune response. The acute-phase protein haptoglobin was reduced in sera of parasitized animals, once again pointing to an anti-inflammatory effect.

Recent estimates of hunger and food insecurity are that 821 million people are undernourished, more than 10% of the world population. This appears to be increasing in almost all regions in Africa and South America, while it is stable in most regions of Asia. A more striking fact is that the number of undernourished has been rising since 2014 (FAO et al., 2018). A low BMI is a risk factor for TB development (Leung et al., 2007), supported by several experimental models. BALB/c mice receiving 80% of the usual amount of food consumed had an impaired response to a TB vaccine (Ishikawa et al., 2009). The administration of a low-protein-diet in Mtb infected mice increased lung BL, both in BCG-vaccinated or control animals (Hoang et al., 2015). Another approach to this issue has been the study of Mtb infection in mice not expressing leptin, an adipokine reduced in

malnutrition and fasting (Maffei et al., 1995). This Th1 inducer increases with Mtb infection and mice lacking leptin have higher BL in advanced stages of infection (Wieland et al., 2005). This was accompanied by increased numbers of PMNs in lungs. Little is known of the impact of intermittent fasting, a circumstance resembling an aspect of food insecurity. Food deprivation experiments have been carried out in mice. In our case, 48 hours deprivation induced weight reduction, which was recovered with a normal diet for five days, and an increase in oxidative stress. This regimen did not cause a reduction in albumin levels, suggesting that reduction of protection was not due to protein deprivation. Interestingly, intermittent fasting was related to a cortisol increase, probably caused by stress. Elevated levels of cortisol are linked to neutrophilia (Ronchetti et al., 2018), which can increase neutrophilic infiltration in Mtb lesions, as has already been described in asthma patients (Fukakusa et al., 2005). However, hkMm appears to balance this reaction as hkMm-Parasitized-Fasting animals had the same levels of cortisol as control mice.

ADA has a proinflammatory effect, by inducing Th1 response and reducing extracellular levels of adenosine (Cortés et al., 2015). Parasitized-Fasting animals were the only ones that did not present lower levels of ADA than control. This matches the results in lung pathology and BL, but once again animals treated with hkMm had a distinct outcome. It appears that hkMm gave some level of protection against intermittent fasting, although it was not strong enough to be evidenced in the progression of TB in this model.

Even though the goodness of fit our PLS-PM model could be considered moderate (28, 29), it is enough to evidence the causal relationship among starvation, parasite load, environmental mycobacteria, exudative lesions (ExL), and bacillary load. Heat-killed *M. manresensis* and helminths contributed equally to diminish exudative lesions and thus bacillary load as suggested by previous research in mice (Gil et al., 2006) and cattle (Menin

et al., 2013). Further investigation, however, should be conducted to elucidate the drivers of this protective effects beyond the immunological pathway used in our research.

To our knowledge, this is the first time that the impact of parasitization by intestinal helminths in the progression of active TB has been studied in an experimental model, considering important factors like food fasting and exposure to environmental mycobacteria. Our data shows an unprecedented protective impact of intestinal parasitization, which is abrogated by intermittent food fasting. This data should be considered when contemplating deworming policies and highlights the importance of good nutrition in the fight against TB.

4.4.6. Supplementary material

Table S 3.4.6.1. Description of both latent and manifest variables used for the model. [1]Mean ± SD (Range).

Latent variable	Manifest variable	Descriptive statistics[1]
Fasting	Categorical variable with two modalities: 0 = not food deprivation 1 = food deprivation	0.333 ± 0.475 (0 - 1)
Heat-killed *M. manresensis* (hkMm)	Categorical variable with two modalities: 0 = mannitol treatment 1 = hkMm treatment	0.5 ± 0.504 (0 - 1)
Helminths	*Heligmosomoides polygyrus*	10.00 ± 18.394 (0.00 - 89.00)
	Trichuris muris	6.367 ± 7.474 (0.00 - 30.000)
Cortisol	Cortisol	1.289 ± 0.544 (0.408 - 2.870)
Body condition	Liver weight	1.449 ± 0.274 (0.881 - 1.995)
	Kidneys weight	0.460 ± 0.153 (0.037 - 0.818)
Anti-oxidants	FRAP	0.949 ± 0.155 (0.571 - 1.353)
	TIOL	0.315 ± 0.077 (0.129 - 0.452)
	CUPRAC	0.539 ± 0.072 (0.369 - 0.719)
	TEAC2	1.083 ± 0.053 (0.946 - 1.215)
	TEAC1	0.737 ± 0.087 (0.525 - 0.943)
	PON1	10.214 ± 1.569 (7.230 - 12.930)
Oxidants	ROS	363.0 ± 78.063 (219.0 - 582.0)
	AOPP	128.64 ± 46.495 (37.80 - 293.40)
Proliferative lesions	IL-10	31.03 ± 24.357 (10.16 - 125.35)
	IL-12(p40)	21.29 ± 9.487 (4.26 - 55.42)
	IFN-γ	101.22 ± 51.037 (46.83 - 361.52)
Exudative lesions	IL-1β	202.78 ± 148.678 (27.00 - 813.98)
	IL-6	1202.9 ± 1181.824 (124.0 - 6417.7)
	TNF-α	103.94 ± 63.827 (4.98 - 331.91)
	CXCL5	302.7 ± 123.245 (107.4 - 678.0)
	IL-17	49.32 ± 57.635 (7.12 - 382.12)
	CXCL1	3252.5 ± 1986.255 (558.5 - 8580.7)
	ADA	18.25 ± 4.315 (11.78 - 28.27)
	Haptoglobin	1.921 ± 0.668 (0.430 - 3.070)
Bacillary load	Lung	14064125 ± 13072975 (2400000 - 73000000)
	Spleen	4061116 ± 3546899 (560000 - 18000000)

Table S 3.4.6.2. Direct effects expected between latent variables.

Latent variable	Direct effects on LVs	References
Fasting	Resource limitation could affect helminths, cortisol, body condition, oxidants, antioxidants, proliferative and exudative lesions	Budischak et al., 2015 Int. J. Parasitol 45 (7): 455-463 Budischak et al., 2018 Front. Immunol. 8:1914 Budischak et al., 2018 Front. Immunol. 9:2453. Beldomenico et al., 2008 Proc. R. Soc. B 275:1753-1759 Dang et al., 2014 PLoS ONE 9(10) Rowland, 2007 CM 57(2):149-160 Sinha et al., 2019 J. Infect. Dis. 219:1356–63
Heat-killed *M. manresensis* (hkMm)	hkMm could affect proliferative and exudative lesions, and bacillary load	Cardona et al., 2016 Front. Microbiol. 6:1482 Tukvadze et al., 2016 Int J Mycobacteriol 5(S1):S101-S102
Helminths	Infection with helminths could affect body condition, cortisol, exudative and proliferative lesions, and bacillary load	Fenton, 2013 Parasitology 140: 1119–1132 Maizels et al., 2004 Immunol. Rev. 201:89-116 Mishra et al., 2014 Mucosal Immunol 7(4) Mutapi, 2015 Trends Parasitol. 31(9):405-6 Weinstock, 2014 Clinic Rev Allerg Immunol 49:227–231
Cortisol	Cortisol could affect exudative and proliferative lesions, and bacillary load	Bongiovanni et al., 2015 Tuberculosis 95:562-569
Body condition		
Anti-oxidants	Anti-oxidants could affect oxidants and bacillary load	Lu et al., 2013 Free Radic. Biol. Med. 66:75-87 Mohod et al., 2011 J. Exp. Sci., 2(6): 35-37 Nandi et al., 2009 Immunobiology 215:443–451
Oxidants	Oxidants could affect exudative lesions	Nadeem et al., 2018 Biomed. Pharmacother.107:1196–1204
Proliferative lesions		
Exudative lesions	Exudative lesions could affect bacillary load	Cardona, 2015 Front Microbiol. 6:612
Bacillary load		

Table S 3.4.6.3. Results of the initial outer model before the elimination of non-significative indicators. The output shows the weight, the loading, the communality and the redundancy of each indicator of the latent variable (LV).

	weight	loading	communality	redundancy
Fasting				
Fasting	1.0000	1.0000	1.0000	0.000000
hkMm				
hkMm	1.0000	1.0000	1.0000	0.000000
Helminths				
Heligmosomoides polygyrus	0.0561	0.2530	0.06402	0.004839
Trichuris muris	0.9873	0.9985	0.99698	0.075362
Cortisol				
Cortisol	1.0000	1.000	1.000	0.301867
Body condition				
Liver weight	1.2393	0.9285	0.86205	0.434121
Kidneys weight	-0.4843	0.3110	0.09673	0.048711
Anti-oxidants				
FRAP	0.2247	0.9393	0.88237	0.418144
TIOL	0.1730	0.6540	0.42778	0.202720
CUPRA	0.2356	0.9663	0.93368	0.442460
TAC	0.1948	0.9329	0.87030	0.412423
AAH	0.2155	0.9242	0.85410	0.404748
PON	-0.2962	-0.2270	0.05152	0.024414
Oxidants				
ROS	1.0202	-0.9857	0.97166	0.196688
AOPP	0.1718	0.0327	0.00107	0.000216
Proliferative lesions				
IFNg	0.2367	0.4914	0.24147	0.037776
IL10	0.5046	0.8380	0.70223	0.109858
IL12	0.5135	0.8974	0.80529	0.125981
Exudative lesions				

IL.1b	0.0938	0.8369	0.70048	0.039537
IL.6	0.1633	0.8706	0.75795	0.042781
LIX	0.1605	0.6127	0.37539	0.021188
KC	0.3226	0.9190	0.84465	0.047674
TNFa	0.1574	0.8599	0.73939	0.041734
IL17	0.3698	0.6737	0.45391	0.025620
Bacillary Load				
Lung	1.0018	-0.9910	0.98215	0.146342
Spleen	-0.1340	0.0535	0.00286	0.000426

Table S 3.4.6.4. Results of the initial inner model, summarizing the relationships between each latent variable (LV) and its block of indicators. The output shows the coefficient estimates of each indicator of the LV, standard error, the t-value associated with testing the significance of the parameter listed in the first column and Pr(>\|t\|) that gives you the p-value for that t-test.

	Estimate	**Std. Error**	**t-value**	**Pr(>|t|)**
Helminths				
Intercept	3.98E-16	0.124	3.22E-15	1
Fasting	3.38E-01	0.124	2.74E+00	0.00817
Cortisol				
Intercept	2.32E-16	0.111	2.09E-15	1
Fasting	4.04E-01	0.118	3.43E+00	0.00112
Helminths	2.56E-01	0.118	2.18E+00	0.03369
Body condition				
Intercept	-3.90E-16	0.0972	-4.01E-15	1.00E+00
Fasting	-6.02E-01	0.1033	-5.83E+00	2.76E-07
Helminths	-1.71E-01	0.1033	-1.65E+00	1.04E-01
Anti-oxidants				
Intercept	-5.47E-15	0.11	-4.97E-14	1.00E+00
Fasting	5.45E-01	0.11	4.95E+00	6.71E-06
Oxidants				
Intercept	-2.63E-15	0.114	-2.30E-14	1

Fasting	-1.37E-01	0.136	-1.01E+00	0.31731
Anti-oxidants	-4.18E-01	0.136	-3.07E+00	0.00329
Proliferative lesions				
Intercept	4.05E-16	0.123	3.30E-15	1
Fasting	4.04E-01	0.144	2.81E+00	0.0069
hkMm	1.25E-01	0.129	9.67E-01	0.3377
Helminths	-2.49E-01	0.138	-1.81E+00	0.0764
Cortisol	-1.67E-01	0.153	-1.09E+00	0.2803
Exudative lesions				
Intercept	-8.68E-17	0.13	-6.68E-16	1
Fasting	5.90E-02	0.165	3.58E-01	0.7219
hkMm	-2.37E-01	0.138	-1.71E+00	0.0927
Helminths	-7.36E-02	0.151	-4.88E-01	0.6274
Cortisol	-1.34E-01	0.173	-7.74E-01	0.4422
Oxidants	-7.22E-02	0.151	-4.78E-01	0.6349
Proliferative lesions	1.77E-01	0.143	1.24E+00	0.2204
Bacillary Load				
Intercept	2.29E-15	0.117	1.96E-14	1
hkMm	-8.38E-02	0.125	-6.73E-01	0.50389
Helminths	-1.47E-01	0.13	-1.13E+00	0.26248
Cortisol	-1.55E-01	0.151	-1.03E+00	0.30963
Anti-oxidants	2.84E-01	0.14	2.03E+00	0.04737
Exudative lesions	4.02E-01	0.12	3.34E+00	0.00154

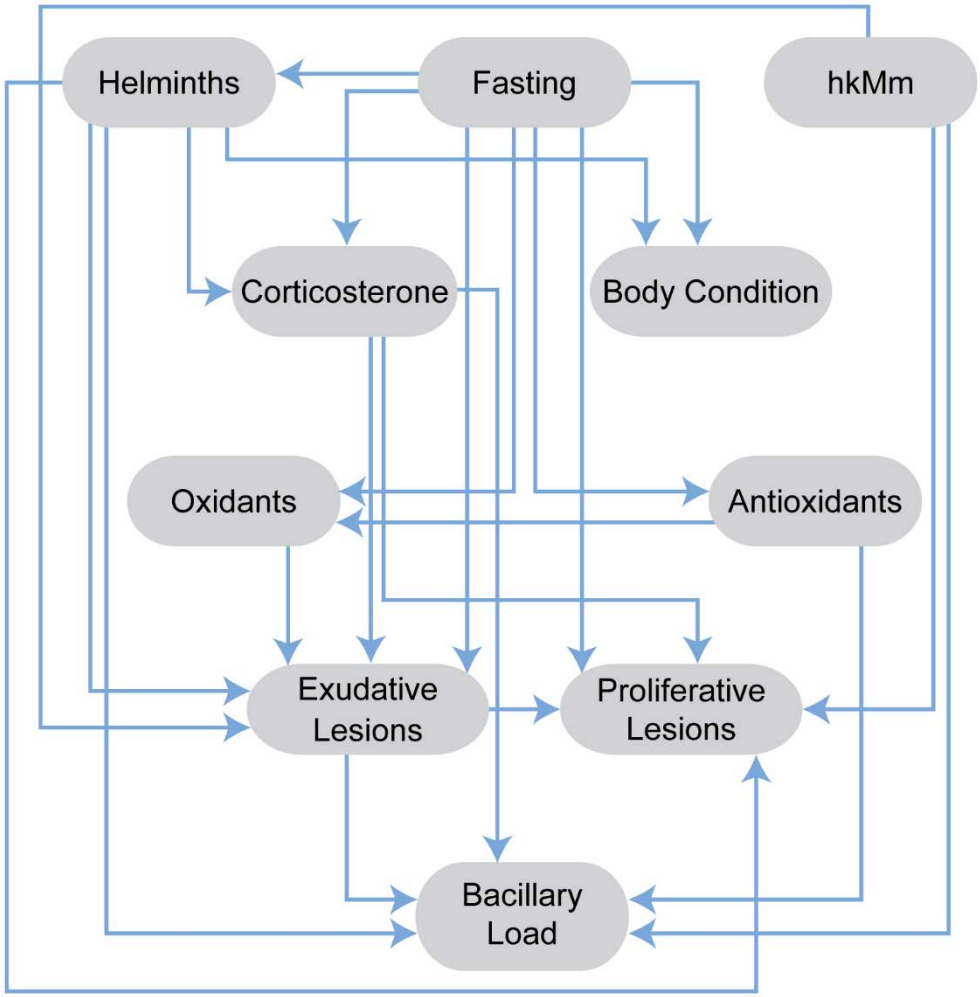

Figure S 3.4.6.1. Initial model, each arrow indicates an assumed relation between the latent variables

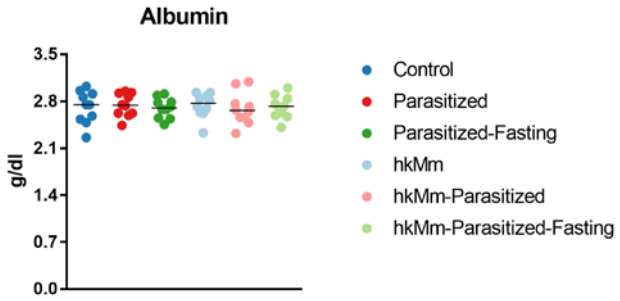

Figure S 3.4.6.2. Albumin levels in serum at week 3 post-infection. Each circle represents an animal and lines are medians. Mann Whitney test. hkMm: heat-killed M. manresensis.

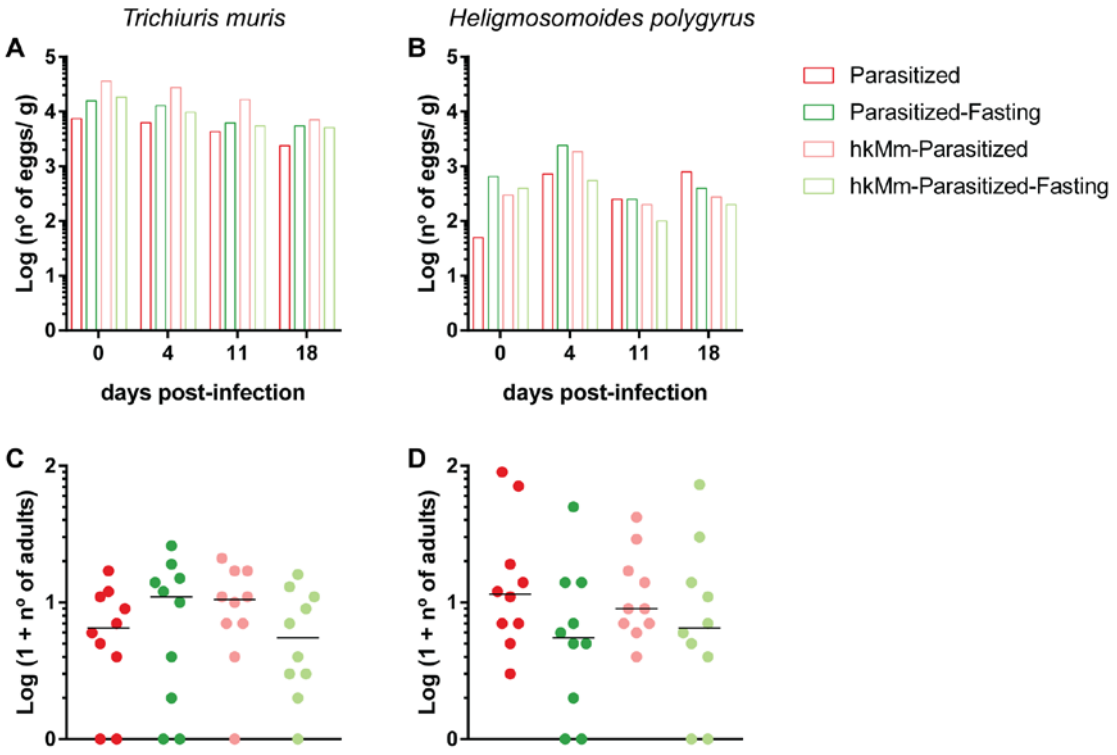

Figure S 3.4.6.3. Recount of helminth eggs in faeces during the experiment (A-B) and adult parasites in intestines at final endpoint (C-D). A and C correspond to T. muris and B and D are H. polygyrus.

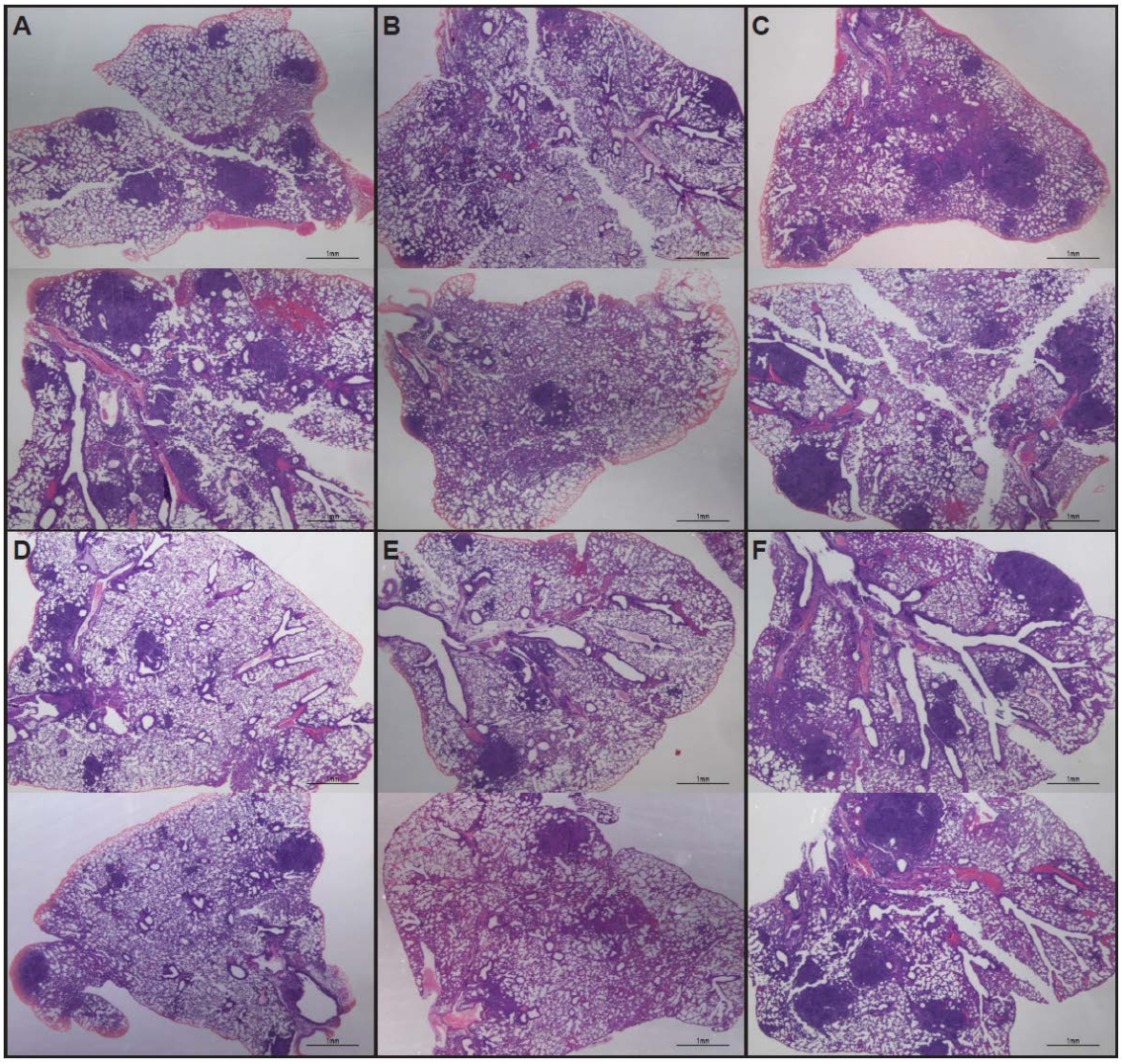

Figure S 3.4.6.4. Representative HE stained lung sections from each experimental group at week 3 post-infection. Lines represent 1 mm. (A) Control, (B) Parasitized, (C) Parasitized-Fasting, (D) hkMm, (E) hkMm-Parasitized, (F) hkMm-Parasitized-Fasting. hkMm: heat-killed M. manresensis.

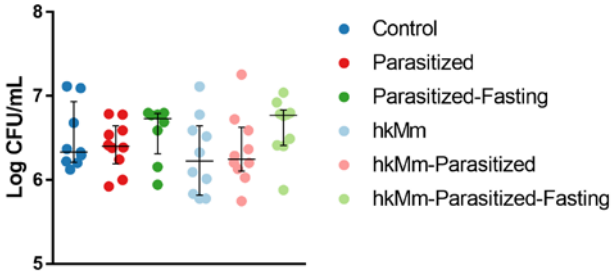

Figure S 3.4.6.5. Spleen bacillary load at week 3 post-infection. Each circle represents an animal. Lines show medians and interquartile range. Mann Whitney test.

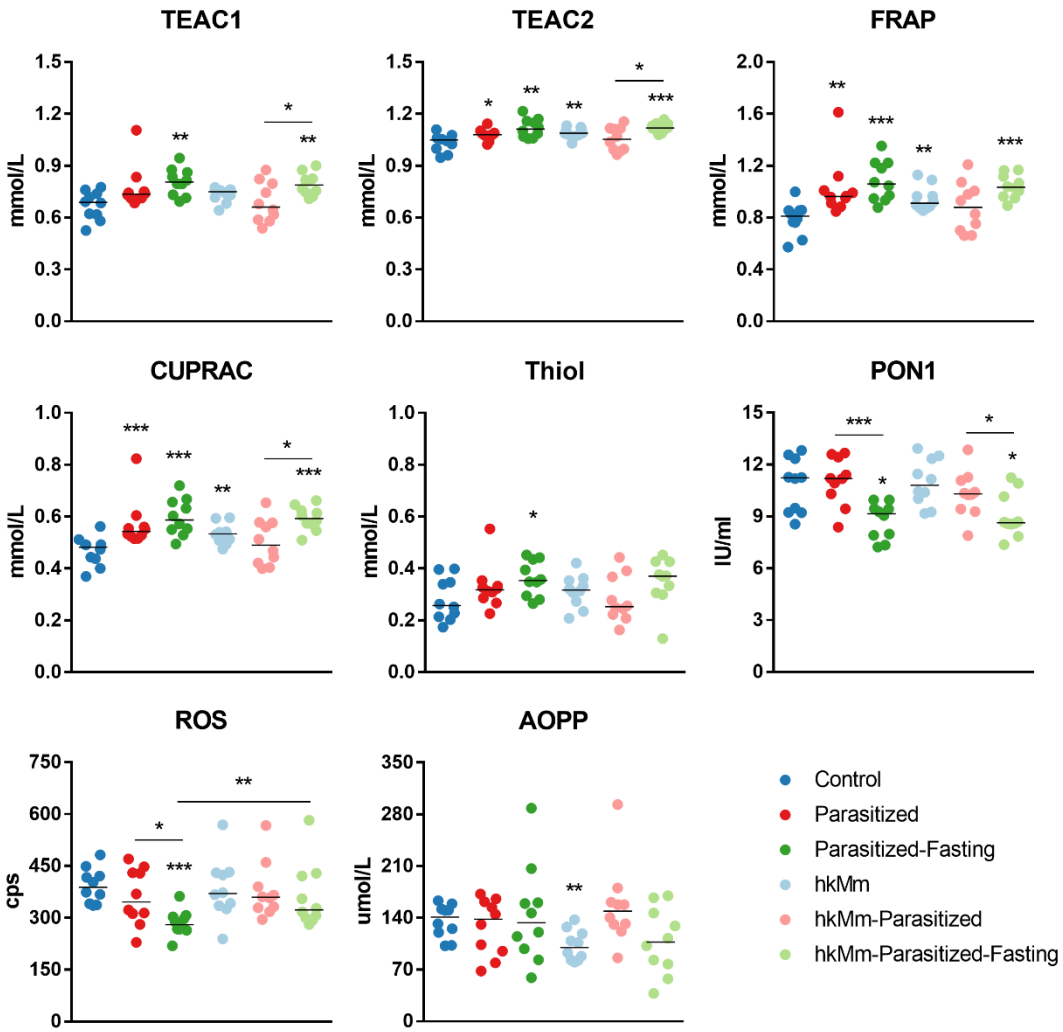

*Figure S 3.4.6.6. Serum levels of oxidative stress mediators at week 3 post-infection. Antioxidants: trolox equivalent antioxidant capacity (TEAC1 and TEAC2), FRAP (ferric reducing antioxidant power), CUPRAC (cupric reducing antioxidant capacity), Thiol, PON1 (paraoxonase 1). Oxidants: ROS (reactive oxygen species), AOPP (advanced oxidation protein products). Each circle represents an animal and lines are medians, Mann Whitney test (*p<0.05, **p<0.01, ***p<0.001). hkMm: heat-killed M. manresensis.*

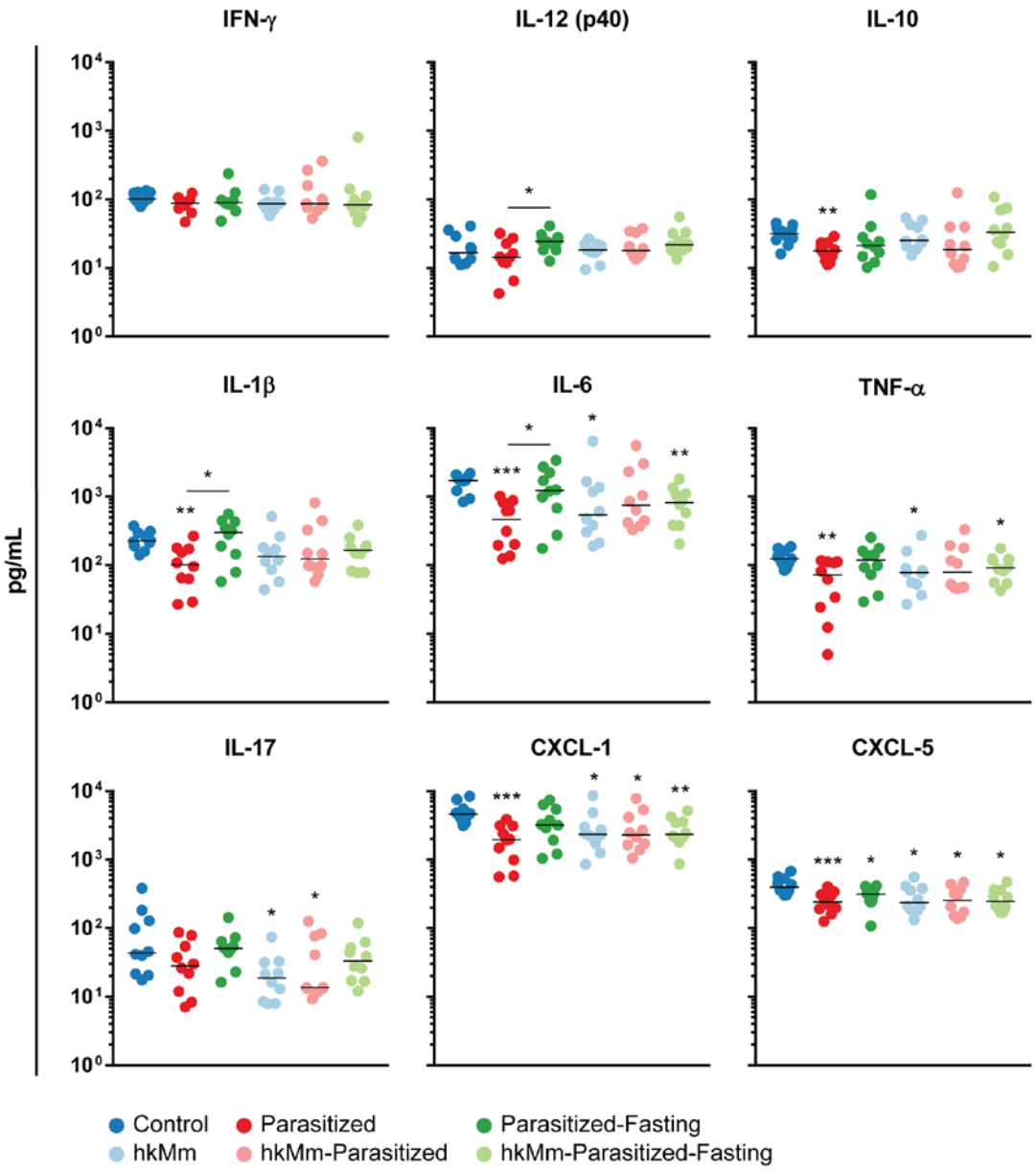

*Figure S 3.4.6.7. Immune mediators in lung homogenates at week 3 post-infection. Each circle represents an animal and lines are medians, Mann Whitney test (*p<0.05, **p<0.01, ***p<0.001). hkMm: heat-killed M. manresensis.*

5. Overall discussion

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In this thesis, we summarize the insights of co-infection research in wildlife during the last century (**study I**), examine the relationship between body condition and infection across a range of wild vertebrate species, (**study II**), study the consequences of multiple disease outbreaks with synzootic potential on growth rates and probabilities of extinction of virtual populations exposed to hard winters, density dependence, and co-occurring infectious disease outbreaks, using modelling techniques (**study III**) and understand the impact of helminthiasis and food shortages on tuberculosis progression and explored the cause-effect relationships among these factors with statistical methods (**study IV**). In the following discussion, we will examine the results obtained from the different studies conducted for this thesis.

A century of co-infection research: A bibliometric study

A systematic review and bibliometric analysis to elucidate the current knowledge on co-infections in animals and to summarize the primary statistical methods used for analysing available data on wildlife, revealed the need to reach a consensus on the appropriate terms, the gaps of literature on certain taxonomic combinations, and a considerable variation in the statistical tests approaches to assess the impact of co-infection on disease outcomes.

Nevertheless, the primary limitation of the bibliometric analysis is that the quality of metadata can vary across databases and may pose challenges when applied to older publications. On the other hand, the interpretation of quantitative data must be interpreted carefully, as they do not always reflect the quality or actual impact of the research.

Because there is a lack of standardization on the co-infection terminology, this inconsistency can create difficulties for researchers trying to identify relevant literature, as

conducting a comprehensive search may require exploring various terms to ensure all pertinent studies are captured.

Host condition and infection in wildlife

A systematic review of scientific literature to evaluate the link between body condition and infection in wild fish, amphibia, reptiles, birds and mammals infected with fungi, viruses, bacteria, protozoa, helminth and arthropod pathogens. Results showed that the overall impact of pathogens varies across taxa, with some groups showing more resilience than others. Most works detect a neutral effect of pathogen infections on the body condition of vertebrates, particularly fungi, viruses, protozoa, helminths and arthropods. A positive relationship was considerable in fungi, bacteria, and protozoa infection. In contrast, the negative effects were mainly observed in helminths and arthropod infections in amphibians, reptiles, birds and mammals.

Contrary to what we traditionally think, we have seen that poor body condition in infected animals is not the rule in wildlife. Our results showed that all wildlife groups showed a positive association between body condition and infection, which means that infected animals may appear outwardly healthy. Some explanation could be as seen in Godkin and Smith (2017), that some pathogens can adapt their strategies at different infection stages to optimize fitness. At first, pathogens maximise their spread through high transmissibility. However, as the infection progresses, they shift their focus towards persistence, extending the duration of infection. This adaptive shift allows pathogens to adapt to varying host population sizes, favouring longer persistence in smaller populations and higher transmissibility in larger ones.

Thereby, the influence of food shortages on disease progression will vary depending on the pathogen type (Eberhardt et al., 2013). Low-virulence pathogens, such as helminths and

ectoparasites, which rely heavily on host resources for survival, may benefit from hosts in good condition.

The nutritional regulation of the adaptive immune response appears to be much more complex than the simple link between good food resources, good condition and low pathogen susceptibility.

The intricate interplay between nutrition and immunity is a key factor influencing host susceptibility to infection. Recent research suggests that the quality and balance of nutrients, rather than simply their quantity, can significantly impact immune function (Cotter et al., 2011)). The immune system's various components exhibit differential responses to different nutritional states (Ponton et al., 2011). More specifically, complex trade-offs in energetic and specific nutritional resources produce cross-regulatory effects on immune system subcomponents, affecting host susceptibility against specific infections (Long and Nanthakumar, 2004). For instance, adequate energy intake, leading to elevated leptin levels, can promote a Th1 immune response, which is beneficial for combating intracellular infections. Conversely, it can suppress the Th2 response, which is important for defending against macroparasites.

Furthermore, the impact of nutritional status becomes even more complex in the context of co-infection. Food restriction can differentially affect the success of different parasite species, with some benefiting from reduced host immunity while others may be disadvantaged. Understanding these complex interactions between nutrition, immunity, and infection is crucial for developing effective strategies to prevent and control infectious diseases.

The synzootic potential of common epidemics in chamois populations

The Population Viability Analysis confirmed that concomitant outbreaks have potential synzootic effects posing an additional threat to the viability of chamois populations previously affected by one of these three studied diseases (sarcoptic mange, keratoconjunctivitis and border disease).

Despite the limitations of our work (e.g., some disease combination outbreaks have not yet been described in natural conditions, and we have only considered demographic consequences, but not transmission or recovery), interactions among co-infecting pathogens not only alter host pathology and disease spread at different levels of biological organization (Johnson et al., 2015) but also the long-term demography of the affected populations.

Our results support the importance of health surveys to forecast the potential consequences of synzootics on the local extinction risk of wild mammal populations. Additionally, managers in charge of chamois populations chronically affected by infectious diseases must take into account the demographic impacts of synzootics increasing efforts in disease surveillance to avoid new disease epidemics even caused by low virulent pathogens.

Protective effect of intestinal helminthiasis against tuberculosis progression is abrogated by intermittent food deprivation

This experimental work and statistical approach showed a protective effect of parasitisation in the outcome of *Mycobacterium tuberculosis* infection, which was abrogated by intermittent food deprivation.

These results are contrary to those found in other studies, on data suggest an increased susceptibility to developing tuberculosis when co-infected. Helminthiasis infection has been linked to pulmonary tuberculosis in a cohort study that compared infected individuals with a control group (Tristão-Sá et al., 2002), or patients who presented a more advanced disease (Resende Co et al., 2007).

The use of experimental models to try to understand the impact of helminthiasis on the progression of tuberculosis has been tried before by several authors, most of them have been carried out in mouse strains (mainly BALB/c and C57Bl/6) that develop chronic infection, and proliferative lesions with a strong Th1 response (Cardona, 2015). The mouse strain used in our study (C3HeB/FeJ) doesn't develop exudative lesions, and is the only mouse strain able to develop liquefaction lesions with the capacity to become cavitated (Marzo et al., 2014).

In our study, we evaluated the parasitisation with *T. muris* and *H. polygyrus*, we used two nematodes since it is a common situation found in natural ecosystems, which only affect intestinal mucosa (Alemu and Mama, 2017; Behnke et al., 2009).

Although both species induce similar immune responses according to other studies (Filbey et al., 2019), our PLS-PM model showed a greater effect of *T. muris* regarding the *H. polygyrus* counterpart.

To point out some limitations of the study, we were unable to detect the parasitisation effect on the Th2 response. However, matching the lower levels of Mtb load, these mice did present a reduction in the pro-inflammatory response in the lungs. This was also observed with the oral administration of hkMm, which additionally reduced IL-17 levels, as already described in Cardona et al. (Cardona et al., 2016).

Moreover, the PLS pathway modelling supported the role of helminths and hkMm in the reduction of exudative lesions and, thus, the reduction of the bacillary load. Parasitized animals exhibited lower IL-10 levels, suggesting a reduced regulatory response (Nel et al., 2014). Additionally, IFN-γ levels stayed practically the same, and the acute-phase protein haptoglobin was reduced in sera of parasitized animals, suggesting an anti-inflammatory effect.

Our understanding of the impact of intermittent fasting is limited, a circumstance resembling an aspect of food insecurity. Food deprivation experiments have been carried out in mice. In our case, 48 hours of deprivation-induced weight reduction, which was recovered with a normal diet for five days, and an increase in oxidative stress. This regimen did not cause a reduction in albumin levels, suggesting that the reduction of protection was not due to protein deprivation. Interestingly, intermittent fasting was related to a cortisol increase, probably caused by stress. However, hkMm appears to balance this reaction as hkMm-parasitised-fasting animals had the same levels of cortisol as control mice.

The results obtained in lung pathology and bacillary load matched the ones found in other studies (Cortés et al., 2015), supporting the proinflammatory effect of ADA in inducing Th1 response and reducing extracellular levels of adenosine. Parasitized and fasting animals were the only ones that did not present lower levels of ADA than control. But once again animals treated with hkMm had a distinct outcome. It appears that hkMm gave some level of protection against intermittent fasting, although it was not strong enough to be evidenced in the progression of tuberculosis in this model.

Even though the goodness-of-fit of our PLS-PM model could be considered moderate (28, 29), it is enough to evidence the causal relationship among starvation, parasite load, environmental mycobacteria, exudative lesions (ExL), and bacillary load. Heat-killed *M.*

manresensis and helminths contributed equally to diminish exudative lesions and thus bacillary load as suggested by previous research in mice (Gil et al., 2006) and cattle (Menin et al., 2013). Further investigation, however, should be conducted to elucidate the drivers of these protective effects beyond the immunological pathway used in our research.

6. Conclusions

6. Conclusions

1. The absence of standardised terminology, the wide range of pathogen combinations that may infect a host, and the absence of consensus on optimal statistical methods for analysing co-infection data can hinder the identification of relevant studies and compromise the comparability of research findings. In light of that, we proposed the following definition of co-infection: the occurrence of more than one simultaneous infection by different infectious agents or strains in individual hosts, populations, or communities.
2. Hosts may maintain good health and condition despite high pathogen burdens. Therefore, it is essential to assess whether pathogen infections lead to disease and consider tolerance as a host defence strategy rather than assuming that low body condition directly indicates disease progression, pathogen infection, or spread.
3. Host populations affected by a second outbreak, even caused by a low virulent pathogen, could experience an increased probability of extinction, probably due to the lack of complete recovery from the first outbreak.
4. Helminth could provide a protective effect against *Mycobacterium tuberculosis* infection, which could be abrogated by fasting. Thus, the nutritional status of the infected host could determine the disease progression of chronic infections and even the outcome of disease treatments.

7. References

7. References

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