THE UNIVERSITY OF SYDNEY

A Holistic Evaluation of Health Indicators in the Koala, Phascolarctos cinereus: Complex Host-Pathogen-Environment Relationships in Rehabilitation and in the Wild.

A thesis submitted in fulfillment of the requirements for the degree of

Doctor of Philosophy by

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Statement of Originality

This thesis is submitted to fulfill the requirements of the degree of Doctor of Philosophy within the Faculty of Science, School of Veterinary Science at The University of Sydney. I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis including unpublished and published sources have been acknowledged and referenced appropriately. This thesis has not been submitted for any other degree or other purposes other than at The University of Sydney.

Yasmine Sophia Sierra Muir

28/2/2025

Generative AI Attribution Statement

No content produced by generative AI tools has been used in the preparation of this thesis.

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24/6/2025

Structure of the thesis

This thesis is composed of six chapters, which include work submitted for publication and unpublished work: an introduction and literature review, four research chapters, and a general discussion. Before each research chapter an author contribution statement is provided. Supplementary information, including tables and figures, is incorporated at the end of each chapter. The overall aim of this thesis is to extend and refine the suite of evidence-based indicators of koala health and disease and, in doing so, identify associations among pathogens and host pathways that can be used as the foundation for targeted mechanistic studies to determine causation, and to drive improvements in health assessments and disease management in wild and rehabilitated koalas. Approaches novel to the field are employed to enable unbiased integrated analysis of the large number of variables involved, and the thesis follows a step-wise approach, first examining frequency and potential relevance of coinfections in rescued koalas, then integrating analysis of host responses to coinfections in the same clinical cohort, then using RNAseq to extend the variables being examined, and then assessing the relevance of the identified risk factors to survival and rehabilitation in a population of free-ranging koalas.

Chapter 1 consists of a general introduction to the thesis and a review of the literature, covering key gaps in our understanding of pathogenesis in the koala, the limitations of current methods of investigations, and presenting the context in which this body of work takes place. This is followed by the aims and hypotheses of the thesis.

Chapter 2 is an investigation of the frequency of co-infections detected in koalas admitted to rehabilitation facilities and their potential relevance as risk factors for disease: *"The relationships of viral and protozoal co-infections to* Chlamydia pecorum infection *and chlamydiosis outcomes in northern koalas."*

Chapter 3 is an investigation that extends analysis of the same cohort to examine the interactions of host immune and physiological gene expression with relevant co-infections and clinical outcomes, using PCA and non-biased clustering; "*Clustering of immune gene transcription identifies immune phenotypes associated with poorer outcomes in koala rehabilitation".*

Chapter 4 is an investigation that extends the panel of immune and physiological markers used in Chapter 2, using next-generation sequencing and differential expression analysis. Distinct phenotypes are described among koalas with chlamydiosis and those euthanised; "*Koala pro-inflammatory genes IL1R2, MARCO, MYO1B, and RARRES1 show upregulated expression in koalas with clinical chlamydiosis and those that are euthanised*".

Chapter 5 is an investigation that re-examines clinical, infectious, and gene expression variables from previous chapters in a new population of free-roaming wild koalas to determine their relevance to short- and long-term clinical, treatment, and survival outcomes in the field; *"Multivariate analysis of host gene expression and population health determinants predict chlamydiosis treatment outcomes and long-term survival at two wild koala sites in south-east Queensland".*

Chapter 6 is a general discussion situating the research findings and key outcomes within the field, to highlight the impact of this work and direct pathways for further investigation. Here we expand upon the significance and importance of accounting for complex relationships among co-infecting agents and markers of the immune response in disease investigations, and the practical implications for biomarkers of health and disease such as adaptive lymphocyte gene activity.

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Authorship attribution statement

Chapter 2 has been submitted for publication within the journal *PLOS Pathogens* and is in review. **Yasmine S. S. Muir** is the main author of this study and has contributed towards every component of the research including the conceptualisation, field work and data curation, methodology, formal analysis, investigation, writing of the original draft and reviewing and editing of the manuscript. Damien P. Higgins and Mark B. Krockenberger supervised and contributed to the conceptualisation, investigation, writing and reviewing of the manuscript and provision of research resources. Belinda R. Wright contributed to the methodology, writing and reviewing of the manuscript, as did Andrea Casteriano in addition to the provision of research resources. Mathew S. Crowther contributed to the statistical supervision, writing and review of the manuscript.

Chapter 3 is intended for publication. **Yasmine S. S. Muir** is the main author of this study and has contributed towards every component of the research including the conceptualisation, field work and data curation, methodology, formal analysis, investigation, writing of the original draft and reviewing and editing of the manuscript. Damien P. Higgins and Mark B. Krockenberger supervised and contributed to the conceptualisation, investigation, writing and reviewing of the manuscript and provision of research resources. Belinda R. Wright contributed to the methodology, writing and reviewing of the manuscript, as did Andrea Casteriano in addition to the provision of research resources. Valentina S.A. Mella contributed to the statistical supervision, writing and review of the manuscript.

Chapter 4 is intended for publication. **Yasmine S. S. Muir** is the main author of this study and has contributed towards every component of the research including the conceptualisation, field work and data curation, methodology, formal analysis, investigation, writing of the original draft and reviewing and editing of the manuscript. Damien P. Higgins and Mark B. Krockenberger supervised and contributed to the conceptualisation, investigation, writing and reviewing of the manuscript and provision of research resources. Belinda R. Wright contributed to the conceptualisation, methodology and data curation, the provision of research resources, writing and reviewing of the manuscript.

Chapter 5 is intended for publication. **Yasmine S. S. Muir** is the main author of this study and has contributed towards every component of the research including the conceptualisation, field work and data curation, methodology, formal analysis, investigation, writing of the original draft and reviewing and editing of the manuscript. Damien P. Higgins and Mark B. Krockenberger supervised and contributed to the conceptualisation, investigation, writing and reviewing of the manuscript and provision of research resources. Belinda R. Wright and Andrea Casteriano contributed to the methodology, the provision of research resources, and reviewing of the manuscript. Deidré L. de Villers and Julien Grosmaire contributed to field work and data curation and reviewing of the manuscript.

Yasmine Sophia Sierra Muir

28/2/2025

Attesting authorship attribution statement

As the supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Damien P. Higgins

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List of manuscripts prepared for publication and related conference presentations

Thesis chapters submitted for publication

Muir, Y.S.S., Wright, B.R., Casteriano, A., Crowther., M., Krockenberger, M.B., Higgins, D.P.. The relationships of viral and protozoal co-infections to *Chlamydia pecorum* infection and chlamydiosis outcomes in northern koalas *(Phascolarctos cinereus)*. *In review, PLOS pathogens.*

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Muir, Y.S.S., Wright, B.R., Casteriano, A., Mella, V.S.A, Krockenberger, M.B., Higgins, D.P.. Clustering of immune gene transcription identifies immune phenotypes associated with poorer outcomes in koala rehabilitation.

Muir, Y.S.S., Wright, B.R., Krockenberger, M.B., Higgins, D.P.. *IL1R2, MARCO, MYO1B*, and *RARRES1* show upregulated expression in koalas with clinical chlamydiosis and in those that are euthanised.

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Conference presentations

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Glossary

The following defines these terms as they are used in this thesis:

Antagonism: The suppression of one infecting microorganism due to infection by another microorganism.

Captive/captivity: An animal that permanently lives under human control or care, including zoos, wildlife parks, and long-term residence in rehabilitation.

Competition: An infecting microbe acquires cellular resources (nutrients, energy, substrates) required for optimal functioning of another infecting microbe.

Detection frequency: The percentage of the sampled cohort of a population in which an infectious agent or other variable was detected.

Interference: The release of products and/or metabolites from one infecting microorganism impedes the function of another infecting microorganism.

Indicator of health: The qualitative and quantitative measures that are associated with health outcomes such as infection, morbidity, and mortality.

Hyperparasitism: Where one infecting microorganism infects another organism within the host.

Rehabilitation: The care of wild animals who have been rescued because they are sick, injured, debilitated, and/or orphaned.

Risk factor / predictor: A parameter that is associated with or predicts a health or disease outcome, but may or may not be causative.

Synergism: The infection by one microorganism augments adverse effects associated with another.

This thesis use of the following terms was chosen to be consistent with those used in the National Koala Disease Risk Analysis:

Acute disease: "A disease occurring in a short timeframe (1-5 days), regardless of severity of clinical signs."

Association: "A statistical relationship between two variables. Two variables may be associated with or without a causal relationship."

Causation: "A situation where the exposure to a hazard is responsible for the effect."

Chronic disease: "A disease occurring over a long timeframe, regardless of the severity of clinical signs."

Clinical sign: "Observed, objective changes in the normal healthy state, bodily function or behaviour of an animal."

Cytokine: "Substances such as interferon, interleukin and growth factors, that are secreted by certain cells of the immune system and have an effect on other cells."

Diagnostic test: "Any procedure used to aid in the characterisation of the cause or nature of an infection or disease."

Disease: "Any disturbance in the structure or function of an animal, which includes those attributed to infectious or non-infectious causes."

Endemic: "A disease or parasite regularly found among particular populations or in a specified geographic area."

Endogenous retrovirus: "Retrovirus which is incorporated into germ cells of the host and therefore is inherited by successive generations."

Exogenous retrovirus: "Retrovirus that is incorporated only into the somatic cells of the host and therefore is not inherited."

Genotype: "The DNA sequence at a specific position within the genome of the pathogen, also known as sequence type, subtype, variant or strain."

KoRV traits/parameters: "The range of characteristics by which KoRV presence in a host may be quantified or described. Includes viral load, proviral load, presence or load of variants and presence or load of certain genetic markers (e.g. pol gene)."

Latent phase (virus): "The stage of the virus lifecycle when it generally lies dormant within host cells."

Lytic phase (virus): "The active phase during which the virus replicates within the host cell and releases a new generation of viruses when the infected host cell lyses."

Northern koala: "Koalas originating from Qld, NSW and ACT populations."

Pol KoRV: "KoRV which possesses the pol gene and is therefore capable of integrating into the koala host DNA and replicating; "replication-competent" KoRV."

Pol-negative koala: "A koala that is infected with KoRV that does not possess the pol gene and is therefore incapable of replication."

Pol-positive koala: "A koala that is infected with KoRV possessing the pol gene ("replication-competent" KoRV)."

Prevalence: "The proportion of the host population with infection, disease or antibody presence, often expressed as a percentage."

Proviral load: "The amount of provirus within a host"

Provirus: "A virus genome that is integrated into the DNA of a host cell. In terms of KoRV, refers to the transcribed DNA copy of KoRV which is incorporated into the koala genome."

RecKoRV: "Defective KoRV elements that appear to be endogenous but lack the pol gene and are therefore not capable of replication."

Rehabilitation facility: "A facility (large or small) for the treatment and care of injured, orphaned, or sick wild animals so that they can be released back to the wild."

Replication competent (retrovirus): "In terms of KoRV: KoRV that contains the full gene complement and is therefore capable of integrating into host DNA and replicating to produce additional virus."

Risk assessment: "The evaluation of the likelihood and the consequences of entry, establishment or spread of a pathogenic agent within a specified animal population or environment."

Southern koala: "Koalas originating from SA and Vic free-ranging populations."

Surveillance: "A systematic ongoing program of investigation designed to establish the presence, extent of or absence of a disease, or of infection, or presence of a pathogen. It includes the examination and testing of animals for clinical signs, antibodies or the presence of a pathogen and the timely dissemination of information so that action can be taken."

Transmission: "The process by which a parasite passes from a source of infection to a new host."

Triage: "The process of organising patients according to the severity of their condition and treating each patient within an appropriate time frame. Triage also includes an assessment of the viability of the patient, and whether euthanasia should be considered due to the severity of disease and associated welfare concerns."

Trypanosomiasis: "Infection of a host by trypanosome parasites.**"**

Wet bottom: "Chronic staining and wetness of the rump of koalas, associated with urinary incontinence and often caused by urinary tract infections, particularly Chlamydia."

Wildlife hospital: "A hospital or clinic that provides veterinary assessment, treatment and care for free-ranging wildlife."

This thesis use of the following terms was chosen to be consistent with those defined by Casadevall, A., & Pirofski, L. A. (2000) and Casadevall, A., & Pirofski, L. A. (2003):

Host: "An entity in which microorganisms reside and/or replicate; an entity in which microbial pathogenesis occurs."

Damage: "Disruptions in the normal homeostatic mechanisms of a host that alter the functioning of cells, tissues or organs; for microorganisms, disruptions in the normal mechanisms that enable host entry, replication and/or the ability to establish residence in a host."

Infection: "Acquisition of a microbe by host; most infections are followed by multiplication of the microbe in the host, but this is not universal because some helminth infections can involve a single organism that does not replicate in the host."

Elimination: "Removal of the microbe from the boundaries of the host by either physical factors, interference by host flora, an immune response, or therapy."

Colonisation: "A state of infection that results in a continuum of damage from none to great, with the latter leading to the induction of host responses that could eliminate or retain the microbe, or progress to chronicity or disease; for organisms that induce no damage during infection this state is indistinguishable from commensalism."

Commensal: "Microbe that induces either no damage or clinically inapparent damage after primary infection; a state that is thought to be established early in life."

Commensalism: "A state of infection that results in either no damage or clinically inapparent damage to the host, though it can elicit an immune response."

Latency: "Synonymous with persistence, this term is often used to describe infections that are asymptomatic over long periods of time but can evolve into overt disease."

Persistence: "A state of infection in which the host response does not eliminate the microbe, resulting in continued damage over time; persistence may evolve into overt disease, depending on the balance of the host-microbe interaction."

Pathogen: "A microbe capable of causing host damage."

Virulence: "The relative capacity of a microorganism to cause damage in a host."

Symbiosis/mutualism: "A state of infection whereby both the host and the microbe benefit as a consequence of infection."

List of Abbreviations

ACT:	Australian Capital Territory
aRB:	Aberrant reticulate body
BCS:	Body condition score
CO_2:	Carbon Dioxide
EB:	Elementary body
Env:	Retroviral envelope gene
FCM:	Faecal cortisol metabolites
FKBP5:	FK506-binding protein 51
Gag:	Viral Group-specific antigen gene
HCPC:	Hierarchical clustering of principle components
HPA:	Hypothalamic-pituitary-adrenal axis
HPE:	Host-pathogen-environment
IHC:	Immunohistochemistry
IL-:	Interleukin
incA:	Inclusion membrane protein A
IUCN:	International Union for Conservation of Nature
KoRV:	Koala retrovirus
LAMP:	Loop-mediated Isothermal Amplification
LOQ:	Limit of quantification
LOD:	Limit of detection
mRNA:	Messenger RNA
NSW:	New South Wales
ompA:	Major outer membrane protein A
PC:	Principal component (also termed dimension)
PCA:	Principal components analysis
PhaHV:	Phascolartid herpesvirus
PhaHV-1:	Phascolartid herpesvirus subtype 1
PhaHV-2:	Phascolartid herpesvirus subtype 2
PIDDS:	Putative immune dysfunction disorders
PKAD:	Putative KoRV associated disease(s)
Pmp:	Polymorphic membrane protein
Pol:	Viral polymerase gene
PSMs:	Plant secondary metabolites
POC:	Point of care
Qld:	Queensland
RB:	Reticulate body
RT- / qPCR:	Reverse transcriptase / quantitative Polymerase Chain Reaction
SA:	South Australia
Th:	T-helper
Treg:	T-regulatory
UGT:	Urogenital
Vic:	Victoria

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Abstract

This thesis demonstrates the greater depth of understanding of complex wildlife diseases that can be gained by application of a holistic multifactorial approach; in this case investigation of host-pathogen-environment interactions in disease of the koala (*Phascolarctos cinereus*), an iconic and endangered Australian marsupial. The multifactorial nature of disease complicates efforts to understand epidemiology, pathogenesis, and disease risk, and develop treatment or risk mitigation strategies. In wildlife disease investigations, small sample sizes, lack of medical histories, broad case definitions and discipline-oriented research often limit the variables that can be included in studies, oversimplifying relationships and sometimes restricting findings to the study context. As a result, while many host, pathogen, and environmental factors have been identified as significant to koala health, their interconnections and relative importance remain unclear. This fragmented understanding hampers the identification of reliable indicators of koala health and disease. Currently, diagnosis, prognosis and definition of disease outcomes in research are largely based on clinical signs, while the complex interactions of coinfections, host responses and underlying stress physiology, all of which may impact fitness and eventual disease and treatment outcomes, remain unseen. The integrated approaches used in this thesis to capture the complexity of disease systems are founded conceptually on two key frameworks: the Damage-Response Framework and the Host-Pathogen-Environment (HPE) interaction (Casadevall & Pirofski, 2003; Fidel *et al.*, 2020; Guégan *et al.*, 2024; McNew, 1960; Scholthof, 2007). Analysis of the large number of variables involved was facilitated by development of novel analytical approaches that deepened our understanding of complex disease threats

facing the koala, and thereby provided new knowledge to inform management of koalas in the clinic and in the wild.

To gain a holistic view, it is necessary to determine the interrelationships among putative significant health indicators, clarify the potential roles and functions of these markers, and understand how this may vary and why. This thesis employs a stepwise approach to address these points: first examining interactions of infectious agents, then integrating this understanding with comprehensive immunophenotypic analysis and then, finally, examining these in the context of environmental factors in a comparative field study. Chapter 1 comprises a literature review that argues the importance and utility of multivariate analysis to identify indicators of health in wildlife and, more specifically in the koala. Chapters 2–5 are experimental chapters. In Chapters 2-4, a heterogeneous cohort of koalas admitted to wildlife hospitals is studied to capture a range of demographics, infections, and co-morbidities. Chapter 5 examines two adjacent free-ranging koala populations with different morbidity and mortality rates to explore how HPE relationships—and thus indicators of health—vary between populations.

Several infectious agents, including *Chlamydia pecorum*, herpesviruses, koala retrovirus (KoRV), and *Trypanosome* species, have each been associated with koala morbidity and mortality. Chapter 2 describes a high frequency of co-infection and varied relationships between infection and disease among koalas in clinical care, highlighting the importance of understanding case-specific co-infections that drive heterogeneity in disease outcomes. Notably, circulating *C. pecorum* was detected in some cases without disease or mucosal shedding; a finding that challenges our approaches to individual and population screening,

treatment and release criteria, and our understanding of the pathogenesis of chlamydiosis. Examination of immune gene transcription and its relationship to koala survival during rehabilitation, showed that immune and physiological profiling are better predictors of triage outcomes than aetiological diagnosis alone, and provides a window into the immunological and physiological changes underpinning decline of koala health. Chapter 4 further validates and refines the immunophenotyping panel, adding new gene targets, such as IL1R2, associated with chlamydiosis and euthanasia, to support further analyses of the immune response. In Chapter 5, key indicators of survival and treatment outcomes for chlamydiosis are determined, while accounting for population-specific characteristics. Here, adaptive immune parameters emerge as a consistent and strong predictor of survival in diseased wild koala populations. Other variables are identified as potential risk factors and low-risk indicators, such as: KoRV gene transcription, and cytokine and innate immune gene transcription.

This research contributes to a more holistic understanding of koala health and the determinants of clinical diagnosis, prognosis and long-term survival. The approaches and perspectives developed here in associative studies can be used as the foundation for targeted mechanistic studies that will clarify the causal significance of health indicators to generate a refined set of evidence-backed indicators of koala health and disease. Improved health assessment is expected to result in better health outcomes in wild and rehabilitated koalas by supporting disease management strategies through developments in disease surveillance and risk assessments, advances to treatments and preventatives, and better informed population management and insurance strategies.

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Chapter 1 Introduction, Literature review and Aims of the thesis

1.1 Introduction:

Increasingly, biodiversity, animal welfare and human health (zoonoses) are of global concern, requiring evidence-based management of wildlife disease. While it is often difficult to attribute extinction or declines to any one cause, due to limited monitoring and under-identification of disease impact (McCallum *et al.*, 2024; Scheele *et al.*, 2019), the cryptic nature of wildlife species, and the complex interactions between factors causing disease (De Castro & Bolker, 2005; McCallum, 2012; McCallum *et al.*, 2024), there is growing evidence of the importance of disease as a threatening process in many different wild animal species. Wildlife disease research and surveillance has established the significance of disease to the extinction of several species including the Polynesian land snail *(Partula turgida),* Christmas Island rodents *(Rattus macleari* & *R. nativiatus),* and ongoing declines in other species including, many amphibians, the Tasmanian devil *(Sarcophilus harrisii),* the koala *(Phascolarctos cinereus),* migratory tundra caribou *(Rangifer tarandus),* sea lions in the Galapagos islands and Australia *(Zalophus wollebaeki & Neophoca cinerea),* and coho, chinook, and sockeye salmon (*Oncorhynchus kisutch, O. tshawytscha, & O. nerka*) (Adams-Hosking *et al.*, 2016; Aguilar *et al.*, 2023; Denkinger *et al.*, 2017; Marcus *et al.*, 2014; McAlpine *et al.*, 2015; McCallum, 2012; McCallum *et al.*, 2024; Miller *et al.*, 2014). Effective disease risk assessment and mitigation requires a sound understanding of the complex drivers of disease and reliable assessment of individual and population health.

This review evaluates the current state of wildlife health assessments and puts the case for an improved approach to investigating indicators of health and disease, using the koala *(Phascolarctos cinereus)* as a model species. Although the multifactorial nature of disease is widely recognised, incorporation of this complexity into disease surveillance, monitoring and management practices is yet to be developed in most wildlife species, including the koala (Scheele *et al.*, 2019; Walton *et al.*, 2016). Here, we present the current state of the koala, highlighting key pressures and diseases contributing to population declines and the results of disease surveillance. The existing koala disease literature is reviewed though the lens of two conceptual frameworks, the damage response framework and host-pathogen-environment interactions, highlighting key gaps in the current understanding of disease dynamics and

management outcomes. This review proposes that alternative methods of analysis that can accommodate increased complexity associated with multivariate relationships, are an essential next step for investigation of complex disease scenarios in the koala and other wildlife species.

1.2 Health assessment in wildlife

Wildlife health assessments help identify individuals and populations at risk and are fundamental to the development of our understanding of disease and approaches to wildlife disease management. Health assessments consist of qualitative and quantitative measures of organ, tissue and cell function (Kophamel *et al.*, 2022; Vitali *et al.*, 2023), by which deviations from normal structure and function (pathology) are detected, that may be the result of infectious disease (caused by living agents of disease) or non-infectious diseases (caused by physical and chemical agents of disease) and other physiological stressors (Kophamel *et al.*, 2022). As such, health assessments are used for many interrelated aspects of wildlife conservation: population monitoring and disease surveillance, wildlife disease management, and investigation of the relationships and ecological mechanisms driving disease dynamics (Fehr *et al.*, 2017; Langmuir, 1963; Organization, 2000; Porta *et al.*, 2014; Thacker & Berkelman, 1988). Additionally, due to the burgeoning interface between humans and wildlife, there is a growing need to incorporate wildlife health assessments in environmental impact assessments to inform sustainable development (Aleuy *et al.*, 2023). Often, integrative management and surveillance systems are not set up, due to lack of resources and species-specific understanding of the factors contributing to pathogenesis, leading to the implementation of management approaches that are not backed by evidence (Joseph *et al.*, 2013). Ideally, disease monitoring and surveillance should be able to support both current decision making and the development of hypotheses to extend understanding of pathogenesis and disease dynamics and thereby continuously improve wildlife disease management (Joseph *et al.*, 2013).

Implementing wildlife disease management without an evidence-base has several risks and could be detrimental to species already vulnerable to extinction. Culling of infected or diseased individuals from a population has been aimed at eradicating wildlife disease or

limiting ongoing transmission (Bolzoni & De Leo, 2013). However, the manipulation of host density changes ecological conditions for the pathogen and can influence transmission and impose strong selective pressures on virulence, leading to a counterintuitive result (Bolzoni & De Leo, 2013; Carter *et al.*, 2007; Prentice *et al.*, 2014). This phenomenon, also termed the 'perturbation effect', was observed in the culling of Eurasian badgers *(Meles meles)* for management of bovine tuberculosis transmission (Carter *et al.*, 2007; Prentice *et al.*, 2019; Schreiner *et al.*, 2020). Additionally, due to an incomplete understanding of pathogenesis and epidemiology, the implementation of selective culling of Tasmanian devils with devil facial tumour disease neither slowed the rate or disease progression nor reduced population-level disease impacts (Lachish *et al.*, 2010). The application of antimicrobials and vaccines are used as a disease management approach in wildlife rehabilitation and although they are effective in some instances – such as for treatment of myasis (Schütte *et al.*, 2025) or the control of rabies (Haydon *et al.*, 2006), in the wrong context these interventions and preventative measures can cause antimicrobial resistance and selection for greater virulence of target and non-target organisms (Joseph *et al.*, 2013; Plumb *et al.*, 2007). For example, immunisation of chickens against Marek's disease virus enhances the fitness of more virulent strains (Read *et al.*, 2015), and anthelmintic treatment of *Heligosomoides polygyrus* in in free-living yellow-necked mice *(Apodemus flavicollis)* caused an increase in infestation by the tick, *Ixodes ricinus* (Ferrari *et al.*, 2009).

Adaptive wildlife disease management that draws from continuous developments in environmental, biomedical, and pathogenesis research can yield more effective strategies for disease intervention. The synthesis of scientific theory and frameworks has improved the management of disease in many species. White nose syndrome was shown to have differential impacts on bat populations based on their size and social behaviour and the humidity and temperature of roosts, informing risk assessments and protection of refuge roosts (Langwig *et al.*, 2012a; Langwig *et al.*, 2012b). In Iberian lynxes *(Lynx pardinus)*, a strong understanding of the transmission dynamics of Feline leukemia virus supported effective management of an outbreak through combined culling of reservoirs, vaccination of negative animals, and removal of viremic lynxes from the population into captivity (López *et al.*, 2009). Haydon et al. (2006) demonstrated that a targeted low-coverage vaccination could successfully manage a rabies outbreak in Ethiopian wolves *(Canis simensis)* by reviewing past

outbreak dynamics considering pack movements and disease fronts. In Switzerland, consideration of the differences in transmission risks among age groups in Classical Swine Fever, an age-focused hunting strategy contributed to the control of an outbreak in wild boar *(Sus scrofa)* (Schnyder et al., 2002). Targeting co-infection interactions through treatment of nematode infection, which modulates host immune responses, in African buffalo *(Syncerus caffer)* improved animal response to tuberculosis treatment (Elias *et al.*, 2006; Ezenwa *et al.*, 2010; Ezenwa & Jolles, 2011). Employment of a structured process of learning and method-optimisation enabled real-time adaptations to improve the efficacy of fluralaner treatment of sarcoptic mange in bare-nosed wombats *(Vombatus ursinus)* through *in situ* surveillance (Mounsey *et al.*, 2022; Wilkinson *et al.*, 2024). Identification of strong relationships between seasonal patterns of ophidiomycosis and ecdysis and thermoregulatory behaviours in pygmy rattlesnakes *(Sistrurus miliarius)* highlighted these interactions as potential drivers of infection outcomes (Lind *et al.*, 2023). The development of an agent-based modelling framework that incorporates real-world heterogeneities in disease drivers and surveillance helped to inform appropriate population-specific sample sizes necessary for prompt detection of chronic wasting disease in cryptic species such as Missouri's white-tailed deer *(Odocoileus virginianus)* (Belsare et al., 2020; Escobar et al., 2020). Collectively, this suite of studies demonstrates how improved understanding of factors that affect transmission, disease susceptibility, and intervention efficacy such as dynamic social behaviours, conspecific and environmental disease reservoirs, stage/age- and climate-dependencies, pathogen life cycles, and immune mediated co-infection interactions can directly improve the efficacy of management approaches through informed delivery, targeting, and timing.

The complexity of these systems and the need to incorporate this complexity in our understanding, poses challenges because many of our approaches to data collection and analysis have been developed for less heterogenous systems (production animals and people) (Artois *et al.*, 2009; Beauvais *et al.*, 2019; Belsare *et al.*, 2020; Dohoo *et al.*, 2003; Walton *et al.*, 2016), and because the complexity increases the degree of multidisciplinarity needed to represent the system. This is recognised by several organizations and authors (Barroso *et al.*, 2023; Joseph *et al.*, 2013; Kock *et al.*, 2018; OIE, 2024; Peters *et al.*, 2019a; Thurman *et al.*, 2024; Travis *et al.*, 2014; Walton *et al.*, 2016) but progress is limited by barriers to multidisciplinary approaches such as limited funding, coordination of research and donor

collaboration, and availability of resources and specialists (Barroso *et al.*, 2024; Mazzamuto *et al.*, 2022; Mysterud *et al.*, 2023; Travis *et al.*, 2014).

The development and application of multivariate approaches in the study of human and wildlife disease has been shown to improve scientific understanding of pathogenesis and can help to identify indicators of health and disease. Importantly, these studies demonstrate the significance of indicators that are not classically included within health assessments such as co-infection, immune gene expression, season, climate, temperature, population-density, microbiome, and stress markers (Aguilar *et al.*, 2023; Barroso *et al.*, 2023; Chapman *et al.*, 2021; Glidden *et al.*, 2018; Grimaudo, 2024; James *et al.*, 2015; Langwig *et al.*, 2012a; Lind *et al.*, 2023; Ribas *et al.*, 2023). For example, several multivariate studies identifying emerging viruses, assessing their relationship with host immune responses, generating indicators of viral infection, and examining the cumulative effects of host-pathogen-environment factors, helped develop the understanding of pathogenesis in multiple Salmon sp., generate indicators of health to support health assessments, and inform changes to farming approaches (Lehman *et al.*, 2020; Miller *et al.*, 2017; Miller *et al.*, 2014; Mordecai *et al.*, 2020; Teffer *et al.*, 2019; Teffer *et al.*, 2022). Overlooking factors that contribute to disease dynamics, and using non-representative biometric ranges, can limit the ability of surveillance to accurately estimate long-term prevalence and detect disease (Belsare *et al.*, 2020; Walton *et al.*, 2016). Surveillance-based estimates of prevalence can be biased by fluctuations in population sizes and disease leading to overconfidence in the precision of estimated prevalence and power to detect disease (Walton *et al.*, 2016). Wildlife disease risk assessments are used to drive policy making, and thus misinformed understanding of species health and can affect the distribution of available resources for disease monitoring as observed in the monitoring of Australian vertebrates (Scheele *et al.*, 2019; Walton *et al.*, 2016).

This thesis proposes a stepwise approach to identify, characterise, and test health indicators to validate their use in health assessments and develop our understanding of biological systems and pathogenesis (Figure 1.1). This approach is similar to the Integrative Causal Paradigm presented by Grace (2024), which conveys the progression of evidence building across multiple studies. Univariate studies generate foundational observations that identify

potential health indicators that are associated with various aspects of animal health and disease such as clinical signs, responses to interventions, and survival outcomes (Di Nardo *et al.*, 2015; Dwyer *et al.*, 2022; Rascón-García *et al.*, 2023). Univariate studies build the foundational understanding of factors that are associated with disease processes and are relied on in wildlife studies due to low sample sizes that result in low statistical power (Bonapersona *et al.*, 2021). However, our approaches must advance beyond univariate associations for several related reasons. Firstly, to address fundamental multifactorial concepts that are key to understanding disease processes, such as the disease triangle and the damage-response framework (Casadevall & Pirofski, 2003; Fidel *et al.*, 2020; Guégan *et al.*, 2024; McNew, 1960; Scholthof, 2007). Because univariate analysis cannot account for the complex interrelationships that can influence disease associations, the significance of associations between health indicators and outcomes can be unclear (Joseph *et al.*, 2013). To reduce contradictory results and clarify the relationships among health indicators, multivariate approaches are needed to generate a holistic assessment that accounts for multiple potential markers and confounding factors. However, we acknowledge that in cases where Koch's postulates are satisfied and a causal disease agent is identified in univariate studies, mechanistic studies are an appropriate next step (Falkow, 1988).

Multivariate studies can holistically assess these potential health indicators to account for collinearity and confounding among indicators and explore the potential roles and functions of these indicators within biological systems and pathogenesis. Multivariate studies can refine the number of health indicators by excluding inconsistent or colinear markers and help form hypotheses to direct further investigations within mechanistic studies, as was done using multivariate factor analysis in a human health study (Klomp & de Haan, 2010). The sequential approach of using univariate analysis to inform multivariate analyses was employed by Kock *et al.* (2018) in a multidisciplinary assessment of the likely contributing factors to a mass mortality event of saiga antelopes *(Saiga tatarica tatarica).* Here putatively associated factors were assessed collectively, better reflecting the multifactorial nature of pathogenesis and providing the means to compare the strength of relationships and generate focused hypotheses to support further investigation of causation (Kock *et al.*, 2018).

Because multivariate studies are associative, additional studies are needed to prove causation. Causation can be determined statistically using mechanistic modelling to determine causal effect estimates (Ezanno *et al.*, 2020; Meza & Jeon, 2022; Plank *et al.*, 2022; Simonetto *et al.*, 2022), and characterisation of causal mechanisms (Clarke *et al.*, 2014; Grace, 2024; Parkkinen *et al.*, 2018). Mechanistic studies test hypotheses built by correlative studies by capturing the underlying mechanism or biological features involved in the development of disease and linking these processes with outcomes (Grace, 2024; Meza & Jeon, 2022; Simonetto *et al.*, 2022). Causative mechanisms can be established through mechanistic studies by direct observation, natural or experimental perturbations, established knowledge from scientific fields, confirmed theory, and simulation results (Clarke *et al.*, 2014), using classical principles of reasoning such as the Bradford Hill criteria (Hill, 1965). By understanding the causal mechanisms, the efficacy of intervention and management approaches can be reliably determined and improved (Hone *et al.*, 2023).

The koala is a great example of a species where the multifactorial nature of disease is acknowledged, but is rarely encapsulated within analyses. It is a strong candidate for progression to multivariate analysis because an abundance of literature focused on specific host-pathogen relationships identifies potential drivers of disease but provides limited understanding of the relative importance of these drivers or their interactions, limiting confidence in management in the clinic and in the wild. It is the aim of this thesis to overcome some of the barriers to integrated multivariate analysis to achieve a more holistic and representative view of the host-pathogen-environment interactions that influence disease risk in the koala.

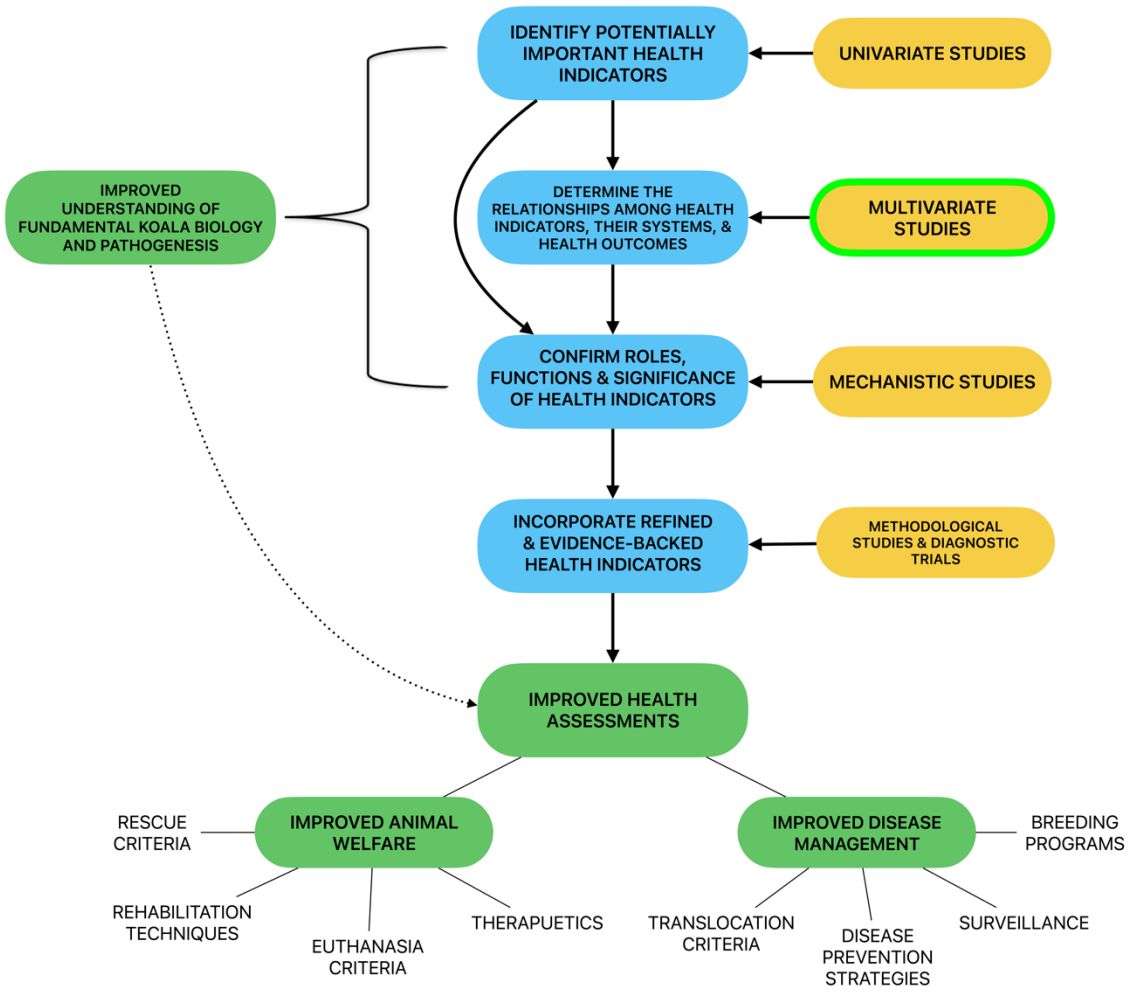


Figure 1.1: Proposed stepwise approach to improving health assessments.

Figure 1.1 illustrates the proposed stepwise progression of disease investigation that demonstrates the type of studies (yellow), examples of what information can be produced (blue), and the hypothesised practical implications of findings (green). This thesis is situated within the multivariate stage (green border).

1.3 The decline of the koala

The koala (*Phascolarctos cinereus*) originally populated Australia in the millions however population decline is a concern for this iconic marsupial. Although accurately surveying this cryptic species is difficult (Crowther *et al.*, 2020; Dissanayake *et al.*, 2021), the estimated national population is now between 144,000-605,000. In South-east Queensland, an 80.3% and 54.3% decline in koala populations was estimated for the koala coast (Redland, Eastern Logan, and South Eastern Brisbane) and pine rivers regions (within Moreton Bay), respectively, between 1996 and 2014 (Rhodes *et al.*, 2015). In New South Wales (NSW), a 26%

decline over the past and next three koala generations (15-21 years) was estimated (Adams-Hosking *et al.*, 2016). Due to the existence of biological and topographical barriers and historical population bottlenecks, the koala's distribution is not continuous, and this species exists as two genetically distinct populations in the north (Queensland, Australian Capital Territory, and New South Wales) and south (Victoria and South Australia) (Cristescu *et al.*, 2009; Fowler *et al.*, 2000; Houlden *et al.*, 1996; Houlden *et al.*, 1999; Ruiz-Rodriguez *et al.*, 2016). Of the total koala population, 65% is estimated to exist within the remaining Mediterranean climate forests, woodlands and scrubs of South Australia (SA) extending into those of Victoria (Vic.) (Whisson *et al.*, 2020). The northern population represents the greatest expanse of this species' range, stretching from the last remaining tropical and subtropical damp broadleaf forests in Queensland (Qld), through the temperate broadleaf and mixed forests along the east coast of Australia and fertile plains and river systems west of the Great Dividing Range, down to the NSW-Victorian border. Its range spans key economical bioregions, fertile farming lands and regions of urban development (IBRA 2012). The koala is recognised as endangered under national and international classifications; the Environmental Protection Biodiversity Conservation (EPBC) Act 1999 and the international ICUN Red List of Threatened Species (DCCEEW, 2022a; ICUN, 2022).

National, regional, and local declines in koala populations can be attributed to several interrelated factors, which are compounding and largely anthropogenic (Lunney *et al.*, 2012a; McAlpine *et al.*, 2015). Disease has been identified as the leading cause of northern koala morbidity and mortality (Gonzalez-Astudillo *et al.*, 2017; Gonzalez-Astudillo *et al.*, 2019; Griffith *et al.*, 2013; Lunney *et al.*, 2023; Rhodes *et al.*, 2011). The National Koala Disease Risk Analysis defines disease as "*any disturbance in the health or function of an animal,* which includes those attributed to *infectious and non-infections causes*" (Vitali *et al.*, 2023). The threat of disease is therefore inextricably linked with all other pressures due to the potential for anthropogenic and climatic impacts to contribute to physical injury (Dissanayake *et al.*, 2023), pathogen distribution and exposure (McAlpine *et al.*, 2017), physiological stress (Davies *et al.*, 2014; Mella *et al.*, 2024; Santamaria *et al.*, 2023), and nutritional imbalances (Chen *et al.*, 2024; Davies *et al.*, 2014). Many studies have examined the impact of environmental and anthropogenic pressures on national, regional, and local koala populations and provide data on the scale of these pressures. For example, recent and historic

drought, heatwaves, fires, and floods have impacted koala populations on a local and regional scale (Cristescu *et al.*, 2023; Filkov *et al.*, 2020; Hyman *et al.*, 2020; Lane *et al.*, 2020; Lunney *et al.*, 2009; Lunney *et al.*, 2012a; Lunney *et al.*, 2012b; Lunney *et al.*, 2020; McAlpine *et al.*, 2015; Tkaczynski & Rundle-Thiele, 2023; Van Eeden *et al.*, 2020; Ward *et al.*, 2020). Large-scale urbanisation of Australia's landscape has resulted in the eradication of local populations in some instances and, in most koala habitats, habitat fragmentation, increased threats from road and rail strikes, domestic animal attacks, and injury and drowning within 'backyard' environments (Adams-Hosking *et al.*, 2016; Lunney *et al.*, 2014; McAlpine *et al.*, 2015; Rhodes *et al.*, 2015; Seabrook *et al.*, 2011; Sullivan *et al.*, 2004). Fragmentation increases risk of predation and injury as koalas traverse between habitats (Whisson *et al.*, 2020); over-population and over-browsing of local niches due to insufficiency of viable habitat for new home ranges (McAlpine *et al.*, 2015; Whisson *et al.*, 2016); and impeded gene flow due to loss of landscape connectivity between populations (Dennison *et al.*, 2016), which in other species has demonstrated knock on effects to host-pathogen immune defence mechanisms (Gonzalez Quevedo, 2014; Perrin *et al.*, 2021). It is widely accepted that, for all species, the extrinsic environment and health are closely linked and that it is therefore likely that the impact of disease will be exacerbated as climatic extremes and natural disasters become more common and natural resources less abundant (Buttke *et al.*, 2021; Caminade *et al.*, 2019; Ebi & Bowen, 2016; Pfenning-Butterworth *et al.*, 2024; Semenza *et al.*, 2022; Weiskopf *et al.*, 2020).

1.3.1. Major infectious diseases of the koala

Like any species, the koala is susceptible to various diseases, some of which are poorly defined. Disease attributed to infection by *Chlamydia pecorum* is well characterised and is the most significant infectious disease (Palmieri *et al.*, 2019; Polkinghorne *et al.*, 2013; Quigley & Timms, 2020), but its drivers are less well established. The role of other prevalent and widely distributed infectious agents, such as Koala Retrovirus (KoRV) and Phascolarctid herpesviruses in causing, or predisposing to, disease are less well known (Gillett, 2023; Greenwood *et al.*, 2023; Kasimov *et al.*, 2020; Quigley & Timms, 2020; Tarlinton & Greenwood, 2024; Vaz *et al.*, 2019b; Wright *et al.*, 2024). Heterogeneity in disease-presentation, responses to treatment, and overall survival outcomes of koalas with these infections, as well as the variation in general and reproductive disease outcomes for koalas in

wild populations, highlight the need for research to improve the current understanding of epidemiology, pathogenesis, and treatment of koala diseases. Because of the complexity of koala disease pathogenesis and the unknown significance of some infectious agents, host characteristics, and environmental variables, here we firstly describe key koala diseases and then follow by focused discussion on the potential contributors to pathogenesis, including agents of unknown pathogenicity.

Chlamydiosis

In northern koalas, chlamydiosis is the most commonly reported disease, comprising 16.5-52.0% of koala admissions to wildlife hospitals (Burton & Tribe, 2016; DEH, 2022; Dutton-Regester, 2024; Gonzalez-Astudillo *et al.*, 2017; Gonzalez-Astudillo *et al.*, 2019; Griffith, 2010; Griffith *et al.*, 2013; Griffith & Higgins, 2012) and 28% to 73% of reported disease in wild populations (Fabijan *et al.*, 2020; Fernandez *et al.*, 2019; Hanger, 2017; Nyari *et al.*, 2017; Robbins *et al.*, 2019, 2020; Simpson *et al.*, 2023). In southern populations, the prevalence of chlamydiosis varies greatly (4 - 53%) but is generally considered less prevalent compared to the north, with some local populations, such as on Kangaroo Island, considered chlamydiosis-free (Fabijan *et al.*, 2019a; Fabijan *et al.*, 2017; Legione *et al.*, 2016; Patterson *et al.*, 2015; Speight *et al.*, 2016). Koala chlamydiosis is almost exclusively caused by the bacterium *Chlamydia pecorum;* although *C. pneumoniae* can also infect koalas and occasionally cause clinical disease (most commonly at ocular and respiratory sites), it is detected very rarely in free ranging koala populations (Devereaux *et al.*, 2003; Hulse *et al.*, 2018; Jackson *et al.*, 1999; Vitali *et al.*, 2023).

Chlamydiosis presents most frequently as disease of the urogenital tract and/or ocular sites (Figure 1.2), comprising cystitis (dysuria, haematuria, incontinence, cloacal eversion, and associated secondary ulceration and cutaneous myasis), renal abnormalities (pyelitis, pyelonephritis, hydroureter and hydronephrosis), reproductive disease (females; salpingitis, vaginitis, endometritis, urogenital sinusitis, uterine cysts, and cystic dilatation of the ovarian bursa. Males; prostatitis), and kerato-conjunctivitis (ocular discharge, conjunctival hyperaemia, chemosis and papillary hypertrophy, corneal oedema and neovascularisation and periocular alopecia (Canfield, 1989; Griffith, 2010; Nyari *et al.*, 2017; Pagliarani *et al.*,

2022; Pagliarani *et al.*, 2024; Palmieri *et al.*, 2019; Polkinghorne *et al.*, 2013; Speight *et al.*, 2016; Wan *et al.*, 2011). The colloquial term for urogenital chlamydiosis, “wet bottom”, denotes the dark brown discolouration of rump pelage due to urinary incontinence. Infection and disease are most commonly persistent and disease can range from mild to severe, persistent or intermittent active inflammatory disease to chronic fibrotic structural disease (Hemsley & Canfield, 1997; Higgins *et al.*, 2005a, 2005b; Vogelnest & Portas, 2019). In severe cases, death ultimately occurs due to organ failure, sepsis, and/or emaciation (Griffith, 2010). Chronic ocular disease can lead to blindness and reproductive disease can cause infertility, which has detrimental consequences for population regeneration (Hemsley & Canfield, 1997; Hulse *et al.*, 2019a; Johnston *et al.*, 2015; Nyari *et al.*, 2017; Pagliarani *et al.*, 2022; Palmieri *et al.*, 2019; Phillips *et al.*, 2021).

Figure 1.2: Gross external and internal clinical signs of chlamydiosis in the koala.

Figure 1.2 demonstrates external and internal signs of chlamydiosis. Top left: male koala with conjunctivitis. Top right: female koala with 'Wet-bottom' and back rider joey with conjunctivitis. Bottom left: female reproductive tract with two fluid filled cysts, both 3 cm in diameter. Bottom middle: right and left kidneys longitudinally sectioned showing dilation of the renal pelvis (hydronephrosis) due to obstruction of distal urine outflow. Bottom right: lower (sectioned) reproductive tract and longitudinally incised bladder displaying fibrotic thickening of the bladder wall. Photo credit: Yasmine S. S. Muir.

Impact of chlamydiosis in the rehabilitation setting

Chlamydiosis remains a dominant cause for admission to wildlife clinics and although rehabilitation techniques and protocols have advanced, trends in overall health outcomes for koalas have not improved greatly. The most recent NSW koala rehabilitation data shows that the proportion of koalas admitted for chlamydiosis is largely unchanged ((DEH, 2022); Figure 1.3). In the 2019-2020 financial year 1613 koala were rescued in NSW(DEH, 2022). Of this, 636 (39.4%) koalas ultimately died, 483 (30%) were released and 494 (30.6%) had unknown outcomes. This regional data, obtained from 29 wildlife rehabilitation organisations and facilities and 11 independent licence holders, demonstrated that, on average, 71% of koalas admitted for chlamydiosis die and 25% are released ((DEH, 2022); Supplementary materials: Table 7.1). These outcome proportions have not improved in nearly a decade.

In Qld, incomplete reporting and follow-up of koala admissions to wildlife clinics limits our understanding of disease trends within the state. However, we do know that disease is frequently severe; of the 1746 reported admissions in 2019, only 25.2% had records indicating release (Kerlin *et al.*, 2022; Queensland Government, 2021). Several retrospective studies assessing koala admissions within major south-east Qld, NSW, and a SA wildlife clinic reported similarly high mortality and disease rates (Supplementary materials: Table 7.1). Burton and Tribe (2016) reported that of the cases classified as 'other medical conditions,' 40% did not have specific diagnoses or additional clinical records available, which likely influenced the resulting lower frequency of chlamydiosis cases. Other retrospective studies of hospital-specific databases show variations in rates of admission, chlamydiosis, and outcomes that reflect population demographics of the catchment areas (Burton & Tribe, 2016; Gonzalez-Astudillo *et al.*, 2017; Griffith *et al.*, 2013; Lunney *et al.*, 2022a; Lunney *et al.*, 2012a; Taylor-Brown *et al.*, 2019). All studies consistently report chlamydiosis as the greatest, or second greatest, cause for admission.

Figure 1.3: Bar chart of annual koala rescues in New South Wales (NSW) obtained from reports from 29 individual koala rehabilitation care groups and clinics submitted to the NSW Government.

Figure 1.3 utilises data that is freely available on the 'Rehabilitation Dashboard'(DEH, 2022). The top chart presents the total number of non-chlamydiosis koala rescued (blue) and the total number of koala rescues attributed to chlamydiosis (orange, counts and percentage (%) of fraction displayed above each column). The bottom dot-plot chart plots three lines which present the percentage of chlamydiosis cases that died (blue), the percentage of chlamydiosis cases that were released (yellow), and the percentage of chlamydiosis cases with other outcomes (grey).

The long-term, post-release, efficacy of treatment and rehabilitation is unclear. Chlamydial infection in koalas can resolve without intervention or recur despite treatment (Robbins *et al.*, 2019; Robbins *et al.*, 2018). Short-term cure rates using antimicrobials have been reported at 81 - 97% (Booth & Nyari, 2020; Markey *et al.*, 2007), but microbial cure rates are likely to be affected by the treatment inclusion criteria, the occurrence of adverse reactions (such as dehydration, weight loss, gut dysbiosis, oxalate nephrosis, and candidiasis), and a wide range of variables many of which remain unknown or undefined. Although drone thermal imaging (Beranek *et al.*, 2020; Beranek *et al.*, 2024; Colombelli-Négrel *et al.*, 2023; Witt *et al.*, 2020), and canine tracking (Cristescu *et al.*, 2020a; Cristescu *et al.*, 2015; Cristescu *et al.*, 2020b; Cristescu *et al.*, 2019; Kent & Cristescu, 2020; Premachandra *et al.*, 2024; Terraube *et al.*, 2023) technology is rapidly evolving, post-rehabilitation monitoring is currently rare due to limited funding (Australian Government, 2021; Leigh *et al.*, 2023). Retrospective assessments indicate a 49.5% re-admission rate for chlamydiosis, with some koalas re-admitted up to four times (Lunney *et al.*, 2022b). Long-term studies of koala populations, e.g. Gunnedah, NSW (Fernandez, 2023); Moreton Bay, QLD (Robbins, 2020), show that chlamydiosis presentations vary over time, infection and disease can regress with or without treatment, and infertility or death can occur without re-exposure to *C. pecorum* (Fernandez et al., 2024a; Fernandez et al., 2019; Robbins et al., 2019; Robbins et al., 2018). Because of the sporadic nature of *C. pecorum* shedding, throughout this thesis cases are defined as having chlamydiosis where disease is observed as described in the beginning of this section. Sub-clinical *C. pecorum* infection - defined here as carriage without clinical signs – has been reported at a frequency of 28.5% in hospital admissions (Chen *et al.*, 2023b) and 34% in a SE-Qld wild population, and doesn't always progress to disease (Robbins *et al.*, 2019). Overall, the mechanisms driving disease development, severity, treatment response and rehabilitation outcomes remain poorly understood, impacting the efficacy with which this disease is managed.

Putative KoRV Associated Diseases

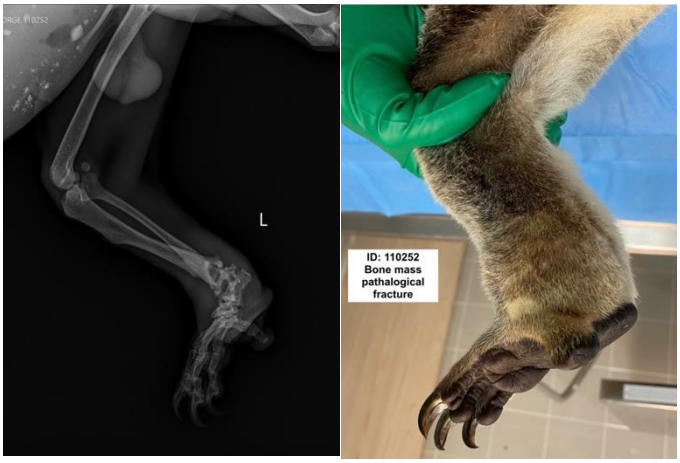
It has been suggested that apparently opportunistic infections of unclear aetiology are linked to immuno-modulative effects of koala retrovirus (KoRV) infection. These have been described previously as 'KoRV-associated diseases' or 'AIDS-like' syndromes (also called Koala immune deficiency syndrome [KIDS]) and, more recently, 'Putative KoRV-associated disease'

(PKAD) (Gillett, 2014, 2023). The association between KoRV and these disease presentations is hypothesised due to similarities in biology and genetic structure between KoRV and other pathogenic retroviruses, such as feline leukemia virus (FeLV) and gibbon ape leukemia virus (GALV) (Hanger *et al.*, 2000; Oliveira *et al.*, 2006; Oliveira *et al.*, 2007; Tarlinton *et al.*, 2008b), and is supported in part by strong associations between KoRV detection or transcription and disease in koalas (Denner & Young, 2013; Stoye, 2006; Tarlinton *et al.*, 2008a), though causative relationships are less certain. Although preliminary measures of prevalence and incidence rates of PKAD are low; 1.16% (8/691; 95% CI 0.5–2.19%) and 3.5 cases/100 koalas/year (95% CI 2.35–4.9), the reported severity of cases and associated high mortality demonstrate the impact on individuals (McKay & Jones, 2023) and, if effects extended to predisposing koalas to development of chlamydial disease, impacts would be much greater.

PKAD is separated into two major classes: Neoplasia and bone marrow dysplasia, and putative immune dysfunction disorders (PIDDS) (Gillett, 2023) (Figure 1.4). In the first class, haematopoietic and oncogenic disorders such as myelodysplasia, leukaemia, aplastic anaemia, lymphoma, osteosarcoma, fibrosarcoma, mesothelioma, and other tumours are included (Greenwood *et al.*, 2023; Imanishi, 2023; McEwen *et al.*, 2021; Speight, 2023; Tarlinton & Greenwood, 2024). As many as 55% of deaths in some captive populations were attributed to lymphoid neoplasia (Hanger, 2000). Following a survey of neoplastic and 'AIDS-like' disease, tumours, largely lymphoma, were overwhelmingly the most prevalent PKAD noted by some koala treatment facilities (56%) (Gillett, 2014). Looking at the clinical records of 264 individual koalas from 16 different koala treating facilities across Qld, NSW, Vic, SA, and WA over a 5 to 28 year period, bone marrow conditions represented 14% of reported PKAD, with leukaemia dominating this category (Gillett, 2014). Among koalas in Japanese zoos (N = 330), "malignant neoplasms" accounted for 16.5% of deaths, with lymphoma reported as the most prevalent (Imanishi, 2023). Cellular dysplasia, which ranged from minimal to moderate dysplastic change, most frequently in erythroid cell lines, was evident in 77% (n = 26) of clinically healthy captive koalas evaluated in North American zoos and 21% and 7% of deaths between 1976-2020 were attributed to lymphoid neoplasia and myelodysplasia, respectively (Singleton & Hamlin-Andrus, 2023). Similarly, another study reported 12.7% of captive koalas from North American and Australian zoos died from leukaemia-lymphoma and 5.6% from other cancers (Zheng *et al.*, 2020b). Although PKAD was historically largely

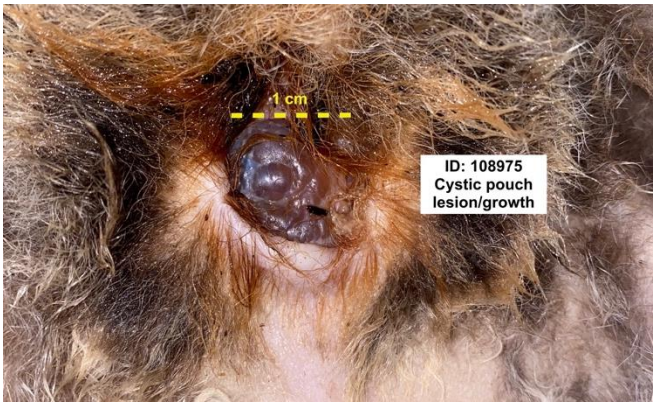
reported in captive koalas, it is an emerging concern for wild populations. A large-scale study that assessed 519 free-ranging koalas that were admitted to wildlife hospitals across SE-Qld, 7.9% had evidence of neoplasia alone or in combination with other conditions at necropsy (Gonzalez-Astudillo *et al.*, 2019).

The second group, PIDDS, is defined as concurrent presence of any two of the following: dermatological, keratinous, oral, gastrointestinal, and haematopoietic conditions, fungal or parasitic infections, treatment refractory conditions, and 'ill-thrift', as well as severe chlamydiosis that is non-responsive to treatment (Gillett, 2023). Gillett (2014) noted that 'AIDS-like' disease accounted for 20% of reported KoRV-associated disease presentations, with dermatitis being most common. In the study of wild-caught and captive koalas, 29.4% of cases died from other causes such as anaemia, pulmonary interstitial fibrosis, and acute respiratory illness (Zheng *et al.*, 2020b). In captive koala populations in Japan, "opportunistic infections", "immunodeficiency", "septicaemia", and "stunted growth of joey" accounted for 3.9%, 0.6%, 3.3%, and 10.9% of deaths (N = 330), respectively (Imanishi, 2023). Monitoring conducted at different times and durations across three free-ranging koala populations in SE-Qld reported 20 cases of "AIDS-like syndrome" (McKay & Jones, 2023). Using retrospective clinical data, poorly defined clinical conditions such as 'wasting', originally termed koala stress syndrome, which is characterised by lassitude, depression, anorexia and coma (Obendorf, 1983), are increasingly reported (Gonzalez-Astudillo *et al.*, 2017; Gonzalez-Astudillo *et al.*, 2019; Kerlin *et al.*, 2022). Because these presentations are not proven diseases of KoRV but are very relevant to this thesis, the potential KoRV -associated co-infection and immunomodulatory interactions will be discussed in detail further down.

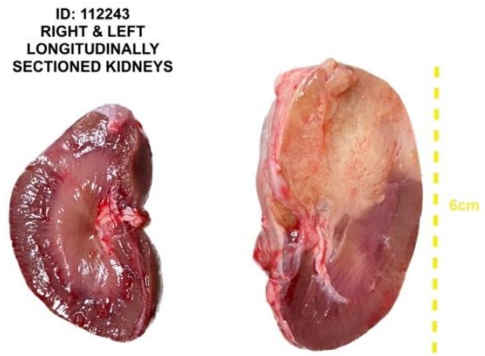


a) Pathological fracture

b) Idiopathic emaciation



c) Cystic growth



d) Fibrous growth

Figure 1.4: Various clinical presentations classed within putative KoRV-associated diseases.

Figure 1.4 provides examples of external and internal signs of disease, encountered during candidature, that were considered by clinicians to be potentially associated with KoRV: a) radiograph and gross presentation of suspected chondrosarcoma, presenting as a pathological fracture of the left hock, b) idiopathic presentation of depressed mentation, emaciation and fatigue in a male koala, c) external cystic pouch lesions (two) that extended into the abdominal cavity, d) right and left kidneys with vascularised fibrotic growth on the left noted on ultrasound and at necropsy; this case had suspect myelodysplasia, poor body condition, 'wet-bottom' with rump ulceration, unilateral reproductive disease, bladder thickening, and unilateral ocular ulceration. Photo credits: Yasmine S. S. Muir. Radiograph: Australia Zoo Wildlife Hospital.

1.4 Towards a more holistic approach to koala health assessment

To advance health assessments for improved approaches to disease surveillance, investigation, treatment, and management, more informative and easily assessed health indicators are needed, and this requires a better understanding of koala biology and pathogenesis. Throughout this thesis, "health indicators" refers to qualitative and quantitative measures that are associated with, or predict, health outcomes such as infection, morbidity, and mortality. Classical veterinary diagnostic tools, such as behavioural and physical examination, radiography, haematology, biochemistry, urinalysis and morphological assessment of biological samples are utilised for koala health assessments (Vogelnest & Portas, 2019). However, physical examination tools are often categorical and subjective, and routine haematology, while providing both categorical (morphological) and objective (cell counts) measures, offers a low resolution of biological states and temporal scales of infection: in SA koalas haematological values were not impacted by sub-clinical *C. pecorum* and KoRV infection; in SA and Qld koalas, haematological values did not differ according to KoRV exogenous subtype (Akter *et al.*, 2023; Hashem *et al.*, 2022; Kayesh *et al.*, 2021b) although some relationships were observed with proviral and viral loads (Fabijan *et al.*, 2020); and over the course of chlamydiosis treatment, haematology and most biochemistry parameters remained within published reference intervals (Canfield *et al.*, 1989; Griffith, 2010).

Extending from haematology to measures of stress and immunological activity can provide an objective assessment of biological states that links environmental stressors, pathogen challenge, and host responses to better elucidate disease dynamics. Incorporation of diverse, yet relevant and informative indicators, such as faecal and hair stress hormone levels, toxicology and pathogen exposure, in the health assessment of an endangered caribou herd helped to determine factors contributing to reduced survival (Aguilar *et al.*, 2023). This approach also reduced human intervention and handling stress through non-invasing sampling (faeces, and hair) (Aguilar *et al.*, 2023). Currently in koalas, many potential indicators of health have been identified but are yet to be assessed collectively to allow their relative importance to be understood sufficiently to incorporate them into health assessments effectively: *C. pecorum* strain type (Fernandez *et al.*, 2024a; Robbins *et al.*, 2020; Sait *et al.*, 2014), presence and activity of other infectious agents (Blyton *et al.*, 2022a; Fabijan *et al.*,

2019b; Fabijan *et al.*, 2020; Fabijan *et al.*, 2017; Hashem *et al.*, 2021; Legione *et al.*, 2017; Maher *et al.*, 2019; McInnes *et al.*, 2011a; Sarker *et al.*, 2020a; Stalder *et al.*, 2015; Stephenson, 2021; Vaz *et al.*, 2019b; Waugh *et al.*, 2017), host genetics (Kidd *et al.*, 2024; Lau *et al.*, 2014; Quigley *et al.*, 2018a; Quigley *et al.*, 2020), host immune response (Fiebig *et al.*, 2015; Higgins, 2004; Higgins *et al.*, 2005a; Kayesh *et al.*, 2022; Kayesh *et al.*, 2021a; Khan *et al.*, 2016; Mathew, 2014; Mathew *et al.*, 2013a; Mathew *et al.*, 2013b; Olagoke *et al.*, 2018; Olagoke *et al.*, 2019; Olagoke *et al.*, 2020a; Pagliarani *et al.*, 2024; Phillips *et al.*, 2019), and environmental factors (Davies *et al.*, 2013; Narayan & Vanderneut, 2019; Narayan & Williams, 2016; Santamaria *et al.*, 2023). These studies are critical to the formation of foundational hypotheses and preliminary identification and assessment of the associations among health indicators and outcomes in the koala. However, research is needed to better understand the veracity of these indicators as biomarkers of disease or disease risk, and the mechanisms of pathogenesis reflected.

The approach of this thesis to considering pathogenesis and disease risk is grounded in two overarching conceptual frameworks. The first, the damage-response framework (DRF) defines disease, in this case infectious disease, as a function of the host-microorganism(s) interactions (Casadevall & Pirofski, 2003; Fidel *et al.*, 2020). This contrasts to earlier definitions of microbial pathogenicity that considered pathogen virulence as only a characteristic of the infectious agent (microorganism-centred) or as a result of failure of host defence mechanisms (host-centred), rather than an interaction between the two (Falkow, 1988; Wassenaar & Gaastra, 2001). The disease triangle is another conceptual model, which complements the DRF by expanding beyond the internal environment to position disease as a function of the interactions between the host, the pathogen(s), and the physical environment (HPE) (McNew, 1960; Scholthof, 2007). Ultimately, these frameworks encompass the reality of pathogenesis; that each host-microorganism interaction is unique and exists within a constantly changing environment (Casadevall & Pirofski, 2003). The diversity of disease presentations in the koala demonstrates the complexities of host-pathogen interactions. Only by accounting for the natural complexities of disease will relevant and consistent health indicators be determined (Casadevall & Pirofski, 2003).

The following sections review current literature pertaining to the elements of the host pathogen environment interactions and damage-response framework as these relate to koala disease. These include koala infectious agents, host responses, and environmental factors that may be associated with disease outcomes and could be investigated further using multivariate approaches to better understand their relationships and potential as indicators of koala health. Given that this thesis focuses solely on northern koalas, greater emphasis is given to pathogen prevalence and relationships identified in koalas from northern regions. However, where relevant we acknowledge the regional differences and direct the reader to further information in southern populations. It is important to note that, although other koala syndromes such as PKADs should be better understood, chlamydiosis represents a considerable proportion of koala disease and focus for disease research in northern populations, which is reflected in this review.

1.4.1 Pathogens, pathogenesis, and co-infection interactions

Chlamydia pecorum is the cause of chlamydiosis in the koala, but disease outcomes vary. The capacity of other microorganisms known to infect the koala, such as KoRV, Phascolarctos herpesvirus -1 and -2, and trypanosomes, to cause or exacerbate disease is unclear, as is the potential for their virulence to alter HPE dynamics. As such, the significance of co-infection interactions to diverse disease outcomes is a major knowledge gap. Damage to the host can result from both the host response and/or microbial factors (Casadevall & Pirofski, 2003) (Figure 1.5). Microbe-induced damage can result from replication, toxin production, and/or destabilisation of host homeostasis and/immune mechanisms. Depending on the microorganism, this can occur at a molecular, cellular, or organ level. Here a 'pathogen' is defined as a microorganism capable of causing host damage and 'virulence' as the relative capacity of a microorganism to cause host damage (Casadevall & Pirofski, 1999). These are not independent microbial variables as it is only within a susceptible host, in a conducive environment, that a microorganism is a pathogen and virulence is expressed (Casadevall & Pirofski, 2003).

Initial host-*C. pecorum* interactions may result in two primary outcomes: elimination of, or colonisation by, the microorganism. Colonisation refers to the "state of host-microorganism

interaction that leads to a variable amount of host damage, from minimal to great, thereby reflecting host immune responses that have the capacity to eliminate the microorganism or to promote the development of another state" (Casadevall & Pirofski, 2003). Other states include commensalism or tolerance, an established host-microorganism synergy, or on the other end of the spectrum, a state of persistence that can progress to disease (subclinical or clinical) (Casadevall & Pirofski, 2003). The factors that influence the balance and shifting along this axis are context-specific and have not been determined in the koala (Figure 1.5). The role of co-infections in driving heterogeneity in disease presentations and responses to treatment through alternations in host-pathogen interactions is an understudied area in koala disease investigations, but this section will demonstrate its potential importance and how the failure to account for co-infections is a potentially significant limitation for many studies investigating the associations between disease outcomes and health indicators, such as immune parameters.

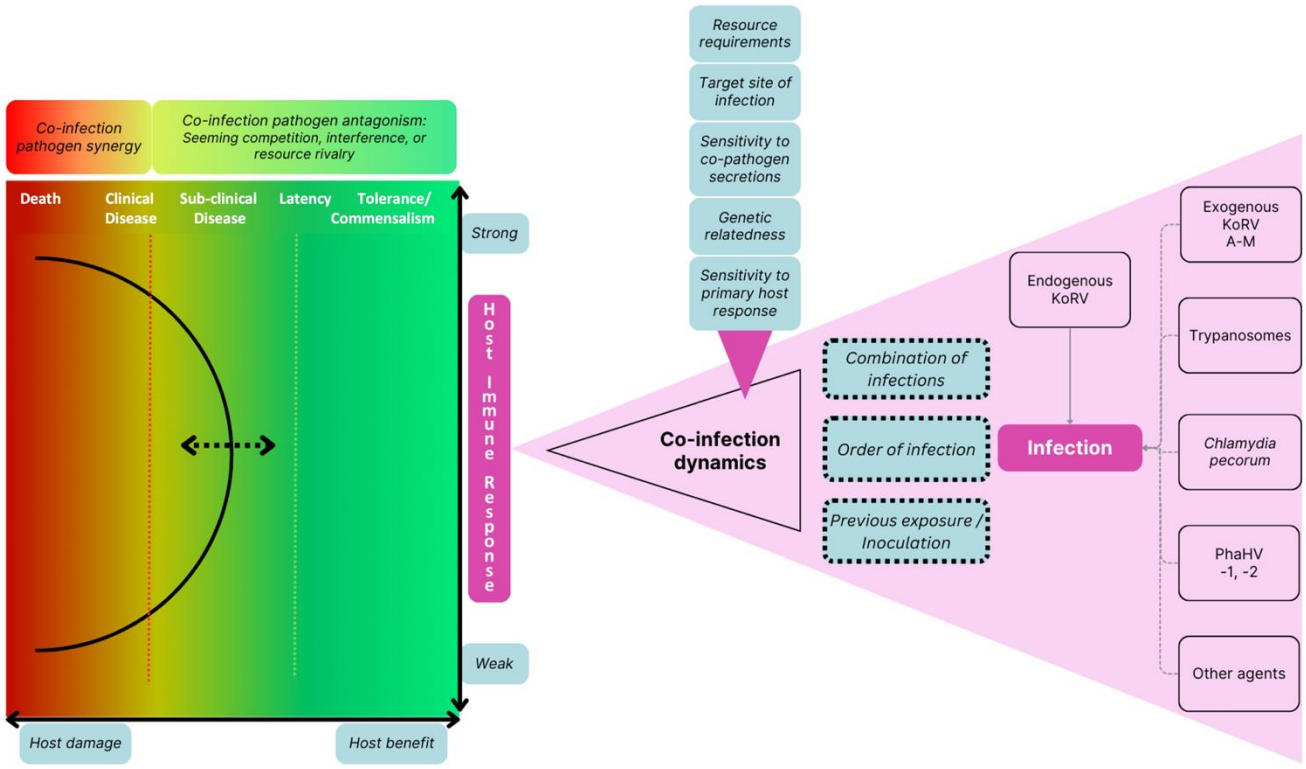


Figure 1.5: The damage-response framework (DRF) in the context of koala disease.

Depicted in Figure 1.5 is the basic parabolic curve central to the DRF that describes variations in host-damage (x-axis) as a function of the host response (y-axis) resulting from the interaction between the host and the microorganism(s). The host response, and thus also host damage, is represented as a continuum (arrow) from 'weak' to 'strong' and from 'damage' to 'benefit'. These terms can all be defined by both quantitative and qualitative characteristics specific to each clinical presentation. At the top of the curve describes several host-microorganism interactions associated with the degree of damage and benefit. Above this, are examples of co-infection interactions. Within the pink triangle to the right are examples of potential pathogens (first column) which are known to infect the koala. The blue boxes describe key factors which can affect the initial host response to these agents (dotted border) and factors that can modify the co-infection dynamic that ultimately alter host-microorganism interactions. Adapted from (Casadevall & Pirofski, 2003).

The impact of coinfecting agents may be negligible, additive, synergistic or antagonistic. For example, in humans, *Chlamydia trachomatis* co-infection with human immunodeficiency virus (HIV) has synergistic effects, enhancing HIV infection by increasing HIV co-receptors on epithelial cells, decreasing cell integrity and increasing HIV migration across the epithelial barrier and increasing the accumulation of HIV co-receptor expressing cells at the site of infection (Buckner *et al.*, 2016; Ghasemian *et al.*, 2023; Schust *et al.*, 2012). Disease progression in HIV co-infection has also been observed with other agents such as

Tuberculosis, through chronic immune activation leading to immune senescence (Bolaji *et al.*, 2024; Saeidi *et al.*, 2015). *C. trachomatis* co-infection with human papillomavirus (HPV) (Koster *et al.*, 2022; Lu *et al.*, 2024) or human herpes viruses (Deka *et al.*, 2006; Deka *et al.*, 2007; Prusty *et al.*, 2012; Prusty *et al.*, 2013; Vanover *et al.*, 2010) is common, with evidence suggesting the induction of a persistent state by the bacteria and either of the viruses through changes in immune environments, and may depend on temporality/order of pathogen infection (Chu *et al.*, 2016; Slade *et al.*, 2016). In contrast, pre-infection of mice with *Trypanosoma brucei brucei* increased host survival by limiting the establishment of coinfection by *Plasmodium berghei*, the agent causing cerebral malaria, through accumulation of hepatic lymphocyte-derived IFN-γ (Sanches-Vaz *et al.*, 2019). Similar or related infectious agents infect the koala and have been detected as coinfections in some individuals (Barbosa *et al.*, 2017; Fernandez, 2023; Fernandez *et al.*, 2024b; Quigley *et al.*, 2018a; Wright *et al.*, 2024). However, we have little understanding of how they might interact to influence disease outcome variability.

Chlamydia pecorum

Chlamydia pecorum is an obligate, intracellular, gram-negative, coccoid bacterium of the family *Chlamydiaceae*. As reported in a review by Sachse and Borel (2020), *Chlamydia* species are globally prevalent, infecting over 400 different animal host species including humans, livestock, domestic animals, marsupials, birds, reptiles, amphibians, and fish. The prevalence of *C. pecorum* in several populations across Australia has been summarised in multiple reviews (Kayesh *et al.*, 2024; Quigley & Timms, 2020). In northern koala populations, infection by *C. pecorum* occurs at variable frequency, up to 91% in some populations, and is absent in few populations (Devereaux *et al.*, 2003; Fernandez *et al.*, 2019; Griffith *et al.*, 2013; Jackson *et al.*, 1999; Kayesh *et al.*, 2024; Kollipara *et al.*, 2013a; Nyari *et al.*, 2017; Polkinghorne *et al.*, 2013; Simpson *et al.*, 2023). Transmission of *C. pecorum* between koalas is assumed to occur horizontally through venereal transmission or through male-male conflict, and vertically through birth and pap-feeding (the ingestion of the dam's caecal contents by the young to establish intestinal flora) (Nyari *et al.*, 2017). There are two main methods of *C. pecorum* detection; the point-of-care (POC) based Loop Mediated Isothermal Amplification (LAMP) assay and the gold-standard laboratory-based quantitative PCR (qPCR). Specificity and sensitivity of qPCR is greater, however the delay in receiving results from specialised

diagnostic laboratories is often not practical in the clinical setting. Hence, LAMP assays are used commonly at the point of care in major koala hospitals as a rapid pathogen detection and surveillance method (Harvey *et al.*, 2019; Hulse *et al.*, 2019c; Jelocnik *et al.*, 2017). Hulse *et al.* (2019b) describes the most used LAMP method in full.

The existence of diverse outcomes of *C. pecorum* infection, including subclinical disease, supports the relevance of the damage-response framework to this disease (Robbins *et al.*, 2019). Detection of mucosally shed *C. pecorum* is not always associated with evidence of disease, indicating that other factors affect the virulence of this pathogen. The level of mucosal chlamydial shedding (DNA copies) has been associated with the detection, severity, and progression of disease at the associated site (Lizárraga *et al.*, 2020b; Phillips *et al.*, 2024b; Quigley *et al.*, 2018a; Robbins *et al.*, 2019; Wan *et al.*, 2011). However, while it is logical that greater replication of an infecting microorganism would generate greater antigenic stimulation of the immune response and thus immune-driven and microorganism-driven damage to the host, contradictory results in other studies suggest that replicative activity of *C. pecorum* is not the only promoter of immune responses and associated damage (Fernandez *et al.*, 2024b; Robbins *et al.*, 2019, 2020; Waugh *et al.*, 2017). For instance, longitudinal monitoring of koalas indicated that high levels of *C. pecorum* shedding was associated with early infection only (Robbins *et al.*, 2019), suggesting that temporal dynamics exist and likely change the relationship and significance of *C. pecorum* replicative activity in pathogenesis.

Under-detection using current swab-based techniques could obscure aspects of the pathogen-disease relationship. *C. pecorum* has been detected in a range of cells and tissues not commonly available to urogenital and conjunctival swab-based sampling; the gastrointestinal tract, hepatic and splenic macrophages (Burach *et al.*, 2014; Higgins *et al.*, 2005b; Phillips *et al.*, 2018; Wedrowicz *et al.*, 2016), pulmonary alveolar macrophages and respiratory epithelium (Mackie *et al.*, 2016), and tarsal tissue and synovial fluid (Burnard *et al.*, 2018). Furthermore, the detection of *C. pecorum* DNA within the bursal fluid of female koalas and in the testis and epididymis of male koalas highlights infection that may not be detected by urogenital swab (Pagliarani *et al.*, 2022; Palmieri *et al.*, 2019).

The ability of chlamydiae to establish latent persistent forms may also impact their detectability. *C. pecorum* is very successful as an infectious agent due to the ability to deviate from its optimal life cycle and maintain viability within sub-optimal host environments. Like other species within this family, *C. pecorum* has a biphasic developmental life cycle (Elwell *et al.*, 2016; Gitsels *et al.*, 2019). The 36-96 hour life cycle (Figure 1.6) begins in the extracellular infectious form, the elementary body (EB) (Bastidas *et al.*, 2013; Corsaro & Danielle, 2004). Invasion of EBs into susceptible cells triggers the EB's differentiation into reticulate bodies (RB), the non-contagious but replication-competent form (Chen *et al.*, 2019a; Grieshaber *et al.*, 2018). Following binary fission, RBs then differentiate back into EBs for release from the cell, by cell lysis. Thus, *C. pecorum* are able to rapidly colonise a host after only a few rounds of replication (Chen *et al.*, 2019a; Chiarelli *et al.*, 2023) (Figure 1.6). Persistence is the term used to describe a stress reaction whereby exposure to particular cytokines, antibiotics, or nutrient deprivation causes the microorganism to reduce intracellular RB division and remain viable and protected from challenges from the extracellular environment (Panzetta *et al.*, 2018). This form, larger than typical RBs and termed aberrant RB (aRB), has slowed DNA replication, with gene transcription occurring without cell division. As pressures cease, the aRBs can transition into infectious EBs and the life cycle can resume (Panzetta *et al.*, 2018). While it has been hypothesised that macrophages could be a potential site of latent persistence for *C. pecorum* in the koala (Higgins *et al.*, 2005b), as macrophages have been observed to sustain the viability of *C. muridium*, *C. pneumoniae* and *C. trachomatis* (Azenabor & Chaudhry, 2003; Gracey et al., 2013; Herweg & Rudel, 2016; Rajaram & Nelson David, 2015; Sun et al., 2012; Tietzel et al., 2019), this has not been established and likely mechanisms driving this state in the koala are unknown.

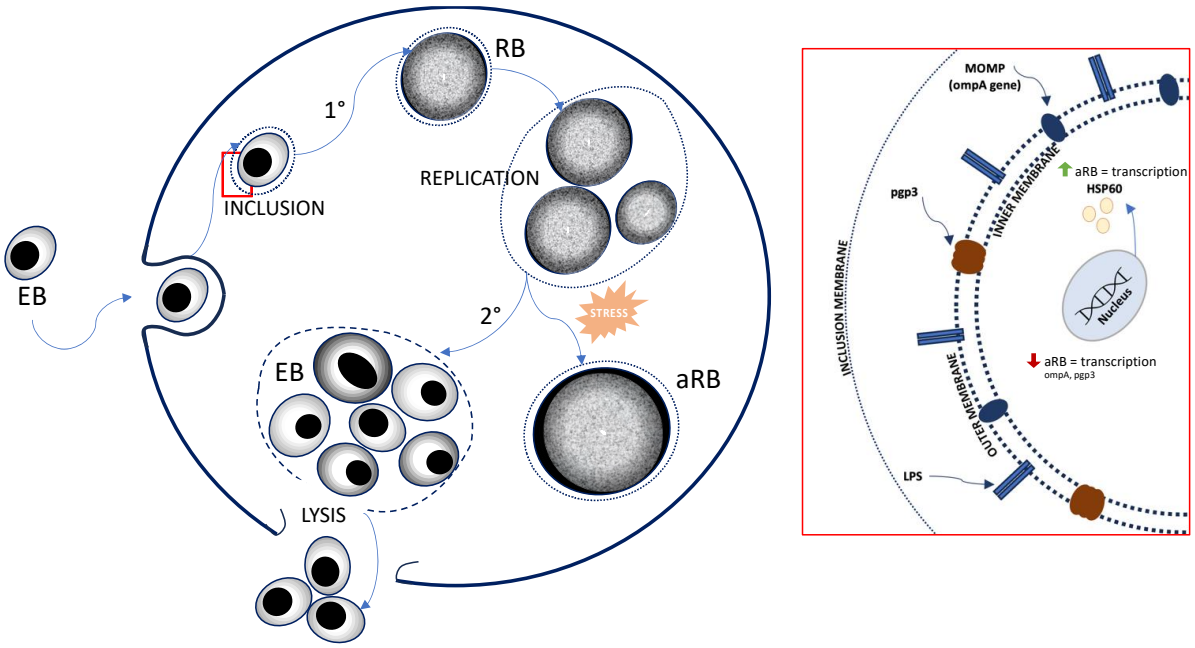


Figure 1.6: Life cycle and selected microbial patterns of Chlamydia.

Figure 1.6 demonstrates the life cycle of Chlamydia sp. *and provides examples of common microbial patterns used to detect and investigate the genetic variation of* Chlamydia sp. *Chlamydial elementary body (EB) gains entry to host cells becoming enclosed within the inclusion membrane allowing for intracellular immune evasion. EBs undergo a primary (1°) differentiation into reticulate bodies (RBs) which then replicate. Under optimal environmental conditions within host cells the RBs undergo a secondary differentiation (2°) into infectious EBs whose maturation triggers host-cell lysis and release into the extracellular matrix allowing for continued infection of the surrounding tissue. Where the host-cell experiences sub-optimal conditions (stress) from resource depletion, immune responses, or co-infection of other pathogens, replicating RBs will undertake a chlamydial stress response by reducing cellular activity and existing/persisting in a latent form termed aberrant RBs (aRBs). In this form, transcription of cell replication and maturation genes such as pgp3 (plasmid glycoprotein 3), LPS (lipopolysaccharide), and ompA (major outer membrane protein) are down regulated, however stress-response genes such as Hsp60 (heat shock protein 60) are maintained or even up-regulated. As stressors are removed and optimal host cell environments return, aRBs revert into replicative RBs and continue the cell-cycle.*

Host-pathogen interactions driving disease may differ according to pathogen genetics. Distinct polymorphic regions, elements and genes exist within the *Chlamydiaceae* genome and include the plasticity zone (PZ), genes encoding Type III secretion system (T3SS), inclusion membrane proteins (*Incs*), polymorphic membrane (*pmps*), and major outer membrane protein (MOMP) (*ompA-C*) (Dimond *et al.*, 2021; Hölzer *et al.*, 2020; Szabo *et al.*, 2020). Strong recombination potential within these genes have resulted in gene variants and - depending on strain typing classification schemes - strain variants that are differentially associated with virulence and pathogenicity (Dimond *et al.*, 2021; Porcella *et al.*, 2015; Rajeeve *et al.*, 2020; Rawre *et al.*, 2019; Somboonna *et al.*, 2019; Song *et al.*, 2013; Turman *et al.*, 2023; Versteeg

et al., 2018; Yang *et al.*, 2020; Zhong, 2017; Zhou *et al.*, 2022). Common *Chlamydia* sp. gene targets associated with virulence include *ompA, pmpG1, IncA,* and *ORF663* (Higgins et al., 2012; Marsh et al., 2011; Mohamad et al., 2014; Mohamad et al., 2008). The major outer membrane protein (MOMP), which is coded by *ompA*, is one of the main targets of the host immune response (Kollipara *et al.*, 2013a; Zhang, 1999; Zheng *et al.*, 2007). Gene products from *pmpG1* and *IncA* are involved in chlamydial attachment to mucosal cell membranes (Becker & Hegemann, 2014; Desclozeaux *et al.*, 2017) and pathogen inclusion and host cytosolic interactions (Mohamad *et al.*, 2008; Scidmore *et al.*, 1996), respectively. *ORF663* includes a 15-nucleotide variant CTR (15-mer) and encodes a hypothetical protein with unknown function but hypothetical involvement in virulence (Mohamad *et al.*, 2008). Inconsistencies among relationships between *C. pecorum* genotypes and disease suggest they are likely not absolute indicators of virulence. Various *ompA* genotypes (Fernandez *et al.*, 2019; Jelocnik *et al.*, 2014; Legione *et al.*, 2016; Nyari *et al.*, 2017; Robbins *et al.*, 2019, 2020), and the type and quantity of coding tandem repeats (CTR) in *IncA* and *ORF633* (Higgins et al., 2012; Mohamad et al., 2014; Mohamad et al., 2008) are differentially associated with disease progression: *ORF663, IncA,* and *ompA* have co-linear relationships with virulence (Mohamad *et al.*, 2014), severe disease is apparent in koala populations despite low sequence diversity of *ompA* (Higgins et al., 2012), and low numbers of *ORF663* CTRs were observed in healthy animals. To unravel the significance of virulence-associated genes, further combined investigation accounting for variations in time, population characteristics, and sample size are required.

Intermittent shedding of *C. pecorum* and recurrent *C. pecorum* infections post-treatment occur in the koala (Booth & Nyari, 2020; Hulse *et al.*, 2019a; Robbins *et al.*, 2018) and in *C. pecorum* infection of other species (Bommana, 2019), and a range of host and external factors likely modify the host-pathogen relationship in *C. pecorum* infection. Some of these might be inferred from other host-chlamydial interactions but not all. Interferon-gamma (IFNy)-induced persistence of *Chlamydia* sp. is observed commonly in *C. trachomatis* infection where, through IDO-mediated catabolism, immune mediated IFNy release drives the depletion of tryptophan, an important nutrient required for *Chlamydia* sp. development (Vollmuth *et al.*, 2022). However, *C. pecorum* does not rely upon host tryptophan and instead can produce its own, suggesting that the induction of *C. pecorum* latency may not be IFN-γ

related (Islam *et al.*, 2018). Quantifying microbial gene expression and protein production can be useful in identifying variations in life-cycle and bacterial activity: an early study identified that in atypical persistent forms of *C. trachomatis*, the production of *Hsp60* proteins remained constant, where MOMP and lipopolysaccharide proteins were greatly reduced under IFN-γ induced stress (Beatty *et al.*, 1993). However, under similar IFN-γ induced stress, *C. pneumoniae* genes *ompA*, *ompB, pyk, nlpD, Cpn0585* were upregulated whereas *Hsp60* – a heat shock protein (hsp) - was not (Mathews *et al.*, 2001). This has potential relevance to pathogenesis as greater serum concentrations of antibodies against *C. trachomatis Hsp60* correlated with more severe manifestations of pelvic inflammatory disease (Debattista *et al.*, 2003; Eckert *et al.*, 1997), and were associated with chronic disease such as asthma, arteriosclerosis, and coronary heart disease in *C. pneumoniae* infection (Betsou *et al.*, 2003; Ciervo *et al.*, 2002; Fong *et al.*, 2002; Huittinen *et al.*, 2001). These findings were corroborated in koala *C. pecorum* infection, where greater anti-hsp IgG titres were associated with koalas with chronic inactive fibrous occlusion in the reproductive tract (Higgins, 2004; Higgins *et al.*, 2005a), though it is unclear whether this reflects a pathogenic role for *Hsp60*, or simply increasing antibody responses with chronic disease.

Koala Retrovirus

Koala Retrovirus (KoRV) was first noted in the 1980's and then described as an infectious agent of the koala in 2000 (Canfield *et al.*, 1988; Hanger *et al.*, 2000). This positive sense RNA gamma retrovirus is most similar to the primate Gibbon Ape Leukemia Virus (GALV) and Feline Leukemia virus (FeLV) (Chiu & VandeWoude, 2021; Hanger *et al.*, 2000; Simmons *et al.*, 2014) and occurs in both endogenous and exogenous subtypes. Endogenous retroviruses (ERV) are retroviruses that become incorporated into the host germ line (Johnson, 2019) and, throughout evolution, become fixed and then eventually lose their ability to replicate as a consequence of random genetic drift and natural selection (Johnson, 2019). Despite their lack of viral functionality, these ERVs have various roles in genomic and biological regulation of the host (Fu *et al.*, 2019; Tokuyama *et al.*, 2018). KoRV exists within the early stages of evolution in its host population; it was estimated to have begun its endogenization into the koala genome recently, between 49,900 and 120 years ago (Ávila-Arcos *et al.*, 2012; Ishida *et al.*, 2015), and endogenous subtypes remain replication-competent. Thus, many KoRV integrations are not fixed (Johnson, 2019).

The epidemiology and taxonomy of KoRV is complex. Deep sequencing of endogenous KoRV A env regions between northern and southern koalas showed that KoRV A first invaded northern koala genomes and spread south (Blyton *et al.*, 2022d). The endogenous KoRV A subtype is endemic (100% prevalence) within northern koalas and is inherited by offspring (vertically transmitted). In southern populations, KoRV genomes are generally missing one or more *gag, pro-pol,* or *env* genes (Sarker *et al.*, 2020a), comprising a range of defective endogenous "RecKoRV" variants that are not fixed between animals (Fabijan *et al.*, 2019b; Legione *et al.*, 2017; Simmons *et al.*, 2012; Tarlinton *et al.*, 2022a) while competent, complete virus is much less common and, at this stage, appears to be exogenous. The regional variations in exogenous subtype infections are summarised graphically in a review by Quigley and Timms (2020). Briefly, exogenous subtypes B – M are locally prevalent and geographically restricted in northern koala populations (Blyton *et al.*, 2022d; Chappell *et al.*, 2017; Joyce *et al.*, 2021; Oliveira *et al.*, 2007; Tarlinton *et al.*, 2006), are highly polymorphic (Simmons *et al.*, 2012), appear to arise independently and diversify within populations due to frequent recombination of the envelope (*env)* (Greenwood et al., 2018; Tarlinton et al., 2008a; Xu et al., 2015), and individuals can be co-infected with multiple subtypes (Blyton *et al.*, 2022d; Fabijan *et al.*, 2019b). Following endogenous KoRV-A, exogenous KoRV-D is the second most common subtype (Quigley *et al.*, 2019) in northern populations. KoRV subtype diversity and transcription varies across local and regional populations. KoRV subtypes vary in the location and frequency of mutations and thus potentially have different interactions with host machinery (Oliveira *et al.*, 2006; Oliveira *et al.*, 2007).

KoRV has been associated with various diseases including chlamydiosis and neoplasia, however our understanding of the mechanisms by which KoRV may drive, or reflect, pathogenesis in koalas is in its infancy. Some other retroviruses either cause or contribute to the development of various cancers and immune-mediated and immunosuppressive diseases (Beatty, 2014; Canto-Valdés *et al.*, 2023; Daradoumis *et al.*, 2023; Mangeney & Heidmann, 1998; Young *et al.*, 2013) through insertional oncogenesis (Bushman, 2020), or immunosuppression due to cytotoxic effects (Attermann *et al.*, 2018; Gibbert *et al.*, 2014; Giraudon *et al.*, 1995; Rycaj *et al.*, 2015), the activity of the immunosuppressive domain (Blinov *et al.*, 2013; Denner, 2016; Haraguchi *et al.*, 1997; Snyderman & Cianciolo, 1984) or

mutation or dysregulation of immune genes by genomic viral integrations (McEwen *et al.*, 2021). The immunosuppressive domain, designated CKS-17, is located within the retroviral transmembrane envelope (p15E) protein, which facilitates fusion between the host cellular membrane and KoRV viral membrane during infection (Fiebig *et al.*, 2006; Kleinerman *et al.*, 1987; Madden *et al.*, 2018; Tarlinton *et al.*, 2005). In KoRV, the immunosuppressive domain is identical to those in FeLV, GALV and MuLV and is hypothesised to be exploited by endogenous retroviral-expressing cancers to escape immunosurveillance (Daradoumis *et al.*, 2023; Mangeney & Heidmann, 1998; Pye *et al.*, 2014). It is suggested that in the koala, similar immunomodulatory effects could increase pathogenicity of co-infecting *C. pecorum* (Fiebig et al., 2006; Kleinerman et al., 1987; Madden et al., 2018; Maher & Higgins, 2016; Maher et al., 2019; Sarker et al., 2020a; Sarker et al., 2020b; Tarlinton et al., 2005; Tarlinton et al., 2008a).

Recent evidence supports an oncogenic role of KoRV in the large proportion of bone marrow and neoplastic diseases seen in both wild and captive koalas. Tarlinton and Greenwood (2024) recently reviewed the relationship between KoRV and neoplasia, highlighting two key studies that have helped to identify several potential mechanisms. Sarker *et al.* (2020b) experimentally infected human cells with KoRV-A and, using Illumina mRNA sequencing, identified differential expression of various genes associated with anti-viral immune responses and oncogenesis. McEwen *et al.* (2021) identified tumour specific KoRV integrations and determined several features: an accumulation in tumour tissue of KoRV-A somatic mutations that were overexpressing oncogenic-associated genes; heritability of these integrations, and associated oncogenic potential; recurrence of KoRV oncogenic-associated integration sites within and among koalas with multiple KoRV integrations; and modified expression of genes associated with retroviral transduction.

The significance of KoRV in the pathogenesis of non-neoplastic disease and its role in immunomodulation is less clear. Modulation of the immune response through dysregulation of expression of genes containing, or adjacent to, KoRV insertion sites, as occurs for oncogenes in neoplasia (McEwen *et al.*, 2021), is thought to be a potential mechanism behind variations in disease outcomes, including chlamydiosis (Tarlinton & Greenwood, 2024). Although differences in immune gene expression have been observed following *in vitro* infection of human and rat cells with KoRV (Fiebig *et al.*, 2006), and among koalas with

differing KoRV subtype diversity, viral load, and KoRV-associated lymphoma (Kayesh *et al.*, 2022; Kayesh *et al.*, 2020b, 2021a; Maher & Higgins, 2016; Maher *et al.*, 2019), the relationship between KoRV and infectious disease, such as chlamydiosis, appears complex. An abundance of studies have observed various, sometimes conflicting associations, depending on the local and regional population characteristics of the study cohort: Total KoRV load (either viraemia or proviral copies per genome) was not associated with chlamydial disease in wild northern koalas (Fabijan *et al.*, 2020; Quigley *et al.*, 2018a; Quigley *et al.*, 2019; Waugh *et al.*, 2017). In SA koalas, KoRV-A infection was not associated with *C. pecorum* or chlamydiosis (Fabijan *et al.*, 2019b) but total viral loads were (Fabijan *et al.*, 2020). In Victorian koalas, competent KoRV infection was associated with wet-bottom (Legione *et al.*, 2017). Previously it was hypothesised that different KoRV subtype-host receptor interactions might result in differences in pathogenesis, which was at times supported by variations in subtype-specific disease associations (Quigley *et al.*, 2018a; Quigley *et al.*, 2018b; Quigley *et al.*, 2019; Waugh *et al.*, 2017). However, it was determined recently that there is a strong correlation between exogenous KoRV transcription and total KoRV viral load (Blyton *et al.*, 2022a; Hashem *et al.*, 2021; Quigley *et al.*, 2018b; Sarker *et al.*, 2019). The diversity of contexts and parameters examined, and the associative nature of most studies (Blyton *et al.*, 2022a; Quigley *et al.*, 2019; Sarker *et al.*, 2019; Tarlinton & Greenwood, 2024) makes it difficult to unravel significance of KoRV to immune function. Considering the impetus to clarify the relationship between KoRV and disease and the range of potential mechanisms employed, it is important that the multifactorial nature of disease is considered and potential confounding factors that can impact host immune responses are accounted for in investigations such as age (Tarlinton *et al.*, 2005), concurrent disease (Butcher *et al.*, 2020; Legione *et al.*, 2017; Sarker *et al.*, 2020a), and co-infections (Stephenson, 2021; Vaz *et al.*, 2019b).

Phascolarctid herpesvirus

Phascolarctid gammaherpesviruses (PhaHV) are enveloped, double-stranded DNA viruses from the family *Herpesviridae*, that infect koalas (Stalder *et al.*, 2015; Vaz *et al.*, 2012; Vaz *et al.*, 2011). Herpesviruses are generally highly coevolved with their hosts, allowing for adapted survival strategies that support lifelong infection. Following the primary infection event, which may or may not induce disease, herpesviruses will persist in a cycle of subclinical latency and virus reactivation and shedding. The fluctuation between states is driven by

changes to the interaction between the virus and the host due to co-infection, stress and immunomodulation (Aneja & Yuan, 2017; Blackbourn *et al.*, 2000; Byrne *et al.*, 2019; Chang *et al.*, 2000; Coskun *et al.*, 2010; Hirsiger *et al.*, 2019; Jiang *et al.*, 2006; Mehta *et al.*, 2014; Rooney *et al.*, 2019; Ueda *et al.*, 2014; Ye *et al.*, 2011a; Ye *et al.*, 2011b). The gammaherpesvirus subfamily are tissue tropic; virions are shed from epithelial cells but latent infections are commonly observed within lymphocytes, and thus can be detected within the spleen, in blood and/or separated buffy coat fractions (Sharma *et al.*, 2016; Van Dyk *et al.*, 2003). There are two known subtypes of gammaherpesviruses infecting koalas, PhaHV-1 and PhaHV-2, first detected in southern koalas (Vaz *et al.*, 2012; Vaz *et al.*, 2011) then in northern koalas (Wright *et al.*, 2024). Regional and local population differences in frequency of detection are apparent for both subtypes (Table 1.1) (Fernandez *et al.*, 2024b; Kasimov *et al.*, 2020; Stalder *et al.*, 2015; Vaz *et al.*, 2019b; Wright *et al.*, 2024), though sample sizes are frequently too low for accurate estimates of prevalence and sensitivity likely differs with sampling site and assay. The relationship between PhaHV detection and sex is unclear: males had greater odds of PhaHV-1 detection than females in one study (Stalder *et al.*, 2015) but no relationship for either PhaHV-1 or PhaHV-2 in other studies (Fernandez *et al.*, 2024b; Kasimov *et al.*, 2020; Stephenson, 2021; Wright *et al.*, 2024). The relationship between PhaHV-1 and age has been more consistent than for PhaHV-2, with greater detection frequencies observed as age increases (Kasimov *et al.*, 2020; Stalder *et al.*, 2015; Stephenson, 2021; Vaz *et al.*, 2019b; Wright *et al.*, 2024).

Table 1.1: Summary of PhaHV detection results from the literature

	Detection frequency	***Sample type***	***Study Population***	***Reference***
Gammaherpesvirus	33.3% (33/99)	Mucosal swabs (Conjunctivae, nasal, oropharynx, cloacal, and prepuce)	10 captive and 89 free-living Victorian koalas	(Stalder *et al.*, 2015)
	72.5% (58/80)	Mucosal swabs (oropharyngeal)	80 wild-caught South Australian koalas	(Kasimov *et al.*, 2020)
	72.45 (63/87)	Spleen	87 euthanised South Australian koalas	(Kasimov *et al.*, 2020)
	54.0% (47/87)	Mucosal swabs (oropharyngeal)	87 euthanised South Australian koalas	(Kasimov *et al.*, 2020)
	32.6% (264/810)	Mucosal swabs (urogenital)	810 wild and captive Victorian koalas	(Vaz *et al.*, 2019b)
PhaHV-1	10% (10/99)	Mucosal swabs (Conjunctivae, nasal, oropharynx, cloacal, and prepuce)	10 captive and 89 free-living Victorian koalas	(Stalder *et al.*, 2015)
	44% (26/58)	Mucosal swabs (oropharyngeal)	80 wild-caught South Australian koalas	(Kasimov *et al.*, 2020)
	48.9% (23/47)	Mucosal swabs (oropharyngeal)	87 euthanised South Australian koalas	(Kasimov *et al.*, 2020)
	17.4% (141/810)	Mucosal swabs (urogenital)	810 wild and captive Victorian koalas	(Vaz *et al.*, 2019b)
	20.0% (4/20)	Mucosal swabs (urogenital)	20 rescued South Australian koalas	(Wright *et al.*, 2024)
	29.0% (2/7)	Mucosal swabs (urogenital)	7 rescued Victorian koalas	(Wright *et al.*, 2024)
	22.0% (26/117)	Mucosal swabs (urogenital)	117 rescued and wild-caught mid- to southern NSW koalas	(Wright *et al.*, 2024)
	30.0% (15/50)	Mucosal swabs (urogenital)	50 rescued mid- to north-west NSW koalas	(Wright *et al.*, 2024)
	27.0% (21/79)	Mucosal swabs (urogenital)	79 wild-caught and rescued northern NSW and SE-Qld koalas	(Wright *et al.*, 2024)
	4% (1/25)	Mucosal swabs (urogenital)	25 wild-caught northern Qld koalas	(Wright *et al.*, 2024)
	55.5% (40/72)	Mucosal swabs (urogenital)	72 wild-caught NSW koalas from Liverpool Plains	(Fernandez *et al.*, 2024b)
	44.4% (16/36)	Mucosal swabs (urogenital)	36 wild-caught NSW koalas from Southern Highlands	(Fernandez *et al.*, 2024b)
PhaHV-2	23% (23/99)	Mucosal swabs (Conjunctivae, nasal, oropharynx, cloacal, and prepuce)	10 captive and 89 free-living Victorian koalas	(Stalder *et al.*, 2015)
	20.7% (12/58)	Mucosal swabs (oropharyngeal)	80 wild-caught South Australian koalas	(Kasimov *et al.*, 2020)
	14.9% (7/47)	Mucosal swabs (oropharyngeal)	87 euthanised South Australian koalas	(Kasimov *et al.*, 2020)
	22.6% (183/810)	Mucosal swabs (urogenital)	810 wild and captive Victorian koalas	(Vaz *et al.*, 2019b)
Both	1% (1/99)	Mucosal swabs (Conjunctivae, nasal, oropharynx, cloacal, and prepuce)	10 captive and 89 free-living Victorian koalas	(Stalder *et al.*, 2015)
	34.5% (20/58)	Mucosal swabs (oropharyngeal)	80 wild-caught South Australian koalas	(Kasimov *et al.*, 2020)
	36.2% (17/47)	Mucosal swabs (oropharyngeal)	87 euthanised South Australian koalas	(Kasimov *et al.*, 2020)
	7.4% (60/810)	Mucosal swabs (urogenital)	810 wild and captive Victorian koalas	(Vaz *et al.*, 2019b)

The clinical significance of herpesviruses in the koala is unclear, however the high frequency of PhaHV and *C. pecorum* co-detection may confound the association between PhaHV and clinical disease if it exists. The presence of genital tract abnormalities including uterine/ovarian bursal cysts and testicular malformation, reduction in female fertility, and urinary incontinence – all known clinical signs of chlamydiosis – were significantly associated with both PhaHV-1 and PhaHV-2 (Vaz *et al.*, 2019b). Reactivation of viral shedding might result from changes in the host-immune environment unrelated to *C. pecorum* co-infection, as is common following stress and trauma in humans (Cohen, 2020). In a retrospective necropsy cohort, PhaHV-2 shedding was negatively correlated with body condition score (Kasimov *et al.*, 2020) and PhaHV-1 was more frequently detected in rescued koalas than free-living koalas (Wright *et al.*, 2024); and in southern female koalas that were KoRV positive (Vaz *et al.*, 2019b). The strong and consistent relationship between PhaHV and *C. pecorum* co-detection prompts several hypotheses: 1) Concomitant transmission of PhaHV and *C. pecorum* is common; 2) Co-infection results in periodic reactivation of shedding and latency/persistence of either agent; 3) A synergistic relationship exists between PhaHV and *C. pecorum* contributing to pathogenesis (Stalder *et al.*, 2015).

Trypanosomes

Trypanosomes are unicellular parasitic flagellate haematoprotozoa from the genus *Trypanosoma* that infect various vertebrate species, including the koala. Currently, the life-cycles of koala-infecting trypanosomes are unknown but are important considerations for pathogenesis. Trypanosome infection occurs following vector bites (salivarian types*:* exposure to contaminated saliva or mouthparts) or exposure to arthropod faeces (stercorarian types) (Stijlemans *et al.*, 2024). Following transfer of trypomastigotes through bite wounds or mucus membranes these immature parasites transform into intracellular amastigotes which, through binary fission, multiplicate in infected tissues and return to trypomastigote forms to burst cells and enter the bloodstream (Stijlemans *et al.*, 2024). Most salivarian trypanosomes of importance to humans and livestock, such as *T. brucei* and *T. cruzi*, are extracellular but stercorarian trypanosomes are often intracellular (Stijlemans *et al.*, 2024). Hence, the pathogenesis of trypanosomiasis is dependent on the infecting species or combination of infecting species as parasite life-cycles and defences, host immune detection and defence mechanisms, and treatment approaches are unique (Stijlemans *et al.*, 2024).

Because of the wide distribution of these *Trypanosome* sp., high occurrence of mixed trypanosome infections, and comparable detection frequency in koalas with and without clinical disease attributable to trypansomiasis, the aetiological significance of trypanosomes in koala disease is unclear (McInnes *et al.*, 2011a; McInnes *et al.*, 2011b). To date, cross sectional studies have identified five species of extracellular trypanosomes in the koala through morphological and phylogenetic analysis: *Trypanosome irwini* (McInnes et al., 2009), *T. gilletti* and *T. copemani* (McInnes et al., 2011b), *T. vegrandis* (Barbosa et al., 2016), *T. noyesi* and a novel species *Trypanosoma* sp. AB-2017 (Barbosa *et al.*, 2017). Although further investigation is needed to confirm the direction of transmission, koala trypanosomes are likely to be of the salivarian type due to the detection among ticks *(Ixodes holocyclus* and *I. tasmani)* removed from trypanosome-positive koala hosts (Barbosa *et al.*, 2017). Geographic range of each trypanosome species differs based on the distribution of its vectors and hosts (Thompson *et al.*, 2014). Detection frequencies appear to be greater in northern koala populations, although southern populations may be understudied (Table 1.2). *T. gilletti* infection has been associated with reduced packed cell volume values and body condition scores (BCS) in koalas with signs of other disease, such as chlamydiosis, PKAD such as bone marrow disease (McInnes *et al.*, 2011a; McInnes *et al.*, 2009); suggesting that *Trypanosome* sp. infection may not be the primary cause of disease but could be a potentiating factor (Barbosa *et al.*, 2017; McInnes *et al.*, 2011a).

Table 1.2: Summary of Trypanosome detection results from the literature

	Detection frequency	Sample type	Study Population	Reference
Trypanosome(s)	20.2% (18/89)	Blood	Mount Lofty, SA	(Howard, 2022)
	80.6% (58/72)	Blood	Moreton Bay, South-east Qld	(Howard, 2022)
	35.1% (59/168)	Blood	South-east Qld & northern NSW	(Barbosa *et al.*, 2017)
	73.8% (439/595)	Blood	South-east Qld & northern NSW	(McInnes *et al.*, 2011a)
	71.4% (5/7)	Liver	South-east Qld & northern NSW	(Ortiz-Baez *et al.*, 2020)
T. irwini	71.1% (423/595)	Blood	South-east Qld & northern NSW	(McInnes *et al.*, 2011a)
	32.1% (54/168)	Blood	South-east Qld & northern NSW	(Barbosa *et al.*, 2017)
	52% (72/139)	Blood	South-east Qld & northern NSW	(McInnes *et al.*, 2011b)
	60% (35/58)	Blood	Moreton Bay, South-east Qld	(Howard, 2022)
	38.2% (26/68)	Blood	South-east Qld & northern NSW	(McInnes *et al.*, 2009)
	36.6% (15/41)	Blood	Southern Highlands, NSW	(Fernandez *et al.*, 2024b)
T. gilletti	21.5% (128/595)	Blood	South-east Qld & northern NSW	(McInnes *et al.*, 2011a)
	25% (42/168)	Blood	South-east Qld & northern NSW	(Barbosa *et al.*, 2017)
	2% (3/139)	Blood	South-east Qld & northern NSW	(McInnes *et al.*, 2011b)
	7% (2/29)	Spleen	South-east Qld & northern NSW	(McInnes *et al.*, 2011b)
	28% (16/58)	Blood	Moreton Bay, South-east Qld	(Howard, 2022)
T. copemani	4.4% (26/595)	Blood	South-east Qld & northern NSW	(McInnes *et al.*, 2011a)
	27.4% (46/168)	Blood	South-east Qld & northern NSW	(Barbosa *et al.*, 2017)
	3% (4/139)	Blood	South-east Qld & northern NSW	(McInnes *et al.*, 2011b)
T. vegrandis	13.6% (6/44)	Blood	South-east Qld & northern NSW	(Barbosa *et al.*, 2016)
	10.1% (17/168)	Blood	South-east Qld & northern NSW	(Barbosa *et al.*, 2017)
T. noyesi	0.6% (1/168)	Blood	South-east Qld & northern NSW	(Barbosa *et al.*, 2017)
T. AB-2017	4.8% (8/168)	Blood	South-east Qld & northern NSW	(Barbosa *et al.*, 2017)
Unidentified Trypanosoma cruzi clade	20.2% (18/89)	Blood	Mount Lofty, (SA)	(Howard, 2022)
Mixed infection	1.5% (2/139)	Blood	South-east Qld & northern NSW	(McInnes *et al.*, 2011b)
	27.4% (/168)	Blood	South-east Qld & northern NSW	(Barbosa *et al.*, 2017)
	21% (125/595	Blood	South-east Qld & northern NSW	(McInnes *et al.*, 2011a)

1.4.2 The Damage Response Framework in the context of koala chlamydiosis

Host responses contribute to the determination of outcomes; hence Immunology is central to resistance, tolerance and pathogenesis. According to the damage-response framework, duality exists in the direction of pathogenesis that is not solely lead by the pathogen, but by damage incurred through the responses of the host (Casadevall & Pirofski, 2003). In koala chlamydiosis, it is accepted that a balance and coordination of both humoral and cellular adaptive immune responses is required for protection against infection and disease (Phillips *et al.*, 2024b; Quigley & Timms, 2020, 2021; Redgrove & McLaughlin, 2014). Therefore, factors such as co-infections and stress that may impact this balance through up or downregulation or inhibition of immune pathways, could increase host susceptibility to infection or lead to immune-mediated damage potentiating disease, as observed in infections by other *Chlamydia* sp. (Di Pietro *et al.*, 2019; Ghasemian *et al.*, 2023). Currently, our understanding of the immune responses resulting from host-*C. pecorum* interactions is fragmented, with

evidence demonstrating the involvement of various immune arms but few studies showing how these pathways interact in the koala (Madden *et al.*, 2018). Despite this, it is clear that diversity in immune responses exists and contributes to the variations in clinical outcomes for koalas; for example, sub-clinical *C. pecorum*-infected koalas have been reported and an estimated 1/3 of these cases are able to resolve infection without intervention (Nyari *et al.*, 2017; Quigley *et al.*, 2018a; Robbins *et al.*, 2019). Furthermore, cases presenting with classical signs of chlamydiosis but without detectable *C. pecorum* by qPCR could represent ongoing immune mediated damage despite resolution of *C. pecorum* infection (Nyari *et al.*, 2017).

Koala immune responses are likely species-specific and thus require in-depth characterisation rather than assuming similarities with the immune systems of placental mammals. Like other marsupial neonates, koalas are born altricial with naïve immune systems as demonstrated by an underdeveloped thymus, which completes development around 8 months of age (Canfield *et al.*, 1996; Symington, 1898). Additionally, in many other marsupial species, postpartum haematopoiesis occurs within the liver until the bone marrow and thymus have matured (Old, 2016), although this needs to be confirmed in the koala. During this time, neonates rely upon maternal strategies such as passive transfer of immunological compounds through milk (Morris *et al.*, 2016), and microbial inoculation via the pouch environment (Maidment *et al.*, 2023) and pap feeding (Blyton *et al.*, 2022c). Although a similar pattern of T and B cell distribution in lymphoid tissues can be observed in koalas compared to eutherian mammals (Mangar *et al.*, 2016; Wilkinson *et al.*, 1995; Wilkinson *et al.*, 1994), immunological variations can also been observed in mature koalas compared to other species; koalas have complex but less gut-associated lymphoid tissues (Hanger & Heath, 1994; Hemsley *et al.*, 1996), B-cells have four Ig heavy (H) loci isotypes including IgA, IgG, IgM, and IgE but lack IgD (Wilkinson *et al.*, 1991), major-histocompatibility complex (MHC) II is predominantly expressed on circulating B lymphocytes and rarely on T lymphocytes *in vitro* (Lau *et al.*, 2012), and a diverse array of innate antimicrobial peptides, including defensins and cathelicidins exist (Jones *et al.*, 2017; Park *et al.*, 2025; Peel *et al.*, 2025; Peel *et al.*, 2024).

Given the significance of host responses to pathogenesis of chlamydiosis, there has been some research focus on examining immune-associated parameters to determine the dominant pathways and intensity of immune responses in koalas. Studies using

immunohistochemistry helped to identify several key immunological molecular and cellular components in koala lymphoid tissues, mitogen stimulated peripheral-blood-mononuclear cells (PBMCs) and lymphocytes: major-histocompatibility complex (MHC) I and II (Canfield *et al.*, 1996; Hemsley & Canfield, 1997; Hemsley *et al.*, 1995, 1996; Pagliarani *et al.*, 2024), T- and B-cell surface markers CD3ε, CD4, CD8α, CD8β, and CD79b (Canfield *et al.*, 1996; Hemsley & Canfield, 1997; Hemsley *et al.*, 1995, 1996; Higgins, 2004; Mangar, 2018; Mangar *et al.*, 2016; Pagliarani *et al.*, 2024), cytokines IFN-γ and IL-4 (Higgins *et al.*, 2004), and immunoglobulins (Higgins *et al.*, 2005a). More recently, deep sequencing, targeted gene sequencing, and cytometric techniques (Cui *et al.*, 2015; Lau *et al.*, 2012; Lau *et al.*, 2013; Olagoke *et al.*, 2020a; Quigley *et al.*, 2018a; Quigley *et al.*, 2020; Robbins *et al.*, 2020; Silver *et al.*, 2022) and transcriptomics (Abts *et al.*, 2015; Cheng *et al.*, 2018; Jobbins *et al.*, 2012) have demonstrated a diverse set of immune genes and proteins with variable expression among koala immune compartments and tissues. This transcriptomic data has helped to identify gene targets for qRT-PCR to assess the relationships between immune gene expression and clinical and intervention outcomes such as for chlamydiosis antibiotic treatment or vaccination: Interleukin (IL)-1β, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17A/F, IL-18, IL-22, IL-32 (Desclozeaux *et al.*, 2017; Khan *et al.*, 2016; Maher *et al.*, 2014; Maher & Higgins, 2016; Maher *et al.*, 2019; Mathew *et al.*, 2013a; Mathew *et al.*, 2013b; Mathew *et al.*, 2014; Simpson *et al.*, 2023), Toll-Like Receptors (TLRs) TLR2-10, and TLR13 (Kayesh *et al.*, 2021b). Importantly for post-vaccination responses, various methods utilising ELISA techniques have been used to quantify immunoglobulin titres in the koala (Desclozeaux *et al.*, 2017; Higgins *et al.*, 2005a; Khan *et al.*, 2016; Nyari *et al.*, 2019; Olagoke *et al.*, 2019; Olagoke *et al.*, 2020a; Phillips *et al.*, 2020; Quigley *et al.*, 2023; Simpson *et al.*, 2023; Waugh *et al.*, 2016).

As the number of known immune parameters grows rapidly, multiplex gene expression technologies such as NanoString nCounter have been used to assess many immune targets to account for multiple levels of the immune response (Fernandez *et al.*, 2024b; Olagoke *et al.*, 2020b; Quigley *et al.*, 2023). While the panel designs differ in the number and types of gene targets, this method allows for a combined analysis of diverse sets of immune targets such as cell receptors and cytokine gene expression (CD4, CD8β, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-17A, IL-18, & IFN-γ) (Fernandez *et al.*, 2024b; Olagoke *et al.*, 2020b; Quigley *et al.*, 2023), alternative cytokine and chemokines: CCL4L (Olagoke *et al.*, 2020b), TNF-α (Fernandez *et al.*, 2024b;

Quigley *et al.*, 2023), IL-12A and IL-22 (Fernandez *et al.*, 2024b), cellular differentiation markers CD3 and CD79b (Fernandez *et al.*, 2024b), koala host restriction factors (BST2, ISG15, RSAD2 and TRIM1) (Olagoke *et al.*, 2020b), TLRs (TLR2, TLR4, & TLR7) (Fernandez *et al.*, 2024b), MHC I and MHC II (Fernandez *et al.*, 2024b; Quigley *et al.*, 2023), stress signalling genes MID2 (Quigley *et al.*, 2023) and FKBP5 (Fernandez *et al.*, 2024b), and pathogen genes for *C. pecorum* (Quigley *et al.*, 2023), KoRV (Fernandez *et al.*, 2024b; Quigley *et al.*, 2023), and Trypanosomes (Fernandez *et al.*, 2024b). While whole transcriptome techniques such as RNAseq can provide a complete assessment of immune gene expression, the affordability and simplicity of the NanoString workflow makes it an increasingly popular method in human and animal studies (Alijagic *et al.*, 2025; Bondar *et al.*, 2020; Chilimoniuk *et al.*, 2024; Hyeon *et al.*, 2017; Pescarmona *et al.*, 2019; Ryan *et al.*, 2023; Veldman-Jones *et al.*, 2015). In the koala, the relationships between the degree of expression or circulation of immune markers and clinical and pathogen parameters have varied considerably among studies and are summarised in Supplementary Table 7.2.

Although the appropriate response to *Chlamydia sp.* infection has not been elucidated, it is evident that a balance between key immunological pathways within the innate and adaptive immune arms is required. The direction of the immune response is established early within the innate phase, and varies based on the types of pathogens, the ligation of receptors to pathogen elements, and the resulting production of cytokines that direct appropriate adaptive responses. In chlamydial infection, pathogen associated molecular patterns (PAMPs), and damage associated molecular patterns (DAMPs), including alarmins released from damaged cells (Chen *et al.*, 2019a) stimulate the fluid and cellular phases of innate inflammation, which then drive adaptive immune responses (Spector & Willoughby, 1963). The fluid phase promotes vascular permeability, vasodilation and chemotaxis through the complement cascade and plasma-derived factors. The cellular phase includes the recognition of PAMPs by resident sentinel cells such as dendritic cells and tissue macrophages that contain pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and C-type lectin receptors (CLRs) (Medzhitov, 2007). PRRs are adapted to bind different ligands, helping to shape downstream responses to the pathogen group involved, for example TLR4 is an extracellular receptor targeting bacterial LPS, while TLR7 is an intracellular receptor targeting viral ssRNA, and CLEC4E/Mincle is a CLR recognising microbial glycolipids (Fisher *et al.*, 2021;

Smith & Williams, 2016). Depending on which PRR ligates with the pathogenic element, several transcription factors can be activated (NF-$_{\kappa}$B, interferon regulatory factors, and AP-1, for example) to direct rapid transcription of pro-inflammatory chemokines and cytokines such as IL-8 (CXCL8), TNF-α, IL-1β, and IL-6 (Fu & Harrison, 2021). These and other cytokines such as IL-12 and IL-18 and a range of other chemotaxins attract additional innate immune cells (monocytes, neutrophils, eosinophils and natural killer cells [NK]) to the site of infection and prime the adaptive immune response through the NK cell production of IFN-γ (Hook *et al.*, 2005; Tseng & Rank, 1998). Antimicrobial peptides such as cathelicidins are produced and released by epithelial cells, a range of mononuclear immune cells, and recruited neutrophils, contributing to the first line of defence through bactericidal effects (Hou *et al.*, 2019; Peel *et al.*, 2021; Tang *et al.*, 2015). These pro-inflammatory, phagocytic and antimicrobial elements of the innate system are critical in establishing a rapid and effective primary response but can be deleterious to the host when in excess.

Professional antigen presenting cells (APCs), e.g. dendritic cells and macrophages, bridge the innate and adaptive immune responses through presentation of antigen on MHC II. Along with co-stimulatory molecules this stimulates the differentiation of antigen-specific naïve T helper (Th) cells (Sagerström *et al.*, 1993). The specific cytokine milieu stimulated by the interactions between PRRs and the pathogen ultimately directs the pathways activated in the adaptive immune response (Campe & Ullrich, 2022; Mangar, 2018; Wilkinson *et al.*, 1995). IL-12 production promotes T-helper 1 (CD3+CD4+Th1) differentiation through IFN-γ, leading to macrophage and CD8 cytotoxic T-cell (Tc) activation for direct elimination of intracellular bacteria and viruses (Johnson *et al.*, 2020). MHC I, which presents endogenous antigens on the surface of all cells, binds to antigen-specific CD8 Tc cells to direct them to infected or neoplastic cells (Norment *et al.*, 1988). A positive feedback loop exists in that IFN-γ promotes greater MHC expression and phagocytosis by APCs and inhibits IL-10 anti-inflammatory functions (Keller *et al.*, 1988). IL-4 production stimulates CD3+CD4+Th2 pathways and B-lymphocyte (CD79b) production to support antibody (humoral) response neutralisation of cell-cell transmission (Egholm *et al.*, 2019). IL-6 is a pleotropic cytokine that drives B-cell differentiation and can promote CD3+CD4+Th17 pathways to produce IL-17, which is particularly important in neutrophil led elimination of bacteria at mucosal sites (Wilton *et al.*,

2025). IL-10 is produced by regulatory-T-lymphocytes (T-regs) to moderate Th1 cytotoxic pathways through the inhibition of IFN-γ production (Omosun *et al.*, 2015).

Classically, severe fibrotic disease associated with chlamydiosis was thought to be due to an immune system that is skewed towards Th2 humoral responses, resulting in the upregulation of pro-fibrotic pathways involved in wound-healing, downregulation of the pro-inflammatory Th1 mechanisms required for bacterial elimination, and allowing for *Chlamydial* persistence (Asquith *et al.*, 2011; Bailey *et al.*, 1995; Gondek *et al.*, 2009; Holland *et al.*, 1996; Holland *et al.*, 1993; Johnson *et al.*, 2014). Current thinking considers the progression to severe disease, such as those resulting in infertility, can be quick due to florid pro-inflammatory innate responses (Crother *et al.*, 2019; Latz *et al.*, 2013; Nagarajan *et al.*, 2012; Prantner *et al.*, 2009), and be cell mediated through Th1 and Th17 led cytotoxicity and neutrophil production of reactive oxygen species that cause damage (Andrew *et al.*, 2013; Murthy *et al.*, 2011; Redgrove & McLaughlin, 2014; Vlcek *et al.*, 2016; Wilton *et al.*, 2025). In koalas, the observation that reproductive damage can develop quickly suggests that host immune responses are a more likely driver rather than prolonged or repeated exposure to chlamydial antigens (Robbins *et al.*, 2019). Ultimately, the host response to *Chlamydia* sp. infection requires balance and coordination of innate and adaptive arms to effectively eliminate or sequester bacterial infection whilst mitigating the cellular damage incurred through clearance mechanisms (Barr *et al.*, 2005; Natividad *et al.*, 2007; Stelzner *et al.*, 2023).

Anti-chlamydial vaccine protocol optimisation and validation is ongoing in the koala and the overall long-term efficacy may not be comparable between populations with varying disease impacts. Current vaccine formulations undergoing assessment include recombinant protein and synthetic peptide types which use *C. pecorum* Major Outer Membrane Protein (MOMP) or MOMP peptide derivatives, respectively. Overall, recombinant MOMP vaccines have been shown to induce systemic and mucosal humoral (Waugh *et al.*, 2016; Waugh *et al.*, 2015) and cellular immune responses (Khan *et al.*, 2016; Khan *et al.*, 2014; Nyari *et al.*, 2019). Synthetic MOMP peptide vaccine has also demonstrated mucosal humoral responses (Nyari *et al.*, 2018). Earlier multi-subunit vaccine designs demonstrated strong antibody and lymphocyte proliferation with no clinical change (Carey *et al.*, 2010; Kollipara *et al.*, 2012), as did mono- and poly-valent MOMP vaccines (Kollipara *et al.*, 2013b). A study compiling the results of five

separate vaccine trials over a 10-year period in the Moreton Bay koala population concluded that there was an observed reduction in the development of chlamydial disease in vaccinated koalas compared with unvaccinated koalas (Phillips *et al.*, 2024a). The vaccines included in this compilation included two full recombinant MOMP vaccines (a one-dose (Desclozeaux *et al.*, 2017; Khan *et al.*, 2016) and a triple-dose (Khan *et al.*, 2016; Kollipara *et al.*, 2012)), two MOMP four-peptide types with one also including a KoRV protein as an additional antigen (Quigley *et al.*, 2023; Waugh *et al.*, 2016). However, using the same synthetic MOMP peptide vaccine design as Waugh *et al.* (2016), Simpson *et al.* (2023) found the vaccine to not be effective in stimulating significant additional systemic humoral or cellular immune responses in a population with pre-existing responses due to a high prevalence of *C. pecorum* infection and chlamydiosis.

1.4.3 Extending to the disease triangle: effects of the environment on host-pathogen relationships

Susceptibility of a host to disease is generally affected by a range of environmental and external factors, through their interaction with immune responses, energy and nutrient reserves or microbiota. These factors are not independent of each other but are often assessed as separate factors (Casadevall & Pirofski, 2018). Here we separate them into two classes: factors that are likely to be involved in host-pathogen interactions (stress, climate and the environment, diet, microbiomes, and genetics) and factors that can confound these relationships (circadian rhythm and seasonality, demographics, and sampling conditions). Due to the complexity of analysis for attributes such as genetics, nutrition, and microbiomes and the rarity in recording accurate animal-specific or population specific histories, climate and environmental data – especially within the clinical setting – the collective investigation of most of these attributes is out of scope for this thesis. However, due to their importance as potential mediators of host responses, here a brief overview of the current literature is provided to demonstrate the connectivity amongst these variables and their implications to the interpretation and understanding of host responses and pathogenesis.

Evidence exists that supports the involvement of host stress, climate and environmental factors, and relatedly, diet and microbiomes, and host and pathogen genetics in pathogenesis

of koala diseases. Stress activates the hypothalamic-pituitary-adrenal (HPA) axis, releasing glucocorticoid steroid hormones such as cortisol, which is detectable in koala circulation and as metabolites in faeces (Johnston *et al.*, 2013). Faecal cortisol metabolites (FCMs) are a non-invasive but generalised measure of stress that is influenced by factors such as climate and environment, population, sex, reproductive status, clinical presentations, length of stay in hospital, time of day and season and can be confounded by handling and sampling stress (Charalambous *et al.*, 2021; Davies *et al.*, 2013; Narayan & Vanderneut, 2019; Narayan *et al.*, 2013; Santamaria *et al.*, 2021a; Santamaria *et al.*, 2021b; Santamaria *et al.*, 2023; Webster *et al.*, 2017). Recently, the *FKBP5* gene, coding for FK506 binding protein 5, was used as a marker of stress in the koala (Fernandez *et al.*, 2024b). Koalas exposed to extreme climatic pressures demonstrated elevated *FKBP5* transcription in circulation (Fernandez *et al.*, 2024b), consistent with studies that present evidence of a role of *FKBP5* in shaping acute stress responses in humans and mice (Daneri-Becerra *et al.*, 2019; Häusl *et al.*, 2021; Kwon *et al.*, 2019; Pei *et al.*, 2009; Staibano *et al.*, 2011; Sun *et al.*, 2014; Zannas *et al.*, 2019). However, while elevation of stress markers is generally assumed to predispose to disease, the thresholds at which this occurs and the impacts on the immunity, inflammation and disease have not been determined in the koala. Furthermore, while several reviews demonstrate ample evidence in human biology of the independent regulatory actions and interactions other steroid hormones such as estrogens, androgens and vitamin D have on the immune response (Bereshchenko *et al.*, 2018; Dupuis *et al.*, 2021; Gotelli *et al.*, 2024; Quatrini *et al.*, 2021), the interplay between these steroid hormones and the immune response is unclear in the koala.

Changes in the environment can directly alter host-pathogen interactions by modulating pathogen and host density and diversity across landscapes (Albery *et al.*, 2019; Meza Cerda *et al.*, 2022), and can directly or indirectly constrain host defences by limiting available nutritional resources, altering feeding behaviours, deviating homeostasis, and inducing physiological stress (Vicente-Santos *et al.*, 2023; Youngentob *et al.*, 2021). For example, during drought and temperature oscillations, greater FCM levels were observed and diets shifted to browse species with greater leaf-moisture content relative to post-flood conditions (Davies *et al.*, 2014), and climate-associated elevations in ambient temperatures and CO_2 exposure altered the nutrient composition of koala feed sources (Duff *et al.*, 1994; Kanowski,

2001). Varied exposure to dietary plant secondary metabolites may impact host responses; these have been shown to modulate cytokine gene expression in koala immune cells in vitro (Marschner *et al.*, 2019b). Supporting the connection between environment, stress, and immune responses, a recent multivariate study showed that greater expression of a stress co-chaperone gene, FKBP5, coincided with a heat stress event, along with a shift from adaptive to pro-inflammatory innate responses (Fernandez *et al.*, 2024b).

In koalas, gut microbiome composition is hypothesised to be a driver of browse selection (Blyton *et al.*, 2023; Blyton *et al.*, 2019) and may therefore impact immune responses and disease outcomes *via* nutrient availability and PSM exposure. The microbiome-gut-brain axis is a bi-directional communication system that enables gut microbes and the brain to communicate using a variety of routes, including cytokines (Dinan & Cryan, 2017). While our understanding of the communicating pathways between the koala microbiome and immune responses is limited, one study noted that age-related changes in microbiomes and immune responses in captive koalas were coordinated: subadult microbiomes associated with T-cell activity (CD4 and CD8b) and adult koala microbiota with macrophage activity (*CLEC4E* gene) (Chen *et al.*, 2023c). Other studies have noted marked variations in gut microbiomes in response to several factors: age and duration of maternal dependency (Blyton *et al.*, 2022b, 2022c), diet (Brice, 2017; Brice *et al.*, 2019), within, between, and across landscapes (Brice, 2017; Littleford-Colquhoun *et al.*, 2022), disease status (Barker *et al.*, 2013), and antibiotic treatment outcomes (Dahlhausen *et al.*, 2018). Whether the link between changes in the microbiome and immune responses is directed by microbe-immune system communication, is a direct effect of age, or other factors such as dietary compounds is unclear. Nonetheless, it is important to determine the factors that can shift microbiomes and the consequences for immune responses and disease outcomes.

The environment and host-pathogen genetics are linked, as genetics evolve in response to environmental change, which can have broad implications for immune responses (Mathew *et al.*, 2013a). In the host, genetic diversity, particularly in the MHC gene, was associated with disease outcomes but significant variation across studies highlights the need for further research (Cheng *et al.*, 2018; Lau *et al.*, 2014; Nyari *et al.*, 2019; Quigley *et al.*, 2018a; Quigley *et al.*, 2020; Robbins *et al.*, 2020; Silver *et al.*, 2022; Silver *et al.*, 2024). Other immune-related

polymorphisms, such as in toll-like receptors and interleukins, are under-researched in koalas but have been shown to affect immune responses to pathogens like *Chlamydia* sp. and the efficacy of quantitative gene expression techniques in other species (Mathew *et al.*, 2013a; Mukherjee *et al.*, 2019; Nieters *et al.*, 2006; Thorolfsdottir *et al.*, 2024). The relationship between pathogen genetic divergence and host immune responses and pathogenesis has yet to be fully elucidated, however genetic diversity in both *C. pecorum* and KoRV across landscapes and between populations has been observed (Fernandez *et al.*, 2023; Fernandez *et al.*, 2024a; Fernandez *et al.*, 2019) and in some studies was associated with distinct immune responses (Desclozeaux *et al.*, 2017; Mathew *et al.*, 2013a).

Although further validation is needed to determine the interrelationships among stress, diet, climate, environment, genetic and microbiome diversity and immune responses, collectively these findings demonstrate the potential for each factor to impact host function independently and through compounding effects. As such, the importance of external or environmental factors in pathogenesis should be given equal consideration to host and pathogen-driven mechanisms.

Complex relationships exist among other factors such as demographics, temporality, and sampling conditions and the immune system, which can confound findings. In koalas there is evidence to support a seasonal fluctuation in immune gene expression (Lau *et al.*, 2012; Maher & Higgins, 2016) and an effect of age on gut microbiota and immune function in captive koalas (Chen *et al.*, 2023c). However, evidence for sex-related differences in koala cytokine gene expression is mixed and could be due to individual hormone fluctuations, population-specific diversity in environment and disease pressures or sampling differences associated with timing and anaesthetic protocols (Maher *et al.*, 2014; Maher & Higgins, 2016; Maher *et al.*, 2019; Mathew *et al.*, 2013a; Pagliarani *et al.*, 2024). In other species, variations in immune function can be observed throughout the day (Bellet *et al.*, 2013; Curtis *et al.*, 2015; Early *et al.*, 2018; Halberg *et al.*, 1960; Keller *et al.*, 2009; Nguyen *et al.*, 2013; Oishi *et al.*, 2017; Schlamp *et al.*, 2021; Silver *et al.*, 2018; Spengler *et al.*, 2012), and in response to different anaesthetic drugs, including those commonly used in the koala during routine health examinations and for invasive sampling (Downey *et al.*, 2020; Vogelnest & Portas, 2019); alfaxalone induction and isoflurane maintenance (Boost *et al.*, 2009; Kotani *et al.*, 1999;

Mazoti *et al.*, 2013; Sayed *et al.*, 2015; Wu *et al.*, 2012; Zhao *et al.*, 2023b). Although drug-associated and daily oscillations in immune gene expression have yet to been investigated in the koala, data from other species suggests that interpreting the significance of immune gene expression variation requires careful consideration of the potential confounding factors, and where able, inclusion of these factors into analyses to allow for better comparison between studies.

1.5 Approaches to koala disease investigations and the examination of immune responses

1.5.1 Univariate approaches and sample treatment

In wildlife disease investigations, immune responses are sometimes measured to better understand the host-pathogen interactions and mechanisms driving pathogenesis. Although mechanistic studies are needed to confirm functions, univariate studies are often employed first, to identify potentially important relationships and risk factors (Grace, 2024; Kock *et al.*, 2018). In the koala, studies have utilised several different experimental and statistical approaches to investigate the relationship between independent variables and immune function, of which the most common are univariate hypothesis testing and modelling in wild or rehabilitated koalas, and *ex vivo* stimulation of peripheral blood mononuclear cells (PBMCs). Both approaches have contributed to fundamental understanding of koala immune responses in relation to host-pathogen-environment factors and therapeutics such as vaccines. Currently, an abundance of studies employing univariate approaches exist that collectively demonstrate the diversity of koala immune responses associated with study contexts, methods and the assessed independent variables (Supplementary materials: Table 7.2) and highlight the complexity of cytokine networks that regulate immune responses. Here we briefly describe the benefits and challenges of each approach and highlight why validation of immune response relationships using non-stimulated samples and the development of multivariate analyses should now follow.

Univariate analyses that test the relationships between immune gene expression targets and host-pathogen-environment variables has formed the basis of our understanding of koala pathogenesis and immunology. In wildlife epidemiology, simpler statistical analyses are often

needed for the identification of relationships as sample sizes often limit statistical power (Bonapersona *et al.*, 2021; Joseph *et al.*, 2013; Kophamel *et al.*, 2022; Peacock *et al.*, 2020). In the koala, these studies identified potentially key immune genes and relationships within the study context that can be used to build hypotheses and be further validated in larger studies (Supplementary materials: Table 7.2) (Desclozeaux *et al.*, 2017; Khan *et al.*, 2016; Maher *et al.*, 2014; Maher & Higgins, 2016; Maher *et al.*, 2019; Mathew, 2014; Mathew *et al.*, 2013a; Mathew *et al.*, 2013b; Mathew *et al.*, 2014; Nyari *et al.*, 2019; Pagliarani *et al.*, 2024). As the field of immunology grows, and so does the understanding of the complexity and diversity of immune gene functions, a broader immunological approach is needed to better understand the mechanisms behind these univariate relationships.

Immune gene expression can be measured in unstimulated or stimulated samples and each of these approaches offers different information. *Ex vivo* stimulation of peripheral blood mononuclear cells (PBMCs) is a common technique in immunology, offering insights by amplifying otherwise undetectable immune responses or indicating which molecular patterns provoke immune activity (Norian *et al.*, 2015; Sullivan *et al.*, 2000). Stimulation of PBMCs offers an opportunity to assess responses in a controlled artificial exposure setting, which is useful as it is unethical to implement *in vivo* infection experiments on an endangered species and it can be challenging to determine the natural infection or disease stage in wildlife (Ohmer *et al.*, 2021; Segner *et al.*, 2022). PBMC stimulation has been used to model koala immune responses to *C. pecorum* before and after the application of vaccination, developing our understanding of the variations in antigen-induced immune gene expression of the koala and the efficacy and immunological effect of various anti-chlamydiosis vaccines (Supplementary materials: Table 7.2) (Desclozeaux *et al.*, 2017; Khan *et al.*, 2016; Lizárraga *et al.*, 2020a; Nyari *et al.*, 2019; Simpson *et al.*, 2023).

Several challenges exist with *ex vivo* PBMC stimulations, which highlight the usefulness of analysing unstimulated samples to help validate findings in a natural experimental setting. Immune responses determined through PBMC stimulation can be challenging to interpret as (1) uncontrolled individual variation in sensitivity to stimulants exists (Maher *et al.*, 2014), (2) stimulants are often of a single antigenic type and thus cannot account for genetic diversity in natural microbial patterns, (3) the impact of diverse co-infections are difficult to replicate

artificially, (4) most stimulation protocols lack representation of later or chronic stages of immune responses as immune gene expression is often measured 3-72 hours post challenge, and (5) validated "true" baselines are needed to accurately interpret fold changes in stimulated immune responses. Furthermore, identifying consistent relationships between studies is difficult due to the study-specific stimulation protocols which can differentially impact immune gene expression (Desclozeaux *et al.*, 2017; Higgins *et al.*, 2004; Kayesh *et al.*, 2020a; Khan *et al.*, 2016; Lau *et al.*, 2012; Lizárraga *et al.*, 2020a; Maher *et al.*, 2014; Maher & Higgins, 2016; Mathew *et al.*, 2013a; Mathew *et al.*, 2013b; Mathew *et al.*, 2014; Nyari *et al.*, 2019; Simpson *et al.*, 2023; Sullivan *et al.*, 2000).

The significance of variations in cytokine gene expression cannot be determined without a deeper understanding of the natural progression of immune responses and the individual kinetics of functional cells and cytokines. Although the interpretation of immune responses in unstimulated samples can be challenged by undetectable/unmeasurable levels of immune gene expression (Fernandez *et al.*, 2024b; Quigley *et al.*, 2023), this in itself improves our understanding of the natural levels and fluctuations in immune gene expression in real-world contexts. Similarly to stimulated experimental designs, identification of natural "baseline" immune gene expression and determination of clear relationships between host responses and independent variables can be challenging. In the koala and other species, "baseline" implies a pre-challenged state (Maher & Higgins, 2016; Nehar-Belaid *et al.*, 2023; Olagoke *et al.*, 2020b), which depends on our understanding of what can challenge or stimulate an immune response and which of these factors are included in the analysis. Longitudinal sampling and analyses of immune responses and infection status in unstimulated samples from a clinically diverse range of cases is an avenue that may clarify host-responses to naturally occurring and dynamic shifts in infection and disease. This approach was previously adopted to investigate *Leptospira interrogans* infection in California sea lions *(Zalophus californianus)*, which utilised several biomarkers to map the trajectory of infection and disease (Prager *et al.*, 2020). Additionally, extending investigations beyond the peripheral blood and into tissues to capture tissue-resident immune cells and inflammatory processes is an important consideration, as these may or may not be related at any given time.

Cytokines and chemokines mediate cell interactions, triggering a range of biological processes from cell survival to apoptosis and further cytokine production. Many of these ligands have multiple binding sites across different cell types, making them versatile but difficult to interpret in isolation (Elenkov & Chrousos, 1999; Fu & Harrison, 2021; Gervassi *et al.*, 2004; Hussain *et al.*, 2007; Mangar, 2018; Xiang *et al.*, 2021). Although cytokines like IFN-γ and IL-17 are traditionally associated with specific immune pathways (e.g., Th1 and Th17 responses), they are actually produced by a range of cell types and influence multiple signalling mechanisms (Hagai *et al.*, 2018; Quigley & Timms, 2021). For example, IFN-γ is produced by cells as diverse as T-cells and macrophages and modulates thousands of genes and pathways related to antigen presentation, cellular survival, and differentiation (Kato, 2020; Rusinova *et al.*, 2012). As human immunological investigations continue to demonstrate, every immune parameter has the potential to interact with virtually the whole immune system and other homeostatic pathways (Gaiffe *et al.*, 2023). Furthermore, as there are limited mechanistic studies of koala immunology, interpretations on the importance and roles of many koala immune genes are based on the assumption that they behave similarly to their eutherian equivalents. However, gene transcription in response to the same stimulation has been shown to vary across cells and species (Hagai *et al.*, 2018). It is imperative that a more global approach that allows immune parameters to be viewed in the context of their associated systems, is developed to consider the complexities of immune responses in the koala, and in other animals with similar investigative limitations (Bossart *et al.*, 2019; Gaiffe *et al.*, 2023; Meza Cerda *et al.*, 2022; Mordecai *et al.*, 2020; Teffer *et al.*, 2022).

1.5.2 Multivariate approaches

As immune responses are shaped by complex interactions between host, pathogen, and environmental factors, holistic approaches should now use the findings from univariate studies to collectively assess potentially important relationships and capture immune system dynamics (Casadevall & Pirofski, 2018; Fernandez, 2023). By accounting for natural complexity, multivariate analysis can help to better understand pathogenesis and determine relevant health indicators that can be used to improve health assessments. Multivariate analytical techniques can range from relatively simple multivariate modelling of key variables to complex machine learning approaches for big data, which require greater sample sizes

(Taylor *et al.*, 2016). As large sample sizes are rarely achieved in wildlife research, dimension reduction methods are valuable tools that enable datasets to be simplified for analysis using small sample sizes without losing complexity (Lubyayi *et al.*, 2021). Dimension reduction techniques can serve both as a method of understanding the roles and relationships of whole systems – rather than single indicators – and as a holistic method of parameter selection for further predictive modelling (Ray *et al.*, 2021).

While studies have demonstrated the multifactorial nature of disease in the koala, only recently have different facets of aetio-pathogenesis been integrated within individual investigations (Chen *et al.*, 2023c; Fernandez *et al.*, 2024b; Quigley *et al.*, 2018a; Robbins *et al.*, 2020). Developing a holistic approach to investigating pathogenesis is a key step in developing a better understanding to support disease management. While a big-data approach using transcriptomic or genomic information would be a powerful method to explore the relationships between host biology and non-host variables, it is an expensive approach that is sensitive to the heterogenous and incomplete data and small sample sizes common in wildlife studies (Hariri *et al.*, 2019; Maugis, 2018; Saidulu & Sasikala, 2017).

A key challenge of this thesis was to navigate a balance between what was desired – which is as integrated approach as possible – with limitations imposed by heterogeneity and limited sample size. Before investing in big-data approaches, which would require stringent data collection and large sample sizes, this thesis provides a stepwise approach that can help to focus future big-data investigations. Importantly, this work determines the strength of relationships among a range of variables of putative significance to koala health and disease, helping to identify factors that might introduce noise in future big-data analyses (Xiong *et al.*, 2006). Based on our current understanding, it could be said that (1) the koala has a diverse range of infectious agents, most of which have an unknown significance to pathogenesis, and (2) the koala immune response is complex and dynamic and is likely impacted by a range of infectious, environmental, and intrinsic host factors. Using sequential multivariate modelling, this thesis begins to breakdown knowledge gaps using separate and targeted investigations. This body of work first assesses infectious agents, their interrelationships and relationships with disease in the koala. Then, an integrated analyses is conducted, incorporating markers

of the koala immune response. Finally, environmental and external factors are considered alongside infectious and immunological variables.

Multivariate modelling can determine relationships between select factors and an outcome but requires in depth understanding of the interrelationships between predictor variables to support parameter selection and inference of relationships. Multivariate modelling approaches are sensitive to overfitting and small sample sizes and so evidence-based selection of predictor variables using confident understanding of the roles, relationships and variations of parameters is required to avoid introducing major biases to interpretations (Gaiffe *et al.*, 2023). As the number of potentially important health indicators grow, holistic assessments are needed to guide inclusion within predictive models. Multivariate modelling approaches used in koala research have included structural equation modelling (Lizárraga *et al.*, 2020b; Quigley *et al.*, 2018a), generalised linear models and regression models (Blyton *et al.*, 2022a; Lau *et al.*, 2014; Santamaria *et al.*, 2023; Sarker *et al.*, 2020a; Stalder *et al.*, 2015; Stephenson, 2021). Most of these studies utilise *a priori* power analysis to determine the maximum number of variables that can be assessed according to their sample size, and backwards elimination to remove variables from models if they do not meet model performance criteria. Whilst this maintains statistical power of the study and produces high-quality models, the resulting relationships are highly specific to the study context and the retained parameters.

Dimension reduction allows for the addition of multiple variables into analyses without costing statistical power in datasets with smaller sample sizes. This generates robust data analyses that simultaneously reduces data complexity whilst maximising its usage and retaining essential variability to identify patterns and relationships which would otherwise not have been readily found using univariate methods or by selective inclusion of parameters into regression models (Ray *et al.*, 2021). Principal Components Analysis (PCA) can reveal complex interactions but is only interpretable within the context of clear and pre-conceived groups. PCA is a dimension reduction method that retains information by summarising the interactions between multiple variables within large datasets into fewer variables or "principal components" (Greenacre *et al.*, 2022). This technique is useful when assessing correlated factors, such as immune gene expression, and has been utilised in the study of

various species such as the Australian sea lion *(Neophoca cinerea)* (Meza Cerda et al., 2022), Berthelot's pipit *(Anthus berthelotii)* (Gonzalez-Quevedo et al., 2016), Florida gopher tortoises *(Gopherus polyphemus)* (Elbers et al., 2018), field voles *(Microtus agrestis)* (Jackson et al., 2011), and more recently in the koala (Fernandez *et al.*, 2024b; McEwen *et al.*, 2021). Collectively, these studies demonstrate the extensive utility of PCA for generating and investigating immunophenotypes among important groups such as different animal populations, demographics, clinical cohorts, and treatment groups.

Clustering of principal components is an alternative method used to allow the data to generate groups based on existing variability within the dataset (Maugeri *et al.*, 2021). Through this, the relationship between pre-conceived definitions of clinical presentation, disease stages and severity with immunophenotypes can be tested without conforming the data into these respective groups. Hence, clustering can reveal relationships which are not readily observed, such as subtle changes in immune responses over time (Gaiffe *et al.*, 2023; Ndoricyimpaye *et al.*, 2023), immunologically similar cases which are clinically distinct and, conversely, clinically similar but immunologically distinct cohorts (Dissanayake *et al.*, 2020). In disease investigations where pathogenesis is unclear and various complex relationships exist between co-infecting agents, host responses and disease outcomes, such as those in the koala, clustering of multivariate parameters can be key in advancing our understanding of key knowledge gaps and identifying strong prognostic indicators of outcomes (Ndoricyimpaye *et al.*, 2023).

Not only do study contexts need to be considered when inferring relationships and comparing findings among investigations, but they should be maximised to support experimental aims. Disease relationships in the koala are mostly studied in the clinical, captive, and field setting (free ranging/wild). These settings can bias a study due to specific environmental, demographic, and genetic characteristics that aren’t reflective of the general koala population. For example, in the clinical setting hospital-admitted cases represent a selective cohort, often biased towards animals displaying severe clinical signs, trauma, or abnormal behaviours linked to proximity to human threats, leading to underrepresentation of healthy individuals or early-stage infections and disease. This introduces a potential bias when assessing relationships with outcomes such as treatment efficacy or survival (Leigh *et al.*,

2023). However, this skewed sample provides the opportunity to capture heterogeneity by assessing a high throughput of cases with diverse clinical presentations, immune responses, demographics, environments of origin, co-infections, treatments and outcomes, which is needed to better understand the varying relationships among these factors.

Field settings are the best setting in which to test relationships and compare between populations. Although some degree of heterogeneity exists among members of a free-ranging population, the genetic, microbiome, environmental, dietary, and pathogen diversity is much reduced compared to clinical cohorts, which are often sourced over a large catchment area. By reducing this variability, important population-specific relationships can be determined to provide a more accurate and refined understanding of koala health. Disease prevalence also varies regionally, with factors such as hospital catchment areas, population density, environment-disease dynamics, and amplified community vigilance/citizen science affecting admission rates (Griffith & Higgins, 2012; Kerlin *et al.*, 2022; Lunney *et al.*, 2022a, 2022b). Without extending surveillance to wild populations, clinic-based research alone cannot capture the true scale of disease prevalence or inform effective intervention strategies across diverse environments.

Longitudinal monitoring in both contexts, throughout and post rehabilitation, and in wild populations is needed to understand how relationships alter over time. Currently, incomplete post-release follow-up restricts the evaluation of disease progression and outcomes to the point of death during rehabilitation or release, which further hinders efforts to evaluate the long-term efficacy and impacts of treatment and rehabilitation on koala health (Burton & Tribe, 2016; DEH, 2022; Haering *et al.*, 2020). Furthermore, longitudinal monitoring is required to elucidate not only the intricacies of various relationships among changing seasons, climatic and environmental conditions, but also changes in the host as they age and how this relates to immune responses and disease outcomes.

Although research is slowly advancing our understanding, additional studies are required to further explore disease dynamics and generate approaches that cohesively assess these various aspects of pathogenesis, which can be adapted further as our understanding grows. Given the seemingly unmitigated impact of koala chlamydiosis and the differing impacts of

this disease on various populations, the long-term efficacy of current therapies and the influence of host, pathogen, and environment factors should be further investigated in both clinical contexts and wild populations to support the development of superior indicators of health and disease, and improve long-term disease mitigation outcomes.

1.6 Aims & Hypotheses

Disease presentations in the koala are diverse and treatment and survival outcomes vary. Current health assessments do not incorporate potentially important indicators of health and disease that are likely to contribute to heterogeneity in pathogenesis. Disease is the result of multifactorial and dynamic interactions between the host, one or more pathogens, and the environment. Many gaps in our understanding of these relationships have been highlighted and are partly due to the ongoing identification of putatively significant indicators and in part due to limiting methods of analysis. The overall aim of this thesis is to contribute to the progression of heath assessments in the koala by applying holistic approaches to assess the interactions among co-infections, immune responses, and key attributes of host susceptibility and determine their relative contribution to disease, treatment, and survival outcomes. Several infectious agents are expected to be differentially associated with disease presentations, which is likely to be reflected by diverse immune responses. It is hypothesised that chlamydiosis, treatment response and survival is influenced by host attributes - including immune responses, population demographics and co-infection diversity.

The following aims address the main aim and hypothesis of the thesis:

1. Determine the relative prevalence of co-infections in koalas admitted to hospital by detecting mucosal and circulating *C. pecorum* and PhaHV-1 and -2, and circulating KoRV, *Trypanosome irwirni*, *T. copemani*, and *T. giletti*
2. Determine the relationships among co-infection parameters, clinical presentation and admission outcome of koalas.
3. Examine how immune responses relate to infection status, clinical presentations, and outcomes
4. Compare the capacity of immune responses and KoRV parameters to predict clinical outcomes on admission and in rehabilitation.
5. Identify genes that are differentially expressed between
    a. koalas with clinical signs of chlamydiosis and koalas without clinical signs of disease

    b. koalas that were euthanised and koalas that survived
6. Validate the NanoString mRNA quantification and transcription counts for immune genes below the quantifiable limit in Chapter 3: IL10, IL17A, IFNγ, and TNFa.
7. Determine if demographics, clinical presentation, co-infection status, and immune gene transcription profiles predict long-term survival in
    a. two wild koala populations with difference prevalence of disease
    b. koalas with chlamydiosis and/or *C. pecorum* infection
    c. koalas that were treated for chlamydiosis and/or *C. pecorum* infection
8. In koalas treated for chlamydiosis and/or *C. pecorum* infection, determine if demographics, clinical presentation, co-infection status, and immune gene transcription profiles predict treatment outcomes including:
    a. Short-term post-treatment mucosal infection clearance
    b. Long-term post-treatment mucosal infection clearance

In **Chapter 2**, the clinical setting is intentionally targeted to maximize heterogeneity, enabling aims 1 and 2 to address key gaps in the current understanding of the prevalence of major infectious agents and their interrelationships in a clinical context. This chapter evaluates the hypothesis by analysing how pathogen detection parameters relate to one another and examining the potential role of co-infections in contributing to variations in clinical outcomes.

In **Chapter 3**, aim 3 builds on the multivariate approach introduced in **Chapter 2**, using a subset of the sample population from the same clinical setting to capture diversity in host responses. Through aim 3 and 4, this chapter investigates whether infectious status, clinical states, and outcomes are reflected in the host immune response. The findings further advance our understanding of immune-physiological responses and contribute to identifying potential health indicators that may inform the biological mechanisms behind conventional clinical classifications.

In **Chapter 4**, aim 5 seeks to enhance future immune-physiological analyses by identifying additional gene targets associated with clinical, infectious, and survival states, thus contributing to the development of a more comprehensive multi-gene panel. Aim 6 confirms the validity of the NanoString panel and the low-level expression patterns of key immune genes—IL10, IL17A, IFNγ, and TNFα—observed in Chapter 3, and provides alternative but associated gene candidates for future analyses. This chapter expands our understanding of these genes' potential functions, roles, and significance in disease dynamics.

Chapter 5 synthesizes findings from the previous chapters to reexamine the relative contributions of co-infections, host responses, and demographics to health outcomes in a field setting. By comparing two populations with differing rates of morbidity and mortality, aims 7 and 8 assess the prognostic capacity of various factors to predict short- and long-term outcomes. This chapter identifies risk factors associated with mortality and treatment outcomes across two distinct environmental contexts.

Finally, **Chapter 6** situates the work of this thesis within the broader field of disease investigation, discussing how its findings address the overarching aims of the study. This chapter highlights the contributions of the research to advancing scientific understanding and improving wildlife health assessments, particularly for koalas, while identifying areas for further study to continue supporting conservation and health outcomes.

Chapter 2 The relationships of viral and protozoal co-infections to *Chlamydia pecorum* infection and chlamydiosis outcomes in northern koalas *(Phascolarctos cinereus)*

2.1 Author contribution statement

Yasmine S. S. Muir is the main author of this study and has contributed towards every component of the research including the conceptualisation, field work and data curation, methodology, formal analysis, investigation, writing of the original draft and reviewing and editing of the manuscript. Damien P. Higgins and Mark B. Krockenberger supervised and contributed to the conceptualisation, investigation, writing and reviewing of the manuscript and provision of research resources. Belinda R. Wright contributed to the methodology, writing and reviewing of the manuscript, as did Andrea Casteriano in addition to the provision of research resources. Mathew S. Crowther contributed to the statistical supervision, writing and review of the manuscript. All authors have read, contributed, and agreed to the final version of the manuscript.

Yasmine Sophia Sierra Muir

28/2/2025

Damien P. Higgins

28/2/2025

2.2 Abstract:

Several infectious agents concurrently infect wild koalas and so, as for similar agents in other species, co-infection interactions could affect disease presentation and clinical outcomes. This study determines the frequency of circulating and mucosal *Chlamydia pecorum* infections along with phascolarctid herpesvirus (PhaHV), Koala retrovirus (KoRV), and trypanosome infections in 115 wild koalas admitted to wildlife hospitals in the states of Queensland and New South Wales. *C. pecorum,* PhaHV, trypanosomes, and KoRV (endogenous subtype A and exogenous subtype D) were detected in 61.1%, 68.9%, 63.3% and 100% of the individuals sampled, respectively. The co-infection relationships identified generate hypotheses for the observed variation in disease presentations in that they resemble co-infection interactions that drive the variations in presentation and response to treatment for chlamydiosis in other species, including humans. PhaHV-1 mucosal shedding positively predicted euthanasia on admission in koalas with chlamydiosis, and accounting for *Trypanosome irwini* infection status improved the model quality. Additionally, the detection of mucosal PhaHV-1 and greater KoRV proviral *pol* loads were equal predictors of chlamydial reproductive disease in female koalas. While the detection frequency of *C. pecorum,* PhaHV-1, PhaHV-2, and *T. gilletti* in circulation were low, cases with circulating *C. pecorum* and without mucosal *C. pecorum* shedding or clinical chlamydiosis were observed presenting an important consideration for future diagnostic testing. This study serves as a basis for investigating co-infection interaction pathways through mechanistic studies to determine their effect on pathogenesis of chlamydiosis, improve our understanding of host-pathogen-environment dynamics impacting the koala, and identify novel intervention and screening methods.

2.3 Introduction:

Koalas are separated broadly into two groups that differ geographically and genetically: the northern population (Queensland [Qld], New South Wales [NSW], & Australian Capital Territory) and the southern population (Victoria & South Australia) (Neaves *et al.*, 2016; Speight *et al.*, 2016). In 2022, the northern koala population was listed as endangered under Australian Commonwealth legislation (DCCEEW, 2022b). In NSW, between 2020-2021, 46.8% of rescued koalas died or were euthanised (DEH, 2022). This rate is 3.1% and 5.6% greater than those recorded in 2019-2020 and 2018-2019, respectively (DEH, 2022). Although the catastrophic 'Black Summer' bushfires of 2019-2020 likely contributed to increases in admissions during that period, on average 71% of koalas admitted for chlamydiosis die and 25% are released (DEH, 2022), suggesting room for improvement in clinical management of disease in koalas. In many regions of Australia, non-traumatic disease is the leading cause for koala admission to wildlife hospitals (Kerlin *et al.*, 2022), with chlamydiosis due to *C. pecorum* the leading cause in NSW (2018-2021) (DEH, 2022). This obligate intracellular bacterium is present within most mainland koala populations and affected animals classically present with conjunctivitis and/or 'wet bottom', a urine-stained rump from incontinence due to cystitis. Both presentations range from mild, acute inflammation to severe and chronic proliferative or fibrotic disease in which the structural tissue damage can result in renal failure, blindness, secondary infection, death or infertility. Infertility is a great concern for population viability, and in females is due to inflammation and associated scarring of the uterus and uterine tubes (pyometra, endometriosis, ovarian bursal cyst formation, obstructive fibrosis and tubal defects) and in males inflammation of the epididymis, prostate or testes, resulting in sperm reduction, damage & abnormal morphology (Higgins *et al.*, 2005a; Hulse *et al.*, 2021; Hulse *et al.*, 2022; Johnston *et al.*, 2015; Palmieri *et al.*, 2019; Phillips *et al.*, 2021).

Due to the high probability of infertility and in some cases poor quality of life (Pagliarani *et al.*, 2022), the presence of ovarian bursal cysts is one criterion for euthanasia supported by the Code of Practice for the Care of Sick, Injured or Orphaned Protected Animals in Queensland and Code of Practice for Injured, Sick and Orphaned Koalas in New South Wales (NSW Government, 2023; WTOB, 2020). In other species the pathogenesis of chlamydiosis is complex, comprising intestinal, urogenital, ocular and systemic activity and interactions with other viruses and bacteria, as well as a range of stressors (Ball *et al.*, 2024; Das & Röst, 2023; Ghasemian *et al.*, 2023; Koster *et al.*, 2022; Leonard *et al.*, 2017; Pérez-Soto *et al.*, 2021; Prusty *et al.*, 2012; Schuchardt & Rupp, 2018; Zhou *et al.*, 2021). Chlamydial sp. infection in humans and animals can also be sub-clinical (Biesenkamp-Uhe *et al.*, 2007; Poudel *et al.*, 2012; Reinhold *et al.*, 2008; Yang *et al.*, 2014), and has been reported as such in the koala (Chen *et al.*, 2023b; Robbins *et al.*, 2019). Overall, the mechanisms behind clinical variation in chlamydiosis outcomes are poorly understood.

In humans and other animals, co-infections are common and influence the severity and progression of chlamydial infection through various mechanisms involving different host, pathogen, and environmental factors (Devi *et al.*, 2021; Ghasemian *et al.*, 2023). As observed in experimental mouse models, of *C. trachomatis* and other bacterial and viral co-infection, specific effects of co-infections on *C. pecorum* activity and chlamydiosis in the koala could be highly dependent on the particular infectious agents involved, the timing and sequence of infections, and the host's immune status (Ghasemian *et al.*, 2023; Schuchardt & Rupp, 2018). The interplay between different agents in a co-infection scenario is complex, causing alterations to pathogenesis through mechanisms such as immunomodulation, competition

for resources, synergistic effects, antagonistic effects, recurrence and reactivation, and disease modulation (Devi *et al.*, 2021). Variations in clinical disease presentations, treatment response, and pathogen detection highlight key gaps in our understanding of pathogenesis and the roles of non-chlamydial infectious agents in driving host-pathogen-co-pathogen interactions. The list of infectious agents of putative significance to the pathogenesis of chlamydiosis in koalas is growing and now includes endogenous and exogenous Koala retrovirus (KoRV) (Madden *et al.*, 2018; Maher & Higgins, 2016; Maher *et al.*, 2019), phascolarctid gamma-herpesvirus (PhaHV) (Stalder *et al.*, 2015; Vaz *et al.*, 2019b), and trypanosomes (McInnes *et al.*, 2011a). In the northern koala population, KoRV subtype A is endogenous and ubiquitous, while exogenous subtypes B-M are locally prevalent (Blyton *et al.*, 2022a; Blyton *et al.*, 2022d; Chappell *et al.*, 2017; Joyce *et al.*, 2021; Oliveira *et al.*, 2006; Tarlinton *et al.*, 2006). Various KoRV characteristics have been associated with chlamydiosis (Blyton *et al.*, 2022a; Fabijan *et al.*, 2020; Joyce *et al.*, 2022; Joyce *et al.*, 2021; Waugh *et al.*, 2017), and it is postulated that these associations exist due to the KoRV immunosuppressive domain (ISD) (Fiebig *et al.*, 2006; Kleinerman *et al.*, 1987; Madden *et al.*, 2018), circular interaction with oxidative stress and inflammation (Rangel *et al.*, 2022), amplification of innate anti-viral immune defences (Srinivasachar Badarinarayan & Sauter, 2021), or insertion adjacent to immune genes as observed in retroviruses in humans (Chuong *et al.*, 2016; McEwen *et al.*, 2021).

Two types of gammaherpesvirus have been identified in the koala so far: PhaHV-1 (Vaz *et al.*, 2011) and PhaHV-2 (Vaz *et al.*, 2012). PhaHV-1 shedding increases with age and PhaHV-2 shedding is more common in euthanased koalas with poorer body condition (Kasimov *et al.*, 2020; Wright *et al.*, 2024). A number of possible pathogen interactions have already been

noted. PhaHV-1 and exogenous KoRV A infection is associated with uterine/ovarian bursal cysts and testicular malformation, reduced female fertility, urinary incontinence, and the co-detection of *C. pecorum* in South Australian (SA) koalas (Vaz *et al.*, 2019b). In humans, herpes simplex virus Type-2 and *Chlamydia trachomatis* have a dynamic relationship that, depending on which is the primary infection, can significantly alter pathogenesis (Slade, 2016).

Of the four species of trypanosome known to infect koalas (*T. copemani, T. Irwini, T. gilletti, and T. vegrandis)*, *T. gilletti* has been suggested to exacerbate anaemia and body condition loss in northern populations (Barbosa *et al.*, 2017; McInnes *et al.*, 2011a). More specifically, koalas with signs of chlamydiosis that were also infected with *T. gilletti* had significantly lower packed cell volumes and body condition scores compared to non-trypanosome infected diseased koalas (McInnes *et al.*, 2011a). These association studies, whilst not yet providing information on causation, are an important and necessary step that guide further focused analyses into potential causal relationships between co-infection risk factors and health outcomes.

With koala populations declining across their northern range, we must establish which infectious agents are likely to impact disease mitigation, prevention, and conservation efforts. Before co-infection associated modulation of the koalas' host response can be explored, the types of co-pathogen interactions and their association with clinical outcomes must be established. To this end, this study utilises gold-standard and novel methods to detect and quantify parameters for four key groups of infectious agents; *C. pecorum*, KoRV, PhaHV-1 & -2, and trypanosomes to: (1) determine the potential for NanoString to detect koala pathogens in circulation, (2) determine the detection frequency of these infectious agents in a sample

population of 115 wild northern koalas with diverse clinical presentations, demographics, and geographical origins, admitted to three wildlife hospitals within NSW and Qld, (3) determine whether co-infections are preferentially associated with *C. pecorum* infection in koalas on admission, and (3) assess the relationships of co-infections with clinical chlamydiosis. Future directions are suggested to further explore the relationship between co-infections to identify pathological mechanisms which may be involved in persistent/recurring infections and the presentation of clinical signs and poor responses to chlamydiosis treatment necessitating euthanasia.

2.4 Methods:

Sample Collection

Sampling was conducted from September 2021 to April 2022 at three koala care facilities servicing South-east Qld , Northern and Mid-North Coast NSW, and Central NSW (Figure 2.1). Sampling was completed at Australia Zoo Wildlife Hospital, Port Macquarie Koala Hospital, and Friends of the Koala between September and December 2021, January to April 2022, and February 2022, respectively. Sampling of koalas was conducted under the University of Sydney Animal Ethics Approval Number 2021/1975, NSW NPWS Scientific License SL102379 and Qld NPWS WA0019256. All koalas admitted during these periods were considered for inclusion, except cases of extreme trauma where the koalas' condition or instability under general anaesthetic (GA) could not support sampling, as advised by the veterinarian; or koalas that were dead on arrival. Sampling was performed under GA in accordance with the hospitals' routine procedures: induction via intramuscular injection of 3 mg/kg alphaxalone (Alfaxan®-CD, RTU; Jurox Pty Ltd., Rutherford, Australia) followed by maintenance using 2%

isoflurane in 100% oxygen delivered either through face mask or via cuffed 4-4.5mm endotracheal tube.

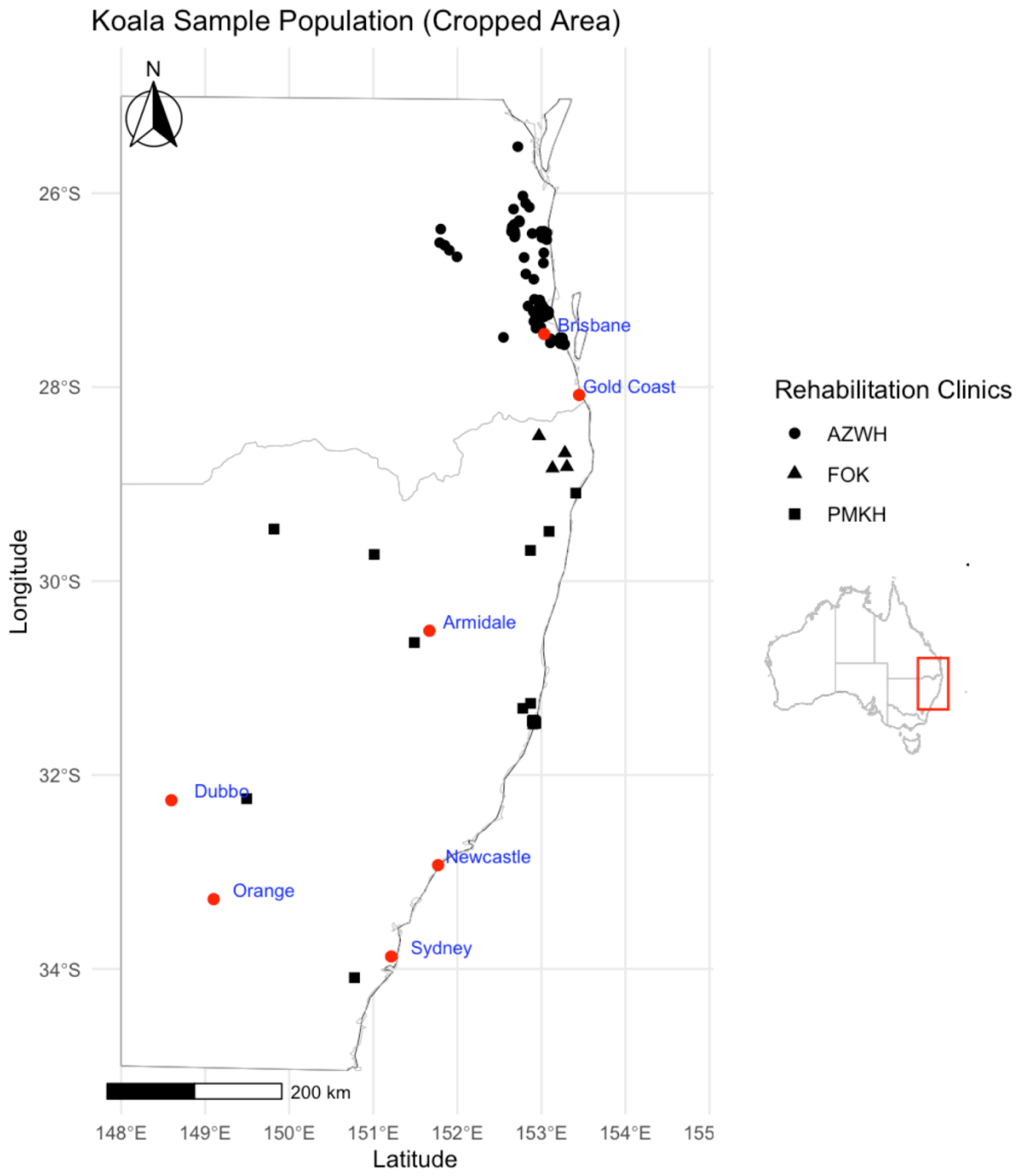


Figure 2.1: Map of Australian states Queensland and New South Wales (truncated) and the distribution of sampled koalas.

In Figure 2.1 Geographical points represent the location (latitude and longitude) from which each individual koala was rescued from before admission to hospital with the shape of points depicting which hospital they were admitted to; Australia Zoo Wildlife Clinic (AZWH, circle), Friends of the Koala (FOK, triangle), and Port Macquarie Koala Hospital (PMKH, square). Red points represent major cities in proximity to koala rescue locations. This map was generated in R using the Natural Earth public domain package.

Blood (3 mL) was collected from the cephalic or saphenous veins into an EDTA tube (Vacuette® Tube, Greiner Bio-One GmBH, Kremsmünster, Austria) and processed into whole blood and buffy coat separations. EDTA whole blood was divided into two 300 µL aliquots, one preserved in 900 µL RNAlater™ (Qiagen). The remaining EDTA blood was then centrifugated at 1,000 x g for 10 min. From the separated EDTA blood, 250 µL of the buffy coat layer was aliquoted with 750 µL RNAlater™ (Qiagen) for RNA analysis. All cryogenic vials containing RNAlater™ (Qiagen) were left at ambient temperature for 24 hours and then stored at -20°C until analysis. All other cryogenic vials were stored at -80°C until analysis. Dry aluminium shaft cotton tipped swabs (Copan Italia, Brasica, Italy) were used to sample the urogenital mucosa (the urogenital sinus in females and the urethra in males), oropharyngeal mucosa, and left and right conjunctivae. Swab tips were cut into respectively labelled cryogenic vials (Biologix Grp Ltd, Kansas, United States) and stored at -80°C.

Clinical information included data recorded from visual assessments, ultrasonography, x-ray, haematology and biochemistry, urinalysis, paracentesis, point of care *C. pecorum* LAMP assays, laparotomy examinations, faecal wet preparations, blood smears, bone marrow aspirates, and post-mortem assessment. When conducted, all clinic-based loop-mediated isothermal amplification (LAMP) testing of ocular and urogenital swabs was performed to detect *C. pecorum* shedding, according to the protocols described by Hulse *et al.* (2019c), with the Genie II (OptiGene, Horsham, South of England, UK). Diagnostic modalities were employed based on the veterinarian's assessment of each individual case and influenced by facility protocols. Clinical examination data was collated by pooling information recorded by the veterinarian and nurses using the standard hospital recording sheets and a standardised

recording sheet designed by the researcher. Some cases of euthanasia were not assessed by radiography due to severity of trauma, emaciated body condition or acute deterioration in clinical condition. In these cases, necropsies were performed to identify internal abnormalities (N = 18). Necropsies were conducted on site at respective hospitals with the majority completed within 24 hours of euthanasia. Most carcasses were stored at 0 – 4°C until necropsy that same day. Three were examined after 48 hours after storage at 0 – 4°C. All necropsies followed the methods outlined by the Koala Health Hub and using their koala necropsy record sheet (Koala Health Hub, 2019). Photos were taken to accompany written records of necropsy findings.

Categorisation of clinical groups:

Clinical groups of interest included the presentation of clinical chlamydiosis, untreatable chlamydiosis, and reproductive disease in females, specifically. These were used as dependent binary outcome variables. As outlined in the clinical criteria table (Supplementary materials: Table 7.3), cases were designated (1) 'clinical chlamydiosis' if presenting with wet-bottom (evidence of recent or current incontinence) and/or conjunctivitis (swelling, proliferation and/or inflammation of the conjunctiva +/- cataracts or ocular ulceration), cystitis (thickened bladder walls and inflammation observed on ultrasound using doppler) +/- other urinary tract abnormalities including renal disease (hyperechoic medulla on ultrasound, hydronephrosis, hydroureter), and/or reproductive disease observed on ultrasound (ovarian bursal cyst(s), pyometra, uterine oedema, endometriosis). It should also be noted that koalas with any of these clinical signs may also have presented with other abnormalities such as candidiasis, anaemia, poor body condition, trauma, etc, and that it is possible for unidentified pathogens to have caused clinical signs of disease. If koalas presented with no abnormalities,

or with abnormalities but without clinical signs attributable to *C. pecorum* infection, they were classed as (0) 'no clinical signs of chlamydiosis'. Koalas with clinical chlamydiosis were sub-classified as (1) 'untreatable chlamydiosis' if they were euthanised on admission or (0) 'admitted for chlamydiosis treatment' to explore the relationships between co-infections and the severity of clinical conditions. Euthanasia was elected on welfare grounds where prognosis was poor due to untreatable/irreparable structural damage to tissues/organs derived from disease and/or trauma, or complex comorbidities in conjunction with emaciation and/or mature age (age > 10 years old). Finally, to determine the importance of co-infections to the prediction of reproductive disease, female koalas were classified as either (1) presenting with evidence of 'reproductive disease' as described above, or (0) 'no evidence of reproductive disease'.

Pathogen detection techniques

Gene transcription

Of the 115 koalas sampled on admission, 101 had buffy coat samples available for RNA extraction. Adequate buffy coat samples are difficult to collect when the initial blood collection is low in volume or becomes clotted. This is often the case in koalas with clinically severe trauma or disease where dehydration and shock decrease the blood pressure. RNA was extracted from available buffy coat samples stored in RNAlater using the RNeasy® Mini Kit (QIAGEN) following the manufacturer's instructions. The purity and concentration of nucleic acid in extracted RNA samples was determined using a Nanodrop Spectrophotometer ND-1000 (Thermo Fisher Scientific Co., Waltham, MA, USA) followed by specific quantification of RNA and measure of RNA integrity and quality (IQ) using the Qubit ® RNA HS Assay Kit (Q32852) and Qubit ® RNA IQ Assay Kit (Q33222) on the benchtop Qubit™ 4 Fluorometer

(Invitrogen, Thermo Fisher Scientific), respectively. The extracted RNA samples were stored at −80 °C until required.

A custom NanoString nCounter plex-set (NanoString Technologies, WA, USA) was designed to quantify transcribed mRNA from a multiplexed set of 72 genes of interest for koala biological and pathological pathways (Supplementary Table 7.4). While different panels may demonstrate variations in sensitivity and specificity due to probe-specific biological variations, overall the NanoString nCounter platform has demonstrated comparable or improved results compared to RT-qPCR (Hyeon *et al.*, 2017; Pescarmona *et al.*, 2019), microarrays (Geiss *et al.*, 2008), immunohistochemistry and fluorescence in situ hybridisation (Hyeon *et al.*, 2017), and RNA-Seq (Bondar *et al.*, 2020; Veldman-Jones *et al.*, 2015). For this study, only pathogen gene targets (14/72) were examined to determine relationships between infection status and clinical outcomes in koalas. Similarly to previous NanoString panel designs for the koala (Olagoke *et al.*, 2020b; Quigley *et al.*, 2023), four housekeeping genes (*GAPDH, ACTB, Stx12, Nckap1l*) were included for normalisation of data (Sarker *et al.*, 2018).

The fourteen infectious agent genes included four KoRV genes targeting three major *env* subtypes (*KoRVAenvRBD, KoRVBenvRBD* and *KoRVDenvRBD),* the immunosuppressive domain *(KoRVenvCKS17),* and the *pol* gene (*KoRVpol)* (Quigley *et al.*, 2023). For *C. pecorum,* the single-copy conserved hypothetical protein *CpecG_0573* was included as a general pathogen detection target (Jelocnik *et al.*, 2015; Jelocnik *et al.*, 2017), the chaperonin GroEL gene encoding the *C. pecorum* heat-shock protein 60 *(Cpec_hsp60)* (White *et al.*, 2021), *C. pecorum* strain L17 major outer membrane protein (*ompA*) gene (Mohamad *et al.*, 2014;

Quigley *et al.*, 2023), and *C. pecorum* strain L1 plasmid pCpecL1 (*Pgp3)* gene was included (Jelocnik *et al.*, 2016). For PhaHV detection, the PhaHV-1 (Vaz *et al.*, 2011) and PhaHV-2 (Vaz *et al.*, 2012) specific DNA dependent DNA polymerase gene (*dpol)* was included. Finally, for trypanosome detection the species-specific 18s rRNA regions were targeted for *Trypanosome copemani* (McInnes *et al.*, 2011b), *T. gilletti* (McInnes *et al.*, 2011b), and *T. irwini* (McInnes *et al.*, 2009). Blasting of the *C. pecorum* gene probes confirmed specificity to the *C. pecorum* species. Given that buffy coat samples were used, detection of transcription for each infectious agent gene is hereafter referred to as 'circulating infection' of each target.

Using the RNA quality and quantification results obtained from the previously described methods, a total concentration of 50 – 100 ng in a total volume of 7-10 µL of eluted RNA was prepared using RNA/DNA free water to adjust concentrations. Additional quality control and sample normalisation was completed by Ramaciotti Centre for Genomics, UNSW, Sydney, Australia preceding mRNA analysis and transcript counting which was performed by the same institute according to the manufacturers protocol (NanoString Technologies, WA, USA). Briefly, this process included mRNA hybridisation with both reporter and capture probes according to the nCounter XT CodeSet Gene Expression Assays Protocol (NanoString Technologies, WA, USA). A NanoString nCounter FLEX Analysis System (NanoString Technologies, WA, USA) was then used as per the manufacturer's recommendations for purification and transcript counting. Final transcript counts were determined using a Digital Analyzer (NanoString Technologies, WA, USA).

Raw data was analysed using nSolver™ 4.0 Analysis Software (NanoString Technologies, WA, USA). Briefly, Reporter Code Count (RCC) files containing barcode counts from each gene and

control within each lane in the CodeSet and Reporter Library Files (RLF) including instrument and gene probe information were loaded into nSolver™. Only samples with housekeeping gene transcription levels exceeding 50 counts were considered for analysis. Using this criterion, 7 samples were omitted from the analysis.

While a *Trypanosome,* PhaHV-1 or PhaHV-2, or KoRV free population was not available to validate the detection threshold for gene mRNA counts, data from a *C. pecorum* free population was. As a preliminary validation, we tested two approaches to thresholding counts for *C. pecorum* gene targets, *Cpec_hsp60* and *CpecG_0573* in a small sample set acquired from a known "Chlamydia free" wild koala population in Campbelltown, NSW, Australia (N = 33): (1) using raw counts compared to (2) normalised counts. For this Campbelltown sample set, samples were obtained, processed, stored, and extracted utilising the same methods reported above. Because the NanoString 72-plex panel design was refined to a 48-plex design following investigations using the sample population of this study (termed 'DAWE'), later investigations using Campbelltown samples do not have data for *C. pecorum ompA* and *pGP3* genes. Raw counts were compared to normalised counts according to gene using box-plot scatter plots visualisation.

Based on the effect of normalisation observed on *C. pecorum* gene targets, which mostly elevated background levels, raw counts were used to classify detection in non-ubiquitous infectious agent genes. As suggested by NanoString Technologies (NanoString Technologies Inc, 2009, 2017), transcription counts below 20 cannot be discerned from background noise so any samples with raw expression levels below this threshold were flagged as below the limit of detection (LOD) and were considered negative. Any sample with raw expression levels

above this threshold were considered positive for circulating infection of the associated infectious agent. The distribution of raw counts above and below the threshold, and the resulting number of samples classified as 'positive' for gene transcription is presented in a box-plot. Quantification of gene transcription was conducted in samples that were considered positive for detection of an infectious agent. In these samples, counts were adjusted against expression thresholds to account for differences in sample content and normalised against housekeeping genes (*GAPDH, ACTB, Stx12 & Nckap1l*).

Mucosal *C. pecorum* DNA qPCR

DNA was extracted from urogenital (UGT) and ocular swabs using the MagMAX™ CORE Nucleic Acid Purification Kit (Thermo Fisher cat# A32702; ThermoFisher Scientific, Waltham, MA, USA) with modifications to the manufacturer's instructions. Swab samples were shaved into a 1.5 mL tube containing 350 µL of MagMAX CORE Lysis Solution and 10 µL of Proteinase K and incubated for 1 hour at 56°C. The lysate was then added to a 96DW-plate containing 350 µL of MagMAX CORE Binding Solution and 20 µL of MagMAX CORE Magnetic Beads, then immediately processed on a KingFisher™ Flex automated extraction instrument using the MagMax_Core_Flex protocol. Each extraction batch contained a sterile unused swab as an extraction blank. DNA was eluted to a final volume of 100 µL and stored at -80°C until analysis.

Extracts were assessed in a multiplex real-time qPCR using a CFX96 Touch™ Real-Time PCR Detection System with the corresponding CFX Maestro software (Bio-Rad, Australia). Briefly, this PCR included a *Chlamydia* genus (23S) and species (*C. pecorum*) *ompB* gene primer set as well as a sample quality control that quantified host DNA by amplification of the koala *β-actin* gene. Detailed information on the primer set adapted from (Hulse *et al.*, 2018) is described in

Supplementary materials Table 7.5. A total PCR reaction volume of 20 µL consisted of 400 nM of each primer, 200 nM of each probe, 10 µL of SensiFAST^{TM} Probe No-ROX (Bioline cat# BIO-86005), 4.4 µL dH_2O and 2 µL of DNA template. Samples were analysed in duplicates and a negative control (no template control; dH_2O) was included. A pUCIDT-AMP vector (Integrated DNA Technologies, USA) containing the 3 target regions and flanking sequences (*β-actin*, *C. pecorum* and *Chlamydia* genus) was used as a synthetic positive control to generate a quantification standard curve at 10-fold dilutions, ranging from 10^3 to 10^7 copies. PCR plates were prepared manually or using a Myra Liquid Handling System (Bio Molecular Systems). qPCR conditions consisted of an initial 3-min denaturation at 95°C (1 cycle) followed by 40 cycles of a 10 s denaturation at 95°C and a 40 s annealing at 58°C.

As a quality control, samples that repeatedly failed to amplify *β-actin* were not included in further analysis (ocular samples, N = 6; UGT samples, N = 7). Samples were considered positive if amplification of *β*-actin and either *C. pecorum ompB or* 23s genus, was achieved in both duplicates. Any sample with discordant results between duplicates was retested and samples that failed to amplify were re-run at 1:10 dilution to dilute inhibitors. LOD was determined using probit regression analysis for this assay and was found to be 86 copies of *C. pecorum* per reaction (95%CI) (Premachandra *et al.*, 2024). Hence, samples with copy counts below this threshold were not included in quantitative analysis. Quantitative results were reported as *β*-actin normalised *C. pecorum* counts by dividing *ompB* gene counts by *β*-actin gene counts. qPCR efficiencies were between 89-100% and intra-assay variation below 5% for all genes.

PhaHV-1 & -2 DNA qPCR

DNA was extracted from oropharyngeal swabs using the same methods described for urogenital and ocular swabs above. Oropharyngeal swab extracts were analysed using the same PhaHV-1 *dpol* and PhaHV-2 *dpol* DNA qPCR design and method described by (Wright *et al.*, 2023) and (Church *et al.*, 2025), respectively. Koala *β-actin* was included as a DNA quality and quantity control (Hulse et al., 2018). The primers listed in Supplementary materials Table 7.5 were used at a concentration of 250 nM in a total reaction volume of 20 µL using 2 µL DNA template and SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Australia #1725270). A pMG-Amp vector (Macrogen, South Korea) synthetic plasmid (positive control) containing the target region and flanking sequence (PhaHV-1: 166 bp, PhaHV-2: 87bp) was used to generate a standard curve at 10-fold dilutions to quantify viral loads (ranging from 10^3 to 10^7 copies/µL). qPCR conditions consisted of an initial 3-min denaturation at 95°C (1 cycle) followed by 40 cycles of a 10 s denaturation at 95°C, and then a 30 s annealing/extension at 56°C. Finally, a melt curve at 56 - 95°C for PhaHV-1 and 65 – 90°C for PhaHV-2 at 0.5°C increments was produced. A sample was considered positive if both duplicates amplified *β-actin* and produced a melt curve at 81 – 81.5°C for PhaHV-1 and/or at 87 – 87.5°C for PhaHV-2. Any sample with discordant results between duplicates was retested and samples that failed to amplify were re-run at 1:10 dilution to dilute potential inhibitors. Samples which failed to amplify koala *β-actin* were excluded from further analysis (PhaHV-1, N = 4; PhaHV-2, N = 1). For quantitative analysis, the limit of quantification (95%CI) of PhaHV-1 and PhaHV-2 was 12 copies and 133 copies per reaction, respectively. Samples resulting in counts for PhaHV1 or PhaHV-2 below this threshold were not included in quantitative analysis. qPCR efficiencies ranged between 90.2–103.9% for PhaHV-1 and 90.2-96.7% for PhaHV-2 and the inter-assay variation was less than 5% for both targets.

KoRV *pol* (cDNA) and proviral (DNA) qPCR

For KoRV *pol* expression, 16 µL of extracted buffy coat RNA, extracted using methods previously described for NanoString, were DNAse treated as neat samples using the DNase I, RNase-free (1U/ µL) kit #EN0521 (Thermo Scientific™) alongside a blank control (dH_2O) following the manufacturer's instructions. A final volume of 20 µL DNAse treated samples underwent cDNA synthesis using RevertAid First Strand cDNA Synthesis Kit (#K1622, ThermoFischer) following the manufacturer's instructions. Cycling was completed using a T100 Thermal Cycle (Bio-Rad) and consisted of 5 min at 25°C, followed by 42°C for 1 hour, then 70°C for 5 min. RT positive and RT negative cDNA products, and DNAse and cDNA blank controls were then assessed in a KoRV *pol* quantitative PCR (RT-qPCR).

For KoRV proviral *pol,* DNA was extracted from EDTA whole blood samples using a MagMAX CORE Nucleic Acid Purification Kit (Thermo Fisher cat# A32702; Thermo Fisher Scientific, Waltham, MA, USA). Briefly, 200 µL of EDTA blood was added into a 1.5 mL tube containing 350 µL of MagMAX CORE Lysis Solution and 10 µL of Proteinase K and incubated at 56 °C for 10 min. The lysate was then added to a 96DW-plate containing 350 µL of MagMAX CORE Binding Solution and 20 µL of MagMAX CORE Magnetic Beads, then immediately processed on a KingFisher™ Flex automated extraction instrument, using the MagMax_Core_Flex protocol. DNA was eluted to a final volume of 100 µL. Each extraction batch contained one no-DNA blank (sterile PBS) to control for potential contamination. DNA extracts were then diluted 1:10 using dH_2O for qPCR.

Both diluted DNA and neat cDNA were assessed using the same KoRV *pol* qPCR protocol. PCR plates were prepared manually and using a Myra Liquid Handling System (Bio Molecular Systems). Separate master mixes were generated for KoRV *pol* gene detection and koala *β-actin* reference gene detection and applied to independent wells so that DNA (for whole blood extracts) and cDNA (for buffy coat extracts) quality and quantity could be assessed in real-time alongside KoRV *pol*. Primer and probe sets used are described in Supplementary materials Table 7.5. For each KoRV *pol* reaction, a total volume of 20 µL comprised of 0.6 µL of primer (10 µM), 0.2 µL of probe (10 µM), 10 µL of SensiFAST^{TM} Probe No-ROX (Bioline cat# BIO-86005), 6.6 µL dH_2O and 2 µL of template. Each 20 µL *β-actin* reaction comprised of 0.8 µL of each primer (10 µM),0.4 µL of the probe (10 µM), 10 µL of SensiFAST^{TM} Probe No-ROX (Bioline cat# BIO-86005), 6 µL dH_2O and 2 µL of template.

Samples were run in duplicates alongside a serial dilution of a positive standard (synthetic KoRV *pol* positive control) and an NTC (no template control; dH_2O). qPCR conditions consisted of an initial 3 min denaturation at 95°C (1 cycle) followed by 40 cycles of a 10 s denaturation at 95°C and a 40 s annealing at 60°C. The limit of quantification for this assay was determined to be 21 copies per reaction and no samples were excluded. For KoRV *pol* qPCR using cDNA, only copy counts from samples with at least a 10 cycle (Ct) difference between the respective RT- and RT+ were retained for further analysis (Laurell *et al.*, 2012). Circulating KoRV *pol* quantities are reported as *β*-actin normalised KoRV *pol* copies per mL of template by taking the ratio of KoRV *pol* counts per µL to koala *β*-actin counts per µL x 1000. qPCR efficiencies for the KoRV *pol* gene ranged between 90.6–100% for analysis of DNA samples and 87.2-103% for cDNA samples and the inter-assay variation being less than 5% and less than 6%, respectively.

Statistical Analysis:

The distribution of age in the population was assessed using histograms and Shapiro-Wilk normality tests. Due to a non-normal distribution, age was reformed as a 3 levelled factor: young adult (1.5-3 years old), adult (>3-9 years old), and aged (>9-12 years old). The proportions of male and female koalas were compared using chi-squared tests of independence and were considered equally distributed between all infectious agent groups but showed significant differences in select clinical groups. Co-infection combinations are presented in a Venn diagram.

For Generalized Linear Models (GLM), all non-ubiquitously detected markers were transformed into binary variables (detected (1), not detected (0)) to improve the assessable sample sizes. To ensure meaningful contributions to model estimations by avoiding low variation and high data skewness, only markers with a minimum of 10 events were included in GLM analysis as independent variables (Courvoisier *et al.*, 2011; Peduzzi *et al.*, 1996; Vittinghoff & McCulloch, 2006). Hence, circulating *C. pecorum,* PhaHV-1, PhaHV-2, and *T. gilletti* transcription status were excluded. Correlations between the five ubiquitous KoRV markers (KoRV *pol* cDNA/mL, KoRV *pol* DNA/mL, KoRV *env* A mRNA, KoRV *env* D mRNA, KoRV *env* CKS17 mRNA) were assessed using Spearman's Rank correlations, firstly without considering KoRV *env* B (N = 95) and then in KoRV *env* B positive koalas (N = 29). In the cases of strong and significant co-correlations ($r > 0.7$, $p < 0.05$), the parameter with the greatest sample size was retained for further analysis (KoRV *pol* cDNA/mL). Residual plots were constructed, and Shapiro Wilk tests performed to check for normality of continuous variables. Log_{10} transformation was applied to non-normal variables.

Four separate GLMs were conducted to test the effect of co-infection variables on (1) mucosal *C. pecorum* detection (N = 87), (2) the presentation of clinical chlamydiosis (N = 87), (3) untreatable chlamydiosis (N = 48), and (4) reproductive disease in females (N = 50). These are referred to as "global models (1-4)". In each global model, age group (young adult, adult, & aged), mucosal PhaHV-1, PhaHV-2, and circulating *T. copemani, T. irwini* and KoRV-B detection were used as independent factor variables and KoRV *pol* transcription and proviral loads as independent continuous variables. Mucosal *C. pecorum* detection was included as an independent factor variable in global models 2-4. Sex (male = 0 or female = 1), was included as an independent factor variable in global models 1-3. Observations with missing data for any of the assessed variables were removed as required for GLMs. To the best models and the relative importance of each independent variable on the outcome, global model coefficients were standardised and model averaging was undertaken using the MuMIn package (Barton, 2023). Comparison of the corrected Akaike's Information Criterion (AIC_c) estimations between models as well as two associated measures of model fit, delta AIC_c (Δ_i) and Akaike weights (w_i), was used to assess the "best" fitting models. Akaike weights (w_i) are a measure of relative strength of evidence for models (Anderson, 2002). Only models returning $\Delta_i < 2$ were considered for model averaging. Precision of model-averaged estimates, also termed the unconditional SE, and unconditional 95% confidence intervals were calculated. All statistical analysis were performed using R Statistical Environment (Version 2022.12.0+353) (R Development Core Team, 2024) and statistical significance threshold of p-value < 0.05 was applied to all tests conducted. A network plot was generated to summarise the relative importance of predictors with RI > 0.5 and the direction of the interaction between predictors and outcome variables. The thickness of arrows between predictors and

outcome variables reflects the magnitude of RI (0.5-1) and the colour indicates a positive (black) or negative (red) interaction.

2.5 Results:

Classification of pathogen 'detection' using NanoString:

Samples taken from koalas originating from a "chlamydia-free" population (n = 33) did not demonstrate raw counts above the LOD threshold (20 mRNA counts) for *Cpec_Hsp60* mRNA counts (Figure 2.2). One koala demonstrated *CpecG_0573* mRNA counts above the LOD (Figure 2.2). Normalisation markedly increased the number of counts above the LOD for both genes in the chlamydia free population, indicating that raw counts should be used to reduce false positives.

Figure 2.2: Raw and normalised mRNA counts for* C. pecorum *gene targets quantified using NanoString nCounter between two datasets obtained from* Chlamydia*-free and* Chlamydia*-affected koala populations.

In Figure 2.2 four scatter-box-plots present the raw (first column) and normalised (second column) mRNA counts for C. pecorum *gene targets: Cpec_hsp60 & CpecG_0573. Counts are separated by dataset and coloured by* Chlamydia *status: Campbelltown – a chlamydia free koala population (pink), and DAWE – sample populations of koalas from* Chlamydia*-affected areas analysed (blue). DAWE refers to the grant providers for the dataset: Department of Air, Water, and Environment (DAWE).*

Using raw counts, most infectious agent genes were detected above the LOD (20 counts) in at least one sample (Figure 2.3). As expected due to their high transcription loads in northern koalas (Blyton *et al.*, 2022d; Sarker *et al.*, 2020a; Sarker *et al.*, 2019; Tarlinton *et al.*, 2022c), all raw counts were above the LOD for several KoRV genes: *KoRVAenvRBD, KoRVDenvRBD, KoRVenvCKS17,* and *KoRVpol.* Therefore, counts for these four genes were normalised for further analysis as continuous variables.

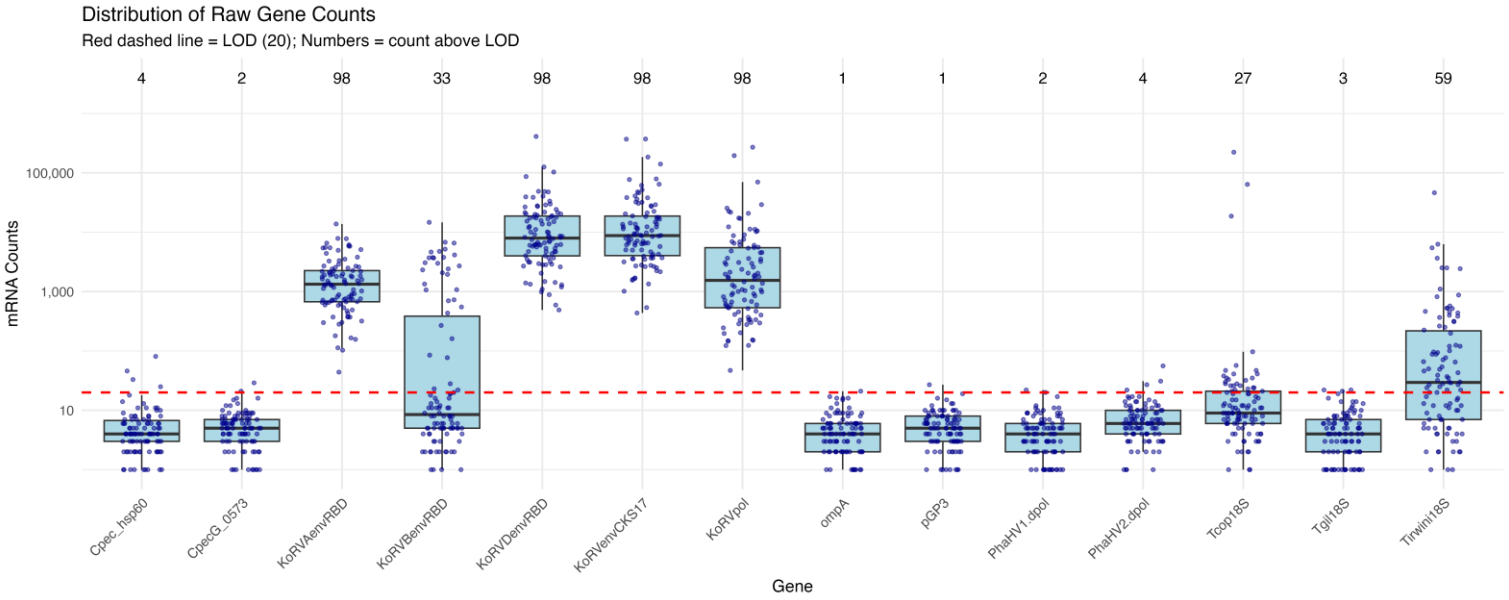


Figure 2.3: Raw mRNA counts according to gene and the number of cases above the LOD.

In Figure 2.3, box-plots with scatter points are displayed demonstrating the distribution of raw counts according to gene. The limit of detection (LOD) for NanoString is plotted as a red dotted line at 20 counts on the y-axis to demonstrate counts that fall above or below the threshold. The number of cases with counts above the LOD, which were classified as positive for gene transcription detection are indicated above each box-plot. In total, 98 koalas had analysable data from NanoString.

Frequency of C. pecorum *detection:*

In the current study, detection of *C. pecorum* mRNA from buffy coats identified cases potentially infected by *C. pecorum* that were not identified using DNA detection at mucosal sites alone. Nine mucosal and 17 buffy coat samples were excluded from analysis due to

either missing or poor-quality extracts. Of 93 koalas with conclusive results for all *C. pecorum* detection parameters, 61.1% (51/93) were positive for at least one *C. pecorum* target (Table 2.1). In these *C. pecorum* positive koalas, mucosal *C. pecorum* was most frequently detected (86.3%, 44/51), followed by combined mucosal and circulating *C. pecorum* (7.8%, 4/51), then by circulating *C. pecorum* only (5.8%, 3/51).

Table 2.1: Detection frequency of infectious agents and specific targets on admission

Infectious agent	*Sample Type*	*Target*	*N samples tested*	Positive count	Positive frequency (%)
C. pecorum	*Mucosal*	*Ocular*	110	25	22.7
		Urogenital	108	56	51.8
		Total Mucosal[1]	106	60	56.5
	Circulating	*G_0573*	98	2	2.0
		Hsp60	98	4	4.1
		OmpA	98	1	1.0
		Pgp3	98	1	1.0
		Total Circulating[2]	98	8	8.2
PhaHV	*Mucosal*	PhaHV-1	112	66	58.9
		PhaHV-2	112	23	20.5
		Total Mucosal[1]	112	71	63.4
	Circulating	PhaHV-1	98	2	2.0
		PhaHV-2	98	4	4.1
		Total Circulating[2]	98	5	5.0
KoRV	*Circulating*	KoRV *pol* DNA	112	112	100
		KoRV *pol* cDNA	113	113	100
		KoRV *pol* mRNA	98	98	100
		KoRV *env* A	98	98	100
		KoRV *env* B	98	33	34.7
		KoRV env D	98	98	100
		KoRV *env* CKS17	98	98	100
Trypanosomes	*Circulating*	*T. irwini*	98	59	60.2
		T. copemani	98	27	27.6
		T. gilletti	98	3	3.1
		Total Circulating[2]	98	62	63.3

1 Grouping all positive results for infectious agent at mucosal sites: *C. pecorum* at UGT or ocular sites, and PhaHV-1 or PhaHV-2 at oropharyngeal site.

2 Grouping all positive results for infectious agent gene transcription to represent circulating presence: *C. pecorum = G_0573, Hsp60, OmpA and/or PgP3,* PhaHV = PhaHV-1 and/or PhaHV-2, Trypanosomes = *T. copemani, T. gilletti,* and/or *T.irwini.*

Frequency of PhaHV-1 & PhaHV-2 detection:

PhaHV DNA and/or mRNA was detected in 68.9% (73/106) of koalas in the study cohort. Irrespective of detection site, PhaHV-1 and PhaHV-2 infection was identified in 62.1% (64/103) and 26.7% (28/105) of koalas, respectively. PhaHV infection largely consisted of mucosal PhaHV-1 and combined mucosal PhaHV-1 and PhaHV-2 (Table 2.2). Overall, there were a total of 95/115 samples with available results for all PhaHV targets (Table 2.2). Single type PhaHV infection was more common than combined PhaHV-1 & PhaHV-2 co-infection; 75.8% (47/62) vs 24.2% (15/62), respectively (Table 2.2). Detection of PhaHV-1 and PhaHV-2 in circulation was rare: 2.1% (2/95) and 4.2% (4/95), respectively (Figure 2.2). Of note, two cases with circulating PhaHV-2 did not have detectable mucosal shedding. PhaHV-2 mucosal detection was significantly associated with geographic origin ($p < 0.001$). The odds of a koala shedding mucosal PhaHV-2 was 6.33 times greater if they originated from NSW compared to Qld (12/24, 50%; vs 12/78, 15.4 %, $c^2 = 13.2$, df = 1, $p < 0.001$; Odds ratio = 6.33, 95%CI = 2.3-17.9). It should be highlighted that 75% (9/12) of PhaHV-2 positive koalas from NSW originated from Port Macquarie, while koalas from Port Macquarie only represented 45% (12/25) of those from NSW in this study.

Table 2.2: Frequency of detection of mucosal and circulating PhaHV -1 & -2 in koalas with complete results for all targets

PhaHV Infection Combinations	Count	%
PhaHV1 Mucosal	40	42.1
PhaHV2 Mucosal	4	4.2
PhaHV2 Circulating	2	2.1
PhaHV1 Mucosal x PhaHV2 Mucosal	13	13.6
PhaHV1 Mucosal x PhaHV1 Circulating	1	1.1
PhaHV1 Mucosal x PhaHV2 Circulating	1	1.1
PhaHV1 Mucosal x PhaHV1 Circulating x PhaHV2 Circulating	1	1.1
None	33	34.7
Total	95	100

Frequency of KoRV detection:

All KoRV parameters, apart from KoRV-B *env* mRNA (detection frequency 33/98, 33.7%), were detected ubiquitously and were quantifiable in the analysed sample population (Table 2.1, Figure 2.4). All KoRV parameters were non-normally distributed and were Log_{10} transformed to assess Spearman's correlations. Strong and significant correlations existed between most KoRV *pol* cDNA (qPCR) and mRNA (Nanostring) parameters (Figure 2.4). Therefore, to avoid effects of multicollinearity in downstream models, KoRV *pol* cDNA/mL was used as a representative for KoRV transcription in further analysis due to greater sample size. KoRV *pol* proviral DNA copies/mL did not correlate with cDNA or mRNA parameters and was retained as an independent predictor in further analysis.

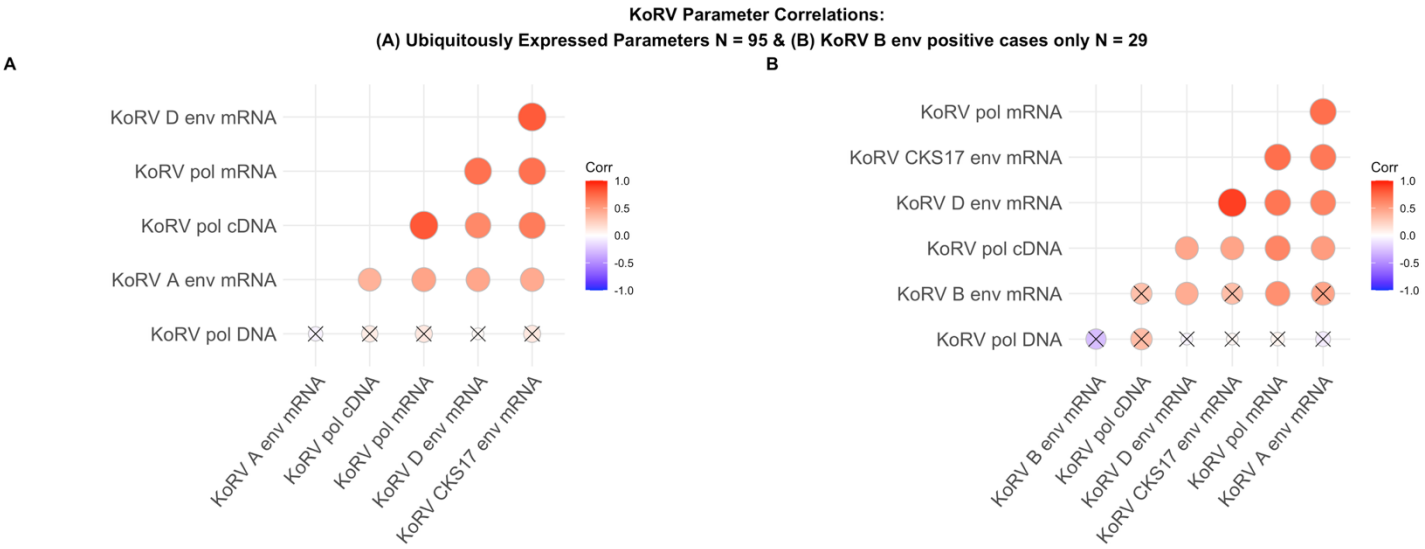


Figure 2.4: Pearson's correlation matrices of KoRV markers.

In Figure 2.4, (A) Depicts correlations between parameters without KoRV B results using 95 observations. (B) Correlations between all markers, inclusive of KoRV B, using 29 observations. Correlations marked X are insignificant ($p > 0.05$).

Frequency of Trypanosome detection:

Trypanosome mRNA was detected in 63.3% of analysed buffy coat samples (62/98) with gene transcription for more than one species detected in 40.3% of those (25/62). *T. irwini* was the most prevalent trypanosome infection, followed by *T. copemani*. *T. gilletti* was the least prevalent (3/98) and 100% of *T. gilletti* detection occurred alongside either *T. copemani* or *T. irwini.* Similarly, 88.9% of samples with *T. copemani* gene transcription also transcribed *T. irwini.* Due to low frequency of detection for *T. gilletti* (Figure 2.3), only *T. copemani* and *T. irwini* were assessed further.

Frequency of chlamydial co-infections:

The detection frequencies of most assessed infectious agents were high. As a result, the frequency of co-infection among chlamydia-infected koalas was also high within the examined population (Figure 2.5). Of the 66 koalas in which mucosal and/or circulating *C. pecorum* was detected (61.1%), only 5 cases (6%) had *C. pecorum* infection alone (irrespective of ubiquitously expressed KoRV, Figure 2.5). The odds that a *C. pecorum* infected koala was co-infected with mucosal or circulating PhaHV -1 and/or PhaHV -2 was 3.1 times the odds of a *C. pecorum* negative koala (49/63, 77.8%; vs 21/40, 52.5%, $\chi^2 = 6.06$, df = 1, p = 0.014; Odds ratio = 3.12, 95%CI = 1.32-7.55, p = 0.009). Specifically, koalas with *C. pecorum* infection had 2.8 times and 2.9 times the odds of shedding PhaHV-1 or PhaHV-2, respectively: 44/65, 67.7%; vs 17/40, 42.5%; $\chi^2 = 5.46$, df = 1, p = 0.019; Odds ratio = 2.79, 95%CI = 1.24-6.45, p = 0.012, and 19/66, 28.7%; vs 5/42, 11.9%; $\chi^2 = 3.31$, df = 1, p = 0.068; Odds ratio = 2.91, 95%CI = 1.04-9.64, p = 0.04. Mucosal loads of *C. pecorum* DNA were not significantly correlated with either PhaHV-1 or PhaHV-2 DNA loads (N = 38, Cor = 0.08, p > 0.05; N = 16, r = 0.05, p > 0.05). When co-infection combinations were assessed in koalas with results for all infectious agent targets

(N = 92), the most common infection combination included *C. pecorum*, PhaHV-1, KoRV B and trypanosome tri-infection irrespective of detection site or trypanosome species (13/92, Figure 2.5).

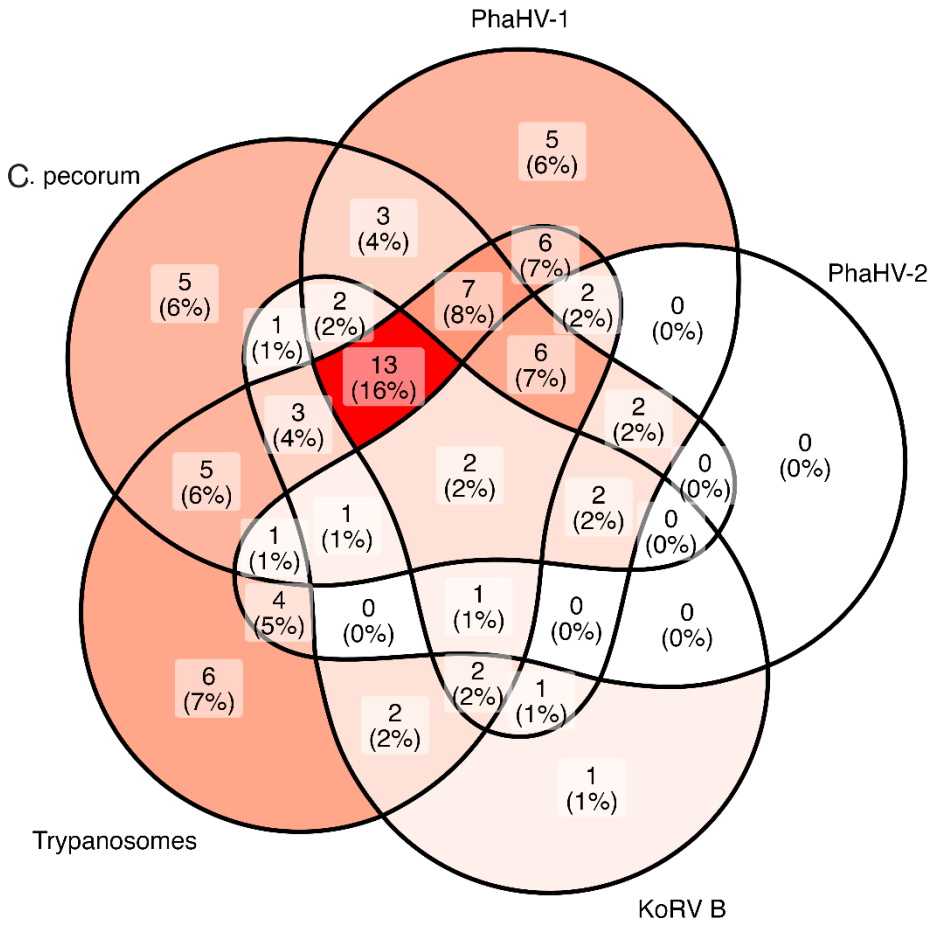


Figure 2.5: Euler venn diagram of complete pathogen detection status.

Euler venn diagram demonstrating the full pathogen detection results for 92 koalas with complete results for all targets. Positive detection of different markers representing the same pathogen were combined so that each case had one result per pathogen: C. pecorum *includes detection at mucosal sites and/or transcription of genes* CpecG_0573, Cpec_hsp60, ompA, or pGP3 *in circulation, PhaHV-1 and PhaHV-2 includes detection of each respective type at mucosal sites or in circulation, Trypanosomes include detection of any of the three species,* T. copemani, T. gilletti, *and/or* T. irwini *in circulation. KoRV B is depicted independently from other KoRV targets as it was not ubiquitously detected. All 92 koalas were positive for KoRV* pol, *KoRV A* env, *KoRV D* env, *and KoRV CKS17* env *transcription in circulation. In total, 23/115 koalas in the whole sample population had missing/inconclusive results for any one pathogen and were exluded.*

General Linear Models: The relationships between co-infections and disease outcomes

Clinical characteristics of tested koalas:

Of the 115 koalas sampled on admission to hospitals, 61% presented with clinical signs of chlamydiosis including any one or combination of cystitis, conjunctivitis and reproductive disease (Figure 2.6B). The remaining koalas presented with trauma (n = 17), disease other than chlamydiosis (n = 8), and no clinical abnormalities (n = 20). In total, 71 koalas (62% of admissions) were retained for rehabilitation and the remainder were euthanised following clinical assessment. Of the euthanised koalas, 84% (N = 37) were classed as 'untreatable chlamydiosis'. Female koalas represented 60% of the sampled population (Figure 2.6A). Over half of female koalas presented with evidence of reproductive disease on admission with or without other signs of chlamydiosis. Reproductive disease was the leading cause for euthanasia in the sample population (45.5%).

The distribution of age groups and sex were unequal within the sampled cohort (Figure 2.6A). Females were more frequently represented by the following categories: Chlamydiosis (χ^2 = 7.94, df = 1, $p < 0.01$), non-treatable chlamydiosis (χ^2 = 12.96, df = 1, $p < 0.001$), cystitis (χ^2 = 5.51, df = 1, $p < 0.05$), and reproductive disease (χ^2 = 32.55, df = 1, $p < 0.001$). Significantly fewer young-adult koalas were shedding mucosal *C. pecorum* in comparison to adults (χ^2 = 8.42, df = 2, adj. $p < 0.05$) and mucosal PhaHV-1 in comparison to adults (χ^2 = 16.55, df = 2, adj. $p < 0.001$) and aged koalas (χ^2 = 16.55, df = 2, adj. $p < 0.01$). Examination for male reproductive disease was not conducted in this study, so modelling for reproductive disease was only performed on the female subset.

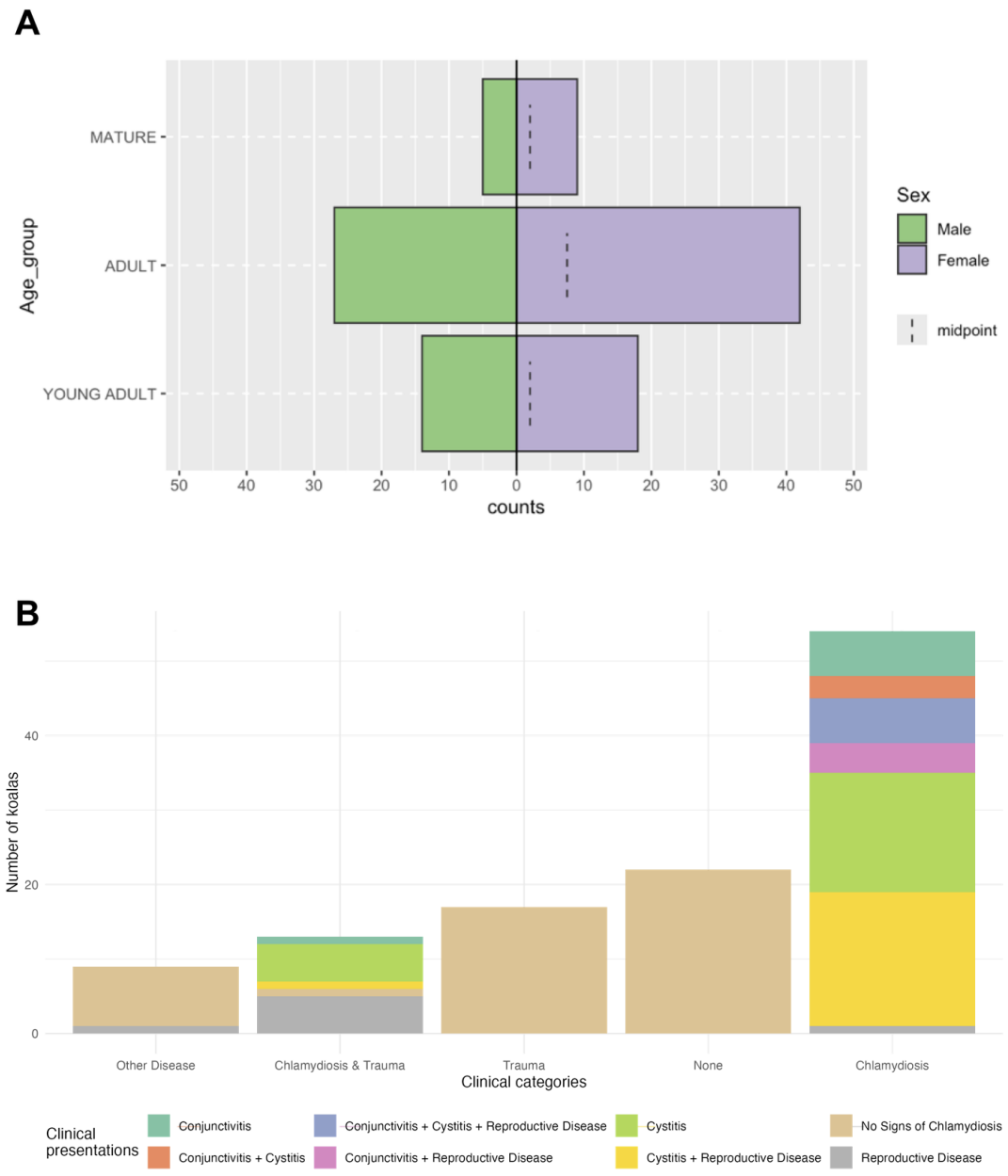


Figure 2.6: Distribution of age, sex, and clinical presentations.

Figure 2.6 presents graphical summaries of demongraphic and clinical status. (A) Pyramid plot showing counts of koalas within each age group according to sex. (B) Bar plot of the clinical allocation count for 115 koalas admitted to hospital including the proportion of specific chlamydiosis-clinical signs detected in cases.

C. pecorum mucosal shedding:

The selected "best model" explaining the differences in likelihood of shedding *C. pecorum* at mucosal sites (urogenital or ocular) included positive relationships with female koalas,

detection of KoRV-B *env,* mucosal PhaHV-2 shedding, and with KoRV *pol* transcription (Table 2.3). Although the top model included *T. copemani* as a predictor, the relative importance < 0.50 and in the next model, which didn't include *T. copemani,* the penalisation of AIC_c and w_i was considered minor (Δ_i = 0.20, and change in w_i = 0.1). After sex, KoRV-B *env* detection was considered the most important pathogen predictor variable, followed by PhaHV-2 mucosal infection, then KoRV *pol* transcription. Of note, the 95% confidence intervals (CIs) for the three pathogen predictors included 1, indicating that while these variables showed positive associations of relative importance with *C. pecorum* mucosal shedding, the increases in odds were not statistically significant in this sample.

Chlamydiosis:

The best model explaining the differences in the likelihood of koalas presenting with chlamydiosis on admission included positive relationships with sex (female), mucosal *C. pecorum* shedding, and mucosal PhaHV-1 shedding. This model had the lowest AIC_c and included all predictors with relative importance > 0.50. *C. pecorum* mucosal shedding and sex (female) were of equal greatest importance to the prediction of chlamydiosis. This was followed by mucosal PhaHV-1 shedding.

Untreatable chlamydiosis:

The best model explaining differences in the probability of koalas with chlamydiosis requiring euthanasia on admission included a positive relationship with sex (female) and PhaHV-1 mucosal shedding, and a negative relationship with circulating *T. irwini* infection. However, the 95% CI for circulating *T. irwini* included 1, indicating that while this variable showed relatively important negative association with koalas with chlamydiosis being untreatable, the decrease in odds was not statistically significant in this sample.

Reproductive disease in females:

The best model explaining differences in the probability of female koalas presenting with reproductive abnormalities on admission included positive relationships with mucosal *C. pecorum* shedding, mucosal PhaHV-1 shedding, and KoRV *pol* proviral loads. This model had superior AIC_c and w_i scores and included all predictors with relative importance > 0.50. In fact, all three predictors demonstrated equally high levels of relative importance to the prediction of reproductive disease in females.

Table 2.3: Best model selections using Akaike Information Criterion corrected for small sample sizes (AICc) and model averaging. Models were generated from a global model and ranked by AICc values. Model averaging was applied using Akaike weights.

Best Model	Number of observations	Number of parameters	AICc	Delta AICc (Δ_i)	Akaike weight (w_i)	Coeff	SE	95% CI		Relative importance
								2.5%	97.5%	
C. pecorum mucosal shedding	87	5	114.96	0.20	0.9					
Sex: Female						1.04	0.48	0.11	2.03	1
Circulating *KoRV B*						1.02	0.55	-0.04	2.14	0.87
Mucosal PhaHV-2 shedding						1.02	0.61	-0.13	2.28	0.62
Log_{10} KoRV *pol* cDNA/ml						0.79	0.46	-0.09	1.75	0.60
Chlamydiosis	87	3	73.44	0.00	0.21					
C. pecorum mucosal shedding						3.39	0.67	2.17	4.87	1
Sex: Female						1.63	0.67	0.37	3.05	1
Mucosal PhaHV-1 shedding						1.24	0.65	0.004	2.59	0.91
Untreatable chlamydiosis	48	3	51.34	0.00	0.17					
Mucosal PhaHV-1 shedding						2.41	0.86	0.84	4.27	1
Sex: Female						2.73	0.90	1.10	4.72	1
Circulating *T. irwini*						-1.42	0.89	-3.36	0.21	0.67
Reproductive disease	50	3	54.40	0.00	0.45					
C. pecorum mucosal shedding						2.49	0.79	1.04	4.24	1
Mucosal PhaHV-1 shedding						1.71	0.80	0.23	3.45	1
Log_{10} KoRV *pol* DNA/ml						3.49	1.87	0.91	8.97	1

Each model presented AIC_c values, Akaike weights (*wi*) and delta AIC_c differences (Δ_i). Standardised model-averaged coefficients (Coeff) weighted unconditional standard errors (SE), 95% confidence intervals (95% CI) and relative importance are provided for each independent variable in the best-supported models. Relative importance values below 0.5 are shown in red.

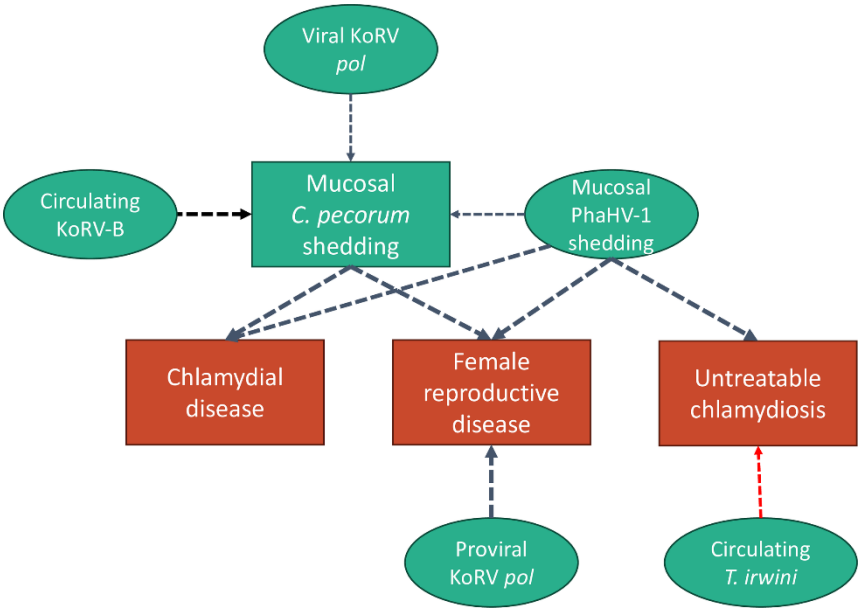


Figure 2.7: Relative Importance (RI) Network Plot demonstrating the degree of importance of infectious predictor variables on clinical outcome variables.

In Figure 2.7, all variables included as predictors in best models are represented in green ovals, and outcome variables in red and green squares. The arrows indicate the direction and positivity (black) or negativity (red) of the interaction. While these arrows are unidirectional to clearly define the model design, the independent and dependent variables, this study does not denote causation and bidirectionality may exist. Only predictors with RI > 0.5 were retained in best model fits and are displayed here. The thickness of the dotted arrows represents the magnitude of RI (0.5-1).

2.6 Discussion:

The findings of this study demonstrate a need to reassess the current understanding of host-pathogen-environment relationships involved in chlamydiosis of koalas. This first collective examination of key infectious agents in northern koalas showed that *C. pecorum*, PhaHV-1, KoRV, were fairly equal in terms of importance to the prediction of chlamydiosis outcomes. Of all the assessed pathogen targets, the relationship between clinical chlamydiosis and *C. pecorum* infection appear to be intertwined with PhaHV-1 shedding, KoRV activity, and - to a lesser extent – *Trypanosome irwini* infection (Figure 2.7). Although the detection of *C. pecorum* and PhaHV-1 and -2 were greater at mucosal sites, the successful – albeit low – detection of circulating pathogen genes suggests that further studies are required to

determine their significance to pathogenesis; *CpecG0573, Cpec_hsp60, ompA, Pgp3, PhaHV-1 dpol, PhaHV-2 dpol,* and *Tgilletti18S.* In particular, the finding of circulating *C. pecorum* gene transcription in koalas without detectable mucosal *C. pecorum* shedding indicates that diagnostic testing of circulatory cell samples should continue to be validated. The strong associations found here between PhaHV, *C. pecorum,* and chlamydiosis mirror those identified in humans and mouse models and the pathogenesis of these relationships is worth further exploration. Finally, given the combination of expected (KoRV B and viral KoRV *pol)* and unexpected (proviral KoRV *pol)* associations of KoRV with mucosal *C. pecorum* and chlamydiosis, we discuss the complexities of KoRV's significance in this condition.

The incorporation of all key koala infectious agents into a single analysis in this study demonstrated equal importance of PhaHV and KoRV to koala chlamydiosis and a high frequency of co-infection. Previously, the relationships of these agents with disease in northern koalas have been studied separately, resulting in an incomplete understanding of the relative importance of co-infecting agents and their interrelationships. Although a study of PhaHV-1, -2, *C. pecorum* and KoRV co-infections was conducted in southern koalas (Vaz *et al.*, 2019b), direct comparison of our results is limited due to the differences host genetic backgrounds and replication competent KoRV activity (Blyton *et al.*, 2022d; Sarker *et al.*, 2020a; Sarker *et al.*, 2019; Tarlinton *et al.*, 2022b). Here, the inclusion of *Trypanosome* detection revealed that the most common co-infection included *C. pecorum*, PhaHV-1, KoRV (including *env* subtypes KoRV A, B, & D), and at least one *Trypanosome* species. Similarly to Vaz *et al.* (2019b), here older koalas and those with *C. pecorum* had high odds of also being co-infected with either PhaHV-1 and/or PhaHV-2. As expected, and corroborating with Vaz *et al.* (2019b), PhaHV-1 shedding was a strong predictor of *C. pecorum* shedding and

chlamydiosis, but PhaHV-1 shedding and KoRV proviral *pol* loads were equally important indicators of reproductive disease in females, which differed to Vaz *et al.* (2019b).

Collectively, our results suggest that in northern koalas, to better understand chlamydiosis both KoRV and PhaHV should be examined alongside *C. pecorum* to account for possible interactions. While co-infection has yet to be investigated as a mechanism driving the variations in the pathogenesis of chlamydiosis in koalas, evidence of co-infection relationships exists in humans, mice models, and other animals infected with other *Chlamydia spp.* (Gieffers et al., 2004; Howe et al., 2019; Hyseni, 2021). Considering similar relationships in chlamydial disease of humans, association of herpesviral and chlamydial co-infection with chlamydial disease outcomes in koalas in this study suggests that these co-infection interactions should be further investigated. In this study, over 70% of *C. pecorum* positive koalas were coinfected with either PhaHV-1 and/or PhaHV-2. Contrasting to a study in southern Australian koalas (Vaz *et al.*, 2019b), mucosal PhaHV-2 was not strongly associated with the detection of *C. pecorum* (circulating and/or mucosal) or clinical disease, whereas mucosal PhaHV-1 was strongly associated with *C. pecorum* infection and clinical disease.

Although Figure 2.7 reflects these interactions with a unidirectional arrow, we emphasise that this study does not denote causation. Given the propensity for herpesviruses to be reactivated with tissue and DNA damage and immunosuppression in humans (Huang *et al.*, 2023; Libert *et al.*, 2015; Shulgina *et al.*, 2025; Simonnet *et al.*, 2021) and other animals (Krstanović *et al.*, 2025; Santos *et al.*, 2020; Tempesta *et al.*, 1998), the relationship between PhaHV-1, *C. pecorum* infection and chlamydiosis may be bi-directional. As in koalas, the pathogenesis of reproductive disease and other fibrotic conditions associated with chronic

chlamydiosis of humans has not been determined (Callan *et al.*, 2021). However, urogenital microbiome and virome composition and, in particular, chlamydial and herpes viral co-infection in people are areas of significant interest (Kaelin Emily *et al.*, 2022; Raimondi *et al.*, 2021; Zhao *et al.*, 2023a).

Given the relationship between PhaHV, *C. pecorum,* and disease severity in this study, these two infectious agents appear likely to be synergistic, but outcomes could depend on which pathogen infected first (Devi *et al.*, 2021). *C. trachomatis* and HSV-2, are currently the leading sexually transmitted pathogens in humans globally (Das & Röst, 2023; Slade, 2016). Disease induced by HSV-2 infection comprises genital ulcers, dysuria, cervicitis, and inguinal lymphadenopathy (Johnston & Corey, 2016). Early epidemiological observations suggested that women positive for both pathogens experience more severe outcomes than are typically experienced during single infections with either pathogen (Lehtinen *et al.*, 1985; Vetter *et al.*, 1990). These observations were supported more recently by studies assessing the incidence of disease in mixed and single *Chlamydia* sp. infection (Kajaia *et al.*, 2006) and the disease outcomes from HSV-2 and *Chlamydia muridarum* single and mixed infections in mouse models (Slade *et al.*, 2019). Studies have also demonstrated an antagonistic relationship between HSV-2 and *C. trachomatis*, with HSV entry into host cells causing down regulation of cell-junction protein nectin-1, on which chlamydial development relies (Slade *et al.*, 2016; Slade, 2016). In murine models, HSV-viral recovery and disease was reduced in *C. trachomatis* pre-infected subjects and this protection ceased when viable *C. trachomatis* was removed (either naturally or through antibiotic treatment) (Slade *et al.*, 2016; Slade, 2016). The interplay between herpesvirus and chlamydial activity and latency/persistence is clearly

complex but strong associations between these two infectious agents and various clinical outcomes warrants further investigation.

Although trypanosome and *Chlamydia spp.* co-infection dynamics are yet to be investigated in other species, trypanosomes can supress the establishment of infection by co-pathogens (Dwinger *et al.*, 1989; Kasozi *et al.*, 2021; Morrison *et al.*, 1982; Sanches-Vaz *et al.*, 2019) and produce trans-sialidase enzymes, which can impact *C. pneumoniae* viability (de Lourdes Higuchi, 2004). Previously, *T. gilletti* was associated with anaemia and poor body conditions in koalas with chlamydiosis (McInnes *et al.*, 2011a). Unfortunately, *T. gilletti* was infrequently detected in this study (3.1%) and was not assessed in general linear models. Although the opposing relationship between *T. irwini* infection and chlamydiosis severity was not significant, accounting for *T. irwini* detection improved the predictive quality of the model. Hense, our study suggests either the possibility of trypanosome species specific interactions with *C. pecorum* or an indirect effect of *T. irwini* infection on chlamydiosis pathogenesis. Transcriptomic analysis of mucosal and buffy coat samples from koalas with no infections, single infections (Trypanosome spp., PhaHV-1, or PhaHV-2, or *C. pecorum*), and co-infections should be utilised to: (1) compare gene expression between sites, (2) identify any differences in pathogen specific persistence/latency associated gene expression, (3) explore the likely mechanisms that drive persistence/latency, and (4) generate a detection method which assesses appropriate gene targets that are indicative of the various phases of activity in PhaHV and *C. pecorum*.

The differing relationships between chlamydiosis outcomes and viral and proviral KoRV *pol* loads in the current study could be a temporal effect. The strong correlations between KoRV

A, KoRV B (in positive cases), KoRV D, KoRV CKS17 and KoRV *pol* transcription is consistent with previous studies demonstrating that increased exogenous subtype transcription is a significant contributor to increased viral KoRV *pol* loads (Blyton *et al.*, 2022a; Hashem *et al.*, 2021; Quigley *et al.*, 2018b; Sarker *et al.*, 2019), which are associated with *C. pecorum* infection (Blyton *et al.*, 2022a; Madden *et al.*, 2018; Maher & Higgins, 2016; Maher *et al.*, 2019). It is possible in early stages of active disease or co-infection that genes involved in cell cycle, innate immunity, adaptive immune responses, and hormone regulation increase KoRV transcription, perhaps leading to greater integrations (Gomez-Lucia *et al.*, 2023). Over time, increases in KoRV integrations would also increase proviral and viral DNA loads, which are often associated to advanced and chronic disease (Chen *et al.*, 2011; Chen *et al.*, 2006; Takenouchi *et al.*, 2003; Yu *et al.*, 2005), In this study, KoRV proviral loads were more associated to reproductive disease in females. While the temporal development of reproductive disease is less clear in koala chlamydiosis, this suggests that in these koalas, mechanisms that support reverse-transcription of replication-competent virus are more active. A similar relationship has been observed in koalas with greater KoRV proviral loads and cell-proliferative disorders (Fabijan *et al.*, 2020; Sarker *et al.*, 2020a). To unravel the intricacies between KoRV and chlamydiosis pathogenesis, mechanistic studies are required. Given that KoRV is endogenous in northern populations, controlled infection studies cannot be conducted. However, longitudinal monitoring of KoRV integrations in koalas before and during acquisition of natural *C. pecorum* infection, while accounting for co-infections, may determine whether integration-driven promotion of immune dysregulation can explain variations in clinical outcomes (McEwen *et al.*, 2021).

Our data suggests that further validation of NanoString nCounter multiplexing is needed to confirm detection of *C. pecorum* in circulation. Given the rarity of observing circulating *C. pecorum* above the limit of detection, a greater sample size is required to support investigations of significance. Nonetheless, the lack of raw transcription of *C. pecorum* genes in a *C. pecorum* free population is suggestive of good specificity of this detection method and that these results are not solely attributed to background noise (Figure 2.2). Given that 50% (4/8) of koalas with *C. pecorum* in circulation did not have detectable shedding at mucosal sites indicates that circulating *C. pecorum* could be considered for inclusion in *Chlamydia pecorum* detection strategies. Current point-of-care and gold-standard laboratory testing, using loop-mediated isothermal amplification (LAMP) and qPCR, target the *C. pecorum mreC*, *ompB* and 23s rRNA genes at either urogenital or ocular mucosal sites (Hulse *et al.*, 2019c). Only koalas with clinical signs of disease and/or detection of *C. pecorum* using one of these tests are considered for antibiotic treatment. Hence, in this study, two of the koalas with circulating *C. pecorum* and without mucosal *C. pecorum* shedding or clinical signs of disease went undetected and were subsequently released. Depending on how prevalent circulating *C. pecorum* is in the general koala population, this presents a perplexing therapeutic problem; firstly, whether these infections impact individuals or populations, and secondly whether currently available treatments are able to eliminate circulating infections. Investigation of whether circulating *C. pecorum* represents a reservoir for recrudescence of mucosal disease is an important topic for future research. Systemically disseminated *C. pneumoniae* resists elimination by standard anti-chlamydial treatment in humans and is hypothesised to initiate reinfection and generate systemic conditions such as atherosclerosis (Gieffers *et al.*, 2001). It is a reasonable proposition to investigate whether the same is true for *C. pecorum*. As a priority for research and diagnosis, we need to determine whether *C. pecorum* can be

detected in blood samples by the more accessible current LAMP and qPCR methods; this would facilitate research to understand the pathogenesis and significance of this finding.

2.7 Conclusion:

The high frequencies of co-infections in koalas admitted to rehabilitation and their important association to *C. pecorum* infection and chlamydiosis in this study shifts the classical paradigm of koala chlamydiosis pathogenesis to one which includes multiple co-infection interactions. It highlights the complexity of pathogenesis of chlamydiosis in koalas that can be altered by various host, pathogen, and environmental factors. Interdisciplinary approaches combining mechanistic in-vitro studies with multivariate analyses incorporating diverse pathogen, environment and host factors are needed to understand these dynamic interactions and improve disease treatment and mitigation strategies.

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Conflict of interest:

This work forms a component of the Australian Government National Koala Health Research Initiative, which is aimed at filling key knowledge gaps to produce immediately applicable outcomes for koala conservation. The sponsors had no role in the design, execution, interpretation, or writing of the study. The authors declare no competing interests.

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Chapter 3 Clustering of immune gene transcription identifies immune phenotypes associated with poorer triage outcomes in the koala.

3.1 Author contribution statement

Yasmine S. S. Muir is the main author of this study and has contributed towards every component of the research including the conceptualisation, field work and data curation, methodology, formal analysis, investigation, writing of the original draft and reviewing and editing of the manuscript. Damien P. Higgins and Mark B. Krockenberger supervised and contributed to the conceptualisation, investigation, writing and reviewing of the manuscript and provision of research resources. Belinda R. Wright contributed to the methodology, writing and reviewing of the manuscript, as did Andrea Casteriano in addition to the provision of research resources. Valentina S.A. Mella contributed to the statistical supervision, writing and review of the manuscript. All authors have read, contributed, and agreed to the final version of the manuscript.

Yasmine Sophia Sierra Muir

28/2/2025

Damien P. Higgins

28/2/2025

3.2 Abstract:

Immune gene transcription panels offer a more comprehensive understanding of immunophenotypes and overall host response than approaches more common in wildlife immunology, using single or limited immune indicators. However, the large number of variables and high individual variation pose challenges for statistical analysis, especially with the limited sample sizes available in wildlife studies. Here, we utilise dimensionality reduction methods in conjunction with clustering of immune components to facilitate the identification of relationships between host biological profiles, infection status, and clinical and survival outcomes for koalas in rehabilitation. We identified an association between poor triage outcomes and an immunophenotype that included reduced adaptive lymphocytic and innate immune and transcription-associated gene expression, providing a biological perspective that may explain the heterogeneity in clinical severity and responses to treatment. Furthermore, upregulation of the gene coding for a transcription intermediary factor (tripartite motif-24, *TRIM24*) was identified as a consistent predictor of positive outcome at triage and post-treatment. Greater KoRV *pol* transcription on admission was associated with negative triage outcomes, corroborating current understanding of KoRV-health outcome relationships. This study demonstrates a suitable approach to enable the incorporation of the complexity of host-pathogen interactions and host responses to direct further research and support disease risk assessment, passive surveillance, and assessments of intervention and disease management efficacy.

3.3 Introduction:

The koala (*Phascolarctos cinereus*) is an iconic Australian marsupial that currently faces significant population declines across most of its range due to habitat loss and fragmentation, and disease, and was re-classified as endangered in the states of New South Wales (NSW), Queensland (Qld) and the Australian Capital Territory (ACT) in 2022 (DCCEEW, 2022b). Previous modelling suggested that controlling disease could prevent further population declines (Rhodes *et al.*, 2011). Chlamydiosis is arguably the most significant disease affecting koalas; not only can it be fatal in severe cases but the development of reproductive disease and infertility limits population regeneration (Hulse *et al.*, 2021; Hulse *et al.*, 2019a; Phillips *et al.*, 2021; Robbins *et al.*, 2018).

Rescue and rehabilitation are significant aspects of koala conservation that provide an opportunity to study wildlife disease in an accessible and controlled environment (Yabsley, 2019). On admission to a veterinary/wildlife clinic, clinical examination and diagnostic techniques determine the course of treatment and predict the likelihood of survival (prognosis) through rehabilitation and subsequent release. To properly support declining populations of northern koalas, and the welfare of individual animals, veterinary and management decisions and comparative analyses must be backed by a solid evidence base. As disease is the product of various host, pathogen, and environmental (HPE) factors, which are often interrelated (Guégan *et al.*, 2024), currently the factors that underpin the heterogeneity of chlamydial disease outcomes in the clinic and the wild are poorly understood. This compromises clinical decision-making in triage and prognostication (Burton & Tribe, 2016), including predicting treatment-induced complications, such as caecal-

dysbiosis-typhlocolitis-syndrome (CDTS), oxalate nephrosis, and candidiasis (Govendir *et al.*, 2012; Robbins, 2020; Robbins *et al.*, 2018).

Many studies have explored factors potentially associated with the pathogenesis of chlamydiosis in the koala: *Chlamydia pecorum* genetic diversity (Fernandez *et al.*, 2019; Robbins *et al.*, 2019, 2020), co-infecting agents (Blyton *et al.*, 2022a; Kasimov *et al.*, 2020; Kayesh *et al.*, 2020b; McInnes *et al.*, 2011a; Vaz *et al.*, 2019b; Wright *et al.*, 2024), host genetics and immune responses (Kayesh *et al.*, 2021b; Quigley *et al.*, 2018a; Robbins *et al.*, 2020; Silver *et al.*, 2022), and stress (Davies *et al.*, 2013; Santamaria *et al.*, 2023). Of note, the complex relationship between koala retrovirus (KoRV) and chlamydiosis is difficult to unravel due to their high concurrent prevalence in many wild koala populations (Quigley *et al.*, 2023), although it is hypothesised that cytotoxic effects, immunomodulating functions of the KoRV immunosuppressive domain or impacts of genomic viral integrations may play a role (Fiebig *et al.*, 2006; Kleinerman *et al.*, 1987; Madden *et al.*, 2018; Maher & Higgins, 2016; Maher *et al.*, 2019; Sarker *et al.*, 2020a; Sarker *et al.*, 2020b; Tarlinton *et al.*, 2005; Tarlinton *et al.*, 2008a). While these studies contribute significantly by identifying important facets of koala biology and disease, the relative importance of these factors is unclear because of the complexity of the interactions and a paucity of integrated multivariate analyses (Burton & Tribe, 2016; Fernandez *et al.*, 2024b; Kerlin *et al.*, 2022; Leigh *et al.*, 2023; Queensland Government, 2021). Multivariate associative studies may generate new perspectives for disease characterisation, identify hypothesis-driven mechanistic studies, and markers with which to evaluate disease risk in individuals and populations.

Clustering of principal components can allow dimension reduction to improve the depth of investigations and characterise heterogeneity into biologically applicable groupings. In the koala, recent multivariate analyses within select captive and wild populations have demonstrated how immune function (Fernandez *et al.*, 2024b); and disease (Chen *et al.*, 2023c; Cristescu *et al.*, 2022; Fernandez *et al.*, 2024b; Silver *et al.*, 2022) can vary significantly among individuals according to their genetics, microbiome diversity, age, sex, and season of sampling, and also between populations with differing population genetics and environmental pressures. In humans, factor analysis and clustering techniques are being applied to understand heterogeneity in biological responses and thereby define variations in disease manifestations and outcomes to several conditions (Gaiffe *et al.*, 2023; Guo *et al.*, 2023; Maugeri *et al.*, 2021; Perea *et al.*, 2021; Robinson *et al.*, 2020; Xu *et al.*, 2021). These new perspectives are made possible because, rather than imposing pre-conceived clinical definitions to assess patterns within a target group, clustering allows cases to be defined by the data (Pina *et al.*, 2020).

This study examines the relationships between immune and stress responses, presence and activity of infectious agents, age, sex, body condition score, and clinical disease states in the context of koala rehabilitation. Through cluster analysis of principle components, this study tests whether host and pathogen gene transcription profiles are associated with disease, infection status, or triage outcomes. To identify potential health indicators, the relationships of genes of significance to survival-associated dimensions are determined using multivariate modelling. In a small subset of koalas that were admitted to rehabilitation for treatment, we determined whether gene transcription at triage or at their final sampling point could predict treatment outcomes. Rehabilitation-based studies provide an alternative perspective to field

studies, where there is often limited diversity in disease presentations (Dutton-Regester, 2024; Gonzalez-Astudillo *et al.*, 2017; Griffith & Higgins, 2012). The analysis will generate new hypotheses for potential mechanistic pathways in the pathogenesis of chlamydiosis in the koala and development of prognostic biomarkers for use in the clinical setting. Methods presented here build upon previous multivariate immunophenotyping studies in wildlife species (Fernandez *et al.*, 2024b; Meza Cerda *et al.*, 2022) to further develop an effective approach for analysis of multivariate data in complex wildlife disease scenarios.

3.4 Methods:

Study cohort and Specimen collection

Under the University of Sydney Animal Ethics Approval Number 2021/1975, NSW NPWS Scientific License SL102379 and Qld NPWS WA0019256, buffy coat samples were collected from 105 koalas on admission to a koala treatment facility (Figure 3.1). Three koala care facilities servicing South-east Qld, Northern NSW extending down the east-coast, and central NSW regions were targeted for cross-sectional sampling between September 2021 to April 2022 (Figure 3.1). Samples were taken under general anaesthesia (GA) at the discretion of the veterinarian using alfaxalone induction (1–3 mg/kg IM) and Isoflurane maintenance with oxygen. Sampling included one swab each of the left and right conjunctiva, the oropharynx, and the urogenital tract (the urogenital sinus in females and the urethra in males) using dry, aluminium shaft, cotton tipped, swabs (Copan Italia, Brasica, Italy) and 3 mL of blood drawn into an EDTA tube. Cut off swab tips were stored at -80 °C in individual cryogenic vials (Biologix Grp Ltd, Kansas, United States). From the EDTA tube, 300 µL of whole blood was stored in a cryogenic vial at -80 °C. Remaining blood in the EDTA tubes was centrifugated at 1,000 x g for

10 mins. From the separated EDTA blood, 250 µL of the buffy coat layer was pipetted into a cryogenic vial containing 750 µL RNAlater™ (Qiagen), incubated at ambient temperature for 24 hrs and then placed into -20 °C for storage until analysis.

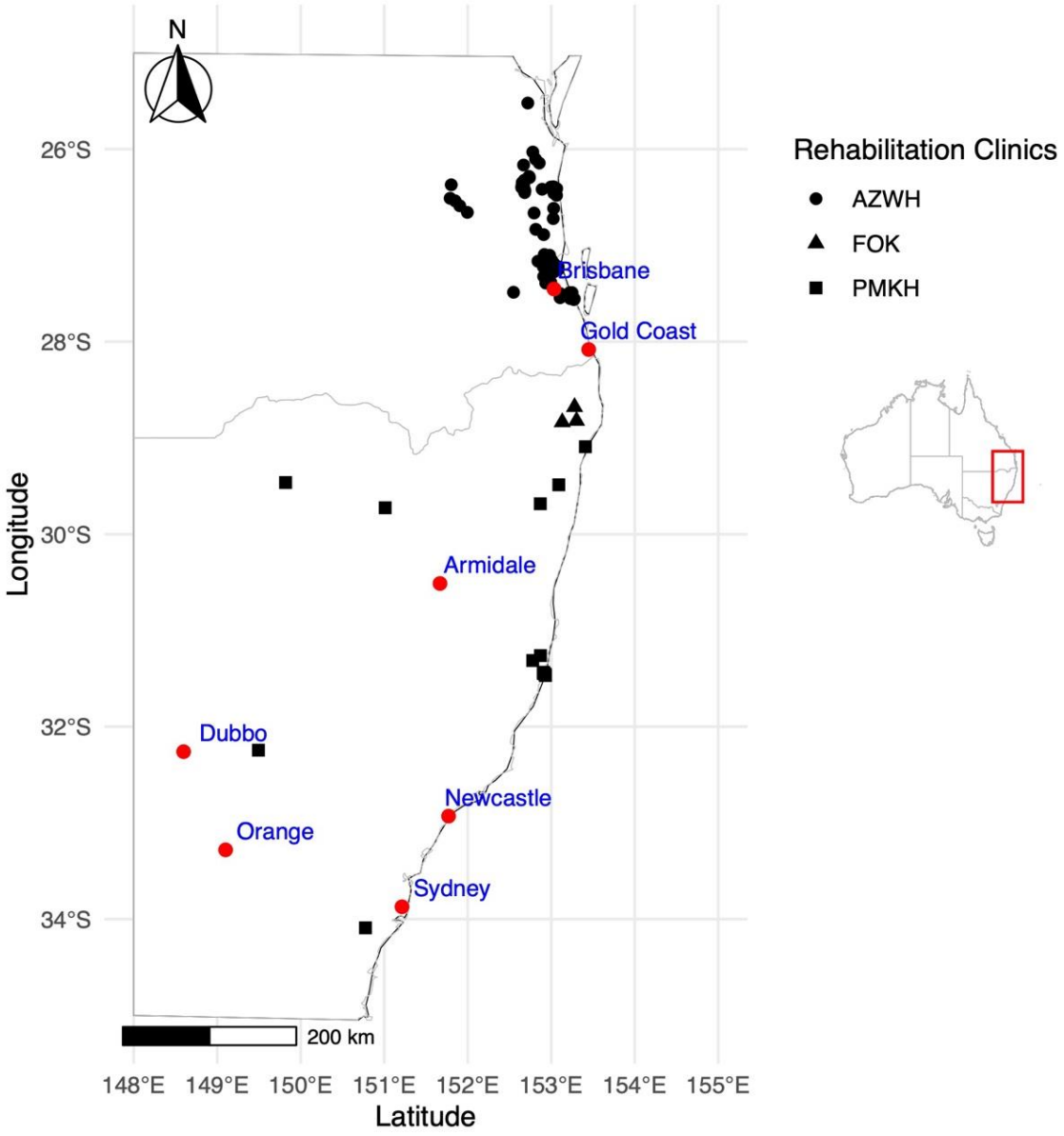

Figure 3.1: Sample population map of 105 koalas admitted to three wildlife clinics across Queensland and New South Wales.

Figure 3.1 is a map illustrating the rescue location of the 105 koalas admitted to wildlife hospitals that were analysed in this study. The map indicates the latitude and longitude coordinates of the point of capture for each analysed koala. Locations include south-east Queensland through to coastal and central New South Wales. Where this region exists within Australia is depicted by red rectangle to the right of the map. The associated clinic where each sampled koala was admitted to is indicated in the legend: Australia Zoo Wildlife Hospital (AZWH, circle), Friends of the Koala (FOK, triangle), and Port Macquarie Koala Hospital (PMKH, square). Red points represent major cities in proximity to koala rescue locations. This map was generated in R using the Natural Earth public domain package.

Health assessment

Health assessment data was extracted from clinical examination records and rehabilitation monitoring sheets. Outcomes were determined on a case-by-case basis by veterinary professionals, independent to this study, based on examination and diagnostic results (Figure 3.2). Euthanasia was elected on welfare grounds, where prognosis was considered poor. Clinical presentations contributing to poor prognosis at triage included: untreatable/ irreparable structural damage to tissues/organs derived from disease and/or trauma; and complex comorbidities in conjunction with emaciation and/or mature age (age > 10 years old). Demographics and health assessment results of the study population at their first sampling event (time zero; T0) were described using counts of observations with percentages (%).

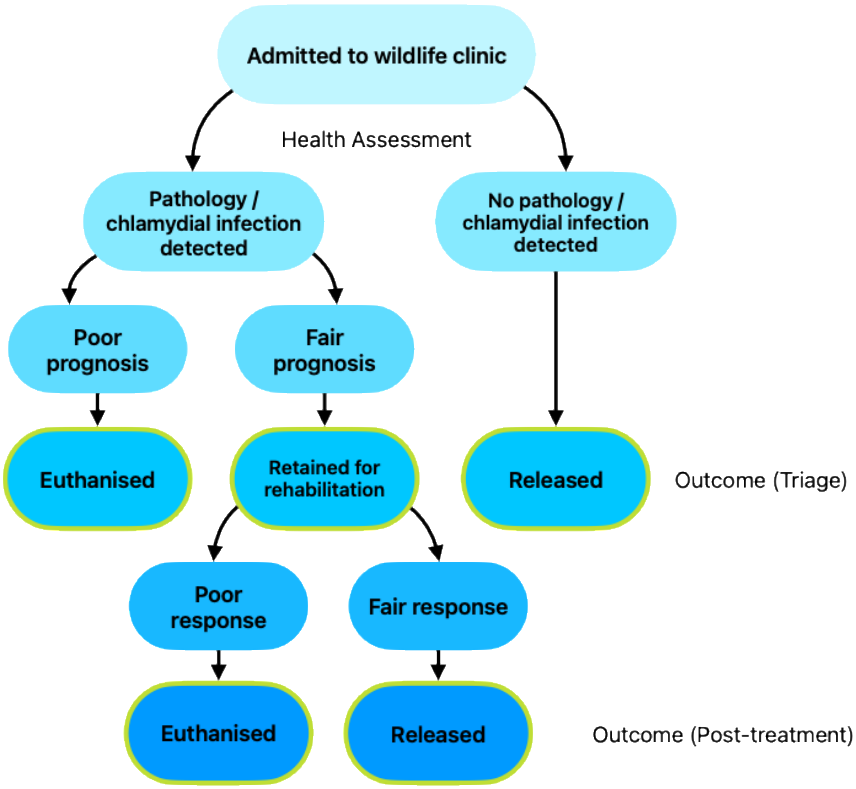

Figure 3.2: Flow diagram of the progression from admission of a koala to a wildlife clinic to triage and post-treatment outcomes (green borders).

Figure 3.2 demonstrates the flow of decision making for survival and euthanasia in wildlife clinics treating koalas. Upon initial health assessment, koalas were released when no disease or infection was detected. Cases with detectable pathology and/or infection were considered for treatment based on their prognosis (fair or poor). Koalas with poor prognosis were euthanised on welfare grounds due to the presentation of untreatable/irreparable structural damage to tissues/organs derived from disease and/or trauma; and complex comorbidities. Koalas with fair prognosis were retained for rehabilitation where follow-up examination repeated the same procedure as on admission to determine whether each cases had a fair or poor prognosis. All cases at follow-up with fair prognosis were released, while those with poor prognosis were euthanised.

Gene transcription analysis

A custom 72 gene NanoString nCounter plex-set (NanoString Technologies, WA, USA) was used to measure pathogen and host gene transcription counts. This same plex-set design was used previously in Fernandez *et al.* (2024b) and Chapter 2 of this thesis. Although a brief description of the 14 pathogen-specific gene targets was provided in Chapter 2, information regarding the full panel design and optimisation is detailed here to support the following

analysis of all gene results. Probe and primer generation, quality control, normalisation, sample concentration determination, and gene transcript counting, was the same for all 72 genes (see Chapter 2). Briefly, probe sequences for these were designed and synthesised according to standard procedure (Kulkarni, 2011) and submitted to Ramaciotti Centre for Genomics (UNSW, NSW, Australia). The CodeSet containing reporter codes and capture probes was manufactured by NanoString Technologies (Supplementary materials Table 7.6).

Gene targets were chosen and designed based on previous studies and putative biological and clinical relevance to koalas (Supplementary materials Table 7.6). Of interest for this study, the following genes were included: reference genes (*GAPDH, ACTB, Stx12, Nckap1l, and Tmem97*) (Sarker *et al.*, 2018); immunological genes including T- and B-cell differentiation markers (*CD3G, CD4, CD79b, CD8beta*) (Mangar, 2018; Pagliarani *et al.*, 2024); MHC I (*MHCIUA*) and II (*PhciDAB & PhciDBB*) (Lau *et al.*, 2014; Pagliarani *et al.*, 2024; Quigley *et al.*, 2018a; Quigley *et al.*, 2020; Robbins *et al.*, 2020); cytokines and cytokine-associated genes representative of key immunological pathways: Th1 (*IFNG, IL18, IL12A,* and *TNFalpha*), Th2 (*IL4*), Th17 (*IL17A, IL22, IL6, IL1beta,* and Caspase recruitment domain family member 9 *[*CARD9*]*), and T-regulatory (*IL10, &* Forkhead box P3 *[FOXP3*) pathways (Maher *et al.*, 2014; Maher & Higgins, 2016; Morris *et al.*, 2014; Olagoke *et al.*, 2020b; Quigley *et al.*, 2023); chemokines *(IL8/CXCL8 &* C-C Motif Chemokine Receptor 4 *[CCR4])* (Sarker *et al.*, 2020b; Tarlinton *et al.*, 2021); macrophage (C-Type Lectin Domain Family 4 Member E *[CLEC4E]*) and natural killer (NK) cell surface receptor (Natural Cytotoxicity Triggering Receptor 3 [*NCR3*]) (Chen *et al.*, 2023c; Morris *et al.*, 2015a); cathelicidin genes (*PhciCATH5* & Cathelicidin-like [*LOC110217150*]) (Peel *et al.*, 2021); toll-like receptors associated with the detection of PAMPS of bacterial *(TLR2 & TLR4)* and viral *(TLR10)* origins (Kayesh *et al.*, 2021b); KoRV

insertion-site associated genes (Tripartite motif-24 *[TRIM24]*, *DICER1,* and Solute Carrier Family 29 Member 1 [SCL29A1]) (Johnson *et al.*, 2018; McEwen *et al.*, 2021) and a potential antiviral immune response marker, SAM And HD Domain Containing protein 1 *[SAMDH1]* (Chen *et al.*, 2019b; Wang *et al.*, 2020)

Other gene transcripts hypothesised to be available in plasma were included in the panel, but only those with high detection frequency and potential associated roles with the immune system were examined in this study. On the panel, genes included those associated with metabolism and detoxification (Resistin *[RETN]* leptin *[LEP],* cytochrome oxidase genes *[CYP2E1, CYP3A4, CYP4A15, & CYP3A78]*) (Johnson *et al.*, 2018); endocrine function (Proopiomelanocortin *[POMC],* corticotropin releasing hormone *[CRH],* melanocortin receptor 2 *[MC2R],* vasopressin receptor 1A *[AVPR1A],* androgen receoptor *[AR],* estrogen receptor 1 *[ESR1],* progesterone receptor *[PGR],* oxytocin/neurophysin I prepropeptide *[OXT],* and the dopamine receptors D1 *[DRD1]* and D3 *[DRD3]*) (Tarlinton *et al.*, 2021); and stress response (mineralocorticoid receptor *[NR3C2],* FKBP prolyl isomerase 5 [*FKBP5*]) (Häusl *et al.*, 2021; Lee, 2016; Touma *et al.*, 2011; Zimmer *et al.*, 2024). In this study, *RETN* and *FKBP5* were retained for analysis.

As described in Chapter 2, fourteen infectious agent targets were included in the analysis for this investigation: four *C. pecorum* markers; the *C.pecG*_0573 general *C. pecorum* species specific gene target derived from the MC/Marsbar strain, chlamydial heat-shock protein gene *Hsp60* (*Cpec_hsp60*), major outer membrane (MOMP) gene *ompA*, and plasmid gene fragment 3 *Pgp3*; two PhaHV markers targeting the *dpol* region of PhaHV-1 & PhaHV-2; five KoRV markers (*KoRV pol, KoRV env A, KoRV env B, KoRV env D, KoRV env CKS17*); and three

trypanosome markers targeting the 18s rRNA species specific regions of *T. copemani*, *T. gilletti*, *T. irwini.*

Prior to running the full set of samples, a trial run using RNA extracted from different sample types was assessed to determine which had optimal gene expression. Overall, buffy coat samples generated the greatest proportion of quantifiable data, compared to plasma, whole blood, urogenital swab and buffy coat samples incubated in cell culture media for 12 h at 37 °C. Buffy coat samples had quantifiable expression across the most samples and genes, whereas in the other sample types – particularly the urogenital swabs and unstimulated mitogen samples, many targets were below detection limits (data not shown). Based on this preliminary data, buffy coats were utilised for RNA extraction and gene expression analysis using NanoString.

In total, RNA was extracted from 129 buffy coat samples stored in RNAlater™ using the RNeasy® Mini Kit (QIAGEN) following the manufacturer's instructions. Purity and concentration of extracted nucleic acid were assessed using a Nanodrop spectrophotometer ND-1000 (Thermo Fisher Scientific Co., Waltham, MA, USA) and the benchtop Qubit™ 4 Fluorometer (Invitrogen, Thermo Fisher Scientific) using the Qubit® RNA HS Assay Kit (Q32852) and Qubit® RNA IQ Assay Kit (Q33222). Extracted RNA was stored at −80 °C until required.

Of the 129 buffy coat RNA extracts, 99 represented time-zero (T0) samples taken individual koalas on admission and 30 were follow-up samples taken throughout rehabilitation. These extracts were assessed using the NanoString nCounter FLEX Analysis System (NanoString

Technologies, WA, USA) as per the manufacturer's protocol. Raw transcript count data was assessed for quality and normalised against housekeeping genes (*GAPDH, ACTB, Stx12 & Nckap1l*) using nSolver™ 4.0 Analysis Software (NanoString Technologies Inc, Washington). Because of low counts, *Tmem97* was not included as a house-keeping gene to normalise mRNA counts and was omitted from further analysis. Four samples were omitted as their housekeeping genes were detected below the threshold of 50 counts, indicating poor quality RNA and unreliable mRNA quantification (NanoString Technologies Inc, 2009).

As indicated by NanoString Technologies Inc (2009), raw counts below 20 were considered below the limit of detection as they cannot be discerned from background noise. To avoid the impact of zero-inflated (or below-threshold) data, which violates assumptions of equal variance and can distort results of principle components analysis (Silverman *et al.*, 2020), genes with raw counts below 20 in over 50% of the sample population were excluded from further analysis (Supplementary materials Table 7.7). As a result, the following host genes and several infectious agent markers were excluded from PCA; *MHCIUA, IFNG, IL12A, IL4, IL17A, IL22, IL10, FOXP3, NCR3, LEP, CYP2E1, CYP3A4, CYP4A15, POMC, CRH, MC2R, AVPR1A, AR, PGR, OXT, DRD1, DRD3, NR3C2, C.pecG_0573, Cpec_hsp60*, *ompA*, *Pgp3*, PhaHV-1 *dpol* & PhaHV-2 *dpol*, KoRV *env* B, and *Trypanosome copemani*, *T. gilletti*, *T. irwini* 18s rRNA regions. Although the frequency of detection above LOD for *TNFalpha* (48.4%) was below 50%, due to its significance as a classical cytokine it was retained for analysis. Furthermore, given the significance of pathogen markers to the study, results below the LOD for these genes were considered negative and were analysed as binary categories (present/ absent) to determine the relationships of immune profiles with infectious status and, in positive cases only, as continuous data representing pathogen loads (Supplementary materials Table 7.7). Given

that these infectious agent markers were detected in buffy coat samples, positive results are referred to as detection in circulation. For genes that were retained for PCA, a threshold was not applied to counts below the limit of detection and normalised counts were used in the analysis as it was considered that the error within this low range was negligible compared to the amplitude of variation of each target (Fernandez *et al.*, 2024b). Only koalas with results from NanoString coinciding with their first examination on admission to hospital following rescue from the wild (N = 95) were included in the PCA and modelling of time-zero immune profiles. In koalas admitted for rehabilitation, box-cox normalised gene mRNA counts from the last opportunistic follow-up sample were included in a separate model analysing follow-up immune profiles post-rehabilitation (N = 28).

DNA extraction:

All samples (whole blood and urogenital, ocular, and oropharyngeal swabs) used in qPCRs for DNA detection and quantification were extracted using the MagMAX CORE Nucleic Acid Purification Kit (Thermo Fisher cat# A32702; Thermo Fisher Scientific, Waltham, MA, USA) using the same methods as Chapter 2, this thesis.

Detection and quantification of infectious agents via qPCR:

Data for mucosal *C. pecorum,* mucosal phascolarctid herpesvirus (PhaHV) -1 and -2, and circulating koala retrovirus (KoRV) *pol* proviral loads determined through qPCR of the first timepoint sample, derived and reported in Chapter 2, this thesis, were used within this analysis; and so Chapter 2 describes the following methods in full: sampling, health assessment data recording, RNA and DNA extraction, quantification of gene transcription using NanoString technologies, *C. pecorum* multiplex qPCR, PhaHV-1 and PhaHV-2 qPCR, and

KoRV *pol* proviral qPCR. All qPCRs were loaded on 96-well PCR plates which were prepared using a Myra Liquid Handling System (Bio Molecular Systems) or manually. qPCRs were performed using a CFX96 Touch™ Real-Time PCR Detection System with the corresponding CFX Maestro software (BioRad, Australia). Primer and probe set information for all qPCRs are described in Supplementary materials Table 7.8.

Proviral KoRV pol DNA qPCR

KoRV proviral *pol* loads were quantified from DNA extracted from 124 whole blood samples in EDTA taken from 105 koalas at admission, and 19 of those again at end of treatment. One no-DNA blank (sterile PBS) was included in each extraction batch to control for potential contamination. dH_2O diluted DNA extracts (1:10) were used for qPCR to prevent PCR inhibition due to high gDNA concentration in extracts of blood samples. DNA quality and quantity were assessed by separate qPCR for the koala *β-actin* gene. KoRV *pol* reaction mixes were made to a total volume of 20 µL and comprised of 0.6 µL of each primer (10 µM), 0.2 µL of probe (10 µM), 10 µL of SensiFAST™ Probe No-ROX (x2; Bioline cat# BIO-86005), 6.6 µL dH2O and 2 µL of template. For each 20 µL *β-actin* reaction mix 0.4 µL of each primer (10 µM) and 0.4 µL of the probe (10 µM), 10 µL of SensiFAST™ Probe No-ROX, 6.8 µL dH2O and 2 µL of template was included. Samples were run in duplicates alongside a serial dilution of a positive standard (synthetic KoRV *pol* positive control) and an NTC (no template control; dH_2O). qPCR conditions were as follows: 1 cycle of denaturation (3 min at 95°C), then 40 cycles of denaturation (10 s at 95°C), and finally an annealing step (40 sat 60°C). The limit of quantification for this assay was determined to be 21 copies per reaction and no samples were excluded. Circulating KoRV *pol* quantities are reported as *β*-actin normalised KoRV *pol* copies per mL of DNA extract by taking the ratio of KoRV *pol* starting copies per µL to koala *β*-

actin starting copies per µL x 1000. As reported in Chapter 2, qPCR efficiencies ranged between 90.6–100% and the inter-assay variation was less than 5%.

Quantification of C. pecorum mucosal shedding

In total, 119 urogenital swabs and 136 ocular swabs from 105 individual koalas at 124 different sampling events were used to quantify mucosal *C. pecorum* shedding (DNA). Of the timepoints included, 77% (n = 95) were admission samples and 23% (n = 29) were repeat samples post-treatment. Extracts were assessed in a multiplex real-time qPCR targeting *Chlamydia* genus (23S) and *C. pecorum* species (*ompB* gene) as well as the koala *β-actin* gene to quantify host DNA (quality control). The PCR reaction mix consisted of 10 µL SensiFAST^{TM} Probe No-ROX (Bioline cat# BIO-86005), 400 nM of each primer, 200 nM of each probe, 4.4 µL of dH_2O and 2 µL of DNA, making a final volume of 20 µL. Cycling conditions were as follows; 1 cycle of denaturation (3 min at 95 °C), followed by 40 cycles of denaturation (10 s at 95 °C), and a combined annealing and extension step (40 s at 58 °C). In each run a synthetic positive control and a negative control (no template control; dH_2O) was included. The positive control, a pUCIDT-AMP vector (Integrated DNA Technologies, USA) containing the 3 target regions and flanking sequences (*β-actin*, *C. pecorum* and *Chlamydia* genus), a standard curve in 10-fold dilution from 10^3 to 10^7 copies, was included in each run. Samples were assessed as neat (undiluted) and diluted (1:10) extracts to account for inhibitors. Samples that repeatedly failed to amplify *β-actin* were not included in further analysis (Ocular samples, N = 6; UGT samples, N = 7). Samples were considered positive for *C. pecorum* shedding if amplification of *ompB* and 23S targets occurred. LOD for this assay was determined to be 86 copies of *C. pecorum* per reaction (95%CI) using probit regression analysis (Premachandra *et al.*, 2024). Quantitative results were reported as *β*-actin normalised *C. pecorum* starting quantities (gene

copies) by dividing *ompB* gene copies by *β*-actin gene copies. Where a koala was shedding at both urogenital and ocular sites, the site with the greatest copies was used as the representative result. qPCR efficiencies were between 89-100% and intra-assay variation below 5% for all genes.

Quantification of PhaHV-1 & -2 mucosal shedding

Oropharyngeal swabs were the primary sample used for the detection of PhaHV. However, where this sample was not obtained or failed quality-control, either urogenital or ocular swabs, if available, were assessed. Although urogenital and ocular swabs are not the optimal sample, PhaHV has been detected at these sites (Kasimov *et al.*, 2020; Vaz *et al.*, 2019b; Wright *et al.*, 2023). In total, oropharyngeal swabs (n = 121), urogenital swabs (n = 2), and ocular swabs (n = 2) from 105 koalas at 125 timepoints were used for the detection of PhaHV-1 *dpol* and PhaHV-2 *dpol* using established qPCR assays (Church *et al.*, 2025; Wright *et al.*, 2023). Of the timepoints included, 76% (n = 95) were admission samples and 24% (n = 30) were repeat samples post-treatment. For PhaHV-1, reaction mixes consisted of 10 µL of SYBR Green Supermix (SsoAdvanced™ Universal SYBR® Green Supermix—BIORAD), 250 nM of each primer, 7 µL of dH_2O and 2 µL of DNA to make up a final volume of 20 µL. PhaHV-1 PCR cycling conditions were as follows: 1 cycle of denaturation (3 min at 98°C), then 40 cycles of denaturation (10 s at 98°C), and a combined annealing and extension step (30 s at 56°C), followed by a PCR product melt curve analysis (56 - 95°C at 0.5°C increments) for PhaHV-1. PhaHV-2 primers were used at a concentration of 250 nM in a total reaction volume of 20 µL using 2 µL DNA template and 10 µL of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Australia). qPCR conditions were as follows: 1 cycle of denaturation (3 min at 95°C) followed

by 40 cycles of denaturation (10 s at 95°C), then an annealing and extension step (30 s at 56°C), and finally a melt curve analysis (65 – 90°C at 0.5°C increments).

For both PhaHV-1 and PhaHV-2 qPCRs, samples were run in duplicate alongside a standard dilution of a pMG-Amp vector (Macrogen, South Korea) synthetic plasmid (positive control) containing the target region and flanking sequence PhaHV-1: 166 bp, PhaHV-2: 87bp). The resulting standard curve at 10-fold dilutions was used to quantify viral loads (ranging from 10^3 to 10^7 copies/µL). A sample was considered positive if both duplicates amplified *β-actin* and produced a melt curve at 81 – 81.5°C for PhaHV-1 and/or at 87 – 87.5°C for PhaHV-2.

Any sample with discordant results between duplicates were reassessed and samples that failed to amplify as neat (undiluted) were re-run at 1:10 dilutions to account for potential inhibitors. Samples which failed to amplify koala *β-actin* were excluded from further analysis (PhaHV-1, N = 2; PhaHV-2, N = 2). For quantitative analysis, the limit of detection (95%CI) of PhaHV-1 and PhaHV-2 was 12 copies and 133 copies per reaction, respectively. Samples resulting in starting quantities for PhaHV1 or PhaHV-2 below this threshold were not included in quantitative analysis. qPCR efficiencies ranged between 90.2–103.9% for PhaHV-1 and 90.2-96.7% for PhaHV-2 and the inter-assay variation were less than 5% for both targets.

Statistical analysis

All statistical analysis were performed using R Statistical Environment (Version 4.3.3) (R Development Core Team, 2024). Gene transcription data did not meet normality assumptions and so Box-cox transformation was employed. Observations at time-zero (admission samples,

n = 95) and repeat observations (post-treatment samples, n = 29) were used in separate analyses.

Multivariate analysis and hierarchical clustering at time-zero

Principal components analysis (PCA) was performed on the scaled and box-cox normalised gene counts for 27 genes on admission to observe gene expression relationships and reduce the number of dimensions for subsequent clustering analysis. Given that the PCA method is capable of handling highly correlated variables, ubiquitously transcribed KoRV parameters (KoRV A *env* RBD, KoRV D *env* RBD, KoRV *env* CKS17, & KoRV *pol*) were included within the PCA to observe marker specific relationships with host genes. A common rule in PCA is to account for ~70% of the variability within the dataset (Jolliffe & Cadima, 2016). Hence, the first 5 dimensions were retained, accounting for 67% of the variation in the dataset (Supplementary materials Table 7.9). A gene contribution matrix and varimax rotation of the PCA was conducted to aid in interpretation of gene contributions to each component (Figures 3.3 & 3.4A). A Hierarchical Clustering on Principle Components (HCPC) approach was used on the scaled PCs 1-5. This approach employs both hierarchical clustering and k-means clustering to generate a robust cluster solution (Husson *et al.*, 2010; Koh *et al.*, 2022; Maugeri *et al.*, 2021). Based on the most common result among five methods of optimal cluster number determination (Supplementary materials Table 7.12), 5 clusters were elected (k = 5). PCA and clustering were performed using the FactoMineR package version 2.8 .

Characterising clusters at time-zero

The validity of clustering results was assessed using Kruskal-Wallis tests to determine whether the clusters differed significantly in the distribution of PC contributions. Clusters were

characterised according to clinical and infectious characteristics listed in Table 3.1 (Supplementary materials Table 7.13 and Table 7.14). Pearson's Chi-Squared tests of independence were conducted to assess the relationship between clusters and categorical data employing both a post-hoc Pearson's Chi-Squared test of independence with Cramers V and pairwise Fisher's T test with false discovery rate (FDR) correction to further assess significant relationships. Kruskal Wallis rank sum tests were utilised for continuous data and post-hoc Dunnett's pairwise testing using Holm's multiple comparison correction to generate adjusted p-values. Significant post-hoc relationships are depicted using 'ggstatsplot' (Patil, 2021). Clusters were then characterised in terms of PC (Supplementary materials Table 7.15 & 7.16) and gene (Supplementary materials Table 7.17) contributions using HCPC V.test statistics, which measures the difference between the mean value of gene expression or PC scores within a cluster and the overall mean across all clusters. A positive or negative v.test statistic accompanied by p-value < 0.05 indicates that a PC score or gene is expressed at a higher level (upregulated) or lower level (downregulated) in the cluster compared to the overall mean. Post-hoc pairwise comparisons were performed using Dunn's test with Bonferroni correction (Supplementary materials Table 7.16), due to non-normal distribution of PC2 and PC3, to identify between cluster differences in the distributions of principal dimensions.

Table 3.1: Clinical and infectious agent variables

	Categorical variable	**Continuous variable**
Clinical characteristics		
Age	Age group: young (1-3) Age group: adult (4-9) Age group: mature (10+)	NA
Sex	Male or female	NA
Body condition score	1-5	NA
Clinical pathology	Yes or no	NA
Chlamydiosis	Yes or no	NA
Reproductive disease	Yes or no	NA
Other disease	Yes or no	NA
Trauma	Yes or no	NA
Outcome at sample	Euthanised or survived	NA
Infectious characteristics		
C. pecorum shedding (mucosal)	Yes or no	*C. pecorum* DNA loads (mucosal)
C. pecorum transcription (circulating)	Yes or no	*C. pecorum G-0573* mRNA loads (circulating) *C. pecorum Hsp_60* mRNA loads (circulating)
PhaHV-1 shedding (mucosal)	Yes or no	PhaHV-1 DNA loads per mL (mucosal)
PhaHV-2 shedding (mucosal)	Yes or no	PhaHV-2 DNA loads per mL (mucosal)
PhaHV-2 transcription (circulating)	Yes or no	PhaHV-2 mRNA (circulating)
Agreggated trypanosome transcription (circulating)	Yes or no	*T. copemani 16s* mRNA (circulating) *T. irwini 16s* mRNA (circulating) *T. gilletti 16s* mRNA (circulating)
Proviral KoRV *pol* (circulating)	NA	KoRV *pol* DNA loads per mL (circulating)
KoRV B transcription (circulating)	Yes or no	KoRV-B *env* mRNA (circulating)

General linear modelling of outcome-associated genes

Multivariate modelling, conducted to determine gene-specific relationships with outcomes, incorporated those genes that strongly contributed to the dimensions that differentially clustered koalas based on survival. Genes that were differentially transcribed between clusters 1 and 5 (Figure 3.7) were selected for general linear modelling to determine the relationships with triage and post-treatment outcomes. Pearson's correlations and variable inflation factors (VIFs) were used for gene model selection. Any gene significantly associated with outcomes with VIFs > 5 was excluded from final models (Gaona *et al.*, 2023; Williams *et al.*, 2008). We used three separate General Linear Models (GLMs) to assess the effect of selected individual gene transcription measured both on admission (time-zero) and post-treatment, on survival outcomes both at triage and post-rehabilitation. Model 1 assessed

whether transcription of KoRV *pol*, *IL18, CD79b, FKBP5, SLC29A1, TLR2, TLR7, TRIM24*, and Cathelicidin-like (*LOC110217150*) gene measured on admission affected triage outcome (N = 95). Model 2 assessed whether transcription of genes measured on admission that affected triage outcome in Model 1 (*TRIM24* and *FKBP5*), also affected post-rehabilitation outcome (N = 29). Model 3 assessed whether transcription of genes measured post-treatment that affected triage outcome in Model 1 also affected post-rehabilitation outcome (N = 29). Akaike's Information Criterion (AIC) compared to other models including other gene iterations.

3.5 Results

Study population characteristics

Characteristics of this sample population are depicted in Table 3.2. The majority (84.2%) of the sample population were admitted to Australia Zoo Wildlife Hospital (Qld) during September-November, which is within the first half of the koala breeding season (Ballantyne *et al.*, 2015). Overall, 59% of koalas were female; 41% male, while 28% were considered young adults (1.5 – 3 years old); 60% of koalas were considered adults (3.5 - 9 years old); and 12% mature (10 – 12 years old). Of the 40 koalas euthanised on admission, 43% had multifocal chlamydiosis including reproductive disease, 28% had multifocal chlamydiosis without reproductive disease, 15% had complicated disease and trauma-related co-morbidities, 10% had complicated disease only, and 5% complex trauma presentations only. Euthanasia post-treatment was applied in cases where no, poor, or adverse response to treatments resulted. Of the 13 cases euthanised post-treatment, 15% had chlamydiosis including reproductive

disease, 23% had chlamydiosis without reproductive disease, 46% had disease and trauma related co-morbidities, and 15% complex trauma presentations.

Table 3.2: Health assessment characteristics of study population

Characteristics	T0 samples (No. = 95)
Age group	
Young adult, n (%)	27 (28)
Adult, n (%)	57 (60)
Mature, n (%)	11 (12)
Sex	
Female, n (%)	56, (59)
Male, n (%)	39 (41)
Body Condition Score (1-5)	
1, n (%)	12, (13)
2, n (%)	16, (17)
3, n (%)	36, (38)
4, n (%)	30, (32)
5, n (%)	1, (1)
Sampling months, n (%) September-April*	S = 24 (25), O = 32 (34), N = 18 (19), D = 6 (6), J = 5 (5), F = 6 (6), M = 1 (1), A = 3 (3)
Sampling state	
Qld, n (%)	80 (84)
NSW, n (%)	15 (16)
No pathology, n (%)	18 (19)
Pathology, n (%)	77 (81)
Chlamydiosis without reproductive disease, n (%)	21 (22)
Chlamydiosis with reproductive disease, n (%)	28 (30)
Other disease, n (%)	6 (6)
Trauma, n (%)	21 (22)
Detection of infectious agents	
Mucosal *C. pecorum* (ocular and/or urogenital swab), n (%)	47 (50)
Circulating *C. pecorum* (buffy coat), n (%)	8 (8)
Mucosal PhaHV-1 (oropharyngeal swab), n (%)	56 (60)
Mucosal PhaHV-2 (oropharyngeal swab), n (%)	19 (20)
Circulating PhaHV-1 or 2 (buffy coat), n (%)	5 (5)
Circulating KoRV-B *env* (buffy coat), n (%)	33 (35)
Circulating trypanosomes (buffy coat), n (%)	58 (61)
Admission Outcomes	
Euthanised post-triage, n (%)	40 (42)
Admitted for rehabilitation, n (%)	34 (37)
Approved for release, n (%)	20 (21)
Koalas with follow-up samples, n (%)	30 (32)

*Months: S = September, O = October, N = November, D = December, J = January, F = February, M = March, A = April

Overall, 81% of analysed koalas had evidence of pathology, of which 52% were characteristic of chlamydial disease. As expected, KoRV *pol* replication and transcription, and KoRV A, D, and CKS17 *env* transcription was detected ubiquitously. Of the non-ubiquitously detected infectious agents, circulating trypanosomes were most frequently detected (61%), followed by mucosal PhaHV-1 (60%), mucosal *C. pecorum* (50%), KoRV B (35%), mucosal PhaHV-2 (20%), and finally circulating agents *C. pecorum* (8%) and PhaHV-1 and/or PhaHV-2 (5%) (Table 3.2).

NanoString performance

The immune targets *CCR4, PhciCATH5, MHCIUA, NCR3, LEP, IL4, IL22, Il12A, IL17A, IL10*, and *IFNG* were omitted from further analysis as they were not detected above threshold levels (> 20 counts, Supplementary materials Table 7.7) in more than 50% of samples. Targets retained for PCA were therefore: *CARD9*, *CD3G, CD4, CD79b, CD8beta, CLEC4E*, *DICER1*, *FKBP5*, *IL18, IL1beta, IL6, IL8, KoRVAenvRBD, KoRVDenvRBD, KoRVenvCKS17, KoRVpol*, Cathelicidin-like (*LOC110217150*), *PhciDAB, PhciDBB, RETN*, *SAMDH1*, *SLC29A1 TLR2, TLR4, TLR7, TNFalpha*, and *TRIM24*. The five dimensions retained from PCA, performed using scaled and Box-Cox normalised transcription counts of 27 host immunological genes, accounted for 67% of the total variance within the dataset (Figure 3.3, Supplementary materials Table 7.9).

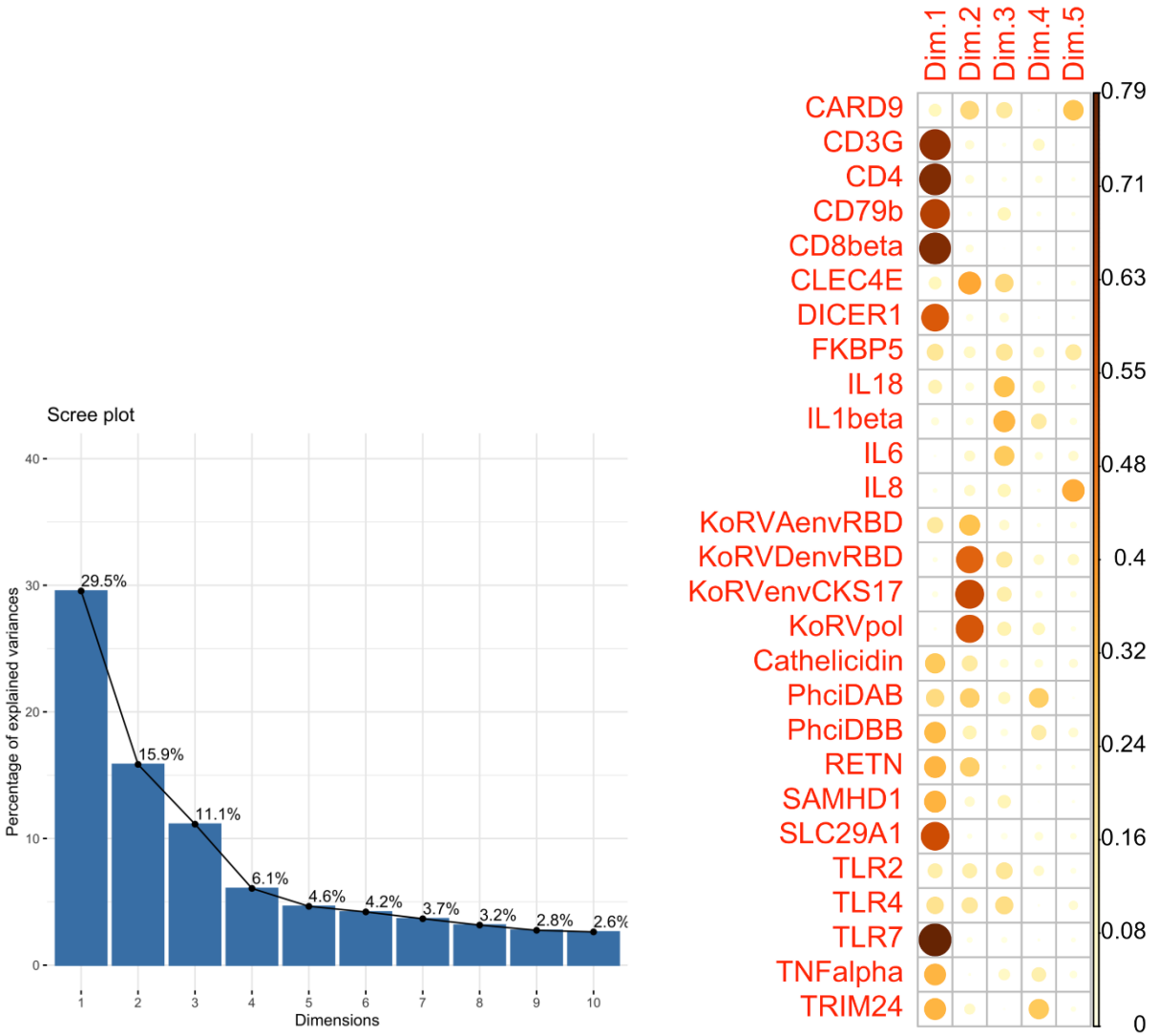

Figure 3.3: Principal components and gene representations amongst dimensions.

In Figure 3.3, the variation described by the first ten principal components and the gene representation among the five retained principal components are presented in the scree plot of first ten dimensions within the PCA of koala gene transcription on admission (bottom left), and the Cos2 plot visualizing the quality of representation for each variable across PCA dimensions (right). The Cos2 values (ranging from 0 to 1) measure the proportion of the variable's variance explained by each principal component, with higher values indicating better representation. Colour intensity corresponds to the magnitude of Cos2, with darker colours representing higher values. The matrix rows represent variables, and the columns correspond to PCA dimensions (e.g., PC1/Dim.1, PC2/Dim.2). This plot highlights the dimensions that most effectively represent the variables based on their squared cosines. In total, 95 samples were included.

Hierarchical clustering of biological gene components

Hierarchical clustering of host gene and KoRV-associated principal components identified major koala immune-physiological phenotypes on admission. Five clusters of koalas were identified based on the distances between each branch of the dendrogram (Figure 3.4B) and by the total within-cluster sum of squares, which was reached at k-clusters = 5 (Supplementary materials Table 7.12). The initial partition was consolidated using the k-means algorithm with the number of clusters defined through unsupervised hierarchical clustering analysis of biological gene expression profiles (Figure 3.4C). The five clusters comprised four larger clusters that each included more than ten biologically similar koalas; and one minor cluster, which comprised six individuals. Post-hoc validation of cluster assignments using Kruskal-Wallis test showed that each cluster derived from the HCPC was statistically distinct in terms of their distributions along the five principal components.

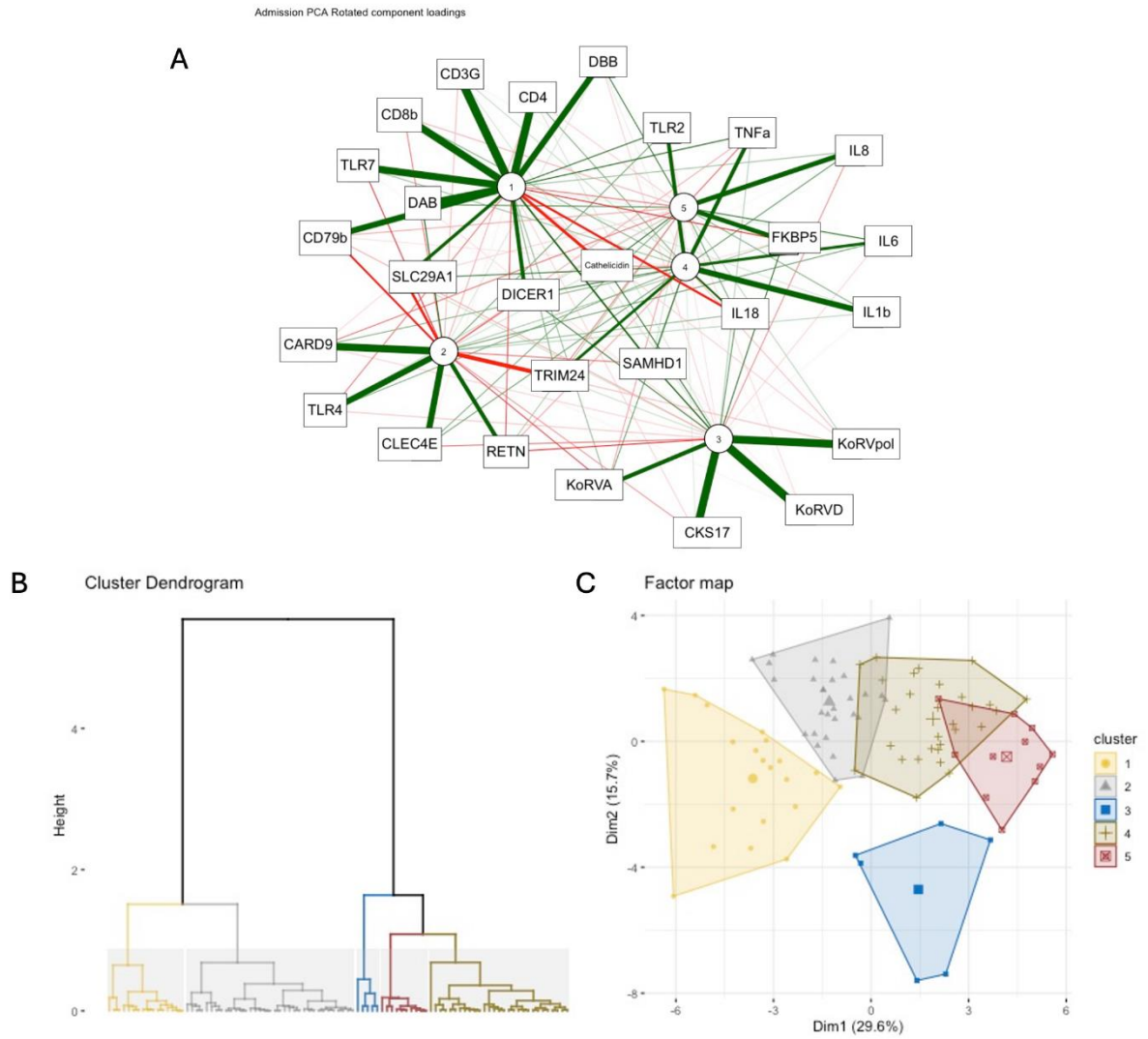

Figure 3.4: Principal Components Analysis (PCA) dimensions used to identify immune profiles of koala immune gene transcription counts on admission, through k-means clustering with Ward's method.

Figure 3.4 demonstrates the host gene dimensions obtained from principal components analysis that were used in hierarchical clustering to partition the dataset. (A) Varimax rotated principal components network plot: Varimax rotation, an orthogonal rotation that minimises the number of high loading variables, is used to simplify the interpretability of retained dimensions and show the characterisation of each PC ultimately used in clustering. (B) Dendrogram and (C) scatter plot of koala clusters: using branch separation, the dendrogram identifies 5 distinct clusters among the immunological gene transcription data of 95 koalas sampled on admission prior to the application of treatments. The assignment of clusters largely reflects differences in the first two dimensions generating five clusters. The five clusters included: cluster 1, n = 20, cluster 2, n = 29, cluster 3, n = 6, cluster 4, n = 29, and cluster 5, n = 11.

Clusters are associated with triage outcome

The proportion of koalas within each age group differed significantly among the clusters ($p < 0.05$) (Supplementary materials Table 7.13). The distribution of males and females differed

among clusters (p = 0.03) (Supplementary materials Table 7.13) and, while chi-squared testing showed that this was associated with an overrepresentation of males in cluster 5 (p = 0.03), post-hoc pairwise Fisher's testing identified no significant differences in the distribution of sexes between clusters. There were no significant differences in the proportions of koalas among clusters according to body condition, the presence of general pathology, presentation of chlamydiosis, reproductive disease, other disease, or trauma (Supplementary materials Table 7.13).

There was a significant difference in the distribution of cases with detectable circulating *C. pecorum* according to cluster (p = 0.0014, Supplementary materials Table 7.13). Although Pearson's Chi-Squared testing suggested that the association between cluster and circulating *C. pecorum* was non-random, it is likely that the lack of cases with detectable circulating *C. pecorum* in cluster 1 drove this association (Figure 3.5A). Overall, small frequencies of detection (N = 8) prevented post-hoc pairwise comparisons. The distribution of outcome (euthanised or survived) on admission (i.e. time-point or triage outcome) was significantly different across clusters (p = 0.004) (Figure 3.5B, Supplementary materials Table 7.13). In cluster 1, the proportion of euthanised koalas was significantly greater compared to cluster 4 (adj. p = 0.04) and cluster 5 (adj. p = 0.006).

For other infectious agents, with all koalas included, there was no significant differences among clusters in the proportions of koalas with and without detectable mucosal *C. pecorum*, mucosal PhaHV-1 or PhaHV-2, circulating PhaHV-1 or PhaHV-2, circulating KoRV B, or circulating trypanosomes (Supplementary materials Table 7.13). Among koalas with loads

above the LOD for respective quantitative tests, no significant differences in pathogen loads for any marker were observed among clusters (Supplementary materials Table 7.14).

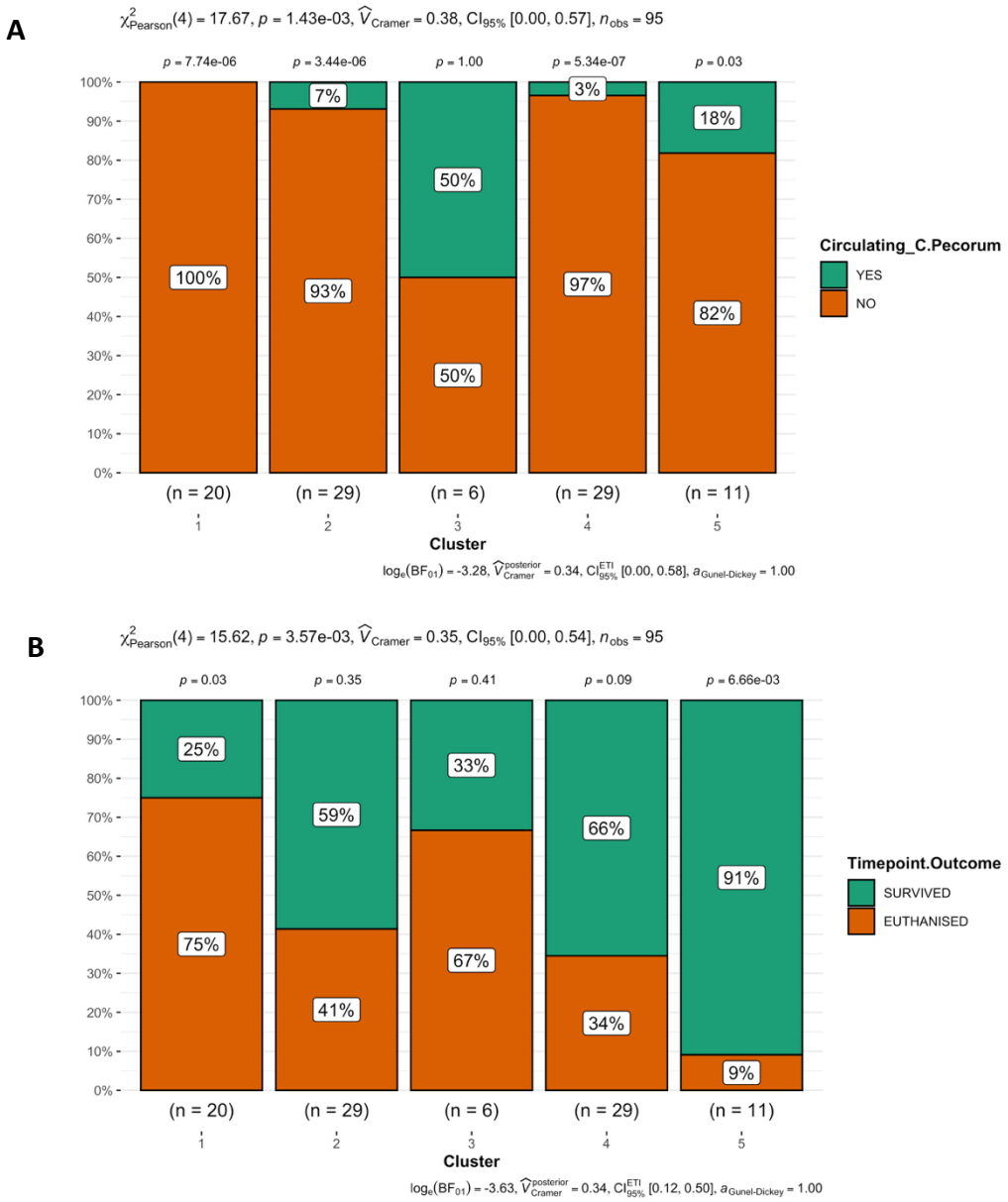

Figure 3.5: Pearson's Chi-Squared test of independence with Cramers V testing the association between (A) detection of circulating* C. pecorum *within clusters and (B) triage outcomes (euthanised or survived) within clusters.

Figure 3.5 presents Pearson's Chi-Squared tests that determine the associated between (A) detection of circulating C. pecorum *and (B) triage outcomes and host clusters. Chi-squared statistic (χ^2) is printed above the graph with degrees of freedom followed by the p-value, Cramer's V effect size score, 95% confidence intervals, and sample size (n).*

Clusters associated with survival have greater PC1 scores

The first dimension/PC accounts for the greatest amount of variation (24.7%) and partitions the dataset along a gradient (Figures 3.4C and 3.6, Supplementary materials Table 7.9-7.11): less influence of PC1 in clusters associated with euthanasia (Supplementary materials: Table 7.15). Clusters 3, 4 and 5 had significantly greater mean scores of PC1 compared to cluster 1; (p adj. < 0.001), (p adj. < 0.001), and (p adj. < 0.001) respectively (Figure 3.6, Supplementary materials Table 7.16). Similarly, clusters 4 and 5 had significantly greater mean scores of PC1 compared to cluster 2; (p adj. < 0.001) and (p adj. < 0.001), respectively (Figure 3.6, Supplementary materials Table 7.16).

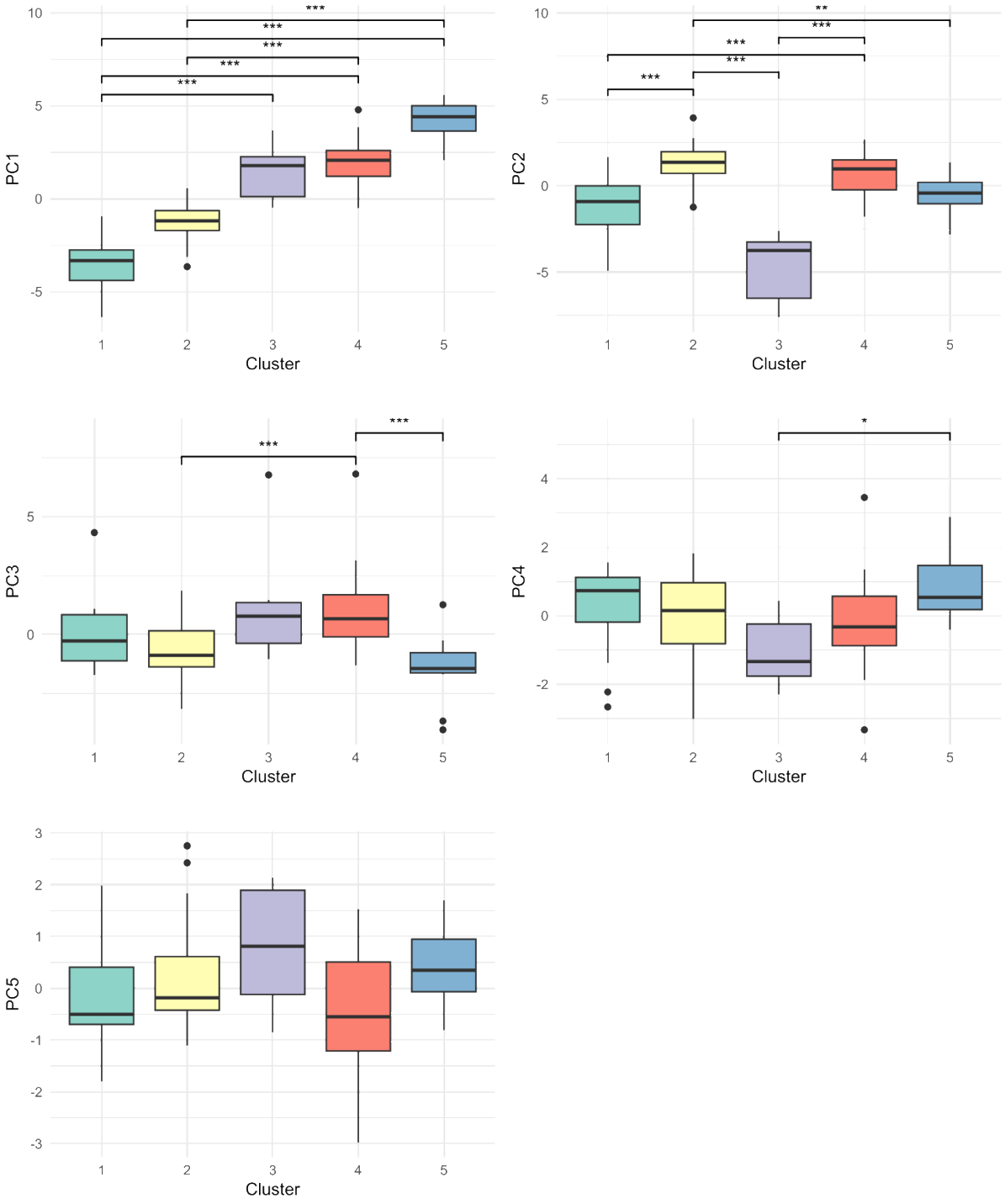

Figure 3.6: Cluster-wise distribution of principal components (dimensions) using Dunn's Test with Bonferroni correction for pairwise comparisons.

*Figure 3.6 presents the distribution of principal component scores among the five clusters using Dunn's Test with Bonferroni correction to determine pairwise comparisons. This test identifies which pairs of clusters differ significantly for each principal component; p > 0.05 = ns, p < 0.05 = *, p < 0.01 = **, p < 0.001 = ***.*

The relationship between PC2-5 scores and clusters with better or poorer outcomes was unclear. The mean score for PC2 was significantly greater in clusters 4 ($p < 0.05$) and 2 ($p < 0.001$) and significantly lower in clusters 1 ($p < 0.01$) and 3 ($p < 0.001$) compared to the mean across all clusters (Supplementary materials Table 7.15). PC3 scores were significantly lower on average in clusters 2 ($p < 0.01$) and 5 ($p < 0.01$) and significantly greater in cluster 4 ($p < 0.001$) compared to the average score across clusters (Supplementary materials Table 7.15). The mean score for PC4 was significantly greater and lower on average in clusters 5 ($p < 0.01$) and 3 ($p < 0.05$), respectively, compared to the average score across clusters (Supplementary materials Table 7.15). Overall, PC5 scores were not differentially distributed among clusters.

Clusters associated with better outcomes have upregulated immune gene and cellular activity gene transcription.

From the HCPC output (Supplementary materials Table 7.17), clusters 4 and 5, which were associated with improved outcomes, are distinguished by significant upregulation ($p < 0.05$) of the genes positively contributing to PC1, relative to the mean transcription of genes across all clusters. This includes greater transcription of MHCII (*PhciDAB*, *PhciDBB*), *TLR7*, adaptive cellular immune genes (*CD3G*, *CD4*, *CD8B*, & *CD79b*), and transcription-associated genes (*DICER1* & *SLC29A1*), and downregulation of an innate antimicrobial peptide gene (Cathelicidin: *LOC110217150*) (Figure 3.7). *IL6* and *CARD9*, both pleotropic immune genes, were downregulated in cluster 5 but upregulated in cluster 4. Furthermore, in cluster 4, *FKBP5* transcription, was downregulated and the transcription of other transcription-associated genes, *TRIM24* and *SAMHD1*, and innate immune genes, *IL1beta*, *TNFalpha*, and *TLR2* was upregulated. In cluster 5, significant downregulation of the adipose tissue associated pro-

inflammatory gene *RETN* along with other innate immune associated genes *IL18*, *TLR4*, and *CLEC4E* was observed (Figure 3.7 & Supplementary materials: Table 7.17).

Like cluster 5, in cluster 3, transcription of *RETN*, *CARD9*, *CLEC4E*, and *TLR4* was downregulated, while *SLC29A1*, *TNFalpha*, *DICER1*, *TRIM24* and *SAMHD1* was upregulated compared to the mean across all clusters (Figure 3.7 & Supplementary materials: Table 7.17). However, unlike clusters 4 and 5 *PhciDAB* was significantly downregulated and *IL18* and KoRV *pol* transcription was significantly upregulated in cluster 3 (Figure 3.7 & Supplementary materials: Table 7.17).

In clusters 1 and 2, a direct contrast is observed in gene expression compared to clusters 4 and 5. Adaptive immune genes, *CD3G* and *CD4*, and transcription-associated genes, *DICER1*, *SAMHD1*, and *SLC29A1*, and cytokine gene *TNFa* were significantly downregulated in clusters 1 and 2 ($p < 0.05$), respectively, compared to the average gene transcription across all clusters (Figure 3.7 & Supplementary materials: Table 7.17). Furthermore, in cluster 1, additional adaptive immune genes such as *CD79b* and *CD8b*, MHCII genes such as *PhciDAB*, *PhciDBB*; innate immune genes such as *TLR2* and *TLR7*, and pleotropic cytokine *IL1b* were significantly downregulated ($p < 0.05$). In contrast, cytokine *IL18*, *TLR4*, KoRV *pol*, and FKBP5 were significantly upregulated in cluster 1 (Figure 3.7 & Supplementary materials: Table 7.17). Downregulation of KoRV markers (KoRV A *env*, KoRV D *env*, KoRV CKS17 *env*, and *pol*) and upregulation of *RETN* and *CLEC4E* was observed in cluster 2 resulting in a significantly greater contribution of PC2 in cluster 2 compared to cluster 1 (Figure 3.7 & Supplementary materials: Table 7.17).

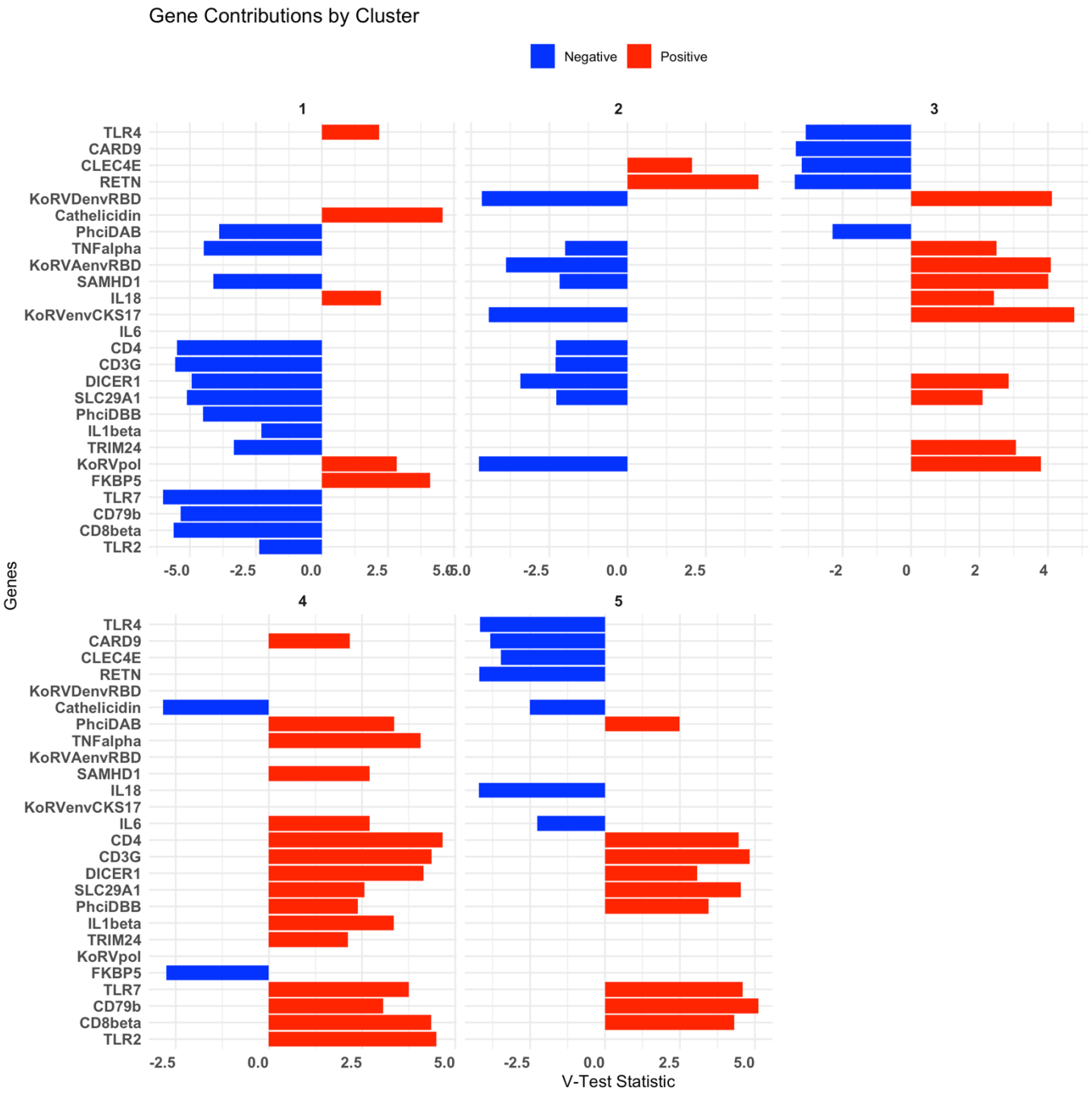

Figure 3.7: Bar chart of V-test scores from hierarchical clustering of principal components (HCPC).

Figure 3.7 illustrates the extent to which a cluster (1-5) is significantly associated with the transcription of certain gene, identifying which genes contribute the most to the characterisation of a given cluster. The null hypothesis of the V-test assumes that the mean gene transcription in the cluster does not differ from the overall mean. Only genes with significantly different mean transcription compared to overall mean are displayed. A positive V-test value (red bars) indicates that the gene is upregulated/increased in the cluster compared to the overall population, similarly a negative V-test value (blue bars) suggests that the gene is downregulated/decreased in the cluster. Larger absolute values of the V-test indicate stronger contributions of the gene to the cluster.

TRIM24, IL18, and KoRV pol gene transcription at admission predicts triage outcomes.

In a GLM assessing the relationship between triage outcome and genes with low collinearity (VIF > 5) that were differentially transcribed between cluster 1 and cluster 5 (Figure 3.7), *IL18, TRIM24*, and KoRV *pol* transcription were significant predictors of triage outcomes on admission (Table 3.3). KoRV *pol* and *IL18* transcription is associated with negative triage outcomes, indicating that greater transcription increases the likelihood of euthanasia. Whereas, *TRIM24* is associated with positive triage outcomes, suggesting that increased transcription of *TRIM24* predicts greater likelihood of survival.

Table 3.3: General linear model predicting triage outcome (euthanised = 0, survived = 1) in N = 95 koalas using Box-cox transformed gene transcription

N = 95	Coefficient	S.E.	Z score	P-value	95%CI
(Intercept)	-19.0	49.1	-0.39	0.70	-117.2 - 78.7
Sex: Male	0.55	0.64	0.87	0.39	-0.71 - 1.84
Age group: Mature	-1.68	1.15	-1.47	0.14	-4.09 - 0.47
Age group: Young adult	-0.83	0.85	-0.98	0.33	-2.57 - 0.80
KoRV pol	-20.9	8.17	-2.56	**0.01**	-38.2 - -5.77
IL18	-5.04	2.57	-1.96	**0.05**	10.5 - -0.24
CD79b	0.44	0.48	0.92	0.36	-0.49 - 1.41
FKBP5	-12.1	6.59	-1.83	0.07	-26.4 - 0.03
SLC29A1	-2.51	2.01	-1.25	0.21	-6.77 - 1.21
TLR2	0.004	0.01	0.49	0.63	-0.01 - 0.02
TLR7	0.58	0.51	1.12	0.26	-0.42 - 1.63
TRIM24	4.12	1.42	2.90	**0.004**	1.57 - 7.22
Cathelicidin	69.5	45.0	1.54	0.12	-15.7 - 163.4
Null deviance: 130.4 on 94 degrees of freedom					
Residual deviance: 77.0 on 82 degrees of freedom					
AIC: 102.9					

TRIM24 gene transcription at triage is associated with improved post-treatment outcomes in koalas admitted to rehabilitation.

In the GLM accounting for age and sex in the subset of 29 koalas that were admitted to rehabilitation, *TRIM24* was associated with positive outcomes, suggesting that greater levels of *TRIM24* at admission resulted in a higher likelihood of being released post-treatment (Table 3.4).

Table 3.4: General linear model predicting post-treatment outcome (euthanised or survived) in N = 29 koalas admitted to rehabilitation using Box-cox transformed gene transcription measured on admission.

N = 29	Coefficient	S.E.	Z score	P-value	95%CI
(Intercept)	18.8	20.3	0.93	0.35	-20.1 - 64.5
Sex: Male	2.20	1.28	1.72	0.08	-0.02 - 5.32
*Age group: Young adult**	1.55	1.24	1.25	0.21	-0.71 - 4.36
TRIM24	6.46	2.78	2.33	**0.02**	2.12 - 13.6
FKBP5	-19.8	10.8	-1.84	0.07	-46.9 - -2.11

Null deviance: 42.9 on 30 degrees of freedom
Residual deviance: 22.5 on 26 degrees of freedom
AIC: 32.5
*Contrasting group is 'adult'. No 'mature' koalas were represented within this cohort.

FKBP5 and IL18 transcription at final veterinary examination predicts post-treatment rehabilitation outcomes.

In the same subset of rehabilitated koalas (N = 29) at their last veterinary examination, the GLM model with the lowest AIC included sex, age group, *IL18* and *FKBP5* (Table 3.5)**.** Contrasting to the relationship between IL18 and negative triage outcomes, IL18 has a positive relationship with post-treatment outcome, meaning higher levels of IL18 are associated with a higher likelihood of being released post-treatment. Male koalas were also associated with higher likelihood of being released post-treatment.

Table 3.5: General linear model predicting post-treatment outcome (euthanised/survived) in N = 29 koalas admitted to rehabilitation using Box-cox transformed gene transcription measured at final veterinary examination.

N = 29	Coefficient	S.E.	Z score	P-value	95%CI
(Intercept)	-4.22	2.50	-1.69	0.09	-12.6 - -0.58
Sex: Male	6.15	2.77	2.22	**0.03**	2.34 - 14.6
*Age group: Young adult**	2.05	2.11	0.97	0.33	-1.55 - 7.84
FKBP5	-0.02	0.01	-1.83	0.07	-0.05 - 0.01
IL18	0.07	0.03	2.40	**0.02**	0.03 - 0.16

Null deviance: 38.2 on 27 degrees of freedom
Residual deviance: 14.3 on 23 degrees of freedom
AIC: 24.3
*****Contrasting group is 'adult'. No 'mature' koalas were represented within this cohort

3.6 Discussion:

To effectively prevent and treat complex diseases such as chlamydiosis, we must first understand the extent to which host, pathogen, and environmental factors determine the severity of disease. In this study, hierarchical clustering of principal components based on immunological variables defined clusters that aligned more closely with clinical decision making at triage than with aetiological diagnosis and generated a novel perspective on the biology associated with varied clinical presentations and rehabilitation outcomes. This biological variation was mostly captured within one component of lymphocytic pathway-associated genes (lymphocyte differentiation markers, MHCII and *TLR7*), which partitioned the clusters along a gradient whereby koalas with greater transcription of associated genes had greater likelihood of release. Additionally, *TRIM24* transcription at triage was associated with improved triage and post-treatment outcomes and should be assessed further in different populations for potential inclusion within health assessments to aid decision making and predict treatment response. Our findings support and build on the current understanding of the relationships between KoRV and clinical outcomes by identifying additional relationships between host-genes and KoRV transcription. Variable associations between *FKBP5* and *IL18* transcription and outcomes highlight an area for further investigation to determine the factors and mechanisms behind stress induced immunomodulation and the inhibition of IL-18 induced functions, respectively.

The clustering approach used here created a novel perspective on the host features associated with koala health decline and the resulting triage outcomes. Clusters derived from the data were independent of the aetiologically based categories and assumed gene functions generally applied in disease association studies in the koala (Blyton *et al.*, 2022a; Hashem *et*

al., 2021; Kayesh *et al.*, 2021a; Legione *et al.*, 2017; Maher & Higgins, 2016; Maher *et al.*, 2019; Quigley *et al.*, 2019; Waugh *et al.*, 2017; Xu *et al.*, 2013) and thereby avoided the potential for those *a-priori* categorizations to obscure relationships or confound outcomes (Loftus *et al.*, 2022). While fewer dimensions could have been used for simplicity to perform clustering, the five dimensions accounting for ~70% of variability were retained due to the large degree of variation in clinical characteristics of koalas within the dataset. The grouping of the comprehensive set of markers into expected host gene systems using PCA also improved our confidence in interpretation of results, relative to our previous approaches that relied on single parameters to represent immunological pathways (Kayesh *et al.*, 2022; Kayesh *et al.*, 2021a; Lizárraga *et al.*, 2020b; Maher *et al.*, 2014; Maher & Higgins, 2016; Maher *et al.*, 2019; Marschner *et al.*, 2019a; Mathew, 2014; Mathew *et al.*, 2013a; Olagoke *et al.*, 2020b). The observed spectrum of declining host gene function with worsening prognosis provides a more holistic picture of why cases with similar aetiological diagnosis have disparate outcomes.

Marked downregulation of adaptive immune and transcription-associated genes was associated with poor outcomes. PC1, which accounted for the greatest variation within the dataset (24.7%), largely reflected adaptive immunity, with upregulation of lymphocyte-associated immune responses associated with better outcomes at triage i.e. transcription of T- & B- cell differentiation marker genes (*CD3G, CD4, CD8b*, & *CD79b*) and MHCII class DAB and DBB genes associated with lymphocyte proliferation (*PhciDAB* & *PhciDBB*) (Lau *et al.*, 2012), upstream innate immune and transcription-associated genes expected to promote them, such as, Toll-like-receptor 7 (*TLR7*), an adenosine transporter (*SLC29A1*), a ribonuclease (*DICER1*), and a dNTP regulating enzyme (*SAMHD1*). Inversely related elements of this PC

included genes encoding products more associated with innate immune responses, such as macrophage-produced cytokine Interleukin-18 (IL-18), a Cathelicidin antimicrobial peptide (*LOC110217150*), and resistin (*RETN*), a hormone secreted from adipose tissue that can act as an innate TLR4 binding chemokine that stimulates macrophages to release 'pro-inflammatory' cytokines TNF-α, IL-1β, IL-8, IL-12, IL-6, (represented by PC4 and PC5) and monocyte-chemotactic protein-1 (MCP-1) secretion (Codoñer-Franch & Alonso-Iglesias, 2015; Filková *et al.*, 2009; Ruiz-Ojeda *et al.*, 2018; Silswal *et al.*, 2005). *RETN* also positively correlated with *TLR4*, *CARD9* and *CLEC4E* transcription in PC2; *CARD9* can transmit signals from *CLEC4E* on macrophages and natural killer cells and TLRs, including *TLR4*, to promote pro-inflammatory responses against bacterial patterns such as LPS (Hattori *et al.*, 2014). The apparently central role for *RETN* in promoting innate and pro-inflammatory pathways over adaptive immunity was evident in its significantly lower expression in clusters associated with survival at triage. A shift from adaptive to proinflammatory innate immunity with worsening outcomes is consistent with comparisons between healthy and declining populations evaluated with the same expression panel and was not related to circulating leucocyte counts (Fernandez *et al.*, 2024b). Further study is needed to longitudinally examine the shift from innate to adaptive immunity post-infection to clarify whether it occurs either due to a down-regulation of adaptive immunity or from a failure to effectively progress from innate immunity. Regardless, the ability to measure an apparent dysregulation or collapse of adaptive immunity provides a way to quantify impacts of potential disease drivers and further elucidate causative mechanisms so these can be targeted in population and clinical management.

The relationship between *TRIM24* transcription and positive outcomes may reflect different co-infection-host interactions, although our dataset could not definitively demonstrate this link. *TRIM24* belongs to a large group of enzymes that not only orchestrate antiviral immunity by regulating pattern recognition receptors through post-translational modifications (Herquel *et al.*, 2013; Sayyad *et al.*, 2024), but have also demonstrated involvement in the host response to chlamydial infection (Stepanenko *et al.*, 2023). The concurrent upregulation of adaptive immune genes (captured in PC1) and *TRIM24* in this study may represent a beneficial host-response to infection as it aligns with immune phenotypes observed in latent infections by herpesviruses and *Wuchereria bancrofti* (Arndts et al., 2012; Reddehase et al., 2008; White et al., 2012). Further supporting this theory, here *TRIM24* was inversely correlated with *TLR4*, a gene that becomes downregulated as infection resolves during antibiotic treatment of koalas with chlamydiosis (Phillips *et al.*, 2019). Additionally, *TRIM24* transcription was strongly inversely correlated with innate immune genes in PC2, including *CLEC4E*, which is upregulated during the acute phases of *Chlamydia* spp. infections (Hattori *et al.*, 2014; Morris *et al.*, 2015b). Collectively, these relationships identify a potential network of communicating host genes that could be involved in the host-pathogen response driving variation in chlamydial pathogenesis. Unlike the relationships demonstrated in mouse models between *TRIM24* and other retroviruses (Margalit *et al.*, 2020) and gamma herpesviruses (De La Cruz-Herrera *et al.*, 2023; Fletcher & Towers, 2013; Sayyad *et al.*, 2024), and bacteria (Stepanenko *et al.*, 2023), we observed no direct associations between *TRIM24* transcription and PhaHV loads, KoRV transcription, or mucosal *C. pecorum* loads. Unfortunately, the relationship between *TRIM24* transcription and circulating *C. pecorum* could not be assessed because of the low detection frequency of circulating *C. pecorum* in this dataset. Using a larger sample size to capture a greater frequency of infections, future studies should longitudinally measure *TRIM24*

transcription in koalas to determine whether a relationship exists between *TRIM24* and the occurrence and development of infection and disease over time.

The relationship between *FKBP5* gene transcription and koala health and disease requires further investigation. *FKBP5* is a co-chaperone gene that modulates the glucocorticoid receptor and directly influences the actions of glucocorticoids and other hormones on the hypothalamic-pituitary-adrenal axis (Nicolaides *et al.*, 2014). It is also an immunophilin; exhibiting high specificity in binding to immunosuppressive agents (Cugliari, 2023; Haughey, 2010; Zgajnar *et al.*, 2019). While a relationship between greater *FKBP5* transcription and reduced adaptive immune responses was observed previously (Fernandez *et al.*, 2024b) and a similar pattern could be observed here (Figure 3.7), *FKBP5* was a marginally non-significant predictor in all outcome models. Fernandez *et al.* (2024b) hypothesised that greater *FKBP5* transcription and immune suppression was driven more-so by environmental pressures rather than disease and so it may be possible that the cross-sectional sampling of koalas from a wide range of environments supressed this relationship.

The varied relationship between *IL18* and outcomes at triage and post-treatment (IL-18 transcription was associated with euthanasia at triage but was associated with successfully treated candidates post-treatment) may reflect the complex function of this cytokine in directing adaptive responses. Increased Interleukin 18 (IL-18), also known as interferon-gamma inducing factor, is downstream of IL-1 and has similar proinflammatory functions but can also mediate inflammation by regulating the secretion of IFN-γ in the presence of IL-12 and IL-10 (Nakanishi, 2018; Zheng *et al.*, 2020a). Although the small sample size utilised in these post-rehabilitation models present a clear limitation, considering the potential for IL-18

to mediate different adaptive responses depending on what co-stimulation exists in the surrounding milieu warrants further investigation to confirm these mechanisms.

Our findings support and build upon current thinking around the relationship between KoRV and disease outcomes by expanding the picture to include host-gene expression. Keeping with present understanding, KoRV *pol* transcription was more strongly correlated with exogenous KoRV *env* D than KoRV *env* A supporting the predominant contribution of exogenous transcription to overall KoRV transcription (Hashem *et al.*, 2021; Quigley *et al.*, 2018b; Sarker *et al.*, 2019). Similar to the association found between exogenous KoRV D transcription and secondary disease (Blyton *et al.*, 2022a), here KoRV *pol* and, by proxy, exogenous KoRV D transcription was a predictor of negative triage outcomes. There was no association between KoRV A transcription and outcomes and instead, a weak positive correlation with PC1 was observed. The positive relationship between KoRV *pol, env* -A, -D and CKS17 with *SAMHD1* and KoRV *env* -A, -D and CKS17 with *DICER1* might indicate active host-viral interactions. *SAMHD1* depletes dNTP pools available for reverse transcription in viral cDNA synthesis, preventing viral replication (Wang *et al.*, 2020); knock-down of *SAMHD1* in mouse monocytic cell-lines and macrophages increased their susceptibility to HIV-1 infection (Zhang *et al.*, 2014). The DICER enzyme cleaves double-stranded RNA and pre-microRNA facilitating the activation of RNA-induced silencing complexes essential for RNA interference (Komori *et al.*, 2020); in HIV-1 infection this can inhibit viral replication and downregulate cellular genes to protect infected cells from apoptosis, contributing to viral latency (Klase *et al.*, 2007; Klase *et al.*, 2009; Komori *et al.*, 2020; Li *et al.*, 2016b). The positive relationship among these markers might indicate cells responding to viral transcription. To better understand the function and significance of these viral-host interactions, we suggest

that future investigation determines whether knockdown of *SAMHD1* and *DICER1* in koala cell lines elevate dNTP pools and how this effects KoRV markers (Franzolin *et al.*, 2013), or whether the transition between lytic-latent-lytic stages is affected by these genes in endogenous and exogenous KoRV and other viruses such as PhaHV.

Although nonstimulated samples provide a realistic indication of biological events in the koala at that point of time, expression of some targets was low, and it would be useful to explore more strongly expressed alternative targets. Like Fernandez *et al.* (2024b), genes producing cytokines of likely significance to koala chlamydiosis: IFN-γ, IL-10, IL-17A (Mathew, 2014; Mathew *et al.*, 2013a), were frequently not quantifiable. Although another study using the same quantification method demonstrated low yet detectable levels of IL-10, IL-17A, IL-4, and IFN-γ in incubated, non-stimulated PBMCs of 10 northern koalas (Olagoke *et al.*, 2020b), no additional health or demographic information was provided in that study to determine why this difference in gene transcription may exist. RNA transcriptomics would be the best method to identify alternative, more strongly expressed genes of relevance.

Overall, NanoString determination of gene transcription was a useful, cost effective, research tool to identify the relationships among measurable markers in population-based studies to assess health status. In the clinical setting, where individual animals are the focus, it is less useful because of its large batch format and timeframes involved in processing. We suggest that a way to apply these indicators could be through integration of select qPCR targets within in a scoring system, similar to those used to predict disease severity cytokine storms in COVID-19 patients (Ndoricyimpaye *et al.*, 2023; Reiff & Cron, 2021), predict survival and response to therapy in cancers (Mezheyeuski *et al.*, 2023), and in critically ill patients with systemic

inflammatory response syndrome (Shimazui *et al.*, 2017). TRIM24 and IL-18 appear to be strong candidates for further investigation of potential host-response scoring systems in the koala.

3.7 Conclusion

The PCA clustering approach used in this study allowed the large number of potentially relevant variables to be incorporated in analysis and thereby identified relationships among major pathways and systems, such as lymphocytic responses, and significant indicators of health and disease outcomes, such as *TRIM24*. New perspectives of the factors associated with clinical variations within the koala developed here can be used to support disease risk assessment and management. Mechanistic investigations are required to determine the funcitonal roles of markers identified within this study, and test their involvement in the pathogenesis of various morbidities including chlamydiosis, trauma, and neoplasia. Through the identification and manipulation of these mechanisms, improved treatment outcomes and disease mitigation strategies can be developed.

Author contributions:

Yasmine S.S. Muir: Conceptualization, methodology, data generation and curation, investigation, formal analysis, visualization, writing – original draft preparation. **Belinda Wright**: Resources, data curation. **Andrea Casteriano**: Resources. **Valentina S. A**. **Mella**: Validation, writing – reviewing and editing. **Mark B. Krockenberger**: Supervision, writing – reviewing and editing. **Damien P. Higgins**: supervision, writing – reviewing and editing.

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Conflict of interest:

This work forms a component of the Australian Government National Koala Health Research Initiative, which is aimed at filling key knowledge gaps to produce immediately applicable outcomes for koala conservation. The sponsors had no role in the design, execution, interpretation, or writing of the study. The authors declare no competing interests.

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Chapter 4 Koala pro-inflammatory genes *IL1R2, MARCO, MYO1B,* and *RARRES1* show upregulated expression in koalas with clinical chlamydiosis and in those that are euthanised.

4.1 Author contribution statement

Yasmine S. S. Muir is the main author of this study and has contributed towards every component of the research including the conceptualisation, field work and data curation, methodology, formal analysis, investigation, writing of the original draft and reviewing and editing of the manuscript. Damien P. Higgins and Mark B. Krockenberger supervised and contributed to the conceptualisation, investigation, writing and reviewing of the manuscript and provision of research resources. Belinda R. Wright contributed to the conceptualisation, methodology and data curation, the provision of research resources, writing and reviewing of the manuscript. All authors have read, contributed, and agreed to the final version of the manuscript.

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28/2/2025

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28/2/2025

4.2 Abstract:

Chlamydiosis is an important disease affecting koala health. Currently, the understanding of host responses at different stages of chlamydial pathogenesis in this wildlife species is limited. To identify candidate biomarkers of health and pathogenesis, this study performed RNAseq transcriptomic analysis on 49 buffy coat samples obtained from koalas admitted to hospitals in New South Wales and Queensland. Here, we firstly validate the use of NanoString for koala gene mRNA quantification and through this, corroborate the low expression of key immune genes, *IL10, IL17A, IFNG*, and *TNF* found previously. Unfortunately, in this study we found no correlation between *IL10, IL17A, IFNG*, and *TNF* gene expression and the expression of their associated receptor genes, indicating that other methods should be explored to quantify lowly expressed cytokine genes in koala circulation. Then we identify *IL1R2, MARCO, MYO1B*, and *RARRES1* as genes that are upregulated in koalas with chlamydiosis and those that are euthanised. The findings of this study provide the foundation to further investigate the roles of various immune genes and cellular pathways potentially involved in koala chlamydiosis.

4.3 Introduction:

The koala (*Phascolarctos cinereus)* is an iconic marsupial of Australia, now classified as endangered in its northern range (Queensland [Qld], New South Wales (NSW), and Australian Capital Territory [ACT]) (DCCEEW, 2022b). This species is currently experiencing local and regional population declines due to collective pressures from disease, vehicle strikes, domestic & feral animal attacks, climate change, habitat destruction and fragmentation (DCCEEW, 2022a; Dissanayake *et al.*, 2023; McAlpine *et al.*, 2015). The investigation of koala disease has largely targeted chlamydiosis and putative koala-retrovirus (KoRV)-associated

diseases (Kayesh *et al.*, 2020b; Madden *et al.*, 2018; Quigley & Timms, 2020; Zheng *et al.*, 2020b). Chlamydiosis, caused by the intracellular obligate bacterium *Chlamydia pecorum*, is the leading infectious disease currently affecting koalas and is also endemic in the koalas' northern range (Vitali *et al.*, 2023). Chlamydiosis presents most commonly as inflammatory and fibrotic urogenital and/or conjunctival pathology, however these presentations range in severity from sub-clinical or mild-moderate inflammation to severe, chronic and irreversible fibrotic structural damage resulting in blindness, incontinence, renal failure, infertility, septicaemia and/or death (Vogelnest & Portas, 2019). Recent discovery of cases with circulating *C. pecorum* but without mucosal *C. pecorum* or clinical disease (Chapter 2, this thesis) presents the hypothesis that beneficial host-*C. pecorum* interactions might exist and may utilise different immune pathways.

Host genes are often used as biomarkers to represent host responses to infection, disease, or clinical interventions. However, the fundamental biology associated with the activity of these genes in the koala, including knowledge of their interrelationships and functional dynamics, is limited and this impedes our understanding of pathogenesis and development of health indicators. Numerous immunological targets have been identified in various koala tissues using *in vivo* and *in vitro* methods such as immunohistochemistry and flow cytometry (Canfield *et al.*, 1996; Hemsley & Canfield, 1997; Hemsley *et al.*, 1995, 1996; Higgins, 2004; Higgins *et al.*, 2004; Mangar, 2018; Mangar *et al.*, 2016; Palmieri *et al.*, 2019), gel electrophoresis and chromatography (Wilkinson *et al.*, 1992a; Wilkinson *et al.*, 1992b), ELISA (Higgins *et al.*, 2005a; Khan *et al.*, 2016; Nyari *et al.*, 2019; Olagoke *et al.*, 2018; Olagoke *et al.*, 2019; Olagoke *et al.*, 2020a; Quigley *et al.*, 2023), cytometric analysis (Lau *et al.*, 2012), and quantification of mRNA transcripts (Abts *et al.*, 2015; Chen *et al.*, 2023c; Desclozeaux *et al.*,

2017; Fernandez *et al.*, 2024a; Kayesh *et al.*, 2020a; Kayesh *et al.*, 2022; Kayesh *et al.*, 2021b; Khan *et al.*, 2016; Lizárraga *et al.*, 2020b; Maher *et al.*, 2014; Maher & Higgins, 2016; Maher *et al.*, 2019; Marschner *et al.*, 2019a; Mathew, 2014; Mathew *et al.*, 2013a; Mathew *et al.*, 2013b; Mathew *et al.*, 2014; Morris *et al.*, 2014; Morris *et al.*, 2015a; Nyari *et al.*, 2019; Olagoke *et al.*, 2020b; Quigley *et al.*, 2023; Simpson *et al.*, 2023). Some, such as mRNA expression of cytokine genes (*IFNG*, *TNFalpha*, *IL17, IL8, IL6, IL10*), NOD-like receptor genes (*NCF12* & *NOX2*), cell-surface marker genes (*CLEC4E*, *CD4*, *CD8b*), and MHC alleles have all been assessed in relation to infection and/or vaccination of koalas with either *C. pecorum* (Desclozeaux *et al.*, 2017; Khan *et al.*, 2016; Kidd *et al.*, 2024; Lizárraga *et al.*, 2020b; Mathew *et al.*, 2013a; Mathew *et al.*, 2013b; Mathew *et al.*, 2014; Nyari *et al.*, 2019; Pagliarani *et al.*, 2024; Phillips *et al.*, 2024b; Silver *et al.*, 2022; Simpson *et al.*, 2023), or KoRV (Kayesh *et al.*, 2020a; Kayesh *et al.*, 2021b; Olagoke *et al.*, 2020b), or both (Fernandez *et al.*, 2024a; Quigley *et al.*, 2023; Robbins *et al.*, 2020). While the individually investigated targets may be useful and can be confirmed by this study, taking a transcriptomics approach may help to identify key linkage points across these networks of gene transcription.

In koala disease investigations, the most assessed immune genes include the cytokines IL-10, IL-17, IFN-γ, and TNF-α due to their significance to chlamydiosis in other species (Gervassi *et al.*, 2004; Gondek *et al.*, 2009; Jendro *et al.*, 2004; Natividad *et al.*, 2007; Rank *et al.*, 2010; Rixon *et al.*, 2022; Schrader *et al.*, 2007; Vats *et al.*, 2007; Xiang *et al.*, 2021; Yang *et al.*, 1999; Yeung *et al.*, 2017), and their expression is measured most commonly in mitogen- or antigen-stimulated samples (Desclozeaux *et al.*, 2017; Khan *et al.*, 2016; Lizárraga *et al.*, 2020a; Maher *et al.*, 2014; Maher & Higgins, 2016; Mathew, 2014; Mathew *et al.*, 2013a; Mathew *et al.*, 2013b; Mathew *et al.*, 2014; Nyari *et al.*, 2018; Simpson *et al.*, 2023). The analysis of gene

expression within unstimulated samples potentially provides a more accurate indication of real-world interactions driving fluctuations in immune responses in the animal at that time (Negash *et al.*, 2024), but quantification of gene targets in unstimulated samples can be challenging due to their low abundance (Fisher *et al.*, 2020). For example, multivariate mRNA quantification hybridization microarrays, such as NanoString (NanoString Technologies, Seattle, WA) (Alijagic *et al.*, 2025; Kim *et al.*, 2018; Kretzschmar *et al.*, 2024; Kulkarni, 2011; Tan *et al.*, 2025; Zarinsefat *et al.*, 2025), offer a holistic method of analysis of koala immune responses that incorporate real-life complexity and generate clearer relationships with disease outcomes compared to single-gene analyses (Fernandez *et al.*, 2024b). As observed in the previous chapter and other studies, due to the assay's higher limit of quantification, detection of genes with low expression such as *IL10, IL17, IFNG,* and *TNFalpha*, has met with variable success (Fernandez *et al.*, 2024b; Quigley *et al.*, 2023). Although other techniques with lower limits of quantification may be used to quantify gene expression, as was done by Fernandez *et al.* (2024b) using previously validated RTqPCR methods (Maher *et al.*, 2014; Mathew *et al.*, 2013a; Mathew *et al.*, 2013b; Mathew *et al.*, 2014), this is labour intensive for multiple genes.

Measuring cytokine receptors could be an alternative approach to understand the activity of their cytokine ligands if their expression is correlated, however this is currently unknown for the koala. As reviewed by (Pestka *et al.*, 2004), classically, IL-10 activity should lead to activation of the IL-10 alpha receptor (IL10RA), and a positive relationship representing this mechanism has been observed in human neoplastic tissue (Zadka *et al.*, 2018). As an inhibitor of T-helper 1 cell functions, studies have also suggested that IL-10 activity leads to decreased TNFα and IFN-γ production (Balcewicz-Sablinska *et al.*, 1999; Gazzinelli *et al.*, 1992; Shibata *et*

al., 1998; Veenbergen *et al.*, 2019), and conversely pro-inflammatory TNFα and IFN-γ inhibit *IL10* gene transcription (Donnelly *et al.*, 1995). In an inflammatory bowel disease *in vitro* model of human colonic mucus, IFN-γ treatment upregulated the IFN-γ receptor 1 (*IFNGR1*) and TNFα receptor 1 (TNFR1, also referred to as TNFRSF1A) gene transcription (Johnson *et al.*, 2022). In the context of human tuberculosis, *IL10*, *IFNGR1*, and *TNFRSF1A* genes shared genetic regulation and were associated with TNFα gene expression in Mtb culture filtrate (Stein *et al.*, 2007). Additionally, a relationship between IL-17, IL-10 and TNF gene family has been observed; TNFR-associated factor (TRAF) 6 and IL-17E (also termed IL-25) receptor (Maezawa *et al.*, 2006), and the IL-10 receptor and Th17 cell responses (Chaudhry *et al.*, 2011; Huber *et al.*, 2011), which may be reflected in their gene expression. It is therefore necessary to assess the relationships amongst cytokines and cytokine receptors specifically in the context of koala chlamydiosis.

Using RNA transcriptomics, all known gene transcripts within a sample can be analysed together to determine average expression levels among koalas and identify genes differentially expressed between key groups. Two studies previously utilised RNA sequencing (RNAseq) to determine the differential expression of genes in conjunctival tissue between 26 koalas with and without ocular disease (Johnson *et al.*, 2018), and in lymph node tissue between 29 koalas originating from “southern” and “northern” genotypes (Tarlinton *et al.*, 2021). Collectively, the findings from these studies show that gene expression significantly differs between disease groups and between populations, demonstrating the use of this technique to increase our understanding of circulating host pathways associated with chlamydiosis.

This study has three main aims. Firstly, the transcriptomic dataset generated here was used to validate the mRNA counts for non-pathogen genes previously assessed using NanoString in Chapter 3 of this thesis and in another study (Fernandez *et al.*, 2024b). Secondly, we compared receptor gene expression with their respective cytokine gene expression for cytokines important to chlamydiosis (*IL10*, *IL17A, IFNG*, and *TNFalpha*) that had previously reported low mRNA counts in unstimulated samples (Fernandez *et al.*, 2024b). Finally, following the identification of an association between improved clinical outcomes and upregulated lymphocytic gene and *TRIM24* (tripartite motif-containing 24) gene transcription (Chapters 2 and 3), this study seeks to determine whether full gene expression differs significantly between (1) koalas with and without signs of chlamydiosis, and (2) koalas that were euthanised compared to released.

4.4 Methods:

Animals

Samples and data used in this study were collected under the University of Sydney Animal Ethics Approval Number 2021/1975, NSW NPWS Scientific License SL102379 and Qld NPWS WA0019256, in conjunction with two other related studies (Chapter 2 and Chapter 3, this thesis). Those studies investigated the significance of co-infections (Chapter 2) and immune response profiles to disease and rehabilitation outcomes (Chapter 3) in koalas admitted to one of three wildlife hospitals: Australia Zoo Wildlife Hospital (AZWH), Qld, Friends of the Koala (FOK), Lismore, NSW, and Port Macquarie Koala Hospital (PMKH), NSW. Overall, 57/115 cases had samples available for RNAseq and formed comparable subsets of koalas (1) with

and without chlamydiosis (chlamydiosis comparison), and (2) koalas that were euthanised or survived triage (outcome comparison) (Table 4.1).

Table 4.1: Case characteristics among sample population and according to comparison groups. *Values are reported as counts with the associated percentage of the respective group in brackets.*

		Sample population	**Comparison 1 (N = 36)[1]**		**Comparison 3 (N = 49)[2]**	
		N = 49	**Chlamydiosis (N = 22)**	**None (N = 14)**	**Survived (N = 26)**	**Euthanised (N = 23)**
Age group	*Young Adult*	18 (37)	6 (27)	8 (57)	11 (42)	7 (30)
	Adult	28 (57)	15 (68)	6 (43)	14 (54)	14 (61)
	Mature	3 (6)	1 (5)	0 (0)	1 (4)	2 (9)
Sex	*Male*	21 (43)	5 (23)	9 (64)	16 (62)	5 (22)
	Female	28 (57)	17 (77)	5 (36)	10 (38)	18 (78)
Body condition score	*≤ 2*	8 (16)	8 (36)	0 (0)	1 (4)	7 (30)
	> 2	41 (84)	14 (64)	14 (100)	25 (96)	16 (70)
Syndrome	*None*	14 (29)	0 (0)	14 (100)	14 (54)	0 (0)
	Chlamydiosis	22 (45)	22 (100)	0 (0)	6 (23)	16 (69)
	Trauma	11 (22)	0 (0)	0 (0)	6 (23)	5 (22)
	Other	2 (4)	0 (0)	0 (0)	0 (0)	2 (9)
Mucosal *C. pecorum* shedding	*Detected*	22 (45)	18 (82)	1 (7)	5 (20)	17 (74)
	Not detected	27 (55)	4 (18)	13 (93)	21 (80)	6 (26)
Outcome	*Survived*	26 (53)	6 (27)	14 (100)	26 (100)	0 (0)
	Euthanised	23 (47)	16 (73)	0 (0)	0 (0)	23 (100)

*NA: cases without results
[1]Comparison 1: comparison of genes between koalas with chlamydiosis and those with no signs of disease
[2]Comparison 2: comparison of genes between koalas that were euthanised and those that survived triage

Briefly, cases used within the three comparisons were defined using clinical data from triage veterinary examinations and gene expression data (Table 4.1). Cases that presented with clinical signs attributed to *C. pecorum* infection were classified as 'chlamydiosis'; these included disease of the urogenital tract (cystitis, renal abnormalities, and reproductive disease) and/or ocular sites (conjunctivitis or keratoconjunctivitis) (Canfield, 1989; Griffith, 2010; Nyari *et al.*, 2017; Pagliarani *et al.*, 2022; Pagliarani *et al.*, 2024; Palmieri *et al.*, 2019; Polkinghorne *et al.*, 2013; Speight *et al.*, 2016; Wan *et al.*, 2011). Cases were also characterised by mucosal *C. pecorum* shedding, mucosal PhaHV-1 shedding, mucosal PhaHV-2 shedding, circulating *C. pecorum,* circulating PhaHV-1 or PhaHV-2 transcription, circulating KoRV B transcription, and circulating trypanosome transcription (Supplementary materials

Table 7.18) (Chapter 2). Triage/Treatment outcomes were determined by veterinarians through case-specific examination and standard diagnostic results used to make informed clinical decisions based on welfare. Cases were euthanised due to poor prognosis, which included the presentation of disease- or trauma-derived irreparable structural damage, or complicated comorbidities concurrent with mature age (age > 10 years old) or emaciation.

Sample preparation and RNA extraction

Blood was collected from koalas and buffy coats prepared and stored in RNALater (Qiagen, Germany) as previously described (Chapters 2 & 3, this thesis). Total RNA was extracted using the Qiagen RNeasy Mini kit (Qiagen, Germany) according to the manufacturer's instructions. RNA extraction quality and yields were measured using both a NanoDrop ND-1000 160 Spectrophotometer and a Qubit® RNA HS Assay Kit (Q32852, Invitrogen, Thermo Fisher Scientific). In total, 57 samples with concentrations above 18 ng/mL were retained for analysis.

RNA extracts were DNAse treated using the TURBO DNA-*free*™ Kit (*Invitrogen™ # AM1907)* according to the manufacturer's instructions with the following modifications: 35 mL RNA extract is heated for 30 minutes at 37°C with 5 mL 10X reaction buffer and 10 mL Turbo DNase (2U/mL). Following this, 5 mL Inactivation agent is added then vortexed every 10-15 seconds over 2 minutes. Tubes were then spun at 10,000 x g for 3 minutes. The top 40 mL of the reaction mix was retained for downstream analysis and the remaining 15 mL was discarded.

Samples were adjusted using DNA/RNA free water to ensure there was less than 50 ng/mL in a total volume of 20 mL. Further quality control (QC) was completed prior to sequencing by

Ramaciotti Centre for Genomics (UNSW, Kensington, NSW, Australia) as follows. QC was conducted measuring purity with Epoch/Nanodrop spectrophotometer to identify samples outside the appropriate range of 260:280, i.e. <1.8 or > 2.2. RNA integrity was measured using either the Agilent TapeStation or Bioanalyser (Agilent Technologies, Santa Clara, CA, USA) to identify samples with desirable RIN values above 7. As a secondary RNA quality measure, the DV200 was calculated when the TapeStation was used. DV200 is a measure of the percentage of RNA fragments above 200 nucleotides. In total, 8 samples failed QC and were removed from further analysis due to irreparably poor RIN values (N = 5), low A260/A230 (N = 3), and one due to large fragments. We note here that the library preparation method can cope with a degree of RNA degradation and so even with a RIN < 7, if the DV200 is above 55% the protocol can be used. All retained samples had a DV200 percentage above 55% and total RNA per sample ranged from 128 ng to 1491 ng.

RNA library preparation and sequencing

Library preparation and sequencing were performed by Ramaciotti Centre for Genomics. RNA sequencing libraries were prepared using Illumina TruSeq Stranded Total RNA with Ribo-Zero Globin kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions, using 1ng total RNA as input. The first step of this protocol included both globulin-encoding mRNA and ribosomal RNA (rRNA) depletion steps to reduce non-target abundant transcripts. Samples were sequenced on a NextSeq 500 150cycle MID (Illumina, USA) run with expected output of ~130M reads, hence ~30M reads/sample. Library preparation for three samples resulted in low yields but were retained for analysis. Each library was indexed with a different sequencing barcode and were sequenced over 49 lanes, i.e. one lane per sample, with 7-10 million read pairs per lane.

Genome mapping and SNP calling

Raw sequences were assessed for quality using *fastQC* (Andrews, 2023). *Trimmomatic* was then used to clean adaptors and trim to 60 base pairs (bp) to remove bias observed within the first 12 bp and last 3 bp (Parameters used: ILLUMINACLIP: All_adaptors.fa:7:25:8:1:true/ SLIDINGWINDOW:20:28 HEADCROP:12 CROP:60 MINLEN:50) (Bolger *et al.*, 2014). Using adapted commands from Chew and Sadsad (2022), reads were mapped to the phaCin_unsw_v4.1 assembly (Johnson *et al.*, 2018) using the *STAR* script with default parameters (overhang of 149bp) (Chew & Sadsad, 2022; Dobin & Gingeras, 2015). *SAMtools* was used to merge and index the paired reads over lanes per sample and *RSeQC infer_experiments* was used as an additional quality check to confirm paired-end, strand-specific data (Li *et al.*, 2009; Wang *et al.*, 2012). The previous tool also confirmed the use of the reverse strand for *htseq count* (Anders et al., 2015). Finally, *bamstat* was used to generate read distribution results (Lindenbaum, 2015).

To investigate pathogen gene expression and validate the quantification of infectious agent gene targets using NanoString in previous chapters, reads were mapped to *C. pecorum*, KoRV, and PhaHV-1 assemblies. For *C. pecorum,* reads were mapped to both the CpecDBDeUG1.0 and CpecMC/MarsBar1.0 strain assemblies (Bachmann *et al.*, 2014). The ASM28885v1 assembly was used to map to KoRV (Hanger *et al.*, 2000), and the ASM1858315v1 assembly for PhaHV-1 (Vaz *et al.*, 2019a). For all infectious agents, the percentage of mapped reads was less than 1% and so the data was not usable.

Agreement between RNAseq and NanoString gene counts in non-stimulated samples

To validate the normalised mRNA counts of genes in unstimulated buffy coat samples previously observed using NanoString in Chapter 3 and in a study by Fernandez *et al.* (2024b), the RNAseq counts for the same genes were compared. Pearson's correlations for raw counts of each gene using the two methods were calculated and tested for and presented in a bar-chart. The average and median correlation across targets was calculated, as was the proportion of statistically significant correlations (using a significance threshold of $p < 0.05$). A scatter plot of the average counts for each gene using the two quantification methods was generated to demonstrate the trend between the results. On this plot, a 1:1 best fit line is plotted along with a regression line, which is calculated using the following formula: $y = a + b \times x$, where a is the intercept and b is the slope.

Comparison between lowly expressed key cytokine genes and their receptors

To determine whether cytokine receptors may be an appropriate alternative indicator of cytokine activity for cytokines that are transcribed below quantifiable levels in NanoString, cytokine receptor and cytokine gene expression was compared. For four key cytokine genes, *IL10, IL17A, IFNG, and TNFa*, which had low mRNA transcript counts in unstimulated buffy coat samples previously observed using NanoString in Chapter 3 and elsewhere (Fernandez *et al.*, 2024b), the expression of their respective receptor genes were investigated using RNAseq. In total, 11 individual genes were examined: *IFNG, IFNGR1, IFNGR2, IL10, IL10RA, LOC110214852 (IL10RB), IL17A, IL17RA, TNF, TNFRSF1A*, and *TNFRSF1B*. Firstly, the average normalised read counts among the whole sample population were assessed to compare those observed in *IL10, IFNG*, and *TNF* with other related genes. Although the average counts of *IL17A* are reported, due to low raw read counts *IL17A* was omitted from further investigation.

Average normalised counts were visualised using bar charts for the sampled population. Finally, to determine the relationships among the 10 genes and identify alternative genes that may be used to infer the activity of *IL10, IFNG*, and *TNF*, Pearson's correlations were generated and visualised in a matrix.

Gene expression analysis

To increase the ability to compare genes across samples, raw gene counts were pre-filtered to retain only genes where the total number of reads across all samples was greater than 5. Gene expression analysis was performed using the standard DESeq2 (version 1.42.1, alpha = 0.05) pipeline on the pre-filtered, raw counts from untrimmed paired-end mapped genes in R (version 4.0.3) (Love *et al.*, 2018; Love *et al.*, 2024). Read quality was assessed using the $\log^2$ transformed raw counts and then again on the DESeq element using dispersion plots. Two DESeq experiments were constructed based on the comparisons previously detailed, the first comparing (1) koalas with chlamydiosis (N = 22) and koalas without signs of disease (N = 14), and (2) koalas that were euthanised (N = 23) and koalas which survived (N = 26).

MA-plots were constructed to visualise the genomic data, evaluate the magnitude of fold changes, and assess the distribution relative to the mean expression using no shrinking and 'ashr' shrinking of the log2 fold changes (LFC) (Stephens, 2017). To account for low count genes, which exhibit high variance and bias measures of significance, the shrunken LFC results were used furthermore. The DESeq2 pipeline employs Benjamin-Hochberg (BH) procedure to control for the false discovery rate (FDR or q-value, 0.05) on the raw p-values generated through Wald testing, resulting in an adjusted p-value. For this study, significant differentially expressed genes for the three sets of comparisons are reported using the following

thresholds: adjusted p-value ≤ 0.01 and LFC cutoff = 0.58 (fold change of 1.5). Normalised differential gene expression results were visualised in box-plots and column plots using 'ggplot2', heatmaps using 'pheatmap', and volcano plots. Counts were normalised using the median of ratios method.

Using the koala genome annotation file available from NCBI under the assembly accession GCF_002099425.1, functions for significant differentially expressed genes in the three comparisons were obtained (Johnson *et al.*, 2018). DE genes were mapped to the gene ontology (GO) IDs from the annotated datafile by matching gene symbols. GO terms were further classified by ontology; biological process (BP), molecular function (MF), and cellular component (CC) by mapping GO IDs to ontology types using the *AnnotationDbi* org.Hs.eg.db database (Carlson *et al.*, 2019) and querying *GO.db* to add GO term descriptions and definitions. Due to small sample sizes within each DEseq model, GO term and pathway analysis could not be conducted.

4.5 Results:

Validation of NanoString gene mRNA transcription using RNAseq

Concordance was observed between RNAseq and NanoString data across targets, with an average Pearson correlation of 0.59, a median correlation of 0.73, and 79.5% of targets showing a significant correlation ($p < 0.05$) (Figure 4.1). On average, RNAseq counts were greater than those quantified using NanoString for the same genes (Figure 4.2).

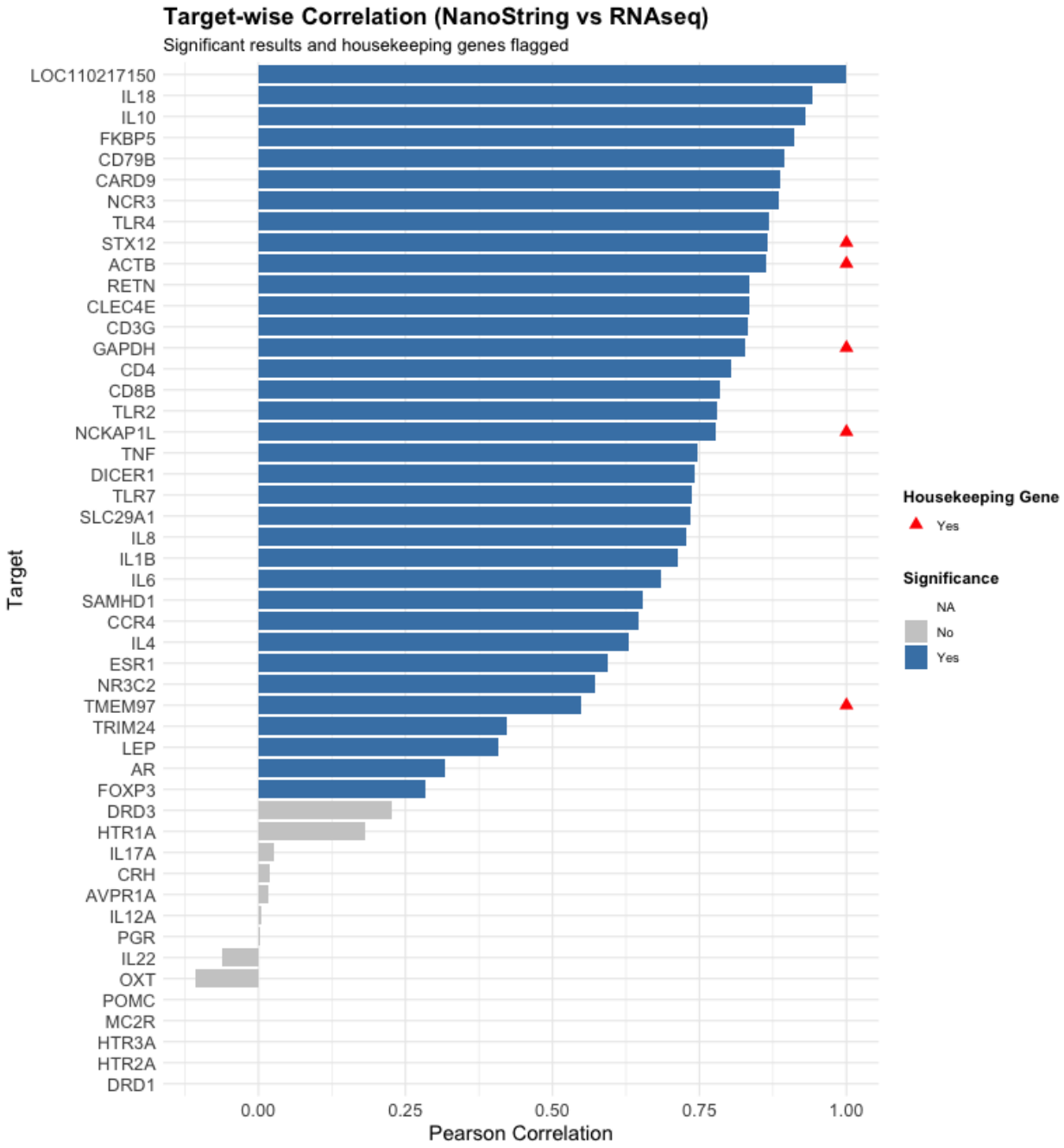

Figure 4.1: Gene target-wise correlation between NanoString and RNAseq counts.

Figure 4.1 presents a bar-plot of Pearson's correlation between NanoString and RNAseq counts for each non-pathogen gene included on the NanoString panel. Bars are colour coded according to statistical significance status: correlations which were statistically significant ($p < 0.05$) in blue, non-significant ($p > 0.05$) correlations are in grey, and targets without a Pearson's correlation result (i.e. NA) have no bar. NA results were due to RNA counts of 0 for all samples on RNAseq, for which no correlation could be calculated. Genes used as house-keeping genes on the NanoString panel are flagged with a red triangle.

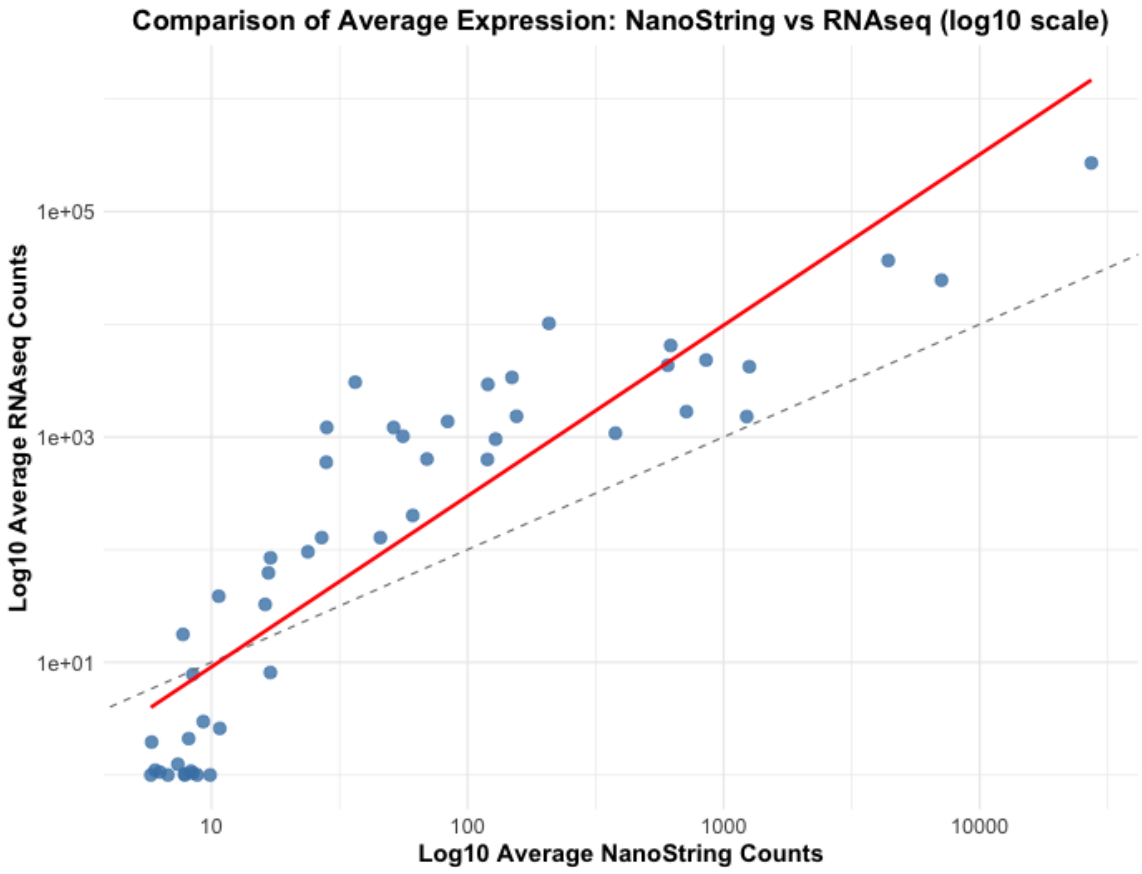

Figure 4.2: Comparison of NanoString gene mRNA transcription counts with gene expression counts from RNAseq.

The scatterplot in Figure 4.2 displays the Log10 transformed average counts for non-pathogen genes with NanoString results on the x-axis and RNAseq results on the y-axis. Two lines are plotted; a 1:1 line (dotted) that demonstrates the expected one to one ratio if RNAseq counts were equal to NanoString counts, and a second regression line (red) which presents the best-fit predictive relationship between the two methods.

Validation of cytokine genes expression in non-stimulated samples

Among all samples analysed using RNAseq, average normalised read counts for IL10, IL17A, IFNG, and TNF were 24.34, 1.04, 17.93, and 50.16. In comparison, the associated receptor genes of IL10, IFNG, and TNF had 51 – 309 fold greater average read counts, while the main receptor gene for IL17A, IL17RA, was expressed at read counts 7555-fold more than that of the ligand gene (Figure 4.3). Among all samples, IL10 gene expression was not significantly correlated with IL10RA nor IL10RB gene expression (Figure 4.3). However, a significant, weak

positive correlation existed between IL10 and IFNGR1 (r = 0.38, adj. p-value = 0.01). IFNG gene expression was not significantly correlated with IFNGR1 but had a significant weak negative correlation with IFNGR2 gene expression (r = -0.28, adj. p-value = 0.05). INFG positively correlated with TNF (r = 0.43, adj. p-value = 2.1e-03) and negatively correlated with TNFRSF1A (r = -0.37, adj. p-value = 0.01). Similarly, TNF gene expression did not significantly correlate with the expression of either of its receptor genes TNFRSF1A or TNFRSF1B.

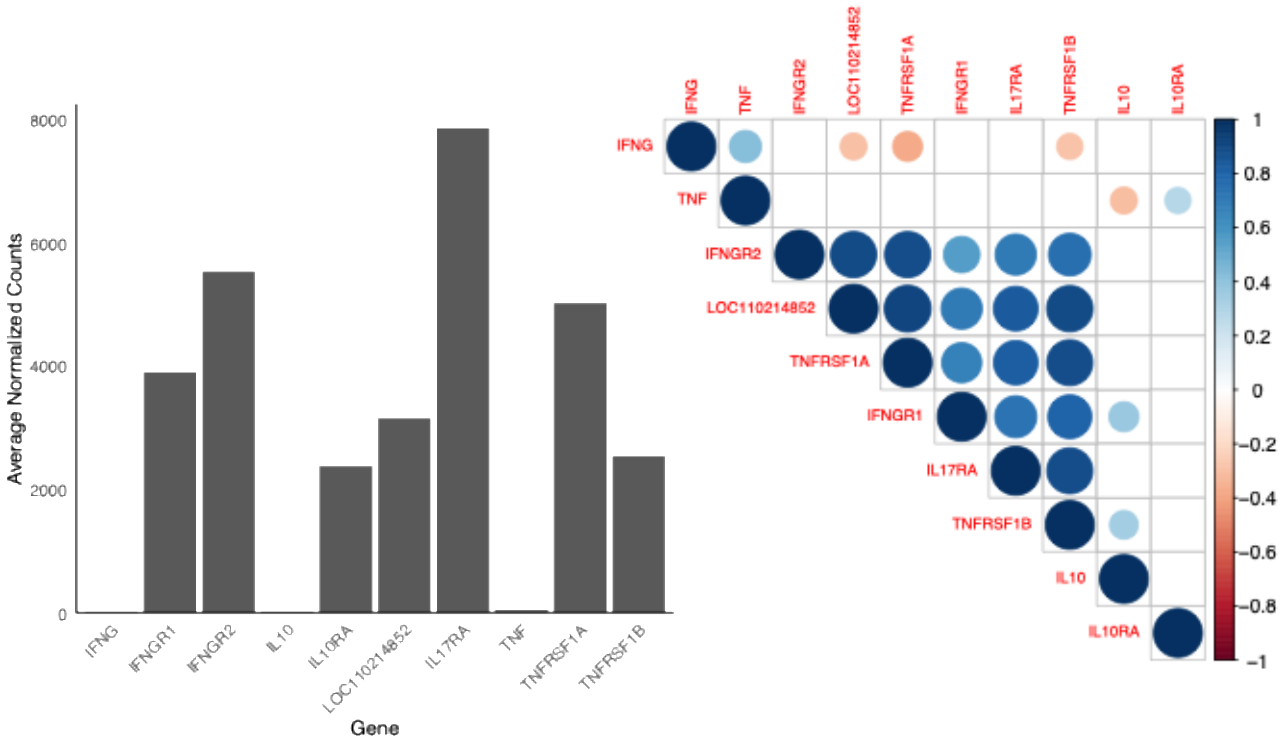

Figure 4.3: Column plot of and Pearson's correlations amongst normalised read counts for key cytokine IL10, TNFa, and IFNγ and their receptors.

Figure 4.3 presents the normalised read counts for IL10, TNF, and IFNG and their receptors along with a correlation matrix among the normalised read counts for these genes. Right: Column plot of the average normalised read counts among the whole sample population for key cytokine genes IL10, IFNG, and TNF and their receptors, including the IL17A receptor. Left: Pearson's correlation plot with hierarchical clustering of normalised gene counts for key koala immune genes IL10, IL17, TNFa, and IFNγ and their associated receptor genes. Non-significant (p-value > 0.05) correlations are not shown. Due to low read counts, IL17A was omitted from the plots.

Differential expression analysis: Comparison group characteristics

Health characteristics of cases included in the sample population and according to each respective comparison group are summarised in Table 4.1. In koalas with clinical chlamydiosis compared to koalas without clinical signs of disease, there was a greater proportion of *C. pecorum* at mucosal sites (χ^2 = 14.7, df = 1, p = $1e^{-04}$), and body condition scores equal to or below 2 (χ^2 = 4.6, df = 1, p = 0.03).

In euthanised koalas, compared to koalas that survived triage, there was a greater proportion of mucosal *C. pecorum* shedding (χ^2 = 12.62, df = 1, p = $4e^{-04}$), KoRV B *env* transcription (χ^2 = 7.39, df = 1, p = 0.006), of koalas with clinical chlamydiosis (χ^2 = 21.2, df = 4, p = $3e^{-04}$), of koalas with body condition scores equal to or below 2 (χ^2 = 4.5, df = 1, p = 0.03), and of females (χ^2 = 6.35, df = 1, p = 0.012). This likely results from the presentation of reproductive cysts (32%, 9/28) in females, which was also associated with euthanasia (χ^2 = 5.86, df = 1, p = 0.02).

Sequence assembly and pre-filtering

Overall, on average approximately 34.7% of reads across samples mapped to 25,743 genes of the koala genome. Across samples, the average number of reads that overlapped with an individual gene was 1230 (range 0 - 2,604,825). Of the mapped genes, 5148 (20%) were filtered out as they had a total number of reads across all samples less than 5. The distribution of read counts across samples, assessed as log2 transformed counts, were not identical (Figure 4.4). The median log2 counts was 6.98 (blue line) and the mean was 9.74. Lower than median counts were observed in two samples, DAWE_047 and DAWE_049. These samples were flagged for careful interpretation in downstream analysis.

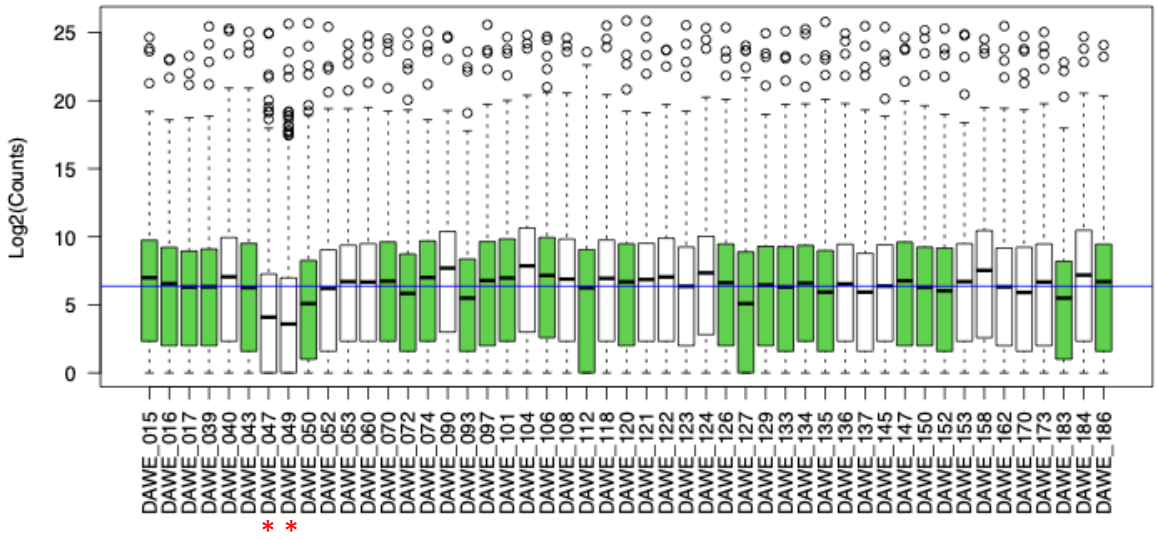

Figure 4.4: Box-plot distribution of raw counts, log 2 transformed, among samples coloured according to their outcome group: green = survived and white = euthanised.

In the box-plot distribution of raw counts displayed in figure 4.4, the median log2 count, 6.98, is plotted as a blue horizontal line. Red asterixis highlight two samples (DAWE_047 and DAWE_049) with counts lower than the median across all samples.

The dispersion plots for the three experiments are visually comparable, displaying similar fitted curves and shrinking of gene-wise dispersion estimates (Supplementary materials: Figure 7.1). Generally, the gene estimates fit well along the curve, with some data points unable to be fitted and are highlighted as potentially differentially expressed genes or outliers. A group of gene estimates that are outside of the fitted trends sit on the left middle of plot.

Normalised read counts ranged from 41,140,665 – 408,320,223 across all samples. The median and mean for normalised read counts was 88,267,669 and 99,437,939 respectively. Significant genes could be observed to be more normally distributed across the full range of expression using ‘ashr’ shrinking compared to no shrinking (Supplementary materials: Figure 7.2). The magnitude and distribution of fold changes relative to mean expression followed a

similar pattern between the two different comparisons. However, significant genes that were differentially expressed between koalas with chlamydiosis compared to koalas without chlamydiosis had smaller fold changes and distribution of fold changes compared to other comparisons.

Differential expression analysis

Following 'ashr' shrinking on the normalised counts in conjunction with an adjusted p-value and LFC threshold of 0.01 and 0.58, respectively, the number of differentially expressed genes between groups reduced compared to the original DESeq: from 185 genes to 27 genes between koalas with chlamydiosis and those without signs of disease, and from 976 genes to 210 genes between koalas that were euthanised and those that survived triage.

Differentially expressed genes between koalas with chlamydiosis and koalas without signs of disease: Chlamydiosis is associated with IL-1 signalling pathways and cell proliferation and migration.

After correction for multiple testing (adjusted p-value ≤ 0.01), there were 27 DE genes between koalas with chlamydiosis compared to koalas without chlamydiosis, of which 81% were upregulated and 19% were downregulated (Supplementary materials: Table 7.19). Genes upregulated in koalas with chlamydiosis were associated with inflammatory and immune responses such as interleukin-1 (type II) blocking receptor activity, cytokine activity and signalling pathways, lymphocyte, monocyte, and neutrophil chemotaxis, cellular response to type II interferon, interleukin-1, and tumour necrosis factor, chemokine mediated signalling pathways, positive regulation of ERK1 and ERK2 cascades, and positive regulation of GTPase activity, phagocytosis, engulfment, pattern recognition receptor activity and

receptor-mediated endocytosis. Of these genes, *IL1R2* was expressed at over 14.8-fold greater levels koalas with chlamydiosis (Figure 4.5A).

In koalas with chlamydiosis, genes associated with cell proliferation and migration were upregulated. In particular, the *MYO1B* gene was over 13.3-fold upregulated in koalas with chlamydiosis. This gene, called Myosin-1B, was associated with the biological processes vesicle transport along actin filaments and actin filament organisation. Other similarly upregulated genes were associated with protein binding, negative regulation of cell population proliferation, and metalloendopeptidase inhibitor activity, proteolysis and serine-type endopeptidase activity, intracellular signal transduction and phosphatidylinositol phosphate biosynthetic process.

Differentially expressed genes between euthanised koalas and those that survived triage: Koalas selected for euthanasia have increased expression of genes associated with immune responses, BMP signalling pathways, and anti-oxidation.

After correction for multiple testing there were 210 DE genes between koalas that were euthanised and koalas that survived (Supplementary materials: Table 7.20), of which 20% were downregulated and 80% were upregulated. The same genes associated with immune responses and cell proliferation in that were upregulated in koalas with chlamydiosis, compared to koalas without signs of disease, were also upregulated in euthanised koalas, compared to koalas that survived: *IL1R2*, *MARCO*, and *RARRES1* (Figure 4.5B). Furthermore, other genes associated with the immune response were upregulated in euthanised koalas and were associated with the following functions: complement cascade, complement receptor mediated signalling pathway, inflammatory response, antimicrobial humoral

immune response mediated by antimicrobial peptide, cellular response to lipopolysaccharide, chemokine-mediated signalling pathway, chemokine activity, neutrophil chemotaxis, innate immune response, and negative regulation of T cell proliferation. Interestingly there was opposing regulation of genes associated with bone morphogenic protein (BMP) signalling pathways that regulate cell proliferation, differentiation and apoptosis, although one gene was markedly upregulated: *SCUBE3* (Signal peptide, CUB domain and EGF like domain containing 3) 8.2-fold expression in euthanised koalas. Additionally, in euthanised koalas, compared to koalas that survived, genes functioning within NADP binding and glutathione peroxidase activity and glutathione metabolic process were upregulated.

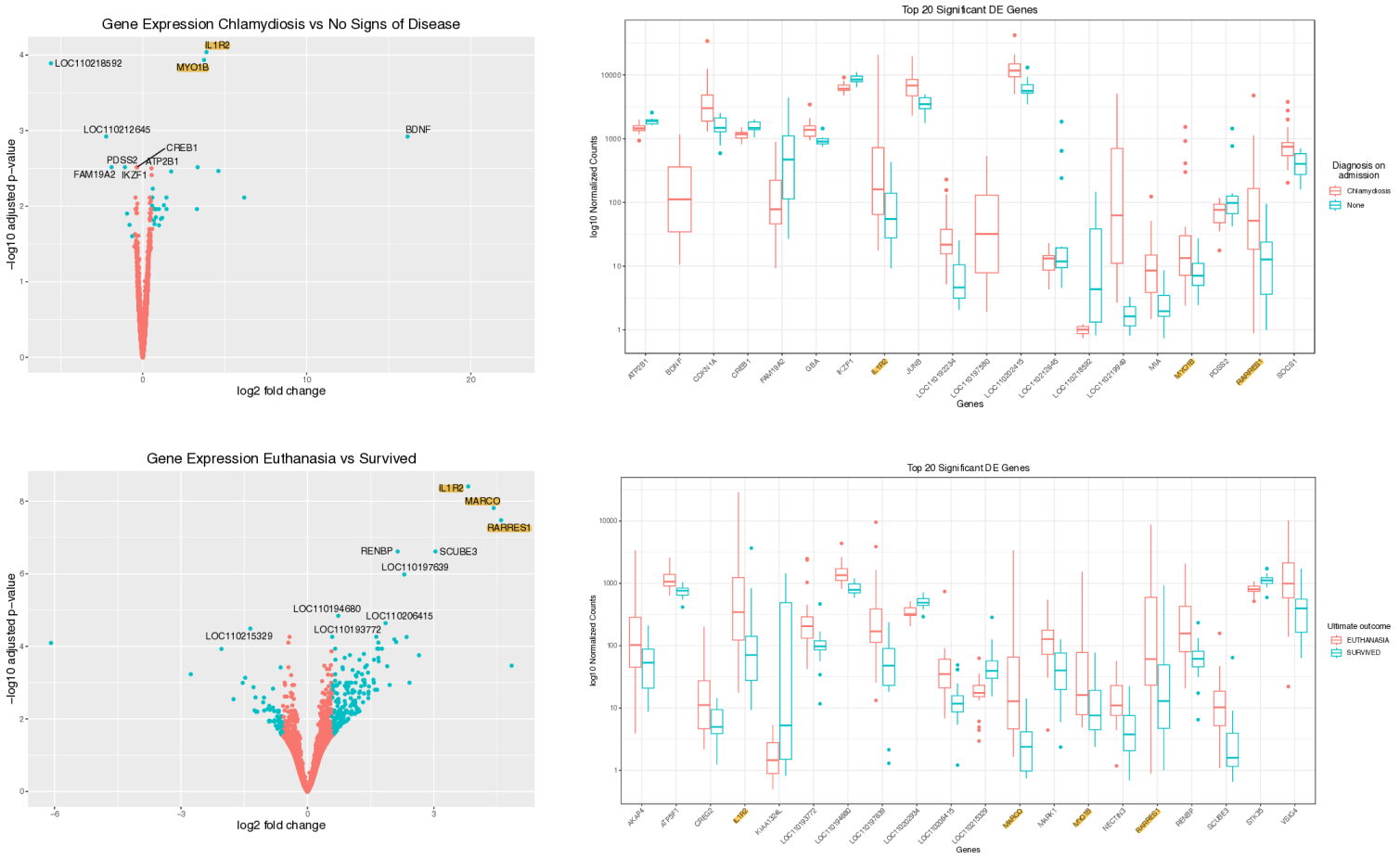

Figure 4.5: Volcano plots (left) of significant (blue points) and non-significant (red points) differentially expressed (DE) genes for the two comparisons.

Figure 4.5 demonstrates significantly differentially expressed genes among the three comparisons. (A) Koalas with chlamydiosis and koalas without signs of disease (first row). (B) Koalas that were euthanised and koalas that survived (second row). The x-axis represents the log2 transformed fold-change of gene expression between the comparison groups and the y-axis shows the -log10 adjusted p-value. The top 10 significant DE genes are labelled. Adjacent to each volcano plot are plots showing boxplots per comparison group of the log10 normalised gene counts (y-axis) for the top 20 DE genes, which are labelled in alphabetical order on the x-axis. Where presented, shared significant genes are highlighted in yellow (IL1R2, MYO1B, RARRES1, & MARCO). No plotting of a box-plot is indicative of no expression present in the group (BDNF & 110197580).

4.6 Discussion:

This investigation generated a transcriptomic dataset that achieved several aims and can be used as a resource for further investigations. The strong correlation between gene expression data generated using RNAseq and gene transcription (mRNA) counts derived from NanoString

nCounter methods suggests that NanoString is a robust and efficient tool for high throughput analyses. Low *IL10, IL17A, IFNG*, and *TNF* expression on NanoString was corroborated in RNAseq, and the lack of strong relationships with respective receptor genes furthers our understanding of cytokine gene relationships. Differential gene expression analysis among two comparisons (chlamydiosis status and survival outcomes) demonstrated significant differential expression of the genes *IL1R2, MARCO, MYO1B,* and *RARRES1* suggesting their potential functional significance in pathogenesis (Supplementary materials Table 7.21). This data helps to identify genes that may be used as indicators to monitor changes in the balance of the host response towards unfavourable (pro-inflammatory) clinical outcomes and higher risk of euthanasia. Similar to previous findings in Chapter 3 and in a previous study (Fernandez *et al.*, 2024b), overexpression of genes associated with the innate immune response and T-cell inhibition provide a broader perspective of the biology behind cases with poor prognosis.

Overall, gene transcription quantified using NanoString, a multi-gene mRNA hybridisation technique (Fernandez *et al.*, 2024b; Quigley *et al.*, 2023), is comparable to RNAseq gene expression. Gene-specific variation exists and is likely to be associated with the sample type. For example, many of the genes with weaker and/or insignificant correlations between the two methods may be better quantified in alternative tissue types, such as adipose tissue for endocrine genes *DRD1, DRD3, AR, ESR1, PGR, OXT, POMC, HTR1A, HTR2A, HTR3A, LEP, CRH* and *MC2R* (Ahmed *et al.*, 2025). Further validation of the pathogen targets used on the NanoString is necessary as mapping to *C. pecorum,* KoRV, and PhaHV-1 genomes did not result in analysable data using this sample set, which likely resulted from low frequency of detection and low abundance of the targets relative to other transcripts. Given the unknown significance of circulating *C. pecorum,* validation of these pathogen gene targets is an

important next step to allow for further optimisation of the NanoString method, epidemiological investigation, and prevalence monitoring.

The limited expression of important cytokine genes representative of classical immune pathways *(IL17A, IL10, IFNG*, and *TNF*) in unstimulated buffy coat samples using both NanoString and RNAseq quantification suggests that alternative methods may be required to determine their significance in pathogenesis. Although receptor genes were expressed at greater levels, *IL10, IFNG*, or *TNF* did not strongly correlate with the expression of their respective receptor genes, which is consistent with highly controlled and restricted expression of potent cytokines compared to their receptors in humans (Jiang *et al.*, 2021; Karlsson *et al.*, 2021; Valente *et al.*, 1992). The lack of correlation between cytokine ligands and their receptors suggests that, in circulating cells, (1) a better understanding of cytokine-receptor relationships is needed, and (2) alternative gene targets or sample sites should be investigated to represent these key pathways. A recent study employing RNAseq to assess gene expression in mucosal epithelium of koalas differed in their observations compared to those found here in blood samples, such as the upregulated expression of *IFNG* in koalas with chlamydial conjunctivitis (Phillips *et al.*, 2024b). Optimally, a comparison of gene expression between koalas that only show circulating *C. pecorum,* those that only show mucosal *C. pecorum,* and those that show both would provide a holistic perspective of the relationships or independence between these states and better inform the succession of events. Additionally, a comparison of cytokine and cytokine receptor expression across various tissues in healthy and diseased koalas would help to inform tissue-specific cytokine activity in pathogenesis.

IL1R2, MARCO, MYO1B, and *RARRES1* have emerged as potential biomarkers for mechanisms underlying disease severity in koalas. In koalas with chlamydiosis and in koalas that were euthanised, the upregulation of *IL1R2* suggests inhibition of IL-1 signalling either following IL-1B upregulation or due to inhibitory mechanisms of the innate immune response such as "M2" polarisation and production of anti-inflammatory cytokines IL-4, IL-13, and IL-10 (Colotta *et al.*, 1993; Colotta *et al.*, 1996; Dickensheets & Donnelly, 1997; Kalliolias *et al.*, 2010; Supino *et al.*, 2022). IL-1 signalling is potentially critical for driving host responses to *Chlamydia pecorum* infection. As a decoy receptor, IL-1R2 binds IL-1A, IL-1B, and IL-1R1 to suppress inflammatory activity, a mechanism observed in other chlamydial infections, such as *C. pneumoniae* (He et al., 2010; Shimada et al., 2011) and *C. muridarum* (Nagarajan et al., 2012). Dysregulation of this pathway can exacerbate inflammation and alter immune cell dynamics, increasing susceptibility to tissue damage and uncontrolled proliferation (Chen *et al.*, 2022; Oelmann *et al.*, 2015). In koalas with chlamydiosis and in koalas that were euthanised, the observed upregulation of *MARCO* (Macrophage Receptor with Collagenous Structure) in buffy coats may reflect shifts in inflammatory responses. *MARCO*, a scavenger receptor typically restricted to tissue-resident macrophages in the spleen, liver, and lymph nodes, plays roles in regulating toll-like receptor-induced inflammation, inducing mast cell TNFα production, and suppressing neutrophil infiltration (Kissick *et al.*, 2014; Kraal *et al.*, 2000). Its heightened expression in circulating monocytes may indicate macrophage activation or tissue damage facilitating macrophage escape into circulation, as suggested by recent studies detecting *MARCO* mRNA in human and murine monocytes (Getts *et al.*, 2014; Ingersoll *et al.*, 2010). Similarly, the upregulation of *MYO1B* (Myosin 1B) in koalas with chlamydiosis and in koalas that were euthanised could be reflective of immune cell infiltration, tissue hypoxia, and inflammation observed in cancer models like Arecoline-

associated oral carcinoma (Sun *et al.*, 2023) and several other cancers (Zhang *et al.*, 2018). Finally, increased expression of *RARRES1* (Retinoic Acid Receptor Responder 1) in koalas with chlamydiosis and in koalas that were euthanised may indicate renal damage and should be validated with histological analysis. This gene encodes a membrane protein critical to podocyte function, where overexpression is linked to podocytopenia, glomerulosclerosis, and apoptosis in glomerular diseases (Chen *et al.*, 2021; Feng *et al.*, 2024). Collectively, these findings are suggestive of an intense innate pro-inflammatory response that *MARCO*, *MYO1B*, *RARRES1* appear to be reflecting and *IL1R2* may be attempting to down-regulate. Longitudinal studies are required to confirm that these genes are upregulated post-damage or if this represents an earlier phase of the innate inflammatory response.

IL18 and *FKBP5* gene transcription may be important markers of koala chlamydiosis and clinical severity. Consistent with the previous findings in Chapter 3, in this study *IL18* and *FKBP5* gene transcription were upregulated amongst koalas with chlamydiosis, and in koalas that were euthanised, respectively. No other host gene included on the NanoString panel in Chapter 3 were identified as being significantly differentially expressed in the current study. This is likely due to the smaller sample set analysed here that was selected to have an equal representation of presence and absence of chlamydiosis disease and outcomes of euthanasia and survival. The identification of novel genes, despite the smaller sample size suggests their effect may be large and might be useful to investigate in future studies. For example, similar to the cell differentiation genes *CD4, CD8beta, CD3, CD79b*, and chemokines *CCR4* and IL8, which were on the NanoString panel, related genes *CD207*, *CD177, CD36, SOCS1, SKOR2, GPR1* and *GPR84* were differentially expressed in this study. These genes have yet to be

analysed in the koala and should be included in future panels to clarify their significance in the pathogenesis of chlamydiosis.

Further investigation of differential gene expression in the koala should prioritise maximising sample sizes with distinct comparison groups. This study was limited in the individual cases and samples available for analysis, resulting in overlap in the samples used to perform the two comparisons. Having distinct, independent comparison groups would reduce the risk of overlapping differentially expressed genes resulting from individual contributions rather than the dependent variables. The findings here should be corroborated using a larger sample set that can control for multiple confounding factors and variables of interest within the one DEseq model (Tarlinton *et al.*, 2024). With a greater sample size, analyses may then support further validation of NanoString panels and GO term and pathway analyses to determine specific system variations according to clinical and infection status in the koala.

4.7 Conclusion

This study identifies several potentially biologically meaningful genes, *IL1R2, MARCO, MYO1B*, and *RARRES1*, that appear likely to be involved in koala chlamydiosis and severe disease warranting euthanasia. While further corroboration of these findings is recommended due to small sample sizes, *IL1R2, MACRO, MYO1B*, and *RARRES1* are promising candidates for inclusion in future multivariate gene target panels. Results here suggest that NanoString is a comparable method for gene transcription quantification. Moreover, the validation of low circulating gene expression levels of *IL10, IL17A*, *IFNG*, and *TNF* highlights the need for alternative methods to support their quantification and further exploration. Overall, this

study lays a solid foundation for generating hypotheses that will underpin targeted mechanistic studies, ultimately guiding efforts to improve health outcomes for this species.

Ethics:

Sampling was conducted under the University of Sydney Animal Ethics Approval Number 2021/1975, NSW NPWS Scientific License SL102379 and Qld NPWS WA0019256.

Acknowledgements:

We would like to acknowledge the veterinary and nursing teams at the following wildlife clinics that contributed to the sampling and data acquisition for this study: Australia Zoo Wildlife Hospital, Friends of the Koala, and Port Macquarie Koala Hospital. This study was supported by the Australian Federal Government, Department of Agriculture, Water, and the Environment, as part of the Bushfire Recovery Multiregional Species Program. We also acknowledge the support from Wildlife Information Rescue and Education Service NSW (WIRES).

Data availability:

The raw sequence reads have been deposited in the NCBI Sequence Read Archive (SRA) under the BioProject accession PRJNA1261631.

Chapter 5 Multivariate analysis of host gene expression and population health determinants predict chlamydiosis treatment outcomes and long-term survival at two wild koala sites in south-east Queensland.

5.1 Author contribution statement

Yasmine S. S. Muir is the main author of this study and has contributed towards every component of the research including the conceptualisation, methodology, formal analysis, investigation, writing of the original draft and reviewing and editing of the manuscript. Damien P. Higgins and Mark B. Krockenberger supervised and contributed to the conceptualisation, investigation, writing and reviewing of the manuscript and provision of research resources. Belinda R. Wright and Andrea Casteriano contributed to the methodology, the provision of research resources, and reviewing of the manuscript. Deidré L. de Villers and Julien Grosmaire contributed to field work and data curation and reviewing of the manuscript. All authors have read, contributed, and agreed to the final version of the manuscript.

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28/2/2025

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28/2/2025

5.2 Abstract

Chlamydiosis, caused by *Chlamydia pecorum*, is the main infectious disease affecting the welfare and population dynamics of koalas. The understanding of chlamydial pathogenesis is increasingly complex as a growing number of co-infecting agents and other host, pathogen, and environmental factors are considered as possible contributors to disease outcomes and treatment success. To overcome the lack of long-term, post-treatment monitoring studies, this study monitored 221 koalas originating from two neighbouring sites over a 2-year period. The two sites are geographically separated by a river, and have differing morbidity, mortality and detection rates of infectious agents. Multivariate analysis was employed to find factors associated with survival and post-treatment outcomes. Greater transcription of adaptive lymphocyte associated genes was associated with survival among koalas with detectable mucosal *C. pecorum* and koalas treated for chlamydiosis, which were more frequent at the high morbidity site. In koalas from the low morbidity site, increased *KoRV* pol and exogenous KoRV D transcription increased the risk of mortality. Indicators of disease, treatment outcomes, and survival identified here offer new perspectives on risk assessment and mitigation.

5.3 Introduction

Treatment and rehabilitation are integral components of wildlife conservation and disease management. When effective, they can help support local populations by returning individuals to health and are an important opportunity for disease surveillance, diagnostic and therapeutic development, wildlife research, community engagement, and rectifying a population's path to local extirpation (Hanger, 2017; Tribe & Orr, 2019). The infectious

disease, chlamydiosis (caused by *Chlamydia pecorum*), has contributed to population declines in koala *(Phascolarctos cinereus)* populations, particularly throughout the northern part of the koala's range (Adams-Hosking *et al.*, 2016; Phillips *et al.*, 2021; Rhodes *et al.*, 2011; Robbins *et al.*, 2018; Silver *et al.*, 2022) where it is classified as endangered (DCCEEW, 2022a, 2022b; ICUN, 2022). Antimicrobial treatments for chlamydiosis are available (Booth & Nyari, 2020), however, their long-term success and the factors that determine survival of rehabilitated and non-rehabilitated koalas are poorly understood (Burton & Tribe, 2016; Kerlin *et al.*, 2022; Leigh *et al.*, 2023; Robbins *et al.*, 2018).

Historically, a paucity of longitudinal monitoring programs in wildlife health has led to a reliance on data generated by wildlife clinics, which report on treatment success in terms of admission and release rates, but only to the point of release (Dutton-Regester, 2024; Griffith *et al.*, 2013; Griffith & Higgins, 2012; Queensland Government, 2021). Whole population veterinary management provides an opportunity for longitudinal study of risk factors in different contexts. For example, longitudinal monitoring studies of a koala population in the Moreton Bay Region, Queensland, demonstrated the efficacy of the standard antimicrobial treatment regime for chlamydiosis (Robbins *et al.*, 2019), and described several outcomes that have been reported previously in wildlife clinics but are not well understood: development of additional illness during treatment, warranting euthanasia; poor response to treatment; recrudescence, and development of iatrogenic conditions post-treatment (Booth & Nyari, 2020; Griffith & Higgins, 2012; Jelocnik, 2019; Jelocnik *et al.*, 2017; Jelocnik *et al.*, 2014; Robbins *et al.*, 2018; Wilson *et al.*, 2015). These studies, and findings in Chapters 2 and 3 of this thesis, point to diverse interactions amongst hosts, pathogens, and environments resulting in varying health outcomes for koalas, suggesting co-infections and host gene

processes should be evaluated in different contexts to better predict the most effective approaches to disease management.

In koalas, the limited understanding of co-infection interactions, diversity in immune responses, and how these factors are affected by the environment, warrants further investigation to understand the differences in disease and treatment responses. In other species, wildlife disease management has been improved by targeting co-infections and identifying individuals and populations at risk using advanced indicators of health (Alizon & van Baalen, 2008; Ezenwa *et al.*, 2010; Ezenwa & Jolles, 2011; Joseph *et al.*, 2013; Lehman *et al.*, 2020; Miller *et al.*, 2017; Miller *et al.*, 2014; Mordecai *et al.*, 2020; Teffer *et al.*, 2019; Teffer *et al.*, 2022). In the koala, previous studies have demonstrated the effect of co-infection status and host gene expression profiles on clinical, triage and post-treatment outcomes in rehabilitation (Chapters 2, 3, and 4 in this thesis) and population health status (Fernandez *et al.*, 2024b). Before these factors are incorporated into routine health assessments, additional investigations are needed to determine the consistency of relationships within different wild populations and contexts.

This study utilises an ongoing comprehensive longitudinal koala monitoring program to investigate the relationships between immune responses, co-infections, disease, treatment response, and survival in 221 koalas originating from two neighbouring sites with differences in morbidity. Using long-term monitoring data, we assess the role of host demographics, co-infection status, immune and stress-associated gene transcription profiles on treatment outcomes. These relationships are expected to enable more effective koala population management procedures (such as risk-assessment, reintroductions, relocations, and breeding

initiatives) and inform population viability and disease mitigation modelling for these populations.

5.4 Methods:

Ethics statement

This study is supported by the Department of Transport and Main Roads (TMR), Queensland Government, through Endeavour Veterinary Ecology Pty (ABN: 21 661 108 916). Animal Ethics was obtained by Endeavour Veterinary Ecology Pty Ltd: Qld Govt DAF: CA 2019/04/1278 and CA 2022/03/1595 Latest Date: 25/04/2025. Sampling was conducted under the research permit obtained by Endeavour Veterinary Ecology Pty Ltd through the department of Environment and Science, under the Nature Conservation (Animals) Regulation 2020: WA20210702-1.

Study sites:

Sampling took place from August 2021 to August 2023 within a koala management program (KMP) in a region of South East-Queensland's Gold Coast from Nerang through Coomera to Pimpama managed by Endeavour Veterinary Ecology (Figure 5.1). The KMP involved capture, veterinary management, and ongoing telemetric monitoring of koalas, with relocation of koalas, located within a development corridor, to other suitable koala habitat. Koalas were captured for veterinary examination every 6 months, or earlier in cases of poor health status. Apart from the following alterations, koala monitoring, clinical examination and treatment protocols were identical to those adopted and described for a previously reported project (Robbins et al., 2018): clinic-based loop-mediated isothermal amplification (LAMP) testing of ocular and urogenital swabs was employed to detect *C. pecorum* shedding according to the

protocols described by Hulse *et al.* (2019c), anti-chlamydial chloramphenicol treatments (Chloramphenicol 150, Ceva, Glenorie, NSW) consisted of a 60mg/kg subcutaneous dose once a day for 14 days, all koalas in care received yoghurt and/or Yomogi® (Pharma-Zentrale GmbH, South Australia, Australia) supplementation, and oral Nystatin (Nilstat®, AUST R 48569, Aspend, Australia) was utilised for fungal infections when required.

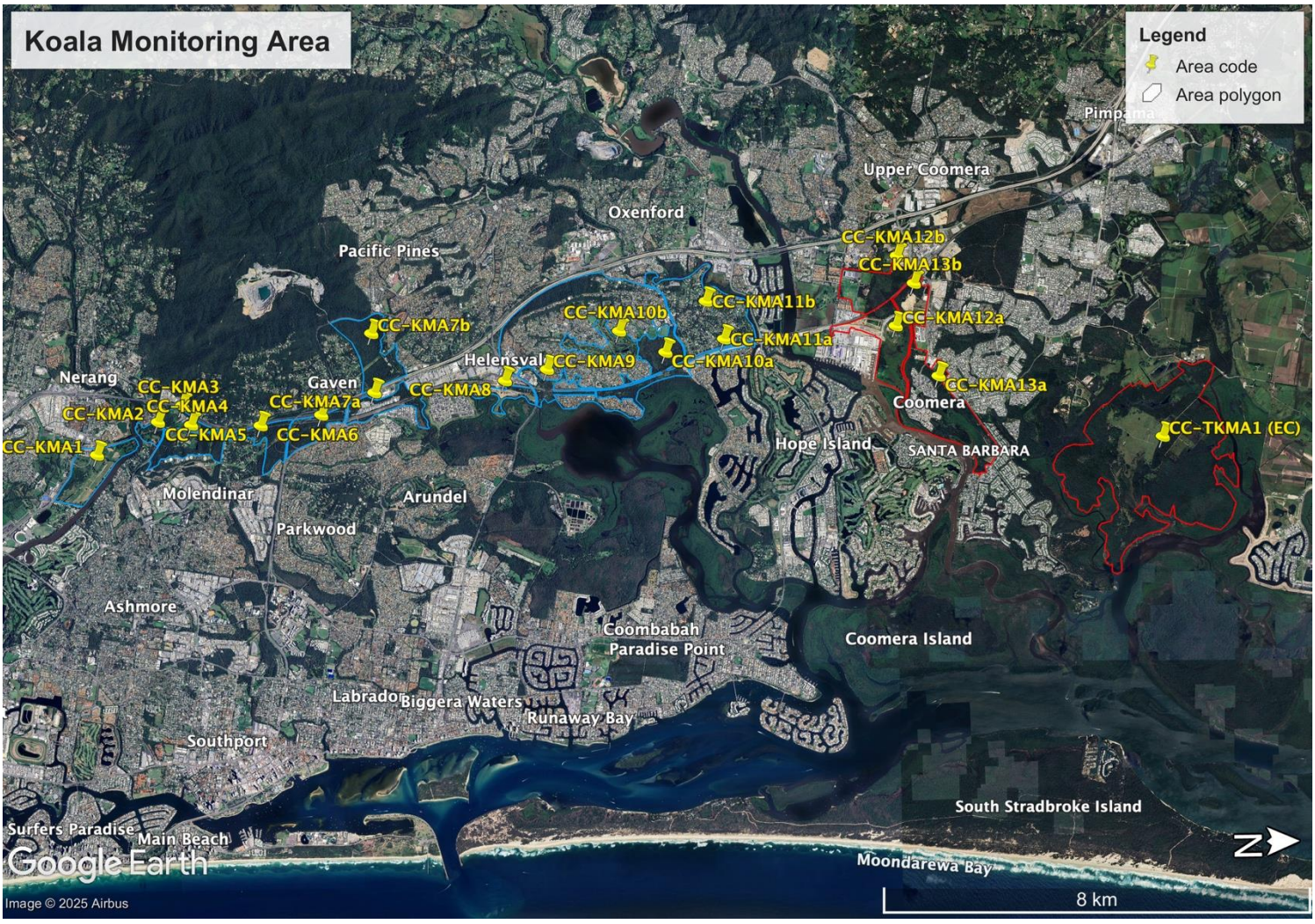

Figure 5.1: Study site map.

Koala management areas (KMA) are indicated by the yellow pins (area codes). KMA's areas (area polygons) within the 'low morbidity' site to the south of the Coomera River are distinguished by light blue borders and those with red borders indicate KMA's included in the 'high morbidity' site to the north of the river. An 8 km ruler is provided in the bottom right corner with the North arrow indicator shown directly above the ruler. Google Earth was used to generate this map.

Sampling

Sampling procedures were similar to those described previously in Chapter 3 and are briefly described here indicating any modifications. Sampling consisted of blood (2 mL) and urogenital, left and right ocular, and oropharyngeal swab collection under general anesthesia (GA). GA was conducted at the discretion of the veterinarian using alfaxalone induction (1–3 mg/kg IM) and Isoflurane maintenance with oxygen. Several pediatric sized tubes were utilised for blood collection as was appropriate for the type of centrifuge: DLAB Palm Micro Centrifuge D1008. Blood was dispensed into each of the following tubes: 1x 0.5 mL and 1x 1.0 mL EDTA, and 1x 0.5 mL serum (Vacuette® Tube, Greiner Bio-One GmBH, Kremsmünster, Austria). Following 20 minutes of gentle inversion, 250 µL of blood in EDTA was dispensed into a 1.5 mL cryovial containing 1 mL RNAlater™ (Qiagen) and another 250 µL of blood in EDTA was dispensed into an empty 1.5 mL cryovial. The remaining blood in EDTA and serum tubes were centrifuged for 1 minute at 7000 rpm (DLAB Palm Micro Centrifuge D1008), 300 µL of plasma dispensed into a 1.5 mL cryovial containing 1 mL RNAlater™ and remaining plasma was dispensed into an empty 1.5 mL cryovial. The buffy coat of 250 µL was then aspirated and dispensed into a 1.5 mL cryovial containing 1 mL RNAlater™. All swabs and samples in plain cryovials were stored immediately at -80°C, or temporarily at -20°C whilst sampling took place at outpatient clinics and transferred to -80°C freezer at the main clinic (Toorbul). All samples in cyrovials with RNAlater™ were stored at room temperature for 24 hours before storing at -20°C. Samples were couriered at -80°C (All swabs and blood samples in plain cryovials) using dry ice and -20°C (all blood samples in cryovials with RNAlater™) to the University of Sydney where they were stored at the respective temperature until processing.

Overall, 221 individual koalas were sampled for this study, with 86% of first samples coinciding with the koalas first veterinary examination (i.e. Time-zero, T = 0), and 14% associated with mid- or end of treatment timepoints. Of the samples taken from the earliest sampling event available for extraction, not all sample types were able to be taken for each koala during sampling, with welfare of the koala prioritised by the treating veterinarian (Table 5.1). Due to differences in sampling constraints for male and female koalas, where additional swab samples were obtained from females compared to males to support multiple research projects, a greater female representation was obtained (Table 5.2). This was consistent between the high morbidity and low morbidity populations.

Table 5.1: Time-zero samples available for DNA or RNA extraction from the sample population (n = 221 koalas)

	Ocular/UGT DNA Extracts		Oropharyngeal DNA Extracts		Buffy Coat RNA Extracts	
	Count	**%**	**Count**	**%**	**Count**	**%**
Total	195	88.2	182	82.3	169	76.5

** Aim was to acquire information about infection and disease status prior to application of treatment. However, 13% of samples were not taken at the time of the first exam (T = 0) – due to circumstances during examination some first samples were taken a few days later, mid-treatment, at the end of treatment prior to release, or at the next exam (in instances where treatments were postponed).*

Data obtained from the whole sample population (N = 221) at the earliest time-point for each case is used to report demographic, clinical examination and treatment outcomes, and detection results for infectious agents. For the remainder of the analyses, data obtained from samples taken at time-zero are considered (N = 191) and, of these, those with results for gene-expression analysis (127/191) are utilised for principal components analysis and the subsequent modelling.

Metadata

Clinical, rehabilitation and monitoring data collected by Endeavour Veterinary Ecology Pty Ltd (EVE) was used to identify factors of interest, characterise demographics according to site, and establish the frequencies of disease and treatment outcomes (Table 5.2). Demographics included site ('high morbidity' or 'low morbidity'), sex (female or male), and age (continuous variable). The classification of sites into 'high' and 'low' morbidity was based initially on population monitoring observations of chlamydiosis and are subsequently validated in the study analysis. Koala management areas (KMA) situated north of the Coomera River (Figure 5.1) were identified as the 'high morbidity' site and those south of the river as the 'low morbidity' site. 'High morbidity' site specific KMA's included: TKMA – 1 (PRCA), KMA12b (Coomera Sports Park), KMA13a (Foxwell Rd), and KMA13b (TAFE). 'Low morbidity' site specific KMA's included: KMA4 (Southport-Nerang Rd), KMA5 (Smith St Motorway), KMA6 (Smith St/M1), KMA7a (Railway Reserve), KMA8 (Country Club Dr), KMA9 (Gold Coast Highway), KMA10a (Coombabah Lake), KMA11a (Monterey Keys), and KMA11b (River Downs Estate). Both sites are within or immediately adjacent to large patches of intact habitat between 30-100 ha but experience fragmentation and reduced connectivity due to bisecting roads. The regional ecosystems and vegetational species of both sites vary from north to south.

Diagnostic records and observations made during veterinary examinations using observational and diagnostic results were used to characterise each case according to clinical signs, body condition score, and general diagnosis. Other factors that may impact treatment outcomes and survival were recorded including vaccination status, duration (days) of chloramphenicol treatment, ovariohysterectomy status, release location status, and evidence

of complications during treatment. Examination of treatment records was conducted to identify a chlamydiosis treatment group and, using LAMP testing results of mucosal swabs, to define treatment outcomes. Inclusion criteria and definitions for these groupings and variables are provided in Table 7.28 (Supplementary materials).

Sample processing, infectious agent detection, and immune gene expression:

Swab DNA extraction

DNA was extracted from urogenital, ocular, and oropharyngeal swabs using the MagMAX™ CORE Nucleic Acid Purification Kit (Thermo Fisher cat# A32702; ThermoFisher Scientific, Waltham, MA, USA) with modifications to the manufacturer's instructions described in Muir et al., 2024. Using the MagMax_Core_Flex protocol, lysates were processed on a KingFisherTM Flex automated extraction instrument. A sterile unused swab was included as an extraction blank in each extraction batch. DNA extracts, eluted to a final volume of 100 µL, were stored at -80°C until analysis.

C. pecorum multiplex qPCR

For the detection of mucosal shedding, ocular and/or urogenital swabs, available from 205/221 koalas, were analysed. DNA extracts from urogenital and ocular swab samples were assessed in a multiplex real-time qPCR using a CFX96 Touch™ Real-Time PCR Detection System with the corresponding CFX Maestro software (BioRad, Australia). Primer sets and qPCR design and conditions used were identical to those described in Chapters 2 and 3 in this thesis, and were was adapted from Hulse *et al.* (2018) (Supplementary materials: Table 7.29). Briefly, this PCR amplified *Chlamydia* genus (23S), the species (*C. pecorum*) *ompB* gene, and koala *β-actin* gene. All samples passed quality control, amplifying *β-actin*. Based on the rarity

of chlamydial infections other than *C. pecorum* in koalas (Devereaux *et al.*, 2003; Jackson *et al.*, 1999; Vitali *et al.*, 2023), samples were considered positive if either *C. pecorum ompB* or 23s genus amplification was achieved in both duplicates. Any sample with discordant results between duplicates was retested and samples that failed to amplify β-actin were re-run at 1:10 dilution to dilute inhibitors. No samples continued to be discordant after retesting with dilutions. qPCR efficiencies were between 90-104%. Of the koalas with analyzable samples, 153 had results for both mucosal *C. pecorum* DNA and circulating *C. pecorum* mRNA of which the latter was determined using NanoString (below).

PhaHV-1 and PhaHV-2 qPCR

For PhaHV detection, 202/221 and 205/221 oropharyngeal mucosal swabs were assayed for the detection of PhaHV-1 and PhaHV-2 shedding, respectively. Oropharyngeal swab extracts were analysed using the same PhaHV-1 *dpol* and PhaHV-2 *dpol* DNA qPCR design and method described in Muir et al., 2024, based on (Wright *et al.*, 2023)) and (Church *et al.*, 2025), respectively (Supplementary materials: Table 7.29). Koala *β-actin* gene was included as a control measure of sample quality. Samples that failed to amplify koala *β-actin* were excluded from further analysis (PhaHV-1, N = 3; PhaHV-2, N = 1). A sample was considered positive if both duplicates amplified *β-actin* and produced a melt curve at 81 – 81.5°C for PhaHV-1 and/or at 87 – 87.5°C for PhaHV-2. Any sample with discordant results between duplicates was retested and samples that failed to amplify β-actin were re-run at 1:10 dilution to dilute potential inhibitors. qPCR efficiencies ranged between 90–99% for PhaHV-1 and 90-102% for PhaHV-2 and the inter-assay variation was less than 5% for both targets. Of the koalas with analysable samples, 149 had results for both mucosal PhaHV-1 and -2 DNA and circulating PhaHV-2 mRNA, of which the latter was determined using NanoString (below).

Buffy coat RNA extraction

Of the 221 koalas sampled, 169 (76%) had buffy coat samples available for RNA extraction. RNA was extracted from available buffy coat samples stored in RNAlater™ using the Rneasy® Mini Kit (Cat. No. 74104, QIAGEN) following the manufacturer's instructions (RNeasy Mini Handbook 2023, QIAGEN). The purity and concentration of nucleic acid in extracted RNA samples was determined using the Qubit ® RNA HS Assay Kit (Q32852) and Qubit ® RNA IQ Assay Kit (Q33222) on the benchtop Qubit™ 4 Fluorometer (Invitrogen, Thermo Fisher Scientific), respectively. The extracted RNA samples were stored at −80 °C until required.

Gene transcription detection and quantification

A custom NanoString nCounter plex-set (NanoString Technologies, WA, USA) was designed to quantify transcribed mRNA from a multiplexed set of 38 genes of interest for koala pathogens, host pathways, and four reference genes (*GAPDH, ACTB, Stx12,* and *Nckap1l*) (Supplementary materials: Table 7.27). Host gene targets were selected based on previously determined performance and relative association with koala health outcomes in Chapters 2 and 3 and in another study (Fernandez *et al.*, 2024b), as well as differential expression results obtained in Chapter 4 of this thesis. To focus the plex-set on immune functions and infectious agents we retained the following genes: *CD3G, CD4, CD79b, CD8beta, CLEC4E, CARD9, IL18, IL1beta, IL6, IL8, MHCIUA, PhciDAB, PhciDBB, RETN, SAMDH1, TLR2, TLR4, TLR7, TNFalpha, KoRVAenvRBD, KoRVBenvRBD, KoRVDenvRBD, KoRVpol, Cpec_Hsp60, CpecG_0573, PhaHV2_dpol*, Cathelicidin-like (*LOC110217150*), *AR, CCR4, PhciCATH5*, and *NCR3*. Gene targets that were detected rarely in previous studies, such as *IL10, IFNG, IL17*, *IL4* and *IL12*, were not included in this study (Fernandez *et al.*, 2024b). Instead to improve our understanding of cytokine-receptor relationships, related cytokine receptor genes were included, as they were

previously found to be expressed at higher levels than their associated cytokines (Chapter 4, this thesis): *IL1R2, IL4R, IFNGR1, IL10RA, IL17RA, IL3RA, IL12RB2*. Additionally, other genes that were significantly differentially expressed amongst koalas with different chlamydiosis status and survival outcomes in rehabilitation were included (Chapter 4, this thesis): *MARCO*, *RENBP*, and *SLCO2A1*.

Using the RNA quality and quantification results obtained from the previously described methods, a total concentration of 50 – 100 ng in a total volume of 7-10 µL of eluted RNA was prepared using RNA/DNA free water to adjust concentrations. Additional quality control and sample normalisation was completed by Ramaciotti Centre for Genomics, UNSW, Sydney, Australia preceding mRNA analysis and transcript counting which was performed by the same institute according to the manufacturers protocol (NanoString Technologies, WA, USA). Briefly, this process included mRNA hybridisation with both reporter codes and capture probes according to the nCounter XT CodeSet Gene Expression Assays Protocol (NanoString Technologies, WA, USA). A NanoString nCounter FLEX Analysis System (NanoString Technologies, WA, USA) was then used as per the manufacturer's recommendations for purification and transcript counting (NanoString Technologies Inc, 2017). Final transcript counts were determined using a Digital Analyser (NanoString Technologies, WA, USA).

Raw data were analysed as described in Chapter 2 and 3 of this thesis using nSolver™ 4.0 Analysis Software (NanoString Technologies, WA, USA). Briefly, Reporter Code Count (RCC) files containing barcode counts from each gene and control within each lane in the CodeSet and Reporter Library Files (RLF) including instrument and gene probe information were loaded into nSolverTM. Count results were normalised against housekeeping genes (*GAPDH*,

ACTB, Stx12 & *Nckap1l*). Counts were also adjusted against expression thresholds to account for differences in sample content. Only samples with housekeeping gene transcription levels exceeding 50 counts were considered for analysis. Using this criterion, 12 samples were omitted from the analysis.

A combination of host genes and infectious agent genes were included in the analysis for this investigation. Detection of *C. pecorum,* PhaHV-2, and KoRV-B env subtype in circulation was not ubiquitous and recorded as binary variables, yes = 1 & no = 0. Positive detection of circulating *C. pecorum* included the detection of either markers; the *CpecG_0573* gene target, derived from the MC/Marsbar strain, or chlamydial heat-shock protein gene Hsp60 (*Cpec_hsp60*). As prescribed by NanoString Technologies, raw counts below 20 cannot be discerned from background noise and so, any samples with expression levels below this threshold were flagged as below the limit of detection and were considered negative. To avoid the impact of zero-inflated (or below-threshold) data, which violates assumptions of equal variance and can distort results of principle components analysis (Silverman *et al.*, 2020), gene targets that resulted in counts above threshold levels (> 20 raw counts) in more than 50% of the samples were retained for analysis (Supplementary materials: Table 7.27). As a result, the following host genes were excluded: *PhciCATH5, SLCO2A1, MARCO, NCR3, AR, MHCIUA, CCR4, FAM19A2, TNFalpha, IL12RB2*. For the same reasons, pathogen genes that were not ubiquitously detected amongst the analysed sample population and house-keeping genes were also excluded from immune gene continuous data analysis: *KoRVBenvRBD, Cpec_Hsp60, CpecG_0573, PhaHV2_dpol, ACTB, GAPDH, Nckap1l, Stx12*. In total, thirty host gene targets were retained for principal components analysis (PCA): *CARD9, CD3G, CD4, CD79b, CD8beta, CLEC4E, FKBP5, IL18, IL1beta, IL1R2, IL4R, IL6, IL8, IFNGR1, IL10RA, IL17RA,*

IL3RA, KoRVAenvRBD, KoRVDenvRBD, KoRVpol, *LOC110197639*, Cathelicidin-like (*LOC110217150*), *PhciDAB, PhciDBB, RENBP, RETN, SAMDH1, TLR2, TLR4*, and *TLR7*.

Gene expression analysis

Of 169 buffy coat RNA extracts analysed using NanoString nCounter technologies, twelve failed due to low mRNA concentration, leaving 157 for statistical analysis (71% of the 221 koalas in the study). Using gene expression results from these samples, PCA was conducted on the thirty host genes to reduce the number of dimensions used in statistical modelling. PCA was performed on the correlation matrix of scaled immune gene mRNA counts. Consistent with the analysis in Chapter 3, to prioritise explained variance the first eight PC's were retained (Supplementary materials: Table 7.23 & Figure 7.4) (Jolliffe & Cadima, 2016). Variable representation (Supplementary materials: Table 7.24 & Figure 7.5), loadings (Supplementary materials: Table 7.25), and contributions (Supplementary materials: Table 7.26) to the eight dimensions were examined visually using firstly a Cos2 matrix on the raw PCA data (Figure 5.3) and then varimax rotated PCA network plot (Figure 5.4), and PCA bi-plots to identify outlier observations (Supplementary materials: Figure 7.6), respectively. No individual outliers were observed, resulting in no further sample exclusions.

Statistical Analysis

The principal components resulting from the PCA of immune gene expression were assessed as continuous variables alongside other demographics, clinical characteristics, examination and treatment outcomes from the first veterinary examination associated with the sampling date (Supplementary materials: Table 7.28). These variables were described as counts and percentages (%) for categorical/observational data and median and interquartile ranges (IQR)

for continuous data. A violin plot was generated to demonstrate the distribution of age and sex. Wilcoxon's test was used to determine whether the distribution of males and females significantly differed ($p \leq 0.05$) at each site (Figure 5.2).

Infectious agent detection frequency results associated with sampling events are reported as detection counts and percentages (%) for each pathogen in three evaluations: (1) the complete results, which include only samples without missing results for any detection method of that pathogen, (2) incomplete results, which include all results and detail missing data, and (3) aggregated results, which separate the methods of detection, include all positives and report an overall detection frequency for the pathogen.

Data are reported for the whole study population and according to site of origin ('High morbidity' vs 'Low morbidity'). Chi-Squared tests of independence and Odds Ratio calculations were performed to assess the relationship between two categorical variables. Fisher's Exact T tests were employed where counts were less than 5 in any category (Courvoisier *et al.*, 2011; Peduzzi *et al.*, 1996; Vittinghoff & McCulloch, 2006). Continuous data were first assessed for residual normal distribution and homogeneity of variances using a range of visual and statistical techniques: plotting residuals in Q-Q plot and standardised residual plot with fitted value line, histogram of residuals, Shapiro-Wilk test of normality, and boxplot to visualise variance and outliers. The residuals of Weight (kg) at examination were normally distributed, and so parametric Two-Sample T-tests were used. Where variances were unequally distributed between groups of interest, a Welch's Two Sample T-test was utilised. The residuals of age (years) at exam and immune gene expression PCA dimensions

(PC1-PC8) were not normal and so non-parametric Mann-Whitney-Wilcoxon's Rank Sum tests were used.

To validate the observation that the two sites differed in morbidity and mortality, Kaplan-Meier survival analysis was conducted for the study population to quantify mortality rates and measure the probability of survival according to site. Following confirmation of site-specific differences in mortality, multivariate cox-proportional hazards modelling for each site (high morbidity site and low morbidity site [models 1 and 2 in Figure 5.7]) was used to determine whether survival probabilities were associated with demographic, infectious, clinical, and genetic covariates (Supplementary materials: Table 7.28). Given that no circulating PhaHV-2 was detected in koalas from the low morbidity site, this variable was excluded from the model. Cox proportional hazards regression modelling was used according to the Andersen-Gill formulation to determine the proportional effect of covariates on the hazard function of koala survival time.

From the whole sample population, two other different cohorts of interest were assessed to determine which covariates (Supplementary materials: Table 7.28) were associated with survival probabilities in: koalas with chlamydiosis and/or mucosal *C. pecorum* shedding, and koalas treated for chlamydiosis at T0 (models 3 and 4 in Figure 5.7). In these two tests, site was included as a co-variate. Because of the overlap between koalas with clinical signs of chlamydiosis and evidence of mucosal *C. pecorum* shedding, these were combined into the one outcome: chlamydiosis and/or mucosal *C. pecorum* shedding (Supplementary materials Figure 7.3). Because of small sample sizes for koalas treated for chlamydiosis at T0 (N = 32), univariate tests were employed to determine the relationship between covariates and the

probability of survival. Here, continuous variables were firstly assessed using univariate cox proportional hazards models, and categorical variables were assessed using log-rank chi-squared tests (Supplementary materials: Table 7.28). Variables with significant univariate relationships to survival were then assessed collectively in a multivariate cox-proportional hazards model. To focus on factors associated with disease-related mortality, koalas that did not die by the end of the monitoring period (25/6/2024) (N = 134), could not be followed-up (collar drop/signal failure; N = 12), or died from non-disease related reasons including trauma (N = 16) were censored (Table 5.2).

Where cox-proportional modelling was employed, covariates were firstly assessed within a full model if sample size permitted (survival at the (1) high morbidity site, and in (3) koalas with chlamydiosis and/or mucosal *C. pecorum* shedding), or in several preliminary models to achieve convergence in cohorts with small sample sizes (survival at the (2) low morbidity site). Covariates that were identified as potentially significant ($p \leq 0.05$) factors associated with the dependent variables were then assessed collectively within a reduced model to scrutinise potential effects of multicollinearity, confounding and overfitting. The removal of insignificant covariates achieves several benefits including a reduction in overfitting by improving model simplicity, an increased sample size by removing insignificant covariates that had missing values, and a reduction in the effect of potentially correlating or confounding variables. Model quality metrics and covariate performance were compared between the full and reduced models. Model quality metrics included the concordance index (C-index) which measures the discriminatory power of the model where a value of 0.5 suggests no predictive discrimination (random chance) and a value of 1 indicates perfect discrimination. The likelihood ratio test, which tests the overall significance of the model, was also assessed in addition to the Wald

test which tests the significance of each coefficient within the model, and the log-rank Score test which tests the proportional hazards assumption and the overall fit of the model.

All statistical analysis were performed using R Statistical Environment (Version 2024.04.1+748) (R Development Core Team, 2024) and statistical significance threshold of p-value < 0.05 was applied to all tests conducted.

5.5 Results

Site-specific characteristics

Demographics: Relative to koalas from the "low morbidity" site to the south of the Coomera River, koalas from the "high morbidity" site, originating north of the Coomera River, were significantly older (χ^2 = 5.8, df = 1, p-value = 0.015), had lower body condition scores (χ^2 = 18.6, df = 5, p-value = 0.002), and had significantly greater odds of chlamydial disease (Odds ratio: 6.9, 95% CI = 3.6-13.9, p < 0.001) (Table 5.2). As a result, these koalas had greater odds of admission for chlamydiosis treatment (36% vs. 12%, Odds ratio: 4.2, 95% CI = 2-8.3, p < 0.001) and odds of disease-related mortality (Odds ratio: 3.3, 95% CI = 1.6-6.6, p < 0.001). Although a potential sampling bias for female koalas existed due to greater availability of samples, there were no significant differences in the proportions of males and females within (Figure 5.2) or between sites, diagnoses, time-zero chlamydial treatment status, or survival outcomes.

Table 5.2: Demographics, clinical characteristics and examination outcomes for koalas originating from high morbidity and low morbidity sites

			High Morbidity (N = 130)	Low Morbidity (N = 91)	Total (N = 221)
Demographics	Male	Count (%)	48 (37)	27 (30)	75 (34)
	Female	Count (%)	82 (63)	64 (70)	146 (66)
	Age (Years)	Median (IQR)	5 (3-6)	3 (2-5.8)	4 (2.5-6)
	Weight (Kg)	Median (IQR)	5.8 (5.2-7.0)	5.5 (4.6-6.3)	5.7 (5.0-6.7)
	BCS ≤ 2	Count (%)	7 (5)	4 (4)	11 (5)
	BCS > 2	Count (%)	123 (95)	87 (96)	210 (95)
T0 Clinical Status	Healthy (NAD)	Count (%)	44 (34)	60 (66)	104 (47)
	Other Disease*	Count (%)	15 (12)	15 (16)	30 (14)
	Clinical Chlamydiosis	Count (%)	71 (54)	16 (18)	87 (39)
	Conjunctivitis	Count (%)	40 (53)	5 (36)	45 (51)
	Cystitis	Count (%)	19 (25)	3 (21)	22 (25)
	Reproductive Disease	Count (%)	42 (56)	9 (64)	51 (57)
T0 Examination Outcomes	Released (healthy)	Count (%)	39 (30)	59 (65)	98 (45)
	Released (other disease)	Count (%)	9 (7)	14 (15)	23 (10)
	Released (chlamydiosis)	Count (%)	23 (18)	3 (3)	26 (12)
	Admitted for chlamydiosis	Count (%)	47 (36)	11 (12)	58 (26)
	Euthanised	Count (%)	12 (9)	4 (5)	16 (7)
Other treatments	OHE**	Count (%)	31 (24)	5 (5)	36 (16)
	Vaccination	Count (%)	51 (39)	49 (54)	100 (45)
Study Outcome (25/6/2024)	Alive	Count (%)	71 (55)	63 (69)	134 (61)
	Ongoing monitoring	Count (%)	53 (75)	60 (95)	113 (84)
	Not being monitored	Count (%)	18 (25)	3 (5)	21 (16)
	Dead	Count (%)	52 (48)	23 (25)	75 (34)
	Disease	Count (%)	37 (59)	13 (52)	50 (67)
	Disease - iatrogenic	Count (%)	9 (13)	0 (0)	9 (12)
	Misadventure	Count (%)	1 (2)	0 (0)	1 (1)
	Predation - carpet python	Count (%)	3 (5)	0 (0)	3 (4)
	Old-age-related	Count (%)	0 (0)	1 (4)	1 (1)
	Trauma	Count (%)	2 (3)	9 (39)	11 (15)
	Loss to follow-up***	Count (%)	7 (5)	5 (5)	12 (5)

*Other disease: inclusive of periodontal disease/gingivitis, candidiasis, oxalate crystals + hyperechoic kidneys, osteoarthritis, generalised lymphadenopathy, ascites, chronic dermatopathy, dysbiosis, mammary tumour, keratinised growth, otitis, pneumonia, subcutaneous mass, microphthalmia, eosinophilia.
**OHE: Ovariohysterectomy performed at any point during monitoring period, i.e. can be before or after sampling event.
***Loss to follow-up refers to koalas that could not be located due to failure of GPS tag or other unknown reasons for disappearance

Figure 5.2: Violin plots of age distribution by sex according to site

In Figure 5.2, the estimated age determined at veterinary examination (y-axis) is plotted by sex (Female = red and Male = blue, x-axis) according to site of origin: High morbidity or Low morbidity site. Within each violin form, a box-plot is plotted to demonstrate the interquartile ranges. The significance result (p-value) from a Wilcoxon's rank-sum test (Mann-Whitney U test) of sex distributions within each site is displayed above each set of violin plots.

Pathogen detection: The overall detection rate of mucosal *C. pecorum*, PhaHV-1, and PhaHV-2 differed between sites (Table 5.3). At the high morbidity site, relative to the low morbidity site, *C. pecorum* within mucosal samples was detected at a significantly greater frequency (χ^2 = 28.67, df = 1, p-value < 0.001). Mucosal PhaHV-1 and PhaHV-2 were both detected at significantly greater frequencies within koalas from the high morbidity site (χ^2 = 10.4, df = 1, p-value = 0.001 and χ^2 = 21.23, df = 1, p-value < 0.001). Circulating PhaHV-2 was only detected in koalas from the high morbidity site. Overall, there was no difference in the detection frequency of circulating *C. pecorum* or KoRV B between sites: χ^2 = 0.5, df = 1, p-value = 0.48 and χ^2 = 0.1, df = 1, p-value = 0.74, respectively. Further detection information including target co-detection frequencies according to infectious agent are outlined in Table 7.22 (Supplementary materials).

Table 5.3: Detection results for infectious agents that were not ubiquitously detected in samples obtained from 221 koalas originating from high morbidity and low morbidity sites

Koala infectious agent detection status		***High morbidity (N = 130)***	***Low morbidity (N = 91)***	***Total (N = 221)***
Chlamydia pecorum	Mucosal *C. pecorum*	82/120 (68%)	25/85 (29%)	107/205 (52%)
	Circulating *C. pecorum*	9/95 (9%)	4/74 (5%)	13/169 (8%)
	C. pecorum	86/118 (73%)	29/77 (37%)	115/195 (59%)
PhaHV - 1	Mucosal PhaHV-1	80/118 (68%)	37/84 (44%)	117/202 (58%)
PhaHV - 2	Mucosal PhaHV-2	52/121 (43%)	10/84 (12%)	62/205 (30%)
	Circulating PhaHV-2	6/95 (6%)	0/74 (0%)	6/169 (3%)
KoRV B	Circulating KoRV B	13/95 (14%)	8/74 (11%)	21/169 (12%)

Gene expression: Host gene targets that failed to meet threshold levels (20 counts) in more than 50% of the samples, were excluded from analysis: *PhciCATH5, SLCO2A1, MARCO, NCR3*, *AR, MHCIUA, CCR4, FAM19A2, TNFalpha, IL12RB2*. Infectious agent genes that weren't ubiquitously detected and house-keeping genes were also excluded from host gene analysis: *KoRVBenvRBD, Cpec_Hsp60, CpecG_0573, PhaHV2_dpol, ACTB, GAPDH, Nckap1l, Stx12*, leaving 30 gene targets for gene expression analysis: *CARD9, CD3G, CD4, CD79b, CD8beta, CLEC4E, FKBP5, IL18, IL1beta, IL1R2, IL4R, IL6, IL8, IFNGR1, IL10RA, IL17RA, IL3RA, KoRVAenvRBD, KoRVDenvRBD, KoRVpol*, Cathelicidin-like (*LOC110217150*), Cathepsin L1-like (*LOC110197639*), *PhciDAB, PhciDBB, RENBP, RETN, SAMDH1, TLR2, TLR4*, and *TLR7* (Supplementary materials: Table 7.27).

Principal components analysis (PCA) with varimax rotation was performed on the correlation matrix of scaled immune gene mRNA counts. Prior to rotation, the first two PCs accounted for 19.3% and 15% of the total variance within the dataset, respectively, with the majority of genes contributing to these dimensions (Figure 5.3).

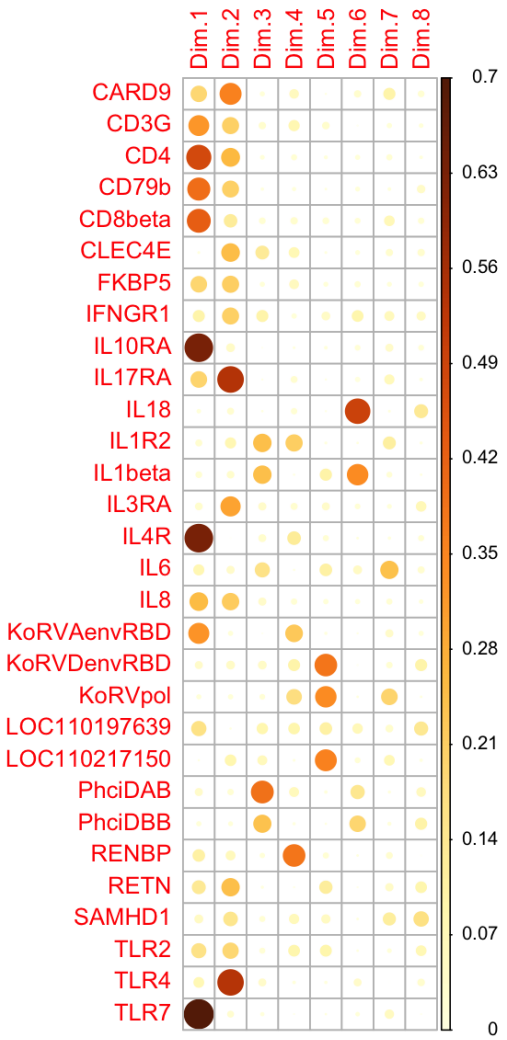

Figure 5.3: Cos^2 (quality of representation) values for genes across each principal component.

Using the 'corrplot' function in R Studio, the cos2 values for each gene are visualized in Figure 5.3 using a custom matrix that demonstrates the quality of representation of each gene within each principal component (1-8). As indicated by the scale on the right side of the plot, greater cos2 values, and thus stronger representations, are coloured dark brown, whereas weaker representations are closer to white (colourless).

Varimax rotation was applied to the standardised scores and loadings from the scaled PCA to maximise high, and minimise low, loadings and generate uncorrelated components of greater interpretability. A network plot was generated displaying the strongest contribution of correlated genes within each component, minimising multi-collinearity between components (Figure 5.4). The rotated components illustrate PCs characterised by the following genes; PC1: *CD3G, CD8B, CD4, CD79B, TLR7,* and *IL8* emphasised, with a negative loading from *RETN*; PC2:

IL10R, *IFNGR1, IL4R, IL17RA, CARD9, TLR2, TLR4*, and *FKBP5*; PC3: *FKBP5, IL1R2, IL3RA, RENBP* and *LOC110197639*; PC4: *SAMHD1* and *KoRV A*, PC5: *KoRV pol, KoRV D* and Cathelicidin-like (*LOC110217150*), PC6: *CLEC4E, DAB* and *DBB*, PC7: *IL1beta* and *IL6*, with a negative loading from *RETN* and Cathelicidin-like (*LOC110217150*), and PC8: *IL1beta* and *IL18*.

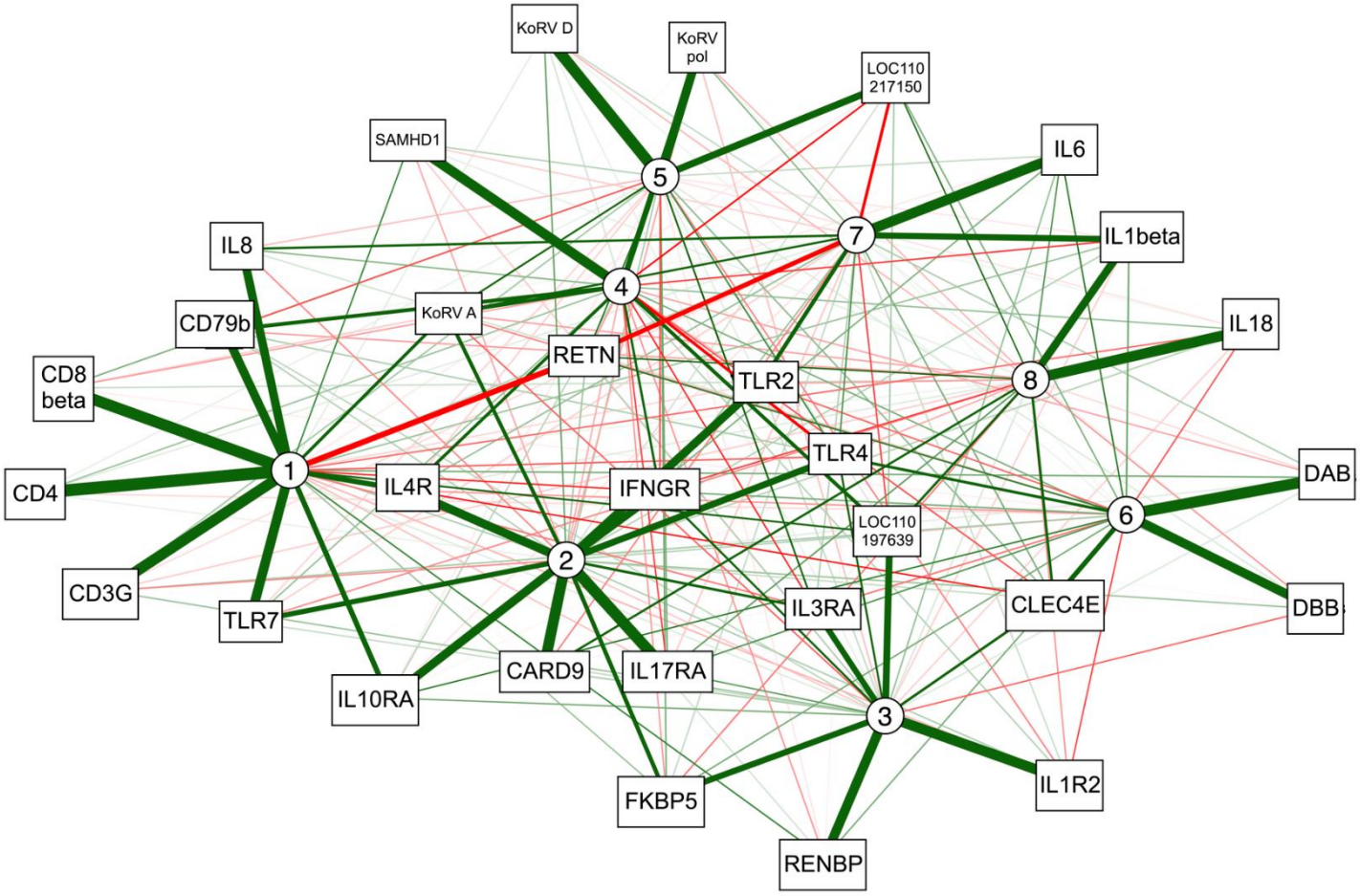

Figure 5.4: Varimax rotated component network plot showing the rotated loadings of each gene within each component (1-8).

Figure 5.4 displays the eight components retained from principal components analysis that collectively account for 68% of the variation explained within the data Rotation to the standardised scores and loadings of genes generate components that are uncorrelated, supporting improved interpretation.

According to Mann-Whitney-Wilcoxon Tests, the distribution of individual koala scores for dimensions 1, 2, 4 and 6 differed significantly according to the site of origin. Koalas originating from high morbidity site had greater scores for PC2 and PC6 and lower scores for PC1 and PC4 compared to koalas from the low morbidity site; PC1: high morbidity median = -1.35 (IQR = 2.41), low morbidity median = 0.51 (IQR = 2.76); W = 1126, p value < 0.001; PC2: high morbidity median = 0.14 (IQR = 1.79), low morbidity median = -0.63 (IQR = 3.43), W = 2443, p value < 0.05; PC4: high morbidity median = -0.22 (IQR = 1.20), low morbidity median = 0.32 (IQR = 1.84), W = 1540, p value < 0.05, and PC6: high morbidity median = 0.09 (IQR = 0.95), low morbidity median = -0.44 (IQR = 1.24), W = 2672, p value < 0.01 (Figure 5.5).

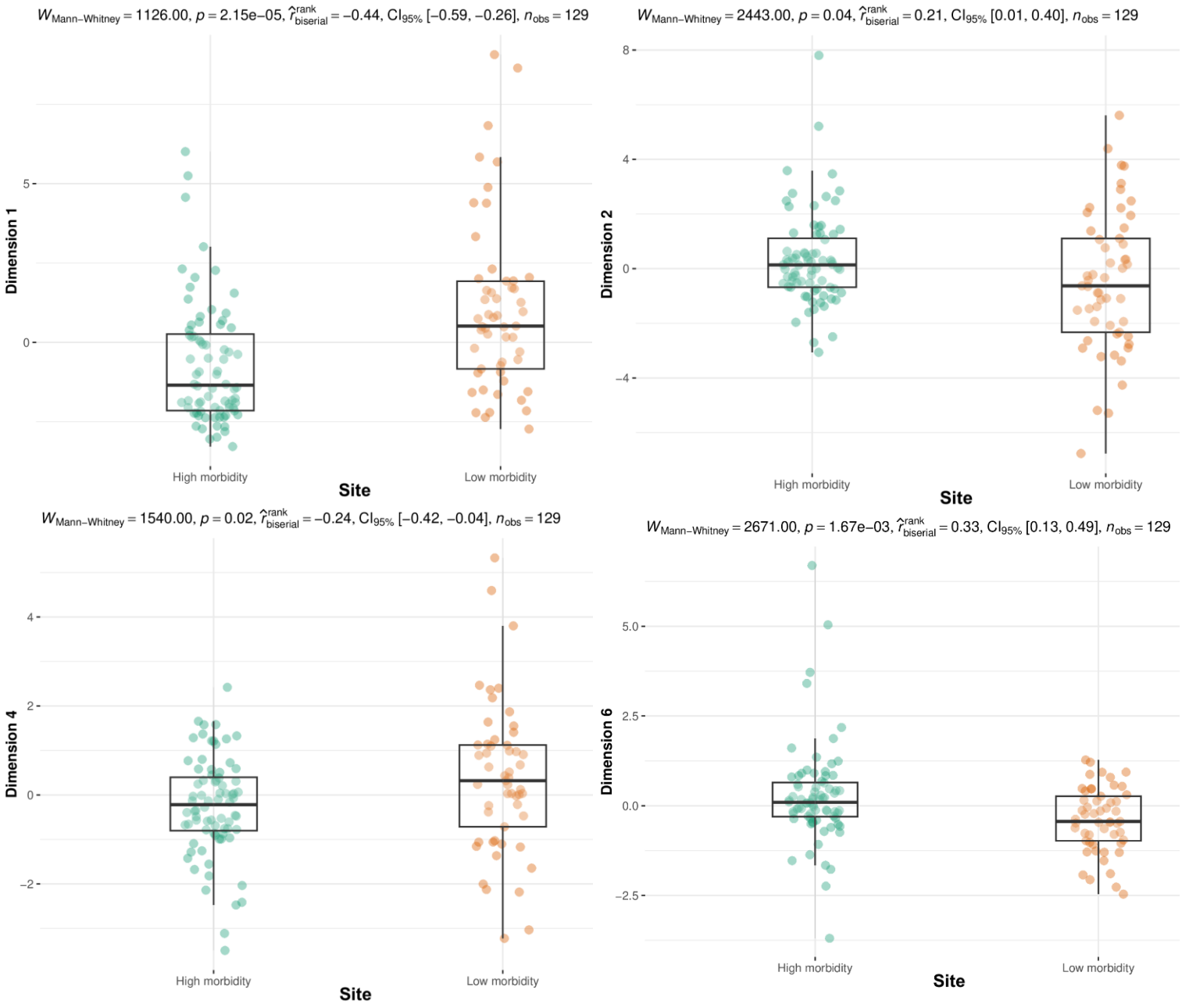

Figure 5.5: Box-plots of principal components 1, 2, 4 and 6, which differed significantly in distribution between the two koala populations with high and low morbidity.

The charts in Figure 5.5 plot the scores of principal components 1, 2, 4, and 6 according to site of origin; the high morbidity site or the low morbidity site. Mann-Whitney-Wilcoxon's tests were conducted to test whether the distributions of the PC scores were identical between the sites.

Factors affecting survival probability

Between sites: The probability of survival differed significantly between the high and low morbidity sites as did the factors associated with survival. Kaplan-Meier survival analysis of

the whole study sample set identified a greater survival probability in koalas originating from the low morbidity site relative to those from the high morbidity site (Figure 5.6, $p < 0.001$). Because of this difference, a cox-proportional hazards model was generated for each population to identify site-specific factors associated with survival.

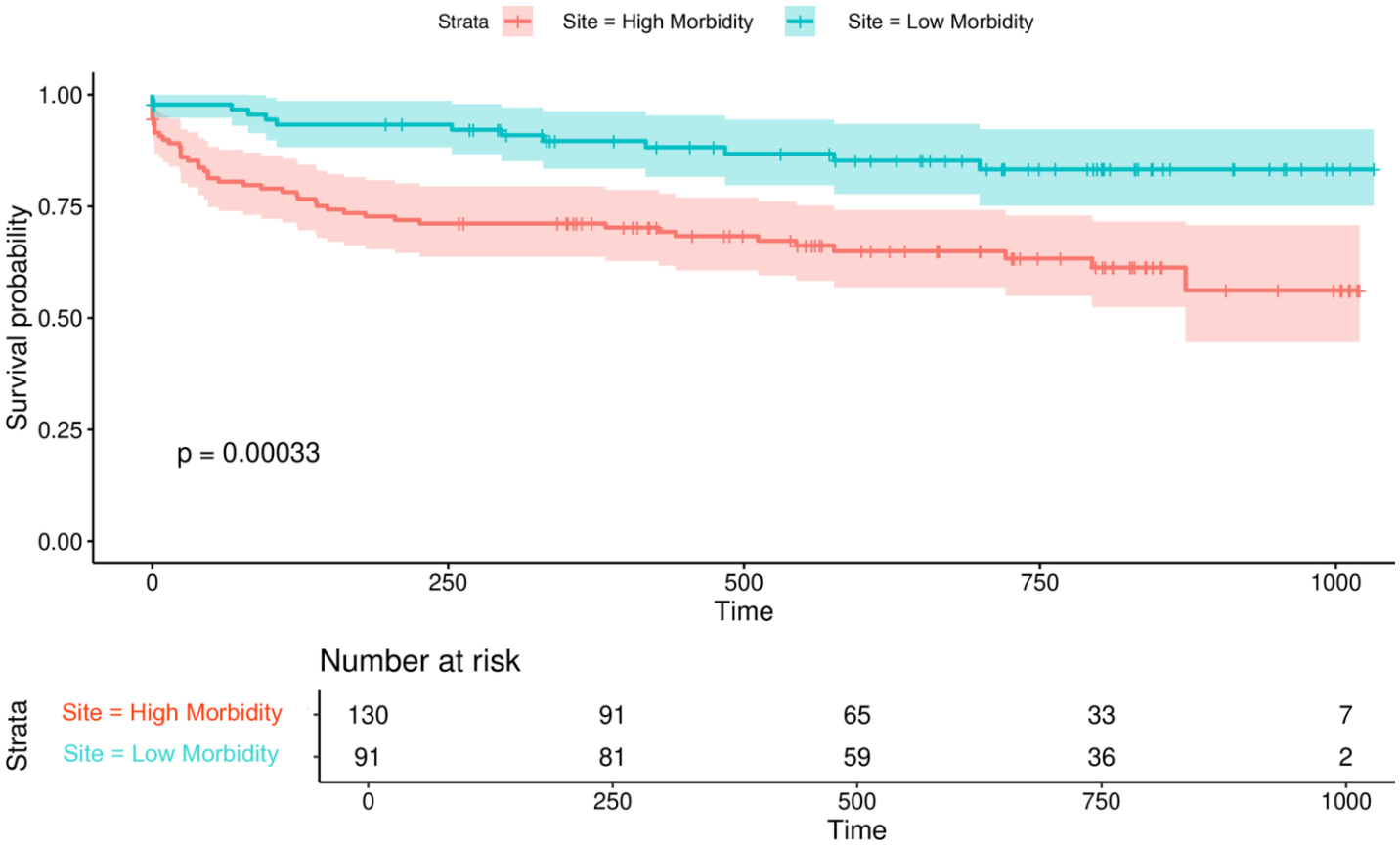

Figure 5.6: Kaplan-Meier Survival Curves for koalas originating from either the low morbidity (blue) or high morbidity (red) sites.

The Kaplan-Meier survival curves in Figure 5.6 represents the disease-associated mortality rate at each site. Overall, 59 cases died from disease-related causes and 162 cases were censored (134 cases alive, 16 cases died from non-disease related causes, and 12 of unknown status [lost to follow-up]). Confidence intervals are represented by the lighter shadowing around each respective curve. The median survival is not shown, as 50% of koalas at either site did not die from disease related causes over the course of the monitoring period. A risk table is presented below the graph detailing the total number of koalas alive at each time interval for each site.

Of the 76 koalas originating from the high morbidity site with a time-zero sample, 68 had a complete data set and so were included within the model, which included 24 events (deaths).

In the final model (Table 5.4), decreases in PC1 was an approaching significant predictor of improved survival in koalas from the high morbidity site. Body condition scores (BCS) below 2 increased the hazard risk significantly. Furthermore, receiving treatment for chlamydiosis at a follow-up examination during the monitoring period, reduced the hazard risk, while having detectable mucosal *C. pecorum* increased the hazard risk. The C-index (concordance) indicates that the model has good predictive accuracy and collectively, the likelihood ratio test, Wald test, and log-rank test scores demonstrate the overall significance of the model and the included variables.

Table 5.4: Final Cox proportional hazards model predicting survival in koalas from the high morbidity site (N = 70, events [deaths] = 26, missing data = 6).

Explanatory Variables [Y]	Estimate	Std. error	HR [†]	95% CI	Z value	P value
PC1	-0.24	0.13	0.78	0.60-1.02	-1.83	0.067
BCS ≤ 2: YES	2.63	1.17	13.93	1.41-138	2.25	0.024
Chlamydiosis treatment: Future	-1.52	0.59	0.22	0.07-0.69	-2.58	0.009
Chlamydiosis treatment: None	0.92	0.53	2.51	0.88-7.19	1.71	0.086
Mucosal *C. pecorum*: NOT DETECTED	-1.54	0.21	0.21	0.06-0.73	-2.46	0.013
Concordance = 0.74 (se = 0.04)						
Likelihood-ratio test = 19.88 on 5 df, p = 0.001						
Wald test = 16.92 on 5 df, p = 0.005						
Score (logrank) test = 18.16 on 5 df, p = 0.003						

[Y] *The categorical reference groups for covariates are: 'NO' for BCS ≤ 2, 'Current' for chlamydiosis treatment status, and 'detected' for mucosal C. pecorum..*

[†] *The hazard risk (HR) calculated from the exponential of the estimate are provided with 95% confidence intervals, and significant covariates are identified by asterisks.*

At the low morbidity site, principal components that best predicted the survival of koalas differed from those for the high morbidity site. Of the 53 koalas in the low morbidity site with a time-zero sample, 49 had complete results and were included in the final cox proportional hazards model. These koalas contributed eight events (deaths) to the measurement of survival probability. In the final model (Table 5.5), increases in PC5 and BCS equal to or below 2 increased the hazard risk in koalas originating from the low morbidity site. The C-index for this model was high and scores for likelihood ratio test, Wald test, and log-rank tests were

significant, indicating the overall accuracy and significance of this model and the final predictors.

Table 5.5: Final Cox proportional hazards model predicting survival in koalas from the low morbidity site (N = 49, events [deaths] = 8, missing data = 4).

Explanatory Variables [Y]	Estimate	Std. error	HR [†]	95% CI	Z value	P value
PC4	0.52	0.25	1.67	1.02-2.77	2.02	0.043
PC5	0.70	0.21	2.02	1.35-3.03	3.41	0.0006
Sex: Male	1.32	1.03	3.75	0.50-28.4	1.28	0.199
Diagnosis at Sample: Healthy	-1.51	1.25	0.22	0.02-2.58	-1.21	0.228
Diagnosis at Sample: Other	-2.43	1.51	0.08	0.00-1.70	-1.61	0.108
Mucosal *C. pecorum*: NOT DETECTED	-2.05	1.25	0.13	0.01-1.50	-1.64	0.101
Concordance = 0.96 (se = 0.02)						
Likelihood ratio test = 28.01 on 6 df, p = 9e-05						
Wald test = 16.17 on 6 df, p = 0.01						
Score (logrank) test = 47.08 on 6 df, p = 2e-08						

[Y] *The categorical reference groups for covariates are 'Female' for sex, 'Chlamydiosis' for diagnosis at sample, and 'detected' for mucosal C. pecorum.*

[†] *The hazard risk (HR) calculated from the exponential of the estimate are provided with 95% confidence intervals, and significant covariates are identified by asterisks.*

Among koalas with chlamydiosis and/or mucosal C. pecorum: Among koalas that presented with clinical chlamydiosis and/or mucosal *C. pecorum* shedding at T0 (N = 69), 91% had complete data for the 22 independent variables and were used in cox proportional hazards modelling (Table 5.6). Risk of death in koalas with clinical chlamydiosis and/or mucosal *C. pecorum* shedding was reduced by increases in PC1 and PC3; having a 'healthy' diagnosis at sampling; and detectable KoRV B. Greater hazard risks and lower survival in koalas with clinical chlamydiosis and/or mucosal *C. pecorum* shedding was associated with greater PC2, PC5, and PC6 scores; greater age; having a BCS equal to or below 2; not experiencing any treatment for chlamydiosis during the monitoring period; originating from the low morbidity site; having detectable circulating *C. pecorum* and PhaHV-2; and a lack of PhaHV-1 detection.

Table 5.6: Final Cox proportional hazards model predicting survival in koalas with detectable C. pecorum shedding with or without clinical chlamydiosis (N = 63, events [deaths] = 24, missing data = 6).

Explanatory Variables [Y]	Estimate	Std. error	HR [†]	95% CI	Z value	P value
PC1	-0.62	0.22	0.53	0.35-0.82	-2.85	0.004
PC2	0.41	0.16	1.51	1.10-2.08	2.55	0.011
Age	0.35	0.13	1.42	1.10-1.84	2.70	0.007
BCS ≤ 2 : YES	1.86	0.72	6.41	1.54-26.6	2.55	0.011
Diagnosis at Sample: Healthy	-1.95	0.96	0.14	0.02-0.94	-2.02	0.043
Diagnosis at Sample: Other	-0.99	1.30	0.37	0.03-4.78	-0.76	0.45
Chlamydiosis treatment: Future	-0.80	0.57	0.45	0.14-1.39	-1.39	0.16
Chlamydiosis treatment: None	2.21	0.84	9.16	1.77-47.4	2.64	0.008
Mucosal PhaHV-1: NOT DETECTED	1.60	0.63	4.98	1.45-17.1	2.55	0.011
Concordance = 0.82 (se = 0.051)						
Likelihood ratio test = 39.74 on 9 df, p = 8e-06						
Wald test = 30.34 on 9 df, p = 4e-04						
Score (logrank) test = 42.63 on 9 df, p = 3e-06						

[Y] *The categorical reference group for is 'no' for BCS ≤ 2, 'Chlamydiosis' for diagnosis at sample, 'Current' for chlamydiosis treatment status, and 'detected' for mucosal PhaHV-1.*

[†] *The hazard risk (HR) calculated from the exponential of the estimate are provided with 95% confidence intervals, and significant covariates are identified by asterisks.*

Indicators among treated koalas

Among koalas that presented with clinical chlamydiosis and/or mucosal *C. pecorum* shedding at T0 (N = 84), 69% were admitted for treatment immediately. For the purposes of predicting treatment outcomes in subsequent modelling, only these cases treated at time-zero, coinciding with sample collection, were considered (N = 58) (Supplementary materials: Figure 7.3). The median chloramphenicol treatment duration was 11 days (range 1 – 17 days) and 37% completed the minimum 14-day regimen.

Chlamydiosis treatment outcomes: Of the 58 koalas treated for chlamydiosis at time-zero, 86% (50/58) obtained a LAMP negative result post-treatment, 9% (5/58) were LAMP positive, and 5% (3/58) did not have post-treatment LAMP results (Supplementary materials: Figure 7.5). In total, 90% (45/50) of koalas that were LAMP negative post-treatment had follow-up LAMP results: 27% had detectable *C. pecorum* shedding at a mucosal site (ocular or urogenital) at a follow-up examination and 73% remained LAMP negative throughout the

remainder of the monitoring period. On average, mucosal *C. pecorum* shedding was detected 169 days post treatment (range 30-589 days). Of the LAMP positive cases post-treatment, one koala received four doses of chloramphenicol then underwent ovariohysterectomy, and was euthanised due to welfare concerns from IV catheter complications. One koala completed the full course (14 days) of chloramphenicol while treatment was terminated early in three cases due to complications such as the development of oxalate nephrosis (n = 2), dysbiosis (n = 1), and extreme stress (n = 1), and all were released.

Post-treatment survival probability in koalas treated for chlamydiosis: Survival was assessed in 55% (32/58) of the cohort of koalas treated for chlamydiosis at time-zero, excluding those with incomplete data. Univariate cox proportional hazard modelling demonstrated significant relationships between survival and PC1 and PC6 scores, while no other continuous or categorical variable was associated; PC2-5 or PC7-8, age, sex, BCS ≤ 2/5, site, diagnosis at sample, vaccination status, ovariohysterectomy status, release location status, mucosal *C. pecorum,* PhaHV-1 and PhaHV-2 status, and circulating *C. pecorum,* PhaHV-2, and KoRV B status. A multivariate cox proportional hazards model showed that one unit increase in PC1 increased the odds of survival, while a one unit increase in PC6 decreased the odds of survival in koalas treated for chlamydiosis (Table 5.7).

Table 5.7: Final Cox proportional hazards model predicting survival in koalas treated for chlamydiosis at sampling (N = 32, events [deaths] = 15, missing data = 6).

Explanatory Variables [Y]	Estimate	Std. error	HR [†]	95% CI	Z value	P value
PC1	-0.52	0.22	0.59	0.38-0.92	-2.32	0.020
PC6	0.51	0.26	1.67	1.01-2.78	1.98	0.047
Concordance = 0.75 (se = 0.058)						
Likelihood ratio test = 10.92 on 2 df, p = 0.004						
Wald test = 7.71 on 2 df, p = 0.02						
Score (logrank) test = 8.34 on 2 df, p = 0.02						

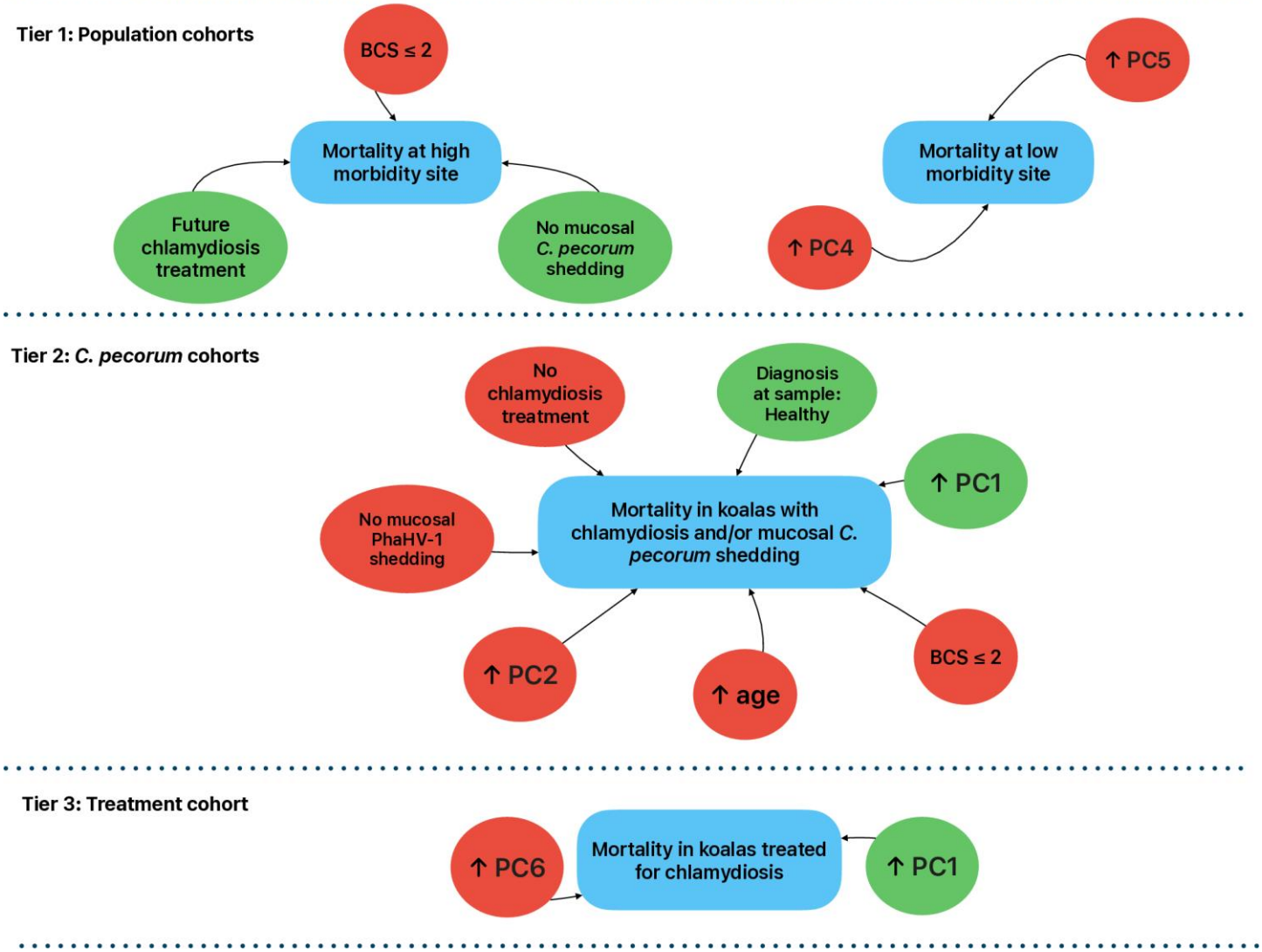

Figure 5.7: Diagram of the significant factors associated with mortality in wild koalas.

In Figure 5.7 the significant factors associated with mortality in four different cohorts of koalas are demonstrated. Each of the four cohorts were assessed in a separate model and organised into three tiers separated by dotted lines: tier 1 assesses factors associated with mortality according to site, tier 2 assesses the same according to C. pecorum infection status, and tier 3 assesses the same in koalas treated for chlamydiosis. Analysed cohorts are represented in the blue boxes, and covariates in the red and green ovals represent increases and decreases in the odds of an outcome occurring, respectively. The presented covariates represent factors that were significantly ($p < 0.05$) associated with the odds of mortality obtained through cox-proportional hazards modelling. The genes strongly correlated to PC1-8 are as follows: PC1: positive loading of CD3G, CD8B, CD4, CD79B, TLR7, and IL8, and negative loading from RETN; PC2: positive loading from IL10R, IFNGR1, IL4R, IL17RA, CARD9, TLR2, TLR4, and FKBP5; PC3: positive loading from FKBP5, IL1R2, IL3RA, RENBP and LOC110197639; PC4: positive loading from SAMHD1 and KoRV A, PC5: positive loading from KoRV pol, KoRV D and Cathelicidin-like (LOC110217150), PC6: positive loading from CLEC4E, DAB and DBB, PC7: positive loading from IL1beta and IL6, and a negative loading from RETN and Cathelicidin-like (LOC110217150), and PC8: positive loading from IL1beta and IL18.

5.6 Discussion

This study used a holistic, multivariate approach to identify indicators of survival and treatment outcomes in wild koalas from two adjacent but discrete sites and, in doing so, identified associations among survival rates, morbidity patterns, site-specific demographics, co-infection, immunophenotype and additional health indicators that provided potential insights into their interactions and relative importance in different contexts. Adaptive lymphocyte markers (PC1) were a consistent indicator of improved survival in koalas with chlamydiosis (clinical disease and/or mucosal *C. pecorum* shedding), and koalas treated for chlamydiosis. Increases in cytokine-receptor gene transcription (PC2) and pathogen/damage detection and presentation gene transcription (PC6) were negative indicators of survival. Lack of detection of mucosal PhaHV-1 shedding and increased transcription of KoRV markers negatively predicted survival in koalas with chlamydiosis and in koalas originating from the low morbidity site, respectively. Due to low detection of circulating *C. pecorum* and PhaHV-2, their significance to koala pathogenesis requires further investigation. This study lays groundwork for new prognostic tools to identify measurable risk factors in *Chlamydia*-affected koalas and highlights the context-specific nature of risk assessment and mitigation, informing future research into drivers of morbidity and mortality, and effective conservation strategies.

Inclusion of immune cytokine receptor genes to the targeted gene expression panel employed in previous studies provided a clearer picture of immune system activity by enhancing the distinction of the adaptive immune response in the rotated components. Gene inclusion can significantly influence PCA results, altering the degree of variance captured by principal components and yielding alternative representations (Tian *et al.*, 2008). In the current study,

low transcribing genes from the NanoString panel used in Chapter 3 and prior studies (Fernandez *et al.*, 2024b) were replaced by cytokine receptor genes: *IL10RA, IL17RA*, *IFNGR1, IL1R2, IL3RA*, and *IL4RA*. Although the expression of *IL10RA, IL17RA*, and *IFNGR1* were not correlated with the expression of their ligands (Chapter 4, this thesis), the reportedly higher transcription levels of these receptors allowed for further examination of their relationships with other immune markers. With the expanded gene set, varimax rotation of scaled principal components revealed a distinct adaptive lymphocyte and associated TLR component (PC1: *CD3G, CD79b, CD4, CD8beta, TLR7, IL8*), consistent with previous studies that examined immune responses in rescued koalas admitted to rehabilitation clinics (Chapter 3, this thesis) and in wild populations in NSW (Fernandez *et al.*, 2024b). Additionally, a separate but aligned PC comprised the novel adaptive pathway cytokine receptors, and previously employed PAMP receptors, and innate immune and stress pathway gene components (PC2: *IL17RA, IFNGR1, IL10RA, IL4R, TLR2, TLR4, CARD9, FKBP5*). Other components represented more distinct separation of system elements than previously observed using the panel design in Chapter 3 and in a previous study (Fernandez *et al.*, 2024b): Innate cytokines (PC3: *IL1R2, IL3RA*, with *RENBP*, cathepsin L1-like [*LOC110197639*] and *FKBP5* and PC7 & 8: *IL6, IL1beta, IL18*); MHCII and a PAMP receptor (PC6: *PhciDAB, PhciDBB, CLEC4E*) and KoRV (PC4: *SAMHD1, KoRV A* and PC5: *KoRV D, KoRV pol*, cathelicidin-like [*LOC110217150*]), allowing clearer distinctions between immune pathways, retroviral activity, and disease relationships.

Increased transcription of adaptive lymphocyte differentiation markers has been a consistent indicator of improved health and survival outcomes in koalas with disease across most cohorts. Greater expression of genes associated with T- and B-lymphocytes (e.g., *CD3G*, *CD4*, *CD8*, *CD79b*), TLR7 and IL-8, was associated with improved survival in the current study,

particularly in koalas with chlamydiosis and/or mucosal *C. pecorum* detection, and koalas that were treated for chlamydiosis (Figure 5.7). This corroborates previous studies that indicated lower expression of these genes were associated with poorer outcomes such as euthanasia during rehabilitation (Chapter 3, this thesis) and a wild koala population under climatic stress (Fernandez *et al.*, 2024b). In human and mouse cells, the activity of Chlamydial Protease-like Activity Factor (CPAF), an integral protease required for *Chlamydial* vacuole integrity, is inversely associated with IL-8 expression (Christian *et al.*, 2010; Jorgensen *et al.*, 2011). Hence, the strong contribution of *IL8* to this dimension may be an indication of reduced *Chlamydial* activity or impaired CPAF expression. *TLR7* is an important PAMP receptor for viral RNA and may be indicative of greater control over infectious viral pressures from either KoRV and/or PhaHV-1 or -2.

Predictors of survival differed with context. Not surprisingly, the strength of lymphocyte differentiation markers as an indicator of survival was greater where the pressure exerted by infectious disease was greatest; in the cohort of koalas with, and being treated for, chlamydiosis. Whereas at the low morbidity site, increased lymphocyte differentiation marker transcription was not a significant indicator of survival in cox proportional hazards modelling, and rather lesser KoRV marker transcription was. This is because koalas from the low morbidity site had significantly greater PC1 scores compared to those from the high morbidity site, trauma-associated mortality was more frequent (39%) and associated with koala movement within an urban environment. Collectively, this suggests at least part of the relationship between exogenous retroviral transcription and survival or disease, noted in other studies (Blyton *et al.*, 2022a; Hashem *et al.*, 2021; Quigley *et al.*, 2018a; Sarker *et al.*, 2019), may be independent of chlamydiosis and mucosal *C. pecorum* shedding.

Adaptive and innate inflammatory markers predicted adverse outcomes. Perhaps reflecting intensity of inflammation and associated pathology, upregulation of cytokine receptors (*IL4R, IFNGR1, IL10RA,* and *IL17RA)*, PAMPs (*TLR2, TLR4*, and *CLEC4E*), MHCII (*PhciDAB* and *PhciDBB*), and a stress marker (FKBP5) in PC2 and PC6 predicted higher risk of mortality in koalas with mucosal *C. pecorum* shedding and/or clinical chlamydiosis and in koalas that were treated for chlamydiosis. Interestingly, upregulation of FKBP5 was observed in conjunction with elevated ambient temperatures and decreased adaptive immune gene expression in the study by Fernandez *et al.* (2024b). Here, increases in PC2 - which FKBP5 most strongly contributed to along with cytokine-receptor genes – was negatively associated with survival in koalas with chlamydiosis and/or mucosal *C. pecorum* shedding. Variations in adaptive immunity could be influenced by factors such as stage of pathogenesis (Andres-Terre *et al.*, 2015; Miller *et al.*, 2017; Woods *et al.*, 2013), co-infections (Ezenwa *et al.*, 2010; Mabbott, 2018; Martini & Champagne, 2021; Yap *et al.*, 2017), immunosenescence (Bajaj *et al.*, 2021; Giefing-Kröll *et al.*, 2015; Peters *et al.*, 2019b), genetics (Elbers *et al.*, 2018; Mangino *et al.*, 2017; Quigley *et al.*, 2020; Robbins *et al.*, 2020; Tarlinton *et al.*, 2021), microbiomes (Grainger *et al.*, 2018; Schachtschneider *et al.*, 2013; Wiertsema *et al.*, 2021), heat stress (Fernandez *et al.*, 2024b), and other environmental and dietary factors (Becker *et al.*, 2020; Maher & Higgins, 2016; Mangino *et al.*, 2017; Marschner *et al.*, 2019a), which alter the immune microenvironment in koalas and other species. Identifying immunophenotypes associated with greater morbidity and mortality allows for the impacts of these putative drivers of disease to be evaluated and more readily quantified than with clinical observations alone.

Age and body condition score were also significant indicators of survival. Having a body condition score below or equal to 2 was associated with decreased survival in koalas from the high morbidity site and in *Chlamydia*-affected koalas. While this finding supports the significance of body condition score as an indicator of health, as suggest by a previous study that found a relationship to survival post-rehabilitation (Leigh *et al.*, 2023), further studies are needed to clarify the pathogenic mechanisms underlying poor body condition, which may be related to reduced ability of animals to access, digest and metabolise food (Blanshard & Bodley, 2008; Lanyon & Sanson, 1986). As the proportion of males and females did not differ significantly among sites or according to diagnosis, treatment or mortality, it is unlikely that the female skewed sampling confounded indicators of survival.

The finding that detection of mucosal PhaHV-1 was associated with greater survival in koalas with chlamydiosis is not consistent with previous studies. Previous findings support mucosal PhaHV-1 shedding and circulating PhaHV-2 transcription as risk factors associated with chlamydiosis, reproductive disease, and euthanasia in koalas admitted to rehabilitation (Chapter 2, this thesis). Mucosal PhaHV-1 and PhaHV-2 shedding were also associated with the presence of reproductive disease in males and females, reduced female fertility, and urinary incontinence (Vaz *et al.*, 2019b). These varied results may resemble a co-infection dynamic previously observed in mouse models where pre-infection or simultaneous infection by *C. muridium* provides protection of the host to disease from herpes simplex virus-2 (HSV-2) disease, which is lost if chlamydia replication stops or replication-incompetent chlamydia infection is applied (Ghasemian *et al.*, 2023; Slade *et al.*, 2016). Although a similar mechanistic study is required to determine whether these dynamics exist in the *C. pecorum* – PhaHV-1 and PhaHV-2 interaction, the current study strongly suggests that the relationship between

koala gammaherpesviruses and chlamydiosis is complex and future studies would need to consider different stages of infection, such as latency and (re-)activation, that currently cannot be distinguished in the koala.

Corroborating with previous studies (Chapter 2 and 3, this thesis), the frequency of detection of circulating *C. pecorum* is low in wild populations and does not always coincide with mucosal *C. pecorum* shedding (Supplementary materials: Table 7.22). Due to the low detection frequency and resultingly low variation and meaningful contribution to models, circulating *C. pecorum* was not a significant indicator of survival in any model. It is clear that the NanoString method of detection requires further validation to determine *C. pecorum* probe-specific sensitivity and specificity compared to gold-standard methods such as qPCR. For this, the first step would be to optimise and validate a *C. pecorum* qRT-PCR to quantify cDNA counts of the same targets. The same is suggested for circulating PhaHV-1 (which was not assessed in this study) and PhaHV-2, which was also rarely detected.

Multivariate models that built by including significant univariate relationships in this study should be re-examined in a larger dataset to clarify their relative importance in the context of other factors. Further studies should also compare the mucosal and systemic immune responses from koalas with mucosal, circulating, and combined infections, given that mucosal immune responses differ to systemic immune responses (Phillips *et al.*, 2024b; Vats *et al.*, 2007; Waugh *et al.*, 2015). This comparison would likely provide a more complete perspective of the koala-*C. pecorum* relationship.

5.7 Conclusion:

A complex interplay of clinical, immunological, and environmental factors influences the survival and treatment outcomes of wild koalas. Our findings highlight the critical role of adaptive lymphocyte immune responses while also emphasizing the diverse interactions among immune markers that may alter their functions. While causation cannot be inferred from this association study, we present compelling evidence that koalas with strong expression of lymphocyte differentiation markers are likely to experience better outcomes and longer survival. Therefore, the potential for site-specific pressures, such as demographics, pathogen factors, co-infections, and genetic factors, to impair this should be further evaluated and considered in both wildlife management and clinical settings. This study highlights the importance of understanding these site-specific risks linked to disease dynamics and co-infection status, reinforcing the need for targeted conservation strategies tailored to regional variations. By identifying measurable risk factors and enhancing our understanding of immune responses, our research establishes an approach that – when applied in a longitudinal setting - can elucidate the mechanisms driving morbidity and mortality in koalas and quantify immunological impacts of potential drivers of disease, ultimately informing effective conservation efforts and enhancing the resilience of this iconic species.

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Data availability statement:

The dataset used and analysed for this study can be available from the corresponding author on reasonable request, and with approval from the Department of Transport and Main Roads via a data licence agreement.

Author contributions:

Yasmine S. S. Muir; conceptualisation, field work and data curation, methodology, formal analysis, investigation, writing of the original draft and reviewing and editing of the manuscript. Damien P. Higgins and Mark B. Krockenberger; supervision, conceptualisation, investigation, writing and reviewing of the manuscript and provision of research resources. Belinda R. Wright and Andrea Casteriano; methodology, the provision of research resources, and reviewing of the manuscript. Deidré L. de Villers and Julien Grosmaire; field work, data curation and reviewing of the manuscript.

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Competing Interests Statement:

Deidré L. de Villers and Julien Grosmaire are employed by Endeavour Veterinary Ecology Pty Ltd and are involved in continuous monitoring and provision of veterinary care for the studied population. Otherwise, the authors declare no competing interests.

Chapter 6 General Discussion

6.1 General discussion of findings and future directions:

Disease can generate significant pressures on wildlife populations. It is the result of complex interactions among multiple factors relating to the host, pathogen(s), and the environment. As environmental factors and pathogen prevalence vary with climate change and urbanisation, it is increasingly important to have strategies in place to monitor these factors and predict disease risk, to help prevent or mitigate its impacts. Using the koala as a model species, the work in this thesis has advanced our understanding of these complex relationships; revealing associations that appear likely to be important in expression and prediction of health and disease (Supplementary materials: Table 7.30). By first exploiting the variety of clinical cases admitted to wildlife hospitals to capture relationships among some of these systems (**Chapters 2-4**), and then re-assessing the consistency of findings in two wild populations (**Chapter 5**), we identified risk factors that may be applicable to predict health, treatment, and survival outcomes within these two different settings. Host gene expression phenotypes were not strongly associated with detection of an infectious agent, nor the presentation of a specific disease, but rather the severity of disease in general, which is reflected in survival and clinical outcomes. As hypothesised, chlamydiosis treatment response, and short and long-term survival were associated with co-infection status, gene transcription, and clinical interventions, highlighting the importance of understanding the variety of individual and population risks that may influence these outcomes. Here we discuss the collective findings from the four experimental chapters of this thesis and demonstrate the significance of this work to the progression of koala conservation and wildlife conservation in general.

The stepwise approach used in this thesis to break down and then integrate pathogen, host and environment systems allowed us to assess the relative strength of associations between a large number of variables and koala health and disease, and is likely to be a useful approach in future wildlife health investigations. Identifying the relative strength of these associations and placing them in the context of current knowledge allows them to be prioritised for investigation of causative relationships through longitudinal and mechanistic studies (Figure 1.1). By first targeting infectious agents in **Chapter 2**, we determined the relative prevalence of potential pathogens in the clinical setting to focus further analysis on agents that were frequently detected or significantly associated with other agents, disease, and survival. In a subset of this same population, we then integrated host gene transcription data in **Chapter 3** and applied an approach, novel to wildlife immunology, to confirm the validity of targets as markers of immune and physiological systems and then assess their relationships with co-infections, disease, and outcomes on admission to rehabilitation. This integration of host immune, stress, transcription/restriction-factor gene expression allowed a more complete evaluation of host-pathogen relationships that predicted, and could be driving, outcomes. The expression panel was then validated and expanded by identification of additional candidate indicators by use of transcriptomics in **Chapter 4,** before integrating environmental factors in **Chapter 5,** by extending beyond the clinical setting into free-ranging koala populations. The strategy employed here was inspired by a similar approach previously used to improve the understanding of host-pathogen-environment relationships and disease in several species of farmed and wild salmon, whereby health indicators, such as thermal stress, infection burden, and pathogen community composition, that could be used in health assessments and as a basis to adjust fishing practices, were identified (Miller *et al.*, 2017; Miller *et al.*, 2014; Mordecai *et al.*, 2020; Teffer *et al.*, 2019; Teffer *et al.*, 2022). Although it

was not feasible for this thesis to incorporate all systems potentially significant to health and pathogenesis, the approaches taken here to assess the relationships among many clinical, infectious, genetic, and demographic variables appears suitable to allow incorporation of additional factors in future studies.

This work showed some discord between detection of circulating and mucosal *C. pecorum* targets that raises questions about the pathogenesis of chlamydiosis. In **Chapter 2**, circulating *C. pecorum* was detected in koalas admitted to wildlife hospitals, sometimes independent of mucosal *C. pecorum*. This is a significant finding as it suggests that a cohort of sub-clinically infected koalas exist undetected and we do not know the risks they pose to perpetuating transmission. Although the NanoString detection method used throughout this thesis requires further validation against gold-standard *C. pecorum* detection techniques such as qPCR, the comparison with results from a *C. pecorum* free population suggests that the *C. pecorum* gene transcripts counted in this study are unlikely to be background noise. The good agreement between NanoString and RNAseq mRNA counts demonstrated in Chapter 4 provides some additional confidence that the NanoString method is robust. However, due to the inability to generate analysable data when mapping to pathogen genomes in this thesis, perhaps due to their low copy number relative to many host and KoRV transcripts, additional studies are needed to compare the transcription of pathogen genes between the two methods.

Circulating *C. pecorum* has been documented in other species (Li *et al.*, 2016a) but this is the first time it has been documented in the koala. *C. pecorum* can infect immune cells of other mammals *in vitro* (Islam *et al.*, 2019), and has been observed in several organs of the koala

that are suggestive of inhalation (Mackie *et al.*, 2016) or systemic spread (Burach *et al.*, 2014; Higgins *et al.*, 2005b; Phillips *et al.*, 2018; Wedrowicz *et al.*, 2016)(Burnard *et al.*, 2018). Naturally, a series of questions were formulated through these findings: (1) How does circulating *C. pecorum* infection occur? (2) Can *C. pecorum* in circulation disseminate to mucosal sites and induce pathology? (3) Does circulating *C. pecorum* represent a stage in the pathogen life-cycle that avoids immunosurveillance, also known as persistence (Hogan *et al.*, 2004)? (4) If so, what mechanisms induce this pathogen response and do these represent a "successful" host response? (5) How do currently applied chlamydiosis vaccinations affect the state and interactions of circulating *C. pecorum* with the host? Future investigations could employ several methods to explore these questions; single-cell RNAseq could indicate which cell types are infected with *C. pecorum,* and how this affects cell phenotype and overall immune phenotype relative to uninfected cells and hosts (Ravindra *et al.*, 2021), and electron microscopy and *Chlamydia* sp. gene expression analysis could determine whether morphologically distinct aberrant bodies (aRBs) can be observed in circulating white blood cells and mucosal cells over the course of infection (Matsumoto & Manire, 1970; Schoborg, 2011) and explore host and pathogen gene expression between the two infection sites to determine functional differences (Mathews *et al.*, 2001; Muramatsu *et al.*, 2016; Ouellette *et al.*, 2006; Vollmuth *et al.*, 2022; Yang *et al.*, 2020). Given the limited number of positive cases in **Chapter 4**, additional sampling should be conducted to build a larger sample size to support gene expression analysis that could provide insight into the interactions between circulating *C. pecorum* and the host.

Through the collective analysis of several key infectious agents of the koala, this thesis not only builds upon previous studies that investigated these agents separately but also provides

the relative importance of different infectious agents in the context of chlamydiosis. The frequency of co-infections detected was far greater than expected and is consistent with the heterogeneity of clinical chlamydiosis and treatment outcomes observed in hospital admissions and in wild populations (Fernandez *et al.*, 2024b; Quigley *et al.*, 2018a). This suggests that, firstly, co-infection relationships should be accounted for in investigations of drivers of disease to avoid confounding of results and, secondly, co-infection interactions are an important avenue for further investigation that may have significant implications for disease management. Here, the key findings that contribute to the development of our understanding of co-infections in the koala are discussed: the association between *Trypanosome* infection and lower likelihood of disease; the complex interactions of PhaHV-1 and PhaHV-2 with chlamydiosis, which appear equally important to KoRV; and the interactions between KoRV, health status, and host gene expression.

The association between *Trypanosome* co-infection and lower likelihood of chlamydial disease appears to be *Trypanosome* species-dependent but requires further investigation using a greater sample size. Previously, McInnes *et al.* (2011a) noted a link between *T. gilletti* infection and anaemia and poor body condition in koalas with chlamydiosis, while no relationships were observed between *T. irwini* or *T. copemani* and clinical outcomes. Although this thesis could not assess the relationship between *T. gilletti* and clinical outcomes due to low frequency of detection, in **Chapter 2** inclusion of *T. irwini* improved the model prediction of chlamydiosis, with the negative relationship approaching significance. The mechanisms behind this are unclear as neither *T. irwini, T. copemani, T. gilletti* nor *Trypanosome* genus (combined variable) was associated with differential immune gene expression in **Chapter 3**. Further investigation of the relationship between trypanosome infection and pathogenesis in

the koala should consider the ability for trypanosome species to employ mechanisms of host-immune system evasion (Cnops *et al.*, 2015; Horn, 2014; Horn & McCulloch, 2010), and alter the direction of pathogenesis based on the diversity of existing co-infecting agents (Dwinger *et al.*, 1989; Lozano *et al.*, 2024; Morrison *et al.*, 1982; Sanches-Vaz *et al.*, 2019).

The relationship between PhaHV-1 and PhaHV-2 and disease or survival is complicated by strong associations with *C. pecorum* infection and chlamydiosis. In **Chapter 2**, detection of mucosal PhaHV-1 was associated with chlamydiosis, reproductive disease, and euthanasia in *C. pecorum*-infected koalas. Although this complements some of the observations in southern koalas by Vaz *et al.* (2019b), where mucosal PhaHV-1 and PhaHV-2 was associated with reproductive disease, here mucosal PhaHV-2 was not associated with infection or disease outcomes and circulating PhaHV-1 and -2 were too rarely detected to meaningfully contribute to modelling. In female koalas, Vaz *et al.* (2019b) observed an association between the detection of PhaHV-1 and KoRV. Whilst this relationship was not directly assessed, in **Chapter 2** both KoRV proviral *pol* loads and the detection of PhaHV-1 were equally important predictors of female reproductive disease. Although these complementary findings would be suggestive of a potentially pathogenic role for PhaHV-1 in koala chlamydiosis, findings in **Chapter 5** of this thesis indicate that the interactions are more complex and likely differ with context: mucosal PhaHV-1 detection predicted better survival in koalas treated for chlamydiosis.

This thesis supports previous reports associating KoRV transcription and disease, and expands our knowledge of associated changes in gene expression *in-vivo*, which will inform investigations of causation. Consistent with Blyton *et al.* (2022a) and Fabijan *et al.* (2020),

strong associations between greater KoRV *pol* transcription and *C. pecorum* detection (**Chapter 2)** and poorer survival outcomes **(Chapter 3)** were observed in koalas in the clinical setting. In free-ranging koalas (**Chapter 5)**, however, KoRV *pol* was not as strongly associated with chlamydiosis or *C. pecorum* infection as were immune gene principal components or demographic variables. Instead, KoRV *pol* was associated with mortality in a low morbidity free-ranging koala population with comparable chlamydial and non-chlamydial drivers of mortality. Also consistent with Blyton *et al.* (2022a), Hashem *et al.* (2021), Quigley *et al.* (2018b), and Sarker *et al.* (2019), in **Chapter 3,** KoRV *pol* transcription was most strongly correlated with exogenous KoRV D *env* transcription, rather than KoRV A, and this was further demonstrated in **Chapter 5** by the separation of KoRV A into a separate principal component; these support exogenous KoRV transcription as the primary contributor to overall KoRV *pol* transcription. In this thesis, principal components analysis revealed positive correlations between endogenous KoRV A and lymphocytic genes and SAMHD1 (**Chapter 3** and **5)** and between exogenous KoRV and a cathelicidin-like gene **(Chapter 5),** demonstrating potentially interacting pathways that could be involved in the host-viral response or driving clinical relationships; relationships not evident when KoRV *pol* transcription was evaluated among populations, independent of host gene expression (Fernandez *et al.*, 2024b).

The use of principal components analysis in this thesis allowed for the immune response to be presented as a collective communicating network. This allowed understanding of the relationships and potential significance of individual markers to be developed in a way that has not been evident in less integrated studies with fewer variables (Desclozeaux *et al.*, 2017; Jobbins *et al.*, 2012; Kayesh *et al.*, 2021a, 2021b; Khan *et al.*, 2016; Lau *et al.*, 2012; Lau *et al.*, 2013; Lizárraga *et al.*, 2020a, 2020b; Maher *et al.*, 2014; Maher & Higgins, 2016; Maher *et al.*,

2019; Marschner *et al.*, 2019a; Mathew, 2014; Mathew *et al.*, 2013a; Nyari *et al.*, 2019; Olagoke *et al.*, 2018; Olagoke *et al.*, 2019; Olagoke *et al.*, 2020a; Olagoke *et al.*, 2020b; Robbins *et al.*, 2020; Simpson *et al.*, 2023). Although many novel relationships were identified throughout this thesis, here we focus our discussion on the most prominent groups: genes associated with the adaptive immune response, the innate 'pro-inflammatory' response, and the stress-response; and their relevance to koala health risk assessment and disease management, and future research on pathogenesis and prognostic indicators.

Aligning with the damage-response framework, which considers the host-response as a key contributor to the development of disease (Casadevall & Pirofski, 2003), the findings of this thesis identify immune genes that are differentially associated with survival in the koala. Consistent with the reduced expression of similar genes in a wild koala population under climatic stress (Fernandez *et al.*, 2024b), reduced expression of lymphocyte differentiation markers consistently predicted poorer survival outcomes, across clinical (**Chapters 3** and **4**) and free-ranging (**Chapter 5**) study cohorts. In addition to these genes, upregulation of *TLR7,* an innate toll-like-receptor gene was correlated with lymphocytic activity and was therefore positively associated with improved survival in **Chapter 3** and **5.** The relationship between *TLR7* and lymphocyte genes is consistent with its role in initiating responses, particularly to viral agents (Kayesh *et al.*, 2021a, 2021b; Russ & Iordanskiy, 2023). On the other hand, transcription of innate pro-inflammatory and cytokine receptor genes of adaptive immunity appears to be associated with host-damage. Upregulation of some innate pro-inflammatory genes (*RETN, TLR2, TLR4, CELC4E, CARD9* and Cathelicidin-like gene (LOC110217150) in clinical and free-ranging koalas in **Chapter 3** and **5**, respectively, and *IL1R2*, *MARCO*, *MYO1B*, and *RARRES1* in clinical koalas in **Chapter 4**), was associated with poor survival. Also in

Chapter 5, greater adaptive cytokine receptor (*IL10R2*, *IFNGR1*, and *IL17RA*) transcription associated with mortality**.** Other genes had more context dependent relationships: MHCII genes *PhciDAB* and *PhciDBB* strongly correlated with lymphocytic differentiation markers and improved outcomes in clinical koalas in **Chapter 3** but predicted mortality in free-ranging koalas treated for chlamydiosis in **Chapter 5;** and greater *IL18* transcription on admission was strongly associated with euthanasia at triage, but greater *IL18* post-treatment in clinical koalas in **Chapter 3** lowered risk. Collectively, these findings likely reflect the interdependence of innate and adaptive responses, the potentially pleotropic functions of immune gene products, and the contexts in which immune responses are productive or pathogenic.

At this stage, immune response indicators could help inform on whole population health status. For example, in bats with white-nose syndrome, population differences in innate-adaptive immune activity impacted treatment efficacy, leading to the recommendation that bat populations are immunologically tested prior to treatment to help inform best management approaches (Maslo *et al.*, 2017). From the findings of this thesis, adaptive immune markers clearly have potential utility in identifying at-risk populations requiring targeted conservation efforts, but it also appears that determining the balance between adaptive and innate pro-inflammatory immune responses could improve accuracy of prognosis and augment triage protocols in clinical settings. Here we propose several hypotheses worth testing to improve our understanding of the roles and interactions of innate and adaptive immune pathways: (1) lower expression of lymphocyte differentiation markers here and in the work by Fernandez *et al.* (2024b), is associated with immune impairment, whereas higher expression represents immunocompetence; (2) lower cytokine receptor expression reflects host responses that limit inflammatory pathology in

chlamydiosis, while higher expression reflects damaging host responses; and (3) overexpression of pro-inflammatory innate immune genes underpins the inflammatory pathology observed in koalas with severe disease. Although we suggest that these hypotheses are best tested by longitudinal studies across multiple populations under varying conditions, cell-culture using koala cell line and next-generation approaches to *in vitro* modelling of immune responses are rapidly developing. Next-generation approaches offer a range of strategies that retain native tissue structures, and can provide a 3D microenvironment with the relevant fluid dimension, biophysical cues and environmental control to test causes and effects (Aleman *et al.*, 2019; de Oliveira *et al.*, 2025; Gosselin *et al.*, 2018; Hammel *et al.*, 2021; Perucca *et al.*, 2025; Sieber *et al.*, 2018). While the adoption of these techniques in veterinary sciences is slow (Ferraz *et al.*, 2020; Nagao *et al.*, 2024; Premachandre *et al.*, 2025; Sakata *et al.*, 2024; Zdyrski *et al.*, 2024), recent reviews have discussed their potential as an ethical, high-throughput, and physiologically relevant approach in this field (Chen *et al.*, 2023a; Yin *et al.*, 2025), which should be considered for future investigations of koala pathogenesis and immune response dynamics.

Stress responses were not a strong indicator of koala health in the clinical or free-ranging setting, suggesting further investigation may be needed. The association between *FKBP5,* a key modulator of the stress response, and koalas' health and survival in this thesis supports previous work that identified a relationship between upregulation of *FKBP5* and climatic extremes and disease (Fernandez *et al.*, 2024b): upregulation of *FKBP5* was associated with euthanasia at triage in the subset of koalas analysed using RNAseq **(Chapter 4)**; but not in the full sample-set analysed using NanoString **(Chapter 3)**; and in free-ranging koalas **(Chapter 5),** *FKBP5* positively correlated with genes that were associated with reduced (PC2) survival in

koalas with chlamydiosis. The relationships between this marker and health and survival observed here suggest that environmental factors may not be the only driver of FKBP5 variation. For instance, despite having significantly different frequencies of morbidity and mortality, the two free-ranging populations studied in **Chapter 5**, although separated, were neighbouring and so it is expected that climatic conditions were similar. In comparison, the populations studied by Fernandez *et al.* (2024b) represented two distinct populations exposed to different climatic and infectious pressures. Thus, we suggest that outcome related variations in *FKBP5* transcription may be associated with more subtle environmental and pathological diversity of the dataset rather than differences in sampling contexts, and that additional factors such as resource availability and population genetics may be influencing stress responses (Davies *et al.*, 2014; Davies *et al.*, 2013; Narayan & Vanderneut, 2019; Santamaria *et al.*, 2023). To clarify the link between stress and immune outcomes, future research should incorporate complementary measures of stress, such as the more common measure of koala stress; faecal cortisol metabolites (FCMs) (Charalambous *et al.*, 2021; Davies *et al.*, 2014; Davies *et al.*, 2013; Narayan & Vanderneut, 2019; Santamaria *et al.*, 2023; Sheriff *et al.*, 2011), and more novel techniques such as leukocyte coping capacity (LCC) (McLaren *et al.*, 2003). These measures provide insights into different aspects of the stress response, from delayed systemic effects to acute immune responses (McLaren *et al.*, 2003; Narayan *et al.*, 2013). Integrating these parameters in longitudinal studies could yield a more comprehensive understanding of how stress interacts with disease progression and immune function.

The findings presented in this thesis have several practical applications for koala conservation and health management. To accurately determine the prevalence of *C. pecorum* in koala populations, detection in circulation should also be conducted. Given its strong relationship

with *C. pecorum* and chlamydiosis, PhaHV-1 and -2 detection in mucosal sites and in circulation should be determined across populations, and routinely in clinics, to help to improve our understanding of this agent. Adaptive and innate immune markers, particularly those associated with long-term immunity and pro-inflammatory responses, could serve as key indicators for assessing population health and identifying at-risk groups. We acknowledge the practical challenge of measuring so many plausible indicators of health, which is where a big-data approach would be appropriate. Alternatively, examples in the literature exist where similar immune parameters have been integrated within a holistic measure or scoring system to provide a simple indicator of immune function that can predict health outcomes: the immune health metric (IHM) (Kubo & Tanaka, 2024; Sparks *et al.*, 2024; Vinuesa *et al.*, 2024), Resistance(R)/Systemic Inflammation(SI)-based endotyping (Brandes-Leibovitz *et al.*, 2024), the cytokine storm (CS) score (Cappanera *et al.*, 2021), and modified APACHE II (Acute Physiology and Chronic Health Evaluation) or MOD (Multiple Organ Dysfunction) scores (Chakraborty & Burns, 2024; Choi *et al.*, 2017; Oberholzer *et al.*, 2005; Zimmerman *et al.*, 2006). Transcription-associated genes such as *TRIM24* and *SAMHD1* comprise promising candidates for predicting treatment outcomes and understanding host-pathogen interactions, particularly with KoRV *pol* transcription. Longitudinal monitoring of free-ranging koalas, combined with *in vitro* studies of immune responses, would provide critical insights into the temporal dynamics of host-pathogen interactions and stress responses. Additionally, integrating complementary stress measures, such as faecal cortisol metabolites and leukocyte coping capacity, could improve our understanding of the links between stress, disease progression, and survival outcomes. The role of cathelicidins in immune modulation and pathogen resistance also warrants further investigation. Understanding the context-

dependent effects of these antimicrobial peptides could inform new therapeutic approaches for managing chlamydiosis and other infectious diseases in koalas.

6.2 Conclusion:

This thesis advances the understanding of host-pathogen-environment interactions and approaches to investigating these relationships in the koala. Collectively, the findings of this thesis demonstrate the complexity of biological responses in koalas and, due to the high frequency of co-infection, the challenge in associating host-responses and pathogenesis to a single infectious agent. The identification of key genes that could be used as indicators of individual and population health, such as those associated with adaptive lymphocytic responses, demonstrates why understanding, and accounting for, complexity is important, particularly in the context of disease management and conservation. To continue to develop the foundation for improving diagnostic, prognostic, and therapeutic strategies, future studies should investigate the key hypotheses generated through this work. In particular, longitudinal studies should elucidate the role of circulating infectious agents in the pathogenesis of chlamydiosis and determine whether circulating *C. pecorum* is a latent state invoked by the host, by co-infecting organisms, or by *C. pecorum* itself. Furthermore, although challenging, longitudinal studies and *in vitro* experimentation should be conducted to determine the relationship between exposure and cellular responses. Through this, the mechanisms driving host-responses associated with improved health or damage can be elucidated and better incorporated into actionable approaches for wildlife health management.

References for the thesis:

Abts, K. C., Ivy, J. A., & DeWoody, J. A. (2015). Immunomics of the koala (Phascolarctos cinereus). *Immunogenetics*, ***67***, 305-321.

Adams-Hosking, C., McBride, M. F., Baxter, G., Burgman, M., de Villiers, D., Kavanagh, R., Lawler, I., Lunney, D., Melzer, A., Menkhorst, P., Molsher, R., Moore, B. D., Phalen, D., Rhodes, J. R., Todd, C., Whisson, D., & McAlpine, C. A. (2016). Use of expert knowledge to elicit population trends for the koala (Phascolarctos cinereus). *Diversity and Distributions*, ***22***(3), 249-262. https://doi.org/https://doi.org/10.1111/ddi.12400

Aguilar, X. F., Leclerc, L.-M., Mavrot, F., Roberto-Charron, A., Tomaselli, M., Mastromonaco, G., Gunn, A., Pruvot, M., Rothenburger, J. L., Thanthrige-Don, N., Jahromi, E. Z., Kutz, S., Kugluktuk Angoniatit, A., Ekaluktutiak, H., Trappers, O., Olokhaktomiut, H., & Trappers, C. (2023). An integrative and multi-indicator approach for wildlife health applied to an endangered caribou herd. *Scientific reports*, ***13***(1), 16524. https://doi.org/10.1038/s41598-023-41689-y

Ahmed, F., Hetty, S., Laterveer, R., Surucu, E. B., Mathioudaki, A., Hornbrinck, E., Patsoukaki, V., Olausson, J., Sundbom, M., Svensson, M. K., Pereira, M. J., & Eriksson, J. W. (2025). Altered Expression of Aromatase and Estrogen Receptors in Adipose Tissue From Men With Obesity or Type 2 Diabetes. *The Journal of Clinical Endocrinology & Metabolism*. https://doi.org/10.1210/clinem/dgaf038

Akter, L., Hashem, M. A., Rakib, T. M., Rashid, M. H. O., Hossain, K. A., Akhter, R., Utsunomiya, M., Kitab, B., Hifumi, T., & Miyoshi, N. (2023). Investigation of koala retrovirus in captive koalas with pneumonia and comparative analysis of subtype distribution. *Arch Virol*, ***168***(12), 298.

Albery, G. F., Becker, D. J., Kenyon, F., Nussey, D. H., & Pemberton, J. M. (2019). The Fine-Scale Landscape of Immunity and Parasitism in a Wild Ungulate Population. *Integrative and Comparative Biology*, ***59***(5), 1165-1175. https://doi.org/10.1093/icb/icz016

Aleman, J., George, S. K., Herberg, S., Devarasetty, M., Porada, C. D., Skardal, A., & Almeida-Porada, G. (2019). Deconstructed microfluidic bone marrow on-A-chip to study normal and malignant hemopoietic cell–niche interactions. *Small*, ***15***(43), 1902971.

Aleuy, O. A., Kutz, S., Mallory, M. L., & Provencher, J. F. (2023). Wildlife health in environmental impact assessments: are we missing a key metric? *Environmental Reviews*, ***31***(2), 348-359. https://doi.org/10.1139/er-2022-0023

Alijagic, A., Russo, R., Scuderi, V., Ussia, M., Scalese, S., Taverna, S., Engwall, M., & Pinsino, A. (2025). Sea urchin immune cells and associated microbiota co-exposed to iron oxide nanoparticles activate cellular and molecular reprogramming that promotes physiological adaptation. *Journal of Hazardous Materials*, ***485***, 136808. https://doi.org/https://doi.org/10.1016/j.jhazmat.2024.136808

Alizon, S., & van Baalen, M. (2008). Multiple infections, immune dynamics, and the evolution of virulence. *The American Naturalist*, ***172***(4), E150-E168.

Anders, S., Pyl, P. T., & Huber, W. (2015). HTSeq—a Python framework to work with high-throughput sequencing data. *bioinformatics*, ***31***(2), 166-169.

Anderson, K. B. D. (2002). Model selection and multimodel inference: a practical information-theoretic approach Springer-Verlag New York. *NY, USA*.

Andres-Terre, M., McGuire, H. M., Pouliot, Y., Bongen, E., Sweeney, T. E., Tato, C. M., & Khatri, P. (2015). Integrated, multi-cohort analysis identifies conserved transcriptional signatures across multiple respiratory viruses. *Immunity*, ***43***(6), 1199-1211.

Andrew, D. W., Cochrane, M., Schripsema, J. H., Ramsey, K. H., Dando, S. J., O'Meara, C. P., Timms, P., & Beagley, K. W. (2013). The duration of Chlamydia muridarum genital tract infection and associated chronic pathological changes are reduced in IL-17 knockout mice but protection is not increased further by immunization. *PLoS One*, ***8***(9), e76664.

Andrews, S. (2023). *FastQC*. In (Version Version 0.12.0) http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Aneja, K. K., & Yuan, Y. (2017). Reactivation and lytic replication of Kaposi's sarcoma-associated herpesvirus: an update. *Frontiers in Microbiology*, ***8***, 613.

Arndts, K., Deininger, S., Specht, S., Klarmann, U., Mand, S., Adjobimey, T., Debrah, A. Y., Batsa, L., Kwarteng, A., Epp, C., Taylor, M., Adjei, O., Layland, L. E., & Hoerauf, A. (2012). Elevated Adaptive Immune Responses Are Associated with Latent Infections of Wuchereria bancrofti. *PLOS Neglected Tropical Diseases*, ***6***(4), e1611. https://doi.org/10.1371/journal.pntd.0001611

Artois, M., Bengis, R., Delahay, R. J., Duchêne, M.-J., Duff, J. P., Ferroglio, E., Gortazar, C., Hutchings, M. R., Kock, R. A., Leighton, F. A., Mörner, T., & Smith, G. C. (2009). Wildlife Disease Surveillance and Monitoring. In R. J. Delahay, G. C. Smith, & M. R. Hutchings (Eds.), *Management of Disease in Wild Mammals* (pp. 187-213). Springer Japan. https://doi.org/10.1007/978-4-431-77134-0_10

Asquith, K. L., Horvat, J. C., Kaiko, G. E., Carey, A. J., Beagley, K. W., Hansbro, P. M., & Foster, P. S. (2011). Interleukin-13 promotes susceptibility to chlamydial infection of the respiratory and genital tracts. *PLOS Pathogens*, ***7***(5), e1001339.

Attermann, A. S., Bjerregaard, A. M., Saini, S. K., Grønbæk, K., & Hadrup, S. R. (2018). Human endogenous retroviruses and their implication for immunotherapeutics of cancer. *Annals of Oncology*, ***29***(11), 2183-2191. https://doi.org/https://doi.org/10.1093/annonc/mdy413

Australian Government. (2021). *Wildlife Care in Australia Report*. Retrieved from https://www.dcceew.gov.au/sites/default/files/documents/wildlife-care-in-australia-report.pdf

Ávila-Arcos, M. C., Ho, S. Y., Ishida, Y., Nikolaidis, N., Tsangaras, K., Hönig, K., Medina, R., Rasmussen, M., Fordyce, S. L., & Calvignac-Spencer, S. (2012). One hundred twenty years of koala retrovirus evolution determined from museum skins. *Molecular biology and evolution*, ***30***(2), 299-304.

Azenabor, A. A., & Chaudhry, A. U. (2003). Chlamydia pneumoniae Survival in Macrophages is Regulated by Free Ca2+ Dependent Reactive Nitrogen and Oxygen Species. *Journal of Infection*, ***46***(2), 120-128. https://doi.org/https://doi.org/10.1053/jinf.2002.1098

Bachmann, N. L., Fraser, T. A., Bertelli, C., Jelocnik, M., Gillett, A., Funnell, O., Flanagan, C., Myers, G. S. A., Timms, P., & Polkinghorne, A. (2014). Comparative genomics of koala, cattle and sheep strains of Chlamydia pecorum. *BMC Genomics*, ***15***(1), 667. https://doi.org/10.1186/1471-2164-15-667

Bailey, R., Holland, M., Whittle, H., & Mabey, D. (1995). Subjects recovering from human ocular chlamydial infection have enhanced lymphoproliferative responses to

chlamydial antigens compared with those of persistently diseased controls. *Infection and immunity*, ***63***(2), 389-392.

Bajaj, V., Gadi, N., Spihlman, A. P., Wu, S. C., Choi, C. H., & Moulton, V. R. (2021). Aging, Immunity, and COVID-19: How Age Influences the Host Immune Response to Coronavirus Infections? [Review]. *Frontiers in Physiology*, ***11***. https://doi.org/10.3389/fphys.2020.571416

Balcewicz-Sablinska, M. K., Gan, H., & Remold, H. (1999). Interleukin 10 produced by macrophages inoculated with Mycobacterium avium attenuates mycobacteria-induced apoptosis by reduction of TNF-α activity. *The Journal of infectious diseases*, ***180***(4), 1230-1237.

Ball, L. M., Bronstein, E., Liechti, G. W., & Maurelli, A. T. (2024). Neisseria gonorrhoeae drives Chlamydia trachomatis into a persistence-like state during in vitro co-infection. *Infect Immun*, ***92***(1), e0017923. https://doi.org/10.1128/iai.00179-23

Ballantyne, K., Lisle, A., Mucci, A., & Johnston, S. D. (2015). Seasonal oestrous cycle activity of captive female koalas in south-east Queensland. *Australian Mammalogy*, ***37***(2), 245-252. https://doi.org/https://doi.org/10.1071/AM14018

Barbosa, A., Austen, J., Gillett, A., Warren, K., Paparini, A., Irwin, P., & Ryan, U. (2016). First report of Trypanosoma vegrandis in koalas (Phascolarctos cinereus). *Parasitology International*, ***65***(4), 316-318. https://doi.org/https://doi.org/10.1016/j.parint.2016.03.004

Barbosa, A. D., Gofton, A. W., Paparini, A., Codello, A., Greay, T., Gillett, A., Warren, K., Irwin, P., & Ryan, U. (2017). Increased genetic diversity and prevalence of co-infection with Trypanosoma spp. in koalas (Phascolarctos cinereus) and their ticks identified using next-generation sequencing (NGS). *PLoS One*, ***12***(7), e0181279. https://doi.org/10.1371/journal.pone.0181279

Barker, C. J., Gillett, A., Polkinghorne, A., & Timms, P. (2013). Investigation of the koala (Phascolarctos cinereus) hindgut microbiome via 16S pyrosequencing. *Veterinary microbiology*, ***167***(3-4), 554-564.

Barr, E. L., Ouburg, S., Igietseme, J. U., Morré, S. A., Okwandu, E., Eko, F. O., Ifere, G., Belay, T., He, Q., Lyn, D., Nwankwo, G., Lillard, J. W., Jr., Black, C. M., & Ananaba, G. A. (2005). Host inflammatory response and development of complications of Chlamydia trachomatis genital infection in CCR5-deficient mice and subfertile women with the CCR5delta32 gene deletion. *J Microbiol Immunol Infect*, ***38***(4), 244-254.

Barroso, P., López-Olvera, J. R., Kiluba wa Kiluba, T., & Gortázar, C. (2024). Overcoming the limitations of wildlife disease monitoring. *Research Directions: One Health*, ***2***, e3, Article e3. https://doi.org/10.1017/one.2023.16

Barroso, P., Relimpio, D., Zearra, J. A., Cerón, J. J., Palencia, P., Cardoso, B., Ferreras, E., Escobar, M., Cáceres, G., & López-Olvera, J. R. (2023). Using integrated wildlife monitoring to prevent future pandemics through one health approach. *One Health*, ***16***, 100479.

Barton, K. (2023). *Package 'MuMIn': Multi-Model Inference*. In [R package version 1.47.5]. https://CRAN.R-project.org/package=MuMIn

Bastidas, R. J., Elwell, C. A., Engel, J. N., & Valdivia, R. H. (2013). Chlamydial intracellular survival strategies. *Cold Spring Harb Perspect Med*, ***3***(5), a010256. https://doi.org/10.1101/cshperspect.a010256

Beatty, J. (2014). Viral causes of feline lymphoma: Retroviruses and beyond. *The veterinary journal*, ***201***(2), 174-180.

Beatty, W. L., Byrne, G. I., & Morrison, R. P. (1993). Morphologic and antigenic characterization of interferon gamma-mediated persistent Chlamydia trachomatis infection in vitro. *Proc Natl Acad Sci U S A*, ***90***(9), 3998-4002. https://doi.org/10.1073/pnas.90.9.3998

Beauvais, W., Zuther, S., Villeneuve, C., Kock, R., & Guitian, J. (2019). Rapidly assessing the risks of infectious diseases to wildlife species. *Royal Society Open Science*, ***6***(1), 181043. https://doi.org/doi:10.1098/rsos.181043

Becker, D. J., Albery, G. F., Kessler, M. K., Lunn, T. J., Falvo, C. A., Czirják, G. Á., Martin, L. B., & Plowright, R. K. (2020). Macroimmunology: The drivers and consequences of spatial patterns in wildlife immune defence. *Journal of Animal Ecology*, ***89***(4), 972-995. https://doi.org/https://doi.org/10.1111/1365-2656.13166

Becker, E., & Hegemann, J. H. (2014). All subtypes of the Pmp adhesin family are implicated in chlamydial virulence and show species-specific function. *Microbiologyopen*, ***3***(4), 544-556.

Bellet, M. M., Deriu, E., Liu, J. Z., Grimaldi, B., Blaschitz, C., Zeller, M., Edwards, R. A., Sahar, S., Dandekar, S., & Baldi, P. (2013). Circadian clock regulates the host response to Salmonella. *Proceedings of the National Academy of Sciences*, ***110***(24), 9897-9902.

Belsare, A. V., Gompper, M. E., Keller, B., Sumners, J., Hansen, L., & Millspaugh, J. J. (2020). An agent-based framework for improving wildlife disease surveillance: A case study of chronic wasting disease in Missouri white-tailed deer. *Ecological Modelling*, ***417***, 108919. https://doi.org/https://doi.org/10.1016/j.ecolmodel.2019.108919

Beranek, C. T., Roff, A., Denholm, B., Howell, L. G., & Witt, R. R. (2020). Trialling a real-time drone detection and validation protocol for the koala (Phascolarctos cinereus). *Australian Mammalogy*, ***43***(2), 260-264.

Beranek, C. T., Southwell, D., Jessop, T. S., Hope, B., Gama, V. F., Gallahar, N., Webb, E., Law, B., McIlwee, A., & Wood, J. (2024). Comparing the cost-effectiveness of drones, camera trapping and passive acoustic recorders in detecting changes in koala occupancy. *Ecology and Evolution*, ***14***(7), e11659.

Bereshchenko, O., Bruscoli, S., & Riccardi, C. (2018). Glucocorticoids, Sex Hormones, and Immunity [Mini Review]. *Frontiers in Immunology*, ***Volume 9 - 2018***. https://doi.org/10.3389/fimmu.2018.01332

Betsou, F., Sueur, J. M., & Orfila, J. (2003). Anti-Chlamydia pneumoniae heat shock protein 10 antibodies in asthmatic adults. *FEMS Immunology & Medical Microbiology*, ***35***(2), 107-111.

Biesenkamp-Uhe, C., Li, Y., Hehnen, H.-R., Sachse, K., & Kaltenboeck, B. (2007). Therapeutic Chlamydophila abortus and C. pecorum vaccination transiently reduces bovine mastitis associated with Chlamydophila infection. *Infection and immunity*, ***75***(2), 870-877.

Blackbourn, D. J., Fujimura, S., Kutzkey, T., & Levy, J. A. (2000). Induction of human herpesvirus-8 gene expression by recombinant interferon gamma. *Aids*, ***14***(1), 98-99.

Blanshard, W., & Bodley, K. (2008). 8. Koalas. In *Medicine of Australian mammals* (pp. 227-329). BioOne.

Blinov, V. M., Krasnov, G. S., Shargunov, A. V., Shurdov, M. A., & Zverev, V. V. (2013). Immunosuppressive domains of retroviruses: Cell mechanisms of the effect on the human immune system. *Molecular Biology*, ***47***(5), 613-621. https://doi.org/10.1134/S0026893313050026

Blyton, M. D. J., Pascoe, J., Hynes, E., Soo, R. M., Hugenholtz, P., & Moore, B. D. (2023). The koala gut microbiome is largely unaffected by host translocation but rather influences host diet. *Frontiers in Microbiology*, ***14***, 1085090.

Blyton, M. D. J., Pyne, M., Young, P., & Chappell, K. (2022a). Koala retrovirus load and non-A subtypes are associated with secondary disease among wild northern koalas. *PLoS Pathog*, ***18***(5), e1010513. https://doi.org/10.1371/journal.ppat.1010513

Blyton, M. D. J., Soo, R. M., Hugenholtz, P., & Moore, B. D. (2022b). Characterization of the juvenile koala gut microbiome across wild populations. *Environmental Microbiology*, ***24***(9), 4209-4219.

Blyton, M. D. J., Soo, R. M., Hugenholtz, P., & Moore, B. D. (2022c). Maternal inheritance of the koala gut microbiome and its compositional and functional maturation during juvenile development. *Environmental Microbiology*, ***24***(1), 475-493.

Blyton, M. D. J., Soo, R. M., Whisson, D., Marsh, K. J., Pascoe, J., Le Pla, M., Foley, W., Hugenholtz, P., & Moore, B. D. (2019). Faecal inoculations alter the gastrointestinal microbiome and allow dietary expansion in a wild specialist herbivore, the koala. *Animal Microbiome*, ***1***(1), 6. https://doi.org/10.1186/s42523-019-0008-0

Blyton, M. D. J., Young, P. R., Moore, B. D., & Chappell, K. J. (2022d). Geographic patterns of koala retrovirus genetic diversity, endogenization, and subtype distributions. *Proc Natl Acad Sci U S A*, ***119***(33), e2122680119. https://doi.org/10.1073/pnas.2122680119

Bolaji, B., Onoja, T., Agbata, C., Omede, B. I., & Odionyenma, U. B. (2024). Dynamical analysis of HIV-TB co-infection transmission model in the presence of treatment for TB. . *Bulletin of Biomathematics*, ***2***(1), 21–56. https://doi.org/https://doi.org/10.59292/bulletinbiomath.2024002

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *bioinformatics*, ***30***(15), 2114-2120.

Bolzoni, L., & De Leo, G. A. (2013). Unexpected Consequences of Culling on the Eradication of Wildlife Diseases: The Role of Virulence Evolution. *The American Naturalist*, ***181***(3), 301-313. https://doi.org/10.1086/669154

Bommana, S. (2019). *Characterisation of Chlamydia pecorum infection and antibody dynamics to inform diagnosis, epidemiology and control* University of the Sunshine Coast].

Bonapersona, V., Hoijtink, H., Sarabdjitsingh, R. A., & Joëls, M. (2021). Increasing the statistical power of animal experiments with historical control data. *Nat Neurosci*, ***24***(4), 470-477. https://doi.org/10.1038/s41593-020-00792-3

Bondar, G., Xu, W., Elashoff, D., Li, X., Faure-Kumar, E., Bao, T.-M., Grogan, T., Moose, J., & Deng, M. C. (2020). Comparing NGS and NanoString platforms in peripheral blood mononuclear cell transcriptome profiling for advanced heart failure biomarker development. *Journal of Biological Methods*, ***7***(1), e123.

Boost, K. A., Leipold, T., Scheiermann, P., Hoegl, S., Sadik, C. D., Hofstetter, C., & Zwissler, B. (2009). Sevoflurane and isoflurane decrease TNF-α-induced gene expression in human monocytic THP-1 cells: Potential role of intracellular IκBα regulation. *International journal of molecular medicine*, ***23***(5), 665-671.

Booth, R., & Nyari, S. (2020). Clinical comparison of five anti-chlamydial antibiotics in koalas (Phascolarctos cinereus). *PLoS One*, ***15***(7), e0236758.

Bossart, G. D., Romano, T. A., Peden-Adams, M. M., Schaefer, A. M., Rice, C. D., Fair, P. A., & Reif, J. S. (2019). Comparative Innate and Adaptive Immune Responses in Atlantic

Bottlenose Dolphins (Tursiops truncatus) With Viral, Bacterial, and Fungal Infections [Original Research]. *Frontiers in Immunology*, ***10***. https://doi.org/10.3389/fimmu.2019.01125

Brandes-Leibovitz, R., Riza, A., Yankovitz, G., Pirvu, A., Dorobantu, S., Dragos, A., Streata, I., Ricaño-Ponce, I., de Nooijer, A., Dumitrescu, F., Antonakos, N., Antoniadou, E., Dimopoulos, G., Koutsodimitropoulos, I., Kontopoulou, T., Markopoulou, D., Aimoniotou, E., Komnos, A., Dalekos, G. N., . . . Netea, M. G. (2024). Sepsis pathogenesis and outcome are shaped by the balance between the transcriptional states of systemic inflammation and antimicrobial response. *Cell Reports Medicine*, ***5***(11). https://doi.org/10.1016/j.xcrm.2024.101829

Brice, K. L. (2017). *Bugs in" bears": Are mismatches between diets and gut microbial communities a bugbear for koalas (Phascolarctos cinereus)?* Western Sydney University (Australia)].

Brice, K. L., Trivedi, P., Jeffries, T. C., Blyton, M. D. J., Mitchell, C., Singh, B. K., & Moore, B. D. (2019). The Koala (Phascolarctos cinereus) faecal microbiome differs with diet in a wild population. *PeerJ*, ***7***, e6534. https://doi.org/10.7717/peerj.6534

Buckner, L. R., Amedee, A. M., Albritton, H. L., Kozlowski, P. A., Lacour, N., McGowin, C. L., Schust, D. J., & Quayle, A. J. (2016). Chlamydia trachomatis infection of endocervical epithelial cells enhances early HIV transmission events. *PLoS One*, ***11***(1), e0146663.

Burach, F., Pospischil, A., Hanger, J., Loader, J., Pillonel, T., Greub, G., & Borel, N. (2014). Chlamydiaceae and Chlamydia-like organisms in the koala (Phascolarctos cinereus)--organ distribution and histopathological findings. *Vet Microbiol*, ***172***(1-2), 230-240. https://doi.org/10.1016/j.vetmic.2014.04.022

Burnard, D., Gillett, A., & Polkinghorne, A. (2018). Chlamydia pecorum in Joint Tissue and Synovial Fluid of a Koala (Phascolarctos cinereus) with Arthritis. *J Wildl Dis*, ***54***(3), 646-649. https://doi.org/10.7589/2017-10-248

Burton, E., & Tribe, A. (2016). The Rescue and Rehabilitation of Koalas (Phascolarctos cinereus) in Southeast Queensland. *Animals (Basel)*, ***6***(9). https://doi.org/10.3390/ani6090056

Bushman, F. D. (2020). Retroviral Insertional Mutagenesis in Humans: Evidence for Four Genetic Mechanisms Promoting Expansion of Cell Clones. *Molecular Therapy*, ***28***(2), 352-356. https://doi.org/10.1016/j.ymthe.2019.12.009

Butcher, R., Pettett, L., Fabijan, J., Ebrahimie, E., Mohammadi-Dehcheshmeh, M., Speight, K., Boardman, W., Bird, P., & Trott, D. (2020). Periodontal disease in free-ranging koalas (Phascolarctos cinereus) from the Mount Lofty Ranges, South Australia, and its association with koala retrovirus infection. *Aust Vet J*, ***98***(5), 200-206.

Buttke, D., Wild, M., Monello, R., Schuurman, G., Hahn, M., & Jackson, K. (2021). Managing Wildlife Disease Under Climate Change. *Ecohealth*, ***18***(4), 406-410. https://doi.org/10.1007/s10393-021-01542-y

Byrne, C. M., Johnston, C., Orem, J., Okuku, F., Huang, M.-L., Selke, S., Wald, A., Corey, L., Schiffer, J. T., & Casper, C. (2019). Increased oral Epstein-Barr virus shedding with HIV-1 co-infection is due to a combination of B cell activation and impaired cellular immune control. *BioRxiv*, 587063.

Callan, T., Woodcock, S., & Huston, W. M. (2021). Ascension of Chlamydia is moderated by uterine peristalsis and the neutrophil response to infection. *PLoS Comput Biol*, ***17***(9), e1009365. https://doi.org/10.1371/journal.pcbi.1009365

Caminade, C., McIntyre, K. M., & Jones, A. E. (2019). Impact of recent and future climate change on vector-borne diseases. *Ann N Y Acad Sci*, ***1436***(1), 157-173. https://doi.org/10.1111/nyas.13950

Campe, J., & Ullrich, E. (2022). T Helper Cell Lineage-Defining Transcription Factors: Potent Targets for Specific GVHD Therapy? [Review]. *Frontiers in Immunology*, ***12***. https://doi.org/10.3389/fimmu.2021.806529

Canfield, P. (1989). A survey of urinary tract disease in New South Wales koalas. *Australian veterinary journal*, ***66***(4), 103-106.

Canfield, P., Hemsley, S., & Connolly, J. (1996). Histological and immunohistological study of the developing and involuting superficial cervical thymus in the koala (Phascolarctos cinereus). *J Anat*, ***189***(Pt 1), 159.

Canfield, P., Sabine, J., & Love, D. (1988). Virus particles associated wth leukaemia in a koala. *Australian veterinary journal*, ***65***(10), 327-328. https://doi.org/https://doi.org/10.1111/j.1751-0813.1988.tb14518.x

Canfield, P. M., O'Neill, M. E., & Smith, E. F. (1989). Haematological and biochemical reference values for the koala (Phascolarctos cinereus). *Australian veterinary journal*, ***66***(10), 324-326.

Canto-Valdés, M. C., Bolio González, M. E., Acevedo-Jiménez, G. E., & Ramírez Álvarez, H. (2023). What role do endogenous retroviruses play in domestic cats infected with feline leukaemia virus? *New Zealand Veterinary Journal*, ***71***(1), 1-7. https://doi.org/10.1080/00480169.2022.2131648

Cappanera, S., Palumbo, M., Kwan, S., Priante, G., Martella, L., Saraca, L., Sicari, F., Vernelli, C., di Giuli, C., Andreani, P., Mariottini, A., Francucci, M., Sensi, E., Costantini, M., Bruzzone, P., D'Andrea, V., Gioia, S., Cirocchi, R., & Tiri, B. (2021). When Does the Cytokine Storm Begin in COVID-19 Patients? A Quick Score to Recognize It. *Journal of Clinical Medicine*, ***10***, 297. https://doi.org/10.3390/jcm10020297

Carey, A. J., Timms, P., Rawlinson, G., Brumm, J., Nilsson, K., Harris, J. M., & Beagley, K. W. (2010). A Multi-Subunit Chlamydial Vaccine Induces Antibody and Cell-Mediated Immunity in Immunized Koalas (Phascolarctos cinereus): Comparison of Three Different Adjuvants. *American Journal of Reproductive Immunology*, ***63***(2), 161-172.

Carlson, M., Falcon, S., Pages, H., & Li, N. (2019). org. Hs. eg. db: Genome wide annotation for Human. *R package version*, ***3***(2), 3.

Carter, S. P., Delahay, R. J., Smith, G. C., Macdonald, D. W., Riordan, P., Etherington, T. R., Pimley, E. R., Walker, N. J., & Cheeseman, C. L. (2007). Culling-induced social perturbation in Eurasian badgers Meles meles and the management of TB in cattle: an analysis of a critical problem in applied ecology. *Proceedings of the Royal Society B: Biological Sciences*, ***274***(1626), 2769-2777.

Casadevall, A., & Pirofski, L.-a. (1999). Host-pathogen interactions: redefining the basic concepts of virulence and pathogenicity. *Infection and immunity*, ***67***(8), 3703-3713.

Casadevall, A., & Pirofski, L.-a. (2018). What Is a Host? Attributes of Individual Susceptibility. *Infection and immunity*, ***86***(2), 10.1128/iai.00636-00617. https://doi.org/doi:10.1128/iai.00636-17

Casadevall, A., & Pirofski, L. A. (2003). The damage-response framework of microbial pathogenesis. *Nat Rev Microbiol*, ***1***(1), 17-24. https://doi.org/10.1038/nrmicro732

Chakraborty, R. K., & Burns, B. (2024). Systemic Inflammatory Response Syndrome. In *StatPearls*.

Chang, J., Renne, R., Dittmer, D., & Ganem, D. (2000). Inflammatory cytokines and the reactivation of Kaposi's sarcoma-associated herpesvirus lytic replication. *Virology*, ***266***(1), 17-25.

Chapman, J. M., Kelly, L. A., Teffer, A. K., Miller, K. M., & Cooke, S. J. (2021). Disease ecology of wild fish: opportunities and challenges for linking infection metrics with behaviour, condition, and survival. *Canadian Journal of Fisheries and Aquatic Sciences*, ***78***(8), 995-1007. https://doi.org/10.1139/cjfas-2020-0315

Chappell, K., Brealey, J., Amarilla, A., Watterson, D., Hulse, L., Palmieri, C., Johnston, S., Holmes, E., Meers, J., & Young, P. (2017). Phylogenetic diversity of koala retrovirus within a wild koala population. *J Virol*, ***91***(3), 10.1128/jvi. 01820-01816.

Charalambous, R., Simonato, T., Peel, M., & Narayan, E. J. (2021). Physiological Stress in Rescued Wild Koalas (Phascolarctos cinereus) Being Held in a Rehabilitation Sanctuary: A Pilot Study. *Animals*, ***11***(10), 2864. https://www.mdpi.com/2076-2615/11/10/2864

Chaudhry, A., Samstein, Robert M., Treuting, P., Liang, Y., Pils, Marina C., Heinrich, J.-M., Jack, Robert S., Wunderlich, F. T., Brüning, Jens C., Müller, W., & Rudensky, Alexander Y. (2011). Interleukin-10 Signaling in Regulatory T Cells Is Required for Suppression of Th17 Cell-Mediated Inflammation. *Immunity*, ***34***(4), 566-578. https://doi.org/10.1016/j.immuni.2011.03.018

Chen, A., Lee, K., & He, J. C. (2021). Autocrine and paracrine effects of a novel podocyte gene, RARRES1. *Kidney Int*, ***100***(4), 745-747. https://doi.org/10.1016/j.kint.2021.07.008

Chen, B., Slocombe, R. F., & Georgy, S. R. (2023a). Advances in organoid technology for veterinary disease modeling. *Frontiers in Veterinary Science*, ***10***, 1234628.

Chen, C.-J., Casteriano, A., Green, A. C., & Govendir, M. (2023b). A retrospective study on antibacterial treatments for koalas infected with Chlamydia pecorum. *Scientific reports*, ***13***(1), 12670. https://doi.org/10.1038/s41598-023-39832-w

Chen, C. F., Lee, W. C., Yang, H. I., Chang, H. C., Jen, C. L., Iloeje, U. H., Su, J., Hsiao, C. K., Wang, L. Y., & You, S. L. (2011). Changes in serum levels of HBV DNA and alanine aminotransferase determine risk for hepatocellular carcinoma. *Gastroenterology*, ***141***(4), 1240-1248. e1242.

Chen, F., Jiang, F., Ma, J., Alghamdi, M. A., Zhu, Y., & Yong, J. W. H. (2024). Intersecting planetary health: Exploring the impacts of environmental stressors on wildlife and human health. *Ecotoxicology and Environmental Safety*, ***283***, 116848. https://doi.org/https://doi.org/10.1016/j.ecoenv.2024.116848

Chen, G., Lin, W., Shen, F., Iloeje, U. H., London, W. T., & Evans, A. A. (2006). Past HBV viral load as predictor of mortality and morbidity from HCC and chronic liver disease in a prospective study. *Official journal of the American College of Gastroenterology| ACG*, ***101***(8), 1797-1803.

Chen, H., Wen, Y., & Li, Z. (2019a). Clear Victory for Chlamydia: The Subversion of Host Innate Immunity [Review]. *Frontiers in Microbiology*, ***10***. https://doi.org/10.3389/fmicb.2019.01412

Chen, J., Lv, W., Zhang, X., Zhang, T., Dong, J., Wang, Z., Liu, T., Zhang, P., Pyne, M., & Dong, G. (2023c). Animal age affects the gut microbiota and immune system in captive koalas (Phascolarctos cinereus). *Microbiol Spectr*, ***11***(1), e04101-04122.

Chen, L., Huang, H., Zheng, X., Li, Y., Chen, J., Tan, B., Liu, Y., Sun, R., Xu, B., Yang, M., Li, B., Wu, C., Lu, B., & Jiang, J. (2022). IL1R2 increases regulatory T cell population in the

tumor microenvironment by enhancing MHC-II expression on cancer-associated fibroblasts. *J Immunother Cancer*, ***10***(9).

Chen, S., Bonifati, S., Qin, Z., St Gelais, C., & Wu, L. (2019b). SAMHD1 Suppression of Antiviral Immune Responses. *Trends Microbiol*, ***27***(3), 254-267. https://doi.org/10.1016/j.tim.2018.09.009

Cheng, Y., Polkinghorne, A., Gillett, A., Jones, E. A., O'Meally, D., Timms, P., & Belov, K. (2018). Characterisation of MHC class I genes in the koala. *Immunogenetics*, ***70***, 125-133.

Chew, T., & Sadsad, R. (2022). Rnaseq-de (Version 1.0)[Computer software]. In: Sydney Informatics Hub.

Chiarelli, T. J., Grieshaber, N. A., Appa, C., & Grieshaber, S. S. (2023). Computational Modeling of the Chlamydial Developmental Cycle Reveals a Potential Role for Asymmetric Division. *mSystems*, ***8***(2), e0005323. https://doi.org/10.1128/msystems.00053-23

Chilimoniuk, J., Erol, A., Rödiger, S., & Burdukiewicz, M. (2024). Challenges and opportunities in processing NanoString nCounter data. *Computational and Structural Biotechnology Journal*, ***23***, 1951-1958. https://doi.org/https://doi.org/10.1016/j.csbj.2024.04.061

Chiu, E. S., & VandeWoude, S. (2021). Endogenous retroviruses drive resistance and promotion of exogenous retroviral homologs. *Annual Review of Animal Biosciences*, ***9***(1), 225-248.

Choi, J. W., Park, Y. S., Lee, Y. S., Park, Y. H., Chung, C., Park, D. I., Kwon, I. S., Lee, J. S., Min, N. E., Park, J. E., Yoo, S. H., Chon, G. R., Sul, Y. H., & Moon, J. Y. (2017). The Ability of the Acute Physiology and Chronic Health Evaluation (APACHE) IV Score to Predict Mortality in a Single Tertiary Hospital. *Korean J Crit Care Med*, ***32***(3), 275-283. https://doi.org/10.4266/kjccm.2016.00990

Christian, J., Vier, J., Paschen, S. A., & Häcker, G. (2010). Cleavage of the NF-KB Family Protein p65/RelA by the Chlamydial Protease-like Activity Factor (CPAF) Impairs Proinflammatory Signaling in Cells Infected with Chlamydiae. *Journal of Biological Chemistry*, ***285***(53), 41320-41327. https://doi.org/10.1074/jbc.M110.152280

Chu, J., Zhang, Q., Zhang, T., Han, E., Zhao, P., Khan, A., He, C., & Wu, Y. (2016). Chlamydia psittaci infection increases mortality of avian influenza virus H9N2 by suppressing host immune response. *Sci Rep*, ***6***, 29421. https://doi.org/10.1038/srep29421

Chuong, E. B., Elde, N. C., & Feschotte, C. (2016). Regulatory evolution of innate immunity through co-option of endogenous retroviruses. *Science*, ***351***(6277), 1083-1087.

Church, C., Casteriano, A., Muir, Y. S. S., Krockenberger, M. B., Vaz, P. K., Higgins, D. P., & Wright, B. R. (2025). New insights into the range and transmission dynamics of a koala gammaherpesvirus, phascolarctid gammaherpesvirus 2. *Sci Rep*, ***In press.***

Ciervo, A., Visca, P., Petrucca, A., Biasucci, L. M., Maseri, A., & Cassone, A. (2002). Antibodies to 60-kilodalton heat shock protein and outer membrane protein 2 of Chlamydia pneumoniae in patients with coronary heart disease. *Clinical and Vaccine Immunology*, ***9***(1), 66-74.

Clarke, B., Gillies, D., Illari, P., Russo, F., & Williamson, J. (2014). Mechanisms and the evidence hierarchy. *Topoi*, ***33***, 339-360.

Cnops, J., Magez, S., & De Trez, C. (2015). Escape mechanisms of African trypanosomes: why trypanosomosis is keeping us awake. *Parasitology*, ***142***(3), 417-427.

Codoñer-Franch, P., & Alonso-Iglesias, E. (2015). Resistin: insulin resistance to malignancy. *Clin Chim Acta*, ***438***, 46-54. https://doi.org/10.1016/j.cca.2014.07.043

Cohen, J. I. (2020). Herpesvirus latency. *J Clin Invest*, ***130***(7), 3361-3369. https://doi.org/10.1172/jci136225

Colombelli-Négrel, D., Sach, I. Z., Hough, I., Hodgson, J. C., Daniels, C. B., & Kleindorfer, S. (2023). Koalas showed limited behavioural response and no physiological response to drones. *Applied Animal Behaviour Science*, ***264***, 105963.

Colotta, F., Re, F., Muzio, M., Bertini, R., Polentarutti, N., Sironi, M., Giri, J. G., Dower, S. K., Sims, J. E., & Mantovani, A. (1993). Interleukin-1 type II receptor: a decoy target for IL-1 that is regulated by IL-4. *Science*, ***261***(5120), 472-475.

Colotta, F., Saccani, S., Giri, J. G., Dower, S. K., Sims, J. E., Introna, M., & Mantovani, A. (1996). Regulated expression and release of the IL-1 decoy receptor in human mononuclear phagocytes. *Journal of immunology (Baltimore, Md.: 1950)*, ***156***(7), 2534-2541.

Corsaro, D., & Danielle, V. (2004). Emerging Chlamydial Infections. *Critical reviews in microbiology*, ***30***, 75-106. https://doi.org/10.1080/10408410490435106

Coskun, O., Sener, K., Kilic, S., Erdem, H., Yaman, H., Besirbellioglu, A. B., Gul, H. C., & Eyigun, C. P. (2010). Stress-related Epstein–Barr virus reactivation. *Clinical and experimental medicine*, ***10***, 15-20.

Courvoisier, D. S., Combescure, C., Agoritsas, T., Gayet-Ageron, A., & Perneger, T. V. (2011). Performance of logistic regression modeling: beyond the number of events per variable, the role of data structure. *Journal of Clinical Epidemiology*, ***64***(9), 993-1000. https://doi.org/https://doi.org/10.1016/j.jclinepi.2010.11.012

Cristescu, R., Cahill, V., Sherwin, W. B., Handasyde, K., Carlyon, K., Whisson, D., Herbert, C. A., Carlsson, B. L. J., Wilton, A. N., & Cooper, D. W. (2009). Inbreeding and testicular abnormalities in a bottlenecked population of koalas (Phascolarctos cinereus). *Wildlife Research*, ***36***(4), 299-308.

Cristescu, R., Frere, C., & Whisson, D. (2020a). Drones, detection dogs, poo spotting: what's the best way to conduct Australia's Great Koala Count? *The Conversation*, ***26***.

Cristescu, R. H., Foley, E., Markula, A., Jackson, G., Jones, D., & Frere, C. (2015). Accuracy and efficiency of detection dogs: a powerful new tool for koala conservation and management. *Scientific reports*, ***5***(1), 8349.

Cristescu, R. H., Gardiner, R., Terraube, J., McDonald, K., Powell, D., Levengood, A. L., & Frère, C. H. (2023). Difficulties of assessing the impacts of the 2019–2020 bushfires on koalas. *Austral Ecology*, ***48***(1), 12-18.

Cristescu, R. H., Miller, R. L., & Frere, C. H. (2020b). Sniffing out solutions to enhance conservation: How detection dogs can maximise research and management outcomes, through the example of koalas. *Australian Zoologist*, ***40***(3), 416-432.

Cristescu, R. H., Miller, R. L., Schultz, A. J., Hulse, L., Jaccoud, D., Johnston, S., Hanger, J., Booth, R., & Frère, C. H. (2019). Developing noninvasive methodologies to assess koala population health through detecting Chlamydia from scats. *Molecular Ecology Resources*, ***19***(4), 957-969.

Cristescu, R. H., Strickland, K., Schultz, A. J., Kruuk, L. E., De Villiers, D., & Frère, C. H. (2022). Susceptibility to a sexually transmitted disease in a wild koala population shows heritable genetic variance but no inbreeding depression. *Molecular ecology*, ***31***(21), 5455-5467.

Crother, T. R., Porritt, R. A., Dagvadorj, J., Tumurkhuu, G., Slepenkin, A. V., Peterson, E. M., Chen, S., Shimada, K., & Arditi, M. (2019). Autophagy Limits Inflammasome During Chlamydia pneumoniae Infection [Original Research]. *Frontiers in Immunology*, ***10***. https://doi.org/10.3389/fimmu.2019.00754

Crowther, M. S., Dargan, J. R., Madani, G., Rus, A. I., Krockenberger, M. B., McArthur, C., Moore, B. D., Lunney, D., & Mella, V. S. (2020). Comparison of three methods of estimating the population size of an arboreal mammal in a fragmented rural landscape. *Wildlife Research*, ***48***(2), 105-114.

Cugliari, G. (2023). FKBP5, a Modulator of Stress Responses Involved in Malignant Mesothelioma: The Link between Stress and Cancer. *Int J Mol Sci*, ***24***(9). https://doi.org/10.3390/ijms24098183

Cui, J., Frankham, G. J., Johnson, R. N., Polkinghorne, A., Timms, P., O'Meally, D., Cheng, Y., & Belov, K. (2015). SNP marker discovery in koala TLR genes. *PLoS One*, ***10***(3), e0121068.

Curtis, A. M., Fagundes, C. T., Yang, G., Palsson-McDermott, E. M., Wochal, P., McGettrick, A. F., Foley, N. H., Early, J. O., Chen, L., & Zhang, H. (2015). Circadian control of innate immunity in macrophages by miR-155 targeting Bmal1. *Proceedings of the National Academy of Sciences*, ***112***(23), 7231-7236.

Dahlhausen, K. E., Doroud, L., Firl, A. J., Polkinghorne, A., & Eisen, J. A. (2018). Characterization of shifts of koala (Phascolarctos cinereus) intestinal microbial communities associated with antibiotic treatment. *PeerJ*, ***6***, e4452.

Daneri-Becerra, C., Zgajnar, N. R., Lotufo, C. M., Ramos Hryb, A. B., Piwien-Pilipuk, G., & Galigniana, M. D. (2019). Regulation of FKBP51 and FKBP52 functions by post-translational modifications. *Biochemical Society Transactions*, ***47***(6), 1815-1831.

Daradoumis, J., Ragonnaud, E., Skandorff, I., Nielsen, K. N., Bermejo, A. V., Andersson, A.-M., Schroedel, S., Thirion, C., Neukirch, L., & Holst, P. J. (2023). An Endogenous Retrovirus Vaccine Encoding an Envelope with a Mutated Immunosuppressive Domain in Combination with Anti-PD1 Treatment Eradicates Established Tumours in Mice. *Viruses*, ***15***(4), 926. https://www.mdpi.com/1999-4915/15/4/926

Das, B., & Röst, G. (2023). Dynamics of herpes and chlamydia co-infection in a population. *Discrete Continuous Dyn Syst Ser B*, ***28***(8), 4366-4398.

Davies, N., Gramotnev, G., Seabrook, L., McAlpine, C., Baxter, G., Lunney, D., & Bradley, A. (2014). Climate-driven changes in diet composition and physiological stress in an arboreal folivore at the semi-arid edge of its distribution. *Biological conservation*, ***172***, 80-88. https://doi.org/https://doi.org/10.1016/j.biocon.2014.02.004

Davies, N. A., Gramotnev, G., McAlpine, C., Seabrook, L., Baxter, G., Lunney, D., Rhodes, J. R., & Bradley, A. (2013). Physiological stress in koala populations near the arid edge of their distribution. *PLoS One*, ***8***(11), e79136.

DCCEEW. (2022a). *Conservation Advice for Phascolarctos cinereus (Koala) combined populations of Queensland, New South Wales and the Australian Capital Territory. In effect under the Environment Protection and Biodiversity Conservation Act 1999 from 12 February 2022.*: Australian Government Retrieved from http://www.environment.gov.au/biodiversity/threatened/species/pubs/85104-conservation-advice-12022022.pdf

DCCEEW. (2022b). *Koala listing under national environmental law*. Australian Government Retrieved from https://www.dcceew.gov.au/environment/biodiversity/threatened/species/koalas/li

sting-under-national-environmental-law#:~:text=The%20koala%20(combined%20populations%20of,have%20any%20impacts%20to%20koalas.
De Castro, F., & Bolker, B. (2005). Mechanisms of disease-induced extinction. *Ecology Letters*, ***8***(1), 117-126.
De La Cruz-Herrera, C. F., Tatham, M. H., Siddiqi, U. Z., Shire, K., Marcon, E., Greenblatt, J. F., Hay, R. T., & Frappier, L. (2023). Changes in SUMO-modified proteins in Epstein-Barr virus infection identifies reciprocal regulation of TRIM24/28/33 complexes and the lytic switch BZLF1. *PLoS Pathog*, ***19***(7), e1011477. https://doi.org/10.1371/journal.ppat.1011477
de Lourdes Higuchi, M. (2004). Trypanosoma cruzi trans-sialidase as a new therapeutic tool in the treatment of chronic inflammatory diseases: possible action against mycoplasma and chlamydia. *Medical hypotheses*, ***63***(4), 616-623.
de Oliveira, D. A., Oliveira, R., Braga, B. V., Straker, L. C., Rodrigues, L. S., Bueno, L. L., Fujiwara, R. T., & Lopes-Torres, E. J. (2025). Experimental trichuriasis: Changes in the immune response and bacterial translocation during acute phase development illustrated with 3D model animation. *PLOS Neglected Tropical Diseases*, ***19***(2), e0012841. https://doi.org/10.1371/journal.pntd.0012841
Debattista, J., Timms, P., Allan, J., & Allan, J. (2003). Immunopathogenesis of Chlamydia trachomatis infections in women. *Fertility and sterility*, ***79***(6), 1273-1287.
DEH, N. G. (2022). *Wildlife rehabilitation data dashboard* https://www2.environment.nsw.gov.au/topics/animals-and-plants/native-animals/rehabilitating-native-animals/wildlife-rehabilitation-data-and-reporting/wildlife-rehabilitation-data-dashboard
Deka, S., Vanover, J., Dessus-Babus, S., Whittimore, J., Howett, M. K., Wyrick, P. B., & Schoborg, R. V. (2006). Chlamydia trachomatis enters a viable but non-cultivable (persistent) state within herpes simplex virus type 2 (HSV-2) co-infected host cells. *Cellular microbiology*, ***8***(1), 149-162.
Deka, S., Vanover, J., Sun, J., Kintner, J., Whittimore, J., & Schoborg, R. V. (2007). An early event in the herpes simplex virus type-2 replication cycle is sufficient to induce Chlamydia trachomatis persistence. *Cell Microbiol*, ***9***(3), 725-737. https://doi.org/10.1111/j.1462-5822.2006.00823.x
Denkinger, J., Guevara, N., Ayala, S., Murillo, J. C., Hirschfeld, M., Montero-Serra, I., Fietz, K., Goldstein, T., Ackermann, M., & Barragán, V. (2017). Pup mortality and evidence for pathogen exposure in Galapagos sea lions (Zalophus wollebaeki) on San Cristobal Island, Galapagos, Ecuador. *Journal of Wildlife Diseases*, ***53***(3), 491-498.
Denner, J. (2016). Immunosuppressive properties of retroviruses. *European Journal of Immunology*, ***46***(1), 253-255. https://doi.org/https://doi.org/10.1002/eji.201545851
Denner, J., & Young, P. R. (2013). Koala retroviruses: characterization and impact on the life of koalas. *Retrovirology*, ***10***, 1-7.
Dennison, S., Frankham, G. J., Neaves, L. E., Flanagan, C., FitzGibbon, S., Eldridge, M. D. B., & Johnson, R. N. (2016). Population genetics of the koala *(Phascolarctos cinereus)* in north-eastern New South Wales and south-eastern Queensland. *Australian journal of zoology*, ***64***(6), 402-412. https://doi.org/https://doi.org/10.1071/ZO16081
Desclozeaux, M., Robbins, A., Jelocnik, M., Khan, S. A., Hanger, J., Gerdts, V., Potter, A., Polkinghorne, A., & Timms, P. (2017). Immunization of a wild koala population with a recombinant Chlamydia pecorum Major Outer Membrane Protein (MOMP) or

Polymorphic Membrane Protein (PMP) based vaccine: New insights into immune response, protection and clearance. *PLoS One*, ***12***(6), e0178786. https://doi.org/10.1371/journal.pone.0178786

Devereaux, L. N., Polkinghorne, A., Meijer, A., & Timms, P. (2003). Molecular evidence for novel chlamydial infections in the koala (Phascolarctos cinereus). *Syst Appl Microbiol*, ***26***(2), 245-253. https://doi.org/10.1078/072320203322346092

Devi, P., Khan, A., Chattopadhyay, P., Mehta, P., Sahni, S., Sharma, S., & Pandey, R. (2021). Co-infections as Modulators of Disease Outcome: Minor Players or Major Players? *Front Microbiol*, ***12***, 664386. https://doi.org/10.3389/fmicb.2021.664386

Di Nardo, A., Libeau, G., Chardonnet, B., Chardonnet, P., Kock, R. A., Parekh, K., Hamblin, P., Li, Y., Parida, S., & Sumption, K. J. (2015). Serological profile of foot-and-mouth disease in wildlife populations of West and Central Africa with special reference to Syncerus caffer subspecies. *Veterinary Research*, ***46***(1), 77. https://doi.org/10.1186/s13567-015-0213-0

Di Pietro, M., Filardo, S., Romano, S., & Sessa, R. (2019). Chlamydia trachomatis and Chlamydia pneumoniae interaction with the host: latest advances and future prospective. *Microorganisms*, ***7***(5), 140.

Dickensheets, H. L., & Donnelly, R. P. (1997). IFN-gamma and IL-10 inhibit induction of IL-1 receptor type I and type II gene expression by IL-4 and IL-13 in human monocytes. *Journal of immunology (Baltimore, Md.: 1950)*, ***159***(12), 6226-6233.

Dimond, Z. E., Suchland, R. J., Baid, S., LaBrie, S. D., Soules, K. R., Stanley, J., Carrell, S., Kwong, F., Wang, Y., & Rockey, D. D. (2021). Inter-species lateral gene transfer focused on the Chlamydia plasticity zone identifies loci associated with immediate cytotoxicity and inclusion stability. *Molecular microbiology*, ***116***(6), 1433-1448.

Dinan, T. G., & Cryan, J. F. (2017). The Microbiome-Gut-Brain Axis in Health and Disease. *Gastroenterology Clinics of North America*, ***46***(1), 77-89. https://doi.org/https://doi.org/10.1016/j.gtc.2016.09.007

Dissanayake, R. B., Giorgi, E., Stevenson, M., Allavena, R., & Henning, J. (2021). Estimating koala density from incidental koala sightings in South-East Queensland, Australia (1997–2013), using a self-exciting spatio-temporal point process model. *Ecology and Evolution*, ***11***(20), 13805-13814. https://doi.org/https://doi.org/10.1002/ece3.8082

Dissanayake, R. B., Stevenson, M., Astudillo, V. G., Allavena, R., & Henning, J. (2023). Anthropogenic and environmental factors associated with koala deaths due to dog attacks and vehicle collisions in South-East Queensland, Australia, 2009–2013. *Scientific reports*, ***13***(1), 14275. https://doi.org/10.1038/s41598-023-40827-w

Dissanayake, T. K., Schäuble, S., Mirhakkak, M. H., Wu, W. L., Ng, A. C., Yip, C. C. Y., López, A. G., Wolf, T., Yeung, M. L., Chan, K. H., Yuen, K. Y., Panagiotou, G., & To, K. K. (2020). Comparative Transcriptomic Analysis of Rhinovirus and Influenza Virus Infection. *Front Microbiol*, ***11***, 1580. https://doi.org/10.3389/fmicb.2020.01580

Dobin, A., & Gingeras, T. R. (2015). Mapping RNA-seq reads with STAR. *Current protocols in bioinformatics*, ***51***(1), 11.14. 11-11.14. 19.

Dohoo, I. R., Martin, W., & Stryhn, H. E. (2003). *Veterinary epidemiologic research*.

Donnelly, R. P., Freeman, S. L., & Hayes, M. P. (1995). Inhibition of IL-10 expression by IFN-gamma up-regulates transcription of TNF-alpha in human monocytes. *Journal of immunology (Baltimore, Md.: 1950)*, ***155***(3), 1420-1427.

Downey, P. M. R., Caraguel, C. G. B., Speight, N., Fabijan, J., & Boardman, W. S. J. (2020). Field immobilization using alfaxalone and alfaxalone–medetomidine in free-ranging

koalas (Phascolarctos cinereus): a randomized comparative study. *Veterinary Anaesthesia and Analgesia*, ***47***(3), 368-376. https://doi.org/https://doi.org/10.1016/j.vaa.2019.10.009

Duff, G., Berryman, C., & Eamus, D. (1994). Growth, biomass allocation and foliar nutrient contents of two Eucalyptus species of the wet-dry tropics of Australia grown under CO 2 enrichment. *Functional Ecology*, 502-508.

Dupuis, M. L., Pagano, M. T., Pierdominici, M., & Ortona, E. (2021). The role of vitamin D in autoimmune diseases: could sex make the difference? *Biology of Sex Differences*, ***12***(1), 12. https://doi.org/10.1186/s13293-021-00358-3

Dutton-Regester, K. J. (2024). Koala admissions to a wildlife hospital in coastal New South Wales, Australia, over a nine-year period, 2014–2022. *Australian journal of zoology*, ***71***(6).

Dwinger, R. H., Murray, M., Luckins, A. G., Rae, P. F., & Moloo, S. K. (1989). Interference in the establishment of tsetse-transmitted Trypanosoma congolense, T. brucei or T. vivax superinfections in goats already infected with T. congolense or T. vivax. *Vet Parasitol*, ***30***(3), 177-189. https://doi.org/10.1016/0304-4017(89)90013-7

Dwyer, R., Goosen, W., Buss, P., Kedward, S., Manamela, T., Hausler, G., Chileshe, J., Rossouw, L., Fowler, J. H., Miller, M., & Witte, C. (2022). Epidemiology of *Mycobacterium bovis* infection in free-ranging rhinoceros in Kruger National Park, South Africa. *Proceedings of the National Academy of Sciences*, ***119***(24), e2120656119. https://doi.org/doi:10.1073/pnas.2120656119

Early, J. O., Menon, D., Wyse, C. A., Cervantes-Silva, M. P., Zaslona, Z., Carroll, R. G., Palsson-McDermott, E. M., Angiari, S., Ryan, D. G., & Corcoran, S. E. (2018). Circadian clock protein BMAL1 regulates IL-1β in macrophages via NRF2. *Proceedings of the National Academy of Sciences*, ***115***(36), E8460-E8468.

Ebi, K. L., & Bowen, K. (2016). Extreme events as sources of health vulnerability: Drought as an example. *Weather and Climate Extremes*, ***11***, 95-102. https://doi.org/https://doi.org/10.1016/j.wace.2015.10.001

Eckert, L., Hawes, S., Wölner-Hanssen, P., Money, D., Peeling, R., Brunham, R., Stevens, C., Eschenbach, D., & Stamm, W. (1997). Prevalence and correlates of antibody to chlamydial heat shock protein in women attending sexually transmitted disease clinics and women with confirmed pelvic inflammatory disease. *The Journal of infectious diseases*, ***175***(6), 1453-1458.

Egholm, C., Heeb, L. E. M., Impellizzieri, D., & Boyman, O. (2019). The Regulatory Effects of Interleukin-4 Receptor Signaling on Neutrophils in Type 2 Immune Responses [Review]. *Frontiers in Immunology*, ***10***. https://doi.org/10.3389/fimmu.2019.02507

Elbers, J. P., Brown, M. B., & Taylor, S. S. (2018). Identifying genome-wide immune gene variation underlying infectious disease in wildlife populations – a next generation sequencing approach in the gopher tortoise. *BMC Genomics*, ***19***(1), 64. https://doi.org/10.1186/s12864-018-4452-0

Elenkov, I. J., & Chrousos, G. P. (1999). Stress, cytokine patterns and susceptibility to disease. *Best Practice & Research Clinical Endocrinology & Metabolism*, ***13***(4), 583-595. https://doi.org/https://doi.org/10.1053/beem.1999.0045

Elias, D., Akuffo, H., & Britton, S. (2006). Helminthes could influence the outcome of vaccines against TB in the tropics. *Parasite Immunology*, ***28***(10), 507-513.

Elwell, C., Mirrashidi, K., & Engel, J. (2016). Chlamydia cell biology and pathogenesis. *Nature Reviews Microbiology*, ***14***(6), 385-400.

Escobar, L. E., Pritzkow, S., Winter, S. N., Grear, D. A., Kirchgessner, M. S., Dominguez-Villegas, E., Machado, G., Townsend Peterson, A., & Soto, C. (2020). The ecology of chronic wasting disease in wildlife. *Biological Reviews*, ***95***(2), 393-408. https://doi.org/https://doi.org/10.1111/brv.12568

Ezanno, P., Andraud, M., Beaunée, G., Hoch, T., Krebs, S., Rault, A., Touzeau, S., Vergu, E., & Widgren, S. (2020). How mechanistic modelling supports decision making for the control of enzootic infectious diseases. *Epidemics*, ***32***, 100398. https://doi.org/https://doi.org/10.1016/j.epidem.2020.100398

Ezenwa, V. O., Etienne, R. S., Luikart, G., Beja-Pereira, A., & Jolles, A. E. (2010). Hidden consequences of living in a wormy world: nematode-induced immune suppression facilitates tuberculosis invasion in African buffalo. *The American Naturalist*, ***176***(5), 613-624.

Ezenwa, V. O., & Jolles, A. E. (2011). From host immunity to pathogen invasion: the effects of helminth coinfection on the dynamics of microparasites. *Integrative and Comparative Biology*, ***51***(4), 540-551.

Fabijan, J., Caraguel, C., Jelocnik, M., Polkinghorne, A., Boardman, W. S. J., Nishimoto, E., Johnsson, G., Molsher, R., Woolford, L., Timms, P., Simmons, G., Hemmatzadeh, F., Trott, D. J., & Speight, N. (2019a). Chlamydia pecorum prevalence in South Australian koala (Phascolarctos cinereus) populations: Identification and modelling of a population free from infection. *Sci Rep*, ***9***(1), 6261. https://doi.org/10.1038/s41598-019-42702-z

Fabijan, J., Miller, D., Olagoke, O., Woolford, L., Boardman, W., Timms, P., Polkinghorne, A., Simmons, G., Hemmatzadeh, F., Trott, D. J., & Speight, K. N. (2019b). Prevalence and clinical significance of koala retrovirus in two South Australian koala (Phascolarctos cinereus) populations. *J Med Microbiol*, ***68***(7), 1072-1080. https://doi.org/10.1099/jmm.0.001009

Fabijan, J., Sarker, N., Speight, N., Owen, H., Meers, J., Simmons, G., Seddon, J., Emes, R. D., Tarlinton, R., Hemmatzadeh, F., Woolford, L., & Trott, D. J. (2020). Pathological Findings in Koala Retrovirus-positive Koalas (Phascolarctos cinereus) from Northern and Southern Australia. *J Comp Pathol*, ***176***, 50-66. https://doi.org/10.1016/j.jcpa.2020.02.003

Fabijan, J., Woolford, L., Lathe, S., Simmons, G., Hemmatzadeh, F., Trott, D. J., & Speight, N. (2017). Lymphoma, Koala Retrovirus Infection and Reproductive Chlamydiosis in a Koala (Phascolarctos cinereus). *J Comp Pathol*, ***157***(2-3), 188-192. https://doi.org/10.1016/j.jcpa.2017.07.011

Falkow, S. (1988). Molecular Koch's postulates applied to microbial pathogenicity. *Reviews of infectious diseases*, S274-S276.

Fehr, R., Alexanderson, K., Favaretti, C., de Jong, J., La Torre, G., Lim, T.-A., Martin-Olmedo, P., Mekel, O. C. L., Michelsen, K., Rosenkötter, N., Verschuuren, M., de Waure, C., & Zeegers Paget, D. (2017). Health assessments for health governance—concepts and methodologies. *European Journal of Public Health*, ***27***(4), 609-616. https://doi.org/10.1093/eurpub/ckx062

Feng, Y., Sun, Z., Fu, J., Zhong, F., Zhang, W., Wei, C., Chen, A., Liu, B. C., He, J. C., & Lee, K. (2024). Podocyte-derived soluble RARRES1 drives kidney disease progression through direct podocyte and proximal tubular injury. *Kidney Int*, ***106***(1), 50-66. https://doi.org/10.1016/j.kint.2024.04.011

Fernandez, C. M. (2023). *The ecology of chlamydiosis in the koala, Phascolarctos cinereus: Interactions between the host, pathogen and environment* Sydney eScholarship. https://hdl.handle.net/2123/32458

Fernandez, C. M., Krockenberger, M. B., Crowther, M. S., Mella, V. S. A., Wilmott, L., & Higgins, D. P. (2023). Genetic markers of Chlamydia pecorum virulence in ruminants support short term host-pathogen evolutionary relationships in the koala, Phascolarctos cinereus. *Infect Genet Evol*, ***116***, 105527.

Fernandez, C. M., Krockenberger, M. B., Ho, S. Y. W., Crowther, M. S., Mella, V. S. A., Jelocnik, M., Wilmott, L., & Higgins, D. P. (2024a). Novel typing scheme reveals emergence and genetic diversity of Chlamydia pecorum at the local management scale across two koala populations. *Veterinary microbiology*, ***293***, 110085. https://doi.org/https://doi.org/10.1016/j.vetmic.2024.110085

Fernandez, C. M., Krockenberger, M. B., Mella, V. S. A., Wright, B. R., Crowther, M. S., & Higgins, D. P. (2024b). A novel multi-variate immunological approach, reveals immune variation associated with environmental conditions, and co-infection in the koala (Phascolarctos cinereus). *Sci Rep*, ***14***(1), 7260. https://doi.org/10.1038/s41598-024-57792-7

Fernandez, C. M., Schmertmann, L. J., Higgins, D. P., Casteriano, A., Irinyi, L., Mella, V. S. A., Crowther, M. S., Meyer, W., & Krockenberger, M. B. (2019). Genetic differences in Chlamydia pecorum between neighbouring sub-populations of koalas (Phascolarctos cinereus). *Vet Microbiol*, ***231***, 264-270.

Ferrari, N., Cattadori, I., Rizzoli, A., & Hudson, P. (2009). Heligmosomoides polygyrus reduces infestation of Ixodes ricinus in free-living yellow-necked mice, Apodemus flavicollis. *Parasitology*, ***136***(3), 305-316.

Ferraz, M. d. A. M. M., Nagashima, J. B., Venzac, B., Le Gac, S., & Songsasen, N. (2020). A dog oviduct-on-a-chip model of serous tubal intraepithelial carcinoma. *Sci. Rep*, ***10***, 1-11.

Fidel, P. L., Yano, J., Esher, S. K., & Noverr, M. C. (2020). Applying the Host-Microbe Damage Response Framework to Candida Pathogenesis: Current and Prospective Strategies to Reduce Damage. *Journal of Fungi*, ***6***(1), 35. https://www.mdpi.com/2309-608X/6/1/35

Fiebig, U., Hartmann, M. G., Bannert, N., Kurth, R., & Denner, J. (2006). Transspecies transmission of the endogenous koala retrovirus. *J Virol*, ***80***(11), 5651-5654. https://doi.org/10.1128/jvi.02597-05

Fiebig, U., Keller, M., Möller, A., Timms, P., & Denner, J. (2015). Lack of antiviral antibody response in koalas infected with koala retroviruses (KoRV). *Virus research*, ***198***, 30-34.

Filkov, A. I., Ngo, T., Matthews, S., Telfer, S., & Penman, T. D. (2020). Impact of Australia's catastrophic 2019/20 bushfire season on communities and environment. Retrospective analysis and current trends. *Journal of safety science and resilience*, ***1***(1), 44-56.

Filková, M., Haluzík, M., Gay, S., & Senolt, L. (2009). The role of resistin as a regulator of inflammation: Implications for various human pathologies. *Clin Immunol*, ***133***(2), 157-170. https://doi.org/10.1016/j.clim.2009.07.013

Fisher, J. R., Chroust, Z. D., Onyoni, F., & Soong, L. (2021). Pattern Recognition Receptors in Innate Immunity to Obligate Intracellular Bacteria. *Zoonoses (Burlingt)*, ***1***(1). https://doi.org/10.15212/zoonoses-2021-0011

Fisher, R., Baselet, B., Vermeesen, R., Moreels, M., Baatout, S., Rahiman, F., Miles, X., Nair, S., du Plessis, P., Engelbrecht, M., Ndimba, R. J., Bolcaen, J., Nieto-Camero, J., de Kock, E., & Vandevoorde, C. (2020). Immunological Changes During Space Travel: A Ground-Based Evaluation of the Impact of Neutron Dose Rate on Plasma Cytokine Levels in Human Whole Blood Cultures [Original Research]. *Frontiers in Physics*, ***8***. https://doi.org/10.3389/fphy.2020.568124

Fletcher, A. J., & Towers, G. J. (2013). Inhibition of retroviral replication by members of the TRIM protein family. *Intrinsic Immunity*, 29-66.

Fong, I. W., Chiu, B., Viira, E., Tucker, W., Wood, H., & Peeling, R. W. (2002). Chlamydial Heat-Shock Protein–60 Antibody and Correlation with Chlamydia pneumoniae in Atherosclerotic Plaques. *The Journal of infectious diseases*, ***186***(10), 1469-1473.

Fowler, E., Houlden, B., Hoeben, P., & Timms, P. (2000). Genetic diversity and gene flow among southeastern Queensland koalas (Phascolarctos cinereus). *Molecular ecology*, ***9***(2), 155-164.

Franzolin, E., Pontarin, G., Rampazzo, C., Miazzi, C., Ferraro, P., Palumbo, E., Reichard, P., & Bianchi, V. (2013). The deoxynucleotide triphosphohydrolase SAMHD1 is a major regulator of DNA precursor pools in mammalian cells. *Proceedings of the National Academy of Sciences*, ***110***(35), 14272-14277.

Fu, B., Ma, H., & Liu, D. (2019). Endogenous retroviruses function as gene expression regulatory elements during mammalian pre-implantation embryo development. *International Journal of Molecular Sciences*, ***20***(3), 790.

Fu, Y. L., & Harrison, R. E. (2021). Microbial Phagocytic Receptors and Their Potential Involvement in Cytokine Induction in Macrophages. *Front Immunol*, ***12***, 662063. https://doi.org/10.3389/fimmu.2021.662063

Gaiffe, E., Colladant, M., Desmaret, M., Bamoulid, J., Leroux, F., Laheurte, C., Brouard, S., Giral, M., Saas, P., Courivaud, C., Degauque, N., & Ducloux, D. (2023). Pre-transplant immune profile defined by principal component analysis predicts acute rejection after kidney transplantation. *Front Immunol*, ***14***, 1192440. https://doi.org/10.3389/fimmu.2023.1192440

Gaona, J., Benito-Verdugo, P., Martínez-Fernández, J., González-Zamora, Á., Almendra-Martín, L., & Herrero-Jiménez, C. M. (2023). Predictive value of soil moisture and concurrent variables in the multivariate modelling of cereal yields in water-limited environments. *Agricultural Water Management*, ***282***, 108280. https://doi.org/https://doi.org/10.1016/j.agwat.2023.108280

Gazzinelli, R. T., Oswald, I. P., James, S. L., & Sher, A. (1992). IL-10 inhibits parasite killing and nitrogen oxide production by IFN-gamma-activated macrophages. *Journal of immunology (Baltimore, Md.: 1950)*, ***148***(6), 1792-1796.

Geiss, G. K., Bumgarner, R. E., Birditt, B., Dahl, T., Dowidar, N., Dunaway, D. L., Fell, H. P., Ferree, S., George, R. D., & Grogan, T. (2008). Direct multiplexed measurement of gene expression with color-coded probe pairs. *Nature biotechnology*, ***26***(3), 317-325.

Gervassi, A., Alderson, M. R., Suchland, R., Maisonneuve, J. F., Grabstein, K. H., & Probst, P. (2004). Differential Regulation of Inflammatory Cytokine Secretion by Human Dendritic Cells upon *Chlamydia trachomatis* Infection. *Infection and immunity*, ***72***(12), 7231-7239. https://doi.org/doi:10.1128/iai.72.12.7231-7239.2004

Getts, D. R., Terry, R. L., Getts, M. T., Deffrasnes, C., Müller, M., van Vreden, C., Ashhurst, T. M., Chami, B., McCarthy, D., & Wu, H. (2014). Therapeutic inflammatory monocyte

modulation using immune-modifying microparticles. *Science translational medicine*, ***6***(219), 219ra217-219ra217.
Ghasemian, E., Harding-Esch, E., Mabey, D., & Holland, M. J. (2023). When Bacteria and Viruses Collide: A Tale of Chlamydia trachomatis and Sexually Transmitted Viruses. *Viruses*, ***15***(9). https://doi.org/10.3390/v15091954
Gibbert, K., Francois, S., Sigmund, A. M., Harper, M. S., Barrett, B. S., Kirchning, C. J., Lu, M., Santiago, M. L., & Dittmer, U. (2014). Friend retrovirus drives cytotoxic effectors through Toll-like receptor 3. *Retrovirology*, ***11***(1), 126. https://doi.org/10.1186/s12977-014-0126-4
Gieffers, J., Füllgraf, H., Jahn, J., Klinger, M., Dalhoff, K., Katus, H. A., Solbach, W., & Maass, M. (2001). Chlamydia pneumoniae infection in circulating human monocytes is refractory to antibiotic treatment. *Circulation*, ***103***(3), 351-356. https://doi.org/10.1161/01.cir.103.3.351
Gieffers, J., Van Zandbergen, G., Rupp, J., Sayk, F., Krüger, S., Ehlers, S., Solbach, W., & Maass, M. (2004). Phagocytes transmit Chlamydia pneumoniae from the lungs to the vasculature. *European Respiratory Journal*, ***23***(4), 506-510.
Giefing-Kröll, C., Berger, P., Lepperdinger, G., & Grubeck-Loebenstein, B. (2015). How sex and age affect immune responses, susceptibility to infections, and response to vaccination. *Aging cell*, ***14***(3), 309-321.
Gillett, A. K. (2014). An examination of disease in captive Australian koalas (Phascolarctos cinereus) and potential links to koala retrovirus (KoRV). *Technical Reports of the Australian Museum (online)*, ***24***, 39-45.
Gillett, A. K. (2023). Defining putative koala retrovirusassociated disease in koalas. Proceedings of the Second Koala Retrovirus Workshop,
Giraudon, P., Bernard, A., Malcus, C., Dufay, N., Desgranges, C., & Belin, M. F. (1995). Retroviral Infection (HTLV-I) Induces Cytokine-regulated Immunomodulation and Cytotoxicity of Medulloblastoma Cells. *Journal of Neuropathology & Experimental Neurology*, ***54***(2), 165-174. https://doi.org/10.1097/00005072-199503000-00003
Gitsels, A., Sanders, N., & Vanrompay, D. (2019). Chlamydial infection from outside to inside. *Frontiers in Microbiology*, ***10***, 2329.
Glidden, C. K., Beechler, B., Buss, P. E., Charleston, B., de Klerk-Lorist, L.-M., Maree, F. F., Muller, T., Pérez-Martin, E., Scott, K. A., & Van Schalkwyk, O. L. (2018). Detection of pathogen exposure in African buffalo using non-specific markers of inflammation. *Frontiers in Immunology*, ***8***, 1944.
Gomez-Lucia, E., Ocaña, J., Benitez, L., & Domenech, A. (2023). In silico analysis of transcription binding site motifs in endogenous and exogenous retroviruses emphasizes their conservation. *Res Sq*. https://doi.org/10.21203/rs.3.rs-2484770/v1
Gondek, D. C., Roan, N. R., & Starnbach, M. N. (2009). T Cell Responses in the Absence of IFN-γ Exacerbate Uterine Infection with Chlamydia trachomatis. *The Journal of Immunology*, ***183***(2), 1313-1319. https://doi.org/10.4049/jimmunol.0900295
Gonzalez Quevedo, C. (2014). *The spatial scale of immune gene variation within and among bottlenecked populations* University of East Anglia].
Gonzalez-Astudillo, V., Allavena, R., McKinnon, A., Larkin, R., & Henning, J. (2017). Decline causes of Koalas in South East Queensland, Australia: a 17-year retrospective study of mortality and morbidity. *Scientific reports*, ***7***(1), 1-11.
Gonzalez-Astudillo, V., Henning, J., Valenza, L., Knott, L., McKinnon, A., Larkin, R., & Allavena, R. (2019). A necropsy study of disease and comorbidity trends in morbidity

and mortality in the koala (Phascolarctos cinereus) in South-East Queensland, Australia. *Scientific reports*, ***9***(1), 17494.
Gonzalez-Quevedo, C., Davies, R. G., Phillips, K. P., Spurgin, L. G., & Richardson, D. S. (2016). Landscape-scale variation in an anthropogenic factor shapes immune gene variation within a wild population. *Molecular ecology*, ***25***(17), 4234-4246. https://doi.org/https://doi.org/10.1111/mec.13759
Gosselin, E. A., Eppler, H. B., Bromberg, J. S., & Jewell, C. M. (2018). Designing natural and synthetic immune tissues. *Nature materials*, ***17***(6), 484-498.
Gotelli, E., Campitiello, R., Hysa, E., Soldano, S., Casabella, A., Pizzorni, C., Paolino, S., Sulli, A., Smith, V., & Cutolo, M. (2024). The epigenetic effects of glucocorticoids, sex hormones and vitamin D as steroidal hormones in rheumatic musculoskeletal diseases. *Clinical and experimental rheumatology*.
Govendir, M., Hanger, J., Loader, J., Kimble, B., Griffith, J., Black, L., Krockenberger, M., & Higgins, D. (2012). Plasma concentrations of chloramphenicol after subcutaneous administration to koalas (Phascolarctos cinereus) with chlamydiosis. *Journal of veterinary pharmacology and therapeutics*, ***35***(2), 147-154.
Grace, J. B. (2024). An integrative paradigm for building causal knowledge. *Ecological Monographs*, ***94***(4), e1628. https://doi.org/https://doi.org/10.1002/ecm.1628
Gracey, E., Lin, A., Akram, A., Chiu, B., & Inman, R. D. (2013). Intracellular survival and persistence of Chlamydia muridarum is determined by macrophage polarization. *PLoS One*, ***8***(8), e69421.
Grainger, J., Daw, R., & Wemyss, K. (2018). Systemic instruction of cell-mediated immunity by the intestinal microbiome. *F1000Res*, ***7***. https://doi.org/10.12688/f1000research.14633.1
Greenacre, M., Groenen, P. J., Hastie, T., d'Enza, A. I., Markos, A., & Tuzhilina, E. (2022). Principal component analysis. *Nature Reviews Methods Primers*, ***2***(1), 100.
Greenwood, A. D., Alquezar-Planas, D. E., McKay, P. A., Mulot, B., Pye, G. W., Robbins, A., Singleton, C. L., Tarlinton, R. E., & Higgins, D. P. (2023). Synthesis of discussions of the Second Koala Retrovirus Workshop, 2021. In Proceedings of the Second Koala Retrovirus Workshop [Online]. *Tech Rep Aust Mus*, ***38***, 53–82. https://doi.org/10.3853/j.1835-4211.38.2023.1842
Greenwood, A. D., Ishida, Y., O'Brien, S. P., Roca, A. L., & Eiden, M. V. (2018). Transmission, evolution, and endogenization: lessons learned from recent retroviral invasions. *Microbiology and molecular biology reviews*, ***82***(1), 10.1128/mmbr. 00044-00017.
Grieshaber, S., Grieshaber, N., Yang, H., Baxter, B., Hackstadt, T., & Omsland, A. (2018). Impact of active metabolism on Chlamydia trachomatis elementary body transcript profile and infectivity. *Journal of bacteriology*, ***200***(14), 10.1128/jb. 00065-00018.
Griffith, J. E. (2010). *Studies into the diagnosis, treatment and management of chlamydiosis in koalas* Sydney eScholarship. http://hdl.handle.net/2123/6836
Griffith, J. E., Dhand, N. K., Krockenberger, M. B., & Higgins, D. P. (2013). A retrospective study of admission trends of koalas to a rehabilitation facility over 30 years. *Journal of Wildlife Diseases*, ***49***(1), 18-28.
Griffith, J. E., & Higgins, D. P. (2012). Diagnosis, treatment and outcomes for koala chlamydiosis at a rehabilitation facility (1995-2005). *Aust Vet J*, ***90***(11), 457-463. https://doi.org/10.1111/j.1751-0813.2012.00963.x
Grimaudo, A. T. (2024). Exploring the drivers and consequences of emerging infectious disease of wildlife.

Guégan, J. F., Poisot, T., Han, B. A., & Olivero, J. (2024). Disease ecology and pathogeography: Changing the focus to better interpret and anticipate complex environment–host–pathogen interactions. *Ecography*, ***2024***(10).

Guo, R., Liu, X., Li, Y., Meng, X., Li, R., Chen, X., & Lu, L. (2023). AOSD endotypes based on immune cell profiles: patient stratification with hierarchical clustering analysis. *Rheumatology*, ***62***(4), 1636-1644.

Haering, R., Wilson, V., Zhuo, A., & Stathis, P. (2020). A survey of veterinary professionals about their interactions with free-living native animals and the volunteer wildlife rehabilitation sector in New South Wales, Australia. *Australian Zoologist*, ***41***. https://doi.org/10.7882/AZ.2020.045

Hagai, T., Chen, X., Miragaia, R. J., Rostom, R., Gomes, T., Kunowska, N., Henriksson, J., Park, J.-E., Proserpio, V., & Donati, G. (2018). Gene expression variability across cells and species shapes innate immunity. *Nature*, ***563***(7730), 197-202.

Halberg, F., Johnson, E. A., Brown, B. W., & Bittner, J. J. (1960). Susceptibility rhythm to E. coli endotoxin and bioassay. *Proceedings of the Society for Experimental Biology and Medicine*, ***103***(1), 142-144.

Hammel, J. H., Cook, S. R., Belanger, M. C., Munson, J. M., & Pompano, R. R. (2021). Modeling Immunity In Vitro: Slices, Chips, and Engineered Tissues. *Annu Rev Biomed Eng*, ***23***, 461-491. https://doi.org/10.1146/annurev-bioeng-082420-124920

Hanger, J. (2000). An investigation of the role of retroviruses in leukaemia and related diseases in koalas.

Hanger, J., de Villiers, D., Forbes, N., Nottidge, B., Beyer, H., Loader, J., & Timms, P. (2017). *Final Technical Report, Moreton Bay Rail Koala Management Program*.

Hanger, J. J., Bromham, L. D., McKee, J. J., O'Brien, T. M., & Robinson, W. F. (2000). The nucleotide sequence of koala (Phascolarctos cinereus) retrovirus: a novel type C endogenous virus related to Gibbon ape leukemia virus. *Journal of Virology*, ***74***(9), 4264-4272.

Hanger, J. J., & Heath, T. J. (1994). The arrangement of gut-associated lymphoid tissues and lymph pathways in the koala (Phascolarctos cinereus). *Journal of Anatomy*, ***185***(Pt 1), 129.

Haraguchi, S., Good, R. A., Cianciolo, G. J., Engelman, R. W., & Day, N. K. (1997). Immunosuppressive retroviral peptides: Immunopathological implications for immunosuppressive influences of retroviral infections. *Journal of Leukocyte Biology*, ***61***(6), 654-666. https://doi.org/https://doi.org/10.1002/jlb.61.6.654

Hariri, R. H., Fredericks, E. M., & Bowers, K. M. (2019). Uncertainty in big data analytics: survey, opportunities, and challenges. *Journal of Big Data*, ***6***(1), 44. https://doi.org/10.1186/s40537-019-0206-3

Harvey, E., Madden, D., Polkinghorne, A., & Holmes, E. C. (2019). Identification of A Novel Picorna-Like Virus, Burpengary Virus, that is Negatively Associated with Chlamydial Disease in the Koala. *Viruses*, ***11***(3), 211. https://www.mdpi.com/1999-4915/11/3/211

Hashem, M. A., Kayesh, M. E. H., Maetani, F., Eiei, T., Mochizuki, K., Ochiai, S., Ito, A., Ito, N., Sakurai, H., Asai, T., & Tsukiyama-Kohara, K. (2021). Koala retrovirus (KoRV) subtypes and their impact on captive koala (Phascolarctos cinereus) health. *Arch Virol*, ***166***(7), 1893-1901. https://doi.org/10.1007/s00705-021-05078-y

Hashem, M. A., Kayesh, M. E. H., Maetani, F., Goto, A., Nagata, N., Kasori, A., Imanishi, T., & Tsukiyama-Kohara, K. (2022). Subtype distribution and expression of the koala retrovirus in the Japanese zoo koala population. *Infect Genet Evol*, ***102***, 105297.

Hattori, Y., Morita, D., Fujiwara, N., Mori, D., Nakamura, T., Harashima, H., Yamasaki, S., & Sugita, M. (2014). Glycerol Monomycolate Is a Novel Ligand for the Human, but Not Mouse Macrophage Inducible C-type Lectin, Mincle *. *Journal of Biological Chemistry*, ***289***(22), 15405-15412. https://doi.org/10.1074/jbc.M114.566489

Haughey, J. P. S. a. N. J. (2010). Immunophilin Ligands. In K. K. a. L. V. Metman (Ed.), *Encyclopedia of Movement Disorders* (pp. 66-68). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-374105-9.00254-9

Häusl, A. S., Brix, L. M., Hartmann, J., Pöhlmann, M. L., Lopez, J.-P., Menegaz, D., Brivio, E., Engelhardt, C., Roeh, S., & Bajaj, T. (2021). The co-chaperone Fkbp5 shapes the acute stress response in the paraventricular nucleus of the hypothalamus of male mice. *Molecular Psychiatry*, ***26***(7), 3060-3076.

Haydon, D. T., Randall, D. A., Matthews, L., Knobel, D. L., Tallents, L. A., Gravenor, M. B., Williams, S. D., Pollinger, J. P., Cleaveland, S., Woolhouse, M. E. J., Sillero-Zubiri, C., Marino, J., Macdonald, D. W., & Laurenson, M. K. (2006). Low-coverage vaccination strategies for the conservation of endangered species. *Nature*, ***443***(7112), 692-695. https://doi.org/10.1038/nature05177

He, X., Mekasha, S., Mavrogiorgos, N., Fitzgerald, K. A., Lien, E., & Ingalls, R. R. (2010). Inflammation and Fibrosis during Chlamydia pneumoniae Infection Is Regulated by IL-1 and the NLRP3/ASC Inflammasome. *The Journal of Immunology*, ***184***(10), 5743-5754. https://doi.org/10.4049/jimmunol.0903937

Hemsley, S., & Canfield, P. (1997). Histopathological and immunohistochemical investigation of naturally occurring chlamydial conjunctivitis and urogenital inflammation in koalas (Phascolarctos cinereus). *J Comp Pathol*, ***116***(3), 273-290.

Hemsley, S., Canfield, P., & Husband, A. (1995). Immunohistological staining of lymphoid tissue in four Australian marsupial species using species cross-reactive antibodies. *Immunol Cell Biol*, ***73***(4), 321-325.

Hemsley, S., Canfield, P., & Husband, A. (1996). Histological and immunohistological investigation of alimentary tract lymphoid tissue in the koala (Phascolarctos cinereus), brushtail possum (Trichosurus vulpecula) and ringtail possum (Pseudocheirus peregrinus). *J Anat*, ***188***(Pt 2), 279.

Herquel, B., Ouararhni, K., Martianov, I., Le Gras, S., Ye, T., Keime, C., Lerouge, T., Jost, B., Cammas, F., & Losson, R. (2013). Trim24-repressed VL30 retrotransposons regulate gene expression by producing noncoding RNA. *Nature structural & molecular biology*, ***20***(3), 339.

Herweg, J. A., & Rudel, T. (2016). Interaction of Chlamydiae with human macrophages. *Febs j*, ***283***(4), 608-618. https://doi.org/10.1111/febs.13609

Higgins, D. (2004). *Chlamydial disease of the koala: a study of pathogenesis and host response* The University of Sydney].

Higgins, D. P., Beninati, T., Meek, M., Irish, J., & Griffith, J. E. (2012). Within-population diversity of koala Chlamydophila pecorum at ompA VD1-VD3 and the ORF663 hypothetical gene. *Vet Microbiol*, ***156***(3-4), 353-358. https://doi.org/10.1016/j.vetmic.2011.11.005

Higgins, D. P., Hemsley, S., & Canfield, P. J. (2004). Assessment of anti-bovine IL4 and IFN gamma antibodies to label IL4 and IFN gamma in lymphocytes of the koala and brushtail possum. *Vet Immunol Immunopathol*, ***101***(3-4), 153-160.

Higgins, D. P., Hemsley, S., & Canfield, P. J. (2005a). Association of uterine and salpingeal fibrosis with chlamydial hsp60 and hsp10 antigen-specific antibodies in Chlamydia-infected koalas. *Clin Diagn Lab Immunol*, ***12***(5), 632-639. https://doi.org/10.1128/cdli.12.5.632-639.2005

Higgins, D. P., Hemsley, S., & Canfield, P. J. (2005b). Immuno-histochemical demonstration of the role of chlamydiaceae in renal, uterine and Salpingeal disease of the koala, and demonstration of chlamydiaceae in novel sites. *J Comp Pathol*, ***133***(2-3), 164-174. https://doi.org/10.1016/j.jcpa.2005.04.005

Hill, A. B. (1965). The environment and disease: association or causation? In: Sage Publications.

Hirsiger, J. R., Fuchs, P. S., Häusermann, P., Müller-Durovic, B., Daikeler, T., Recher, M., Hirsch, H. H., Terracciano, L., & Berger, C. T. (2019). Syphilis reactivates latent epstein-barr virus reservoir via toll-like receptor 2 and B-Cell receptor activation. Open forum infectious diseases,

Hogan, R. J., Mathews, S. A., Mukhopadhyay, S., Summersgill, J. T., & Timms, P. (2004). Chlamydial persistence: beyond the biphasic paradigm. *Infect Immun*, ***72***(4), 1843-1855. https://doi.org/10.1128/iai.72.4.1843-1855.2004

Holland, M., Bailey, R., Conway, D., Culley, F., Miranpuri, G., Byrne, G., Whittle, H., & Mabey, D. (1996). T helper type-1 (Th1)/Th2 profiles of peripheral blood mononuclear cells (PBMC); responses to antigens of Chlamydia trachomatis in subjects with severe trachomatous scarring. *Clinical & Experimental Immunology*, ***105***(3), 429-435.

Holland, M. J., Bailey, R. L., Hayes, L. J., Whittle, H. C., & Mabey, D. C. (1993). Conjunctival scarring in trachoma is associated with depressed cell-mediated immune responses to chlamydial antigens. *Journal of Infectious Diseases*, ***168***(6), 1528-1531.

Hölzer, M., Barf, L.-M., Lamkiewicz, K., Vorimore, F., Lataretu, M., Favaroni, A., Schnee, C., Laroucau, K., Marz, M., & Sachse, K. (2020). Comparative genome analysis of 33 Chlamydia strains reveals characteristic features of Chlamydia psittaci and closely related species. *Pathogens*, ***9***(11), 899.

Hone, J., Drake, V. A., & Krebs, C. J. (2023). Evaluation options for wildlife management and strengthening of causal inference. *BioScience*, ***73***(1), 48-58.

Hook, C. E., Matyszak, M. K., & Gaston, J. S. H. (2005). Infection of epithelial and dendritic cells by Chlamydia trachomatis results in IL-18 and IL-12 production, leading to interferon-γ production by human natural killer cells. *FEMS Immunology & Medical Microbiology*, ***45***(2), 113-120.

Horn, D. (2014). Antigenic variation in African trypanosomes. *Molecular and biochemical parasitology*, ***195***(2), 123-129.

Horn, D., & McCulloch, R. (2010). Molecular mechanisms underlying the control of antigenic variation in African trypanosomes. *Current Opinion in Microbiology*, ***13***(6), 700-705.

Hou, S., Sun, X., Dong, X., Lin, H., Tang, L., Xue, M., & Zhong, G. (2019). Chlamydial plasmid-encoded virulence factor Pgp3 interacts with human cathelicidin peptide LL-37 to modulate immune response. *Microbes and Infection*, ***21***(1), 50-55. https://doi.org/https://doi.org/10.1016/j.micinf.2018.06.003

Houlden, B., England, P., Taylor, A., Greville, W., & Sherwin, W. (1996). Low genetic variability of the koala Phascolarctos cinereus in south-eastern Australia following a severe population bottleneck. *Molecular ecology*, ***5***(2), 269-281.

Houlden, B. A., Costello, B. H., Sharkey, D., Fowler, E. V., Melzer, A., Ellis, W., Carrick, F., Baverstock, P. R., & Elphinstone, M. S. (1999). Phylogeographic differentiation in the mitochondrial control region in the koala, Phascolarctos cinereus (Goldfuss 1817). *Molecular ecology*, ***8***(6), 999-1011.

Howard, E. M. (2022). *Prevalence and molecular characterisation of Trypanosoma spp. in two wild koala populations; Moreton Bay, Queensland and Mount Lofty, South Australia* Murdoch University].

Howe, S. E., Shillova, N., & Konjufca, V. (2019). Dissemination of Chlamydia from the reproductive tract to the gastro-intestinal tract occurs in stages and relies on Chlamydia transport by host cells. *PLOS Pathogens*, ***15***(12), e1008207. https://doi.org/10.1371/journal.ppat.1008207

Huang, L., Zhang, X., Pang, L., Sheng, P., Wang, Y., Yang, F., Yu, H., Huang, X., Zhu, Y., & Zhang, N. (2023). Viral reactivation in the lungs of patients with severe pneumonia is associated with increased mortality, a multicenter, retrospective study. *Journal of Medical Virology*, ***95***(1), e28337.

Huber, S., Gagliani, N., Esplugues, E., O'Connor, W., Jr., Huber, Francis J., Chaudhry, A., Kamanaka, M., Kobayashi, Y., Booth, Carmen J., Rudensky, Alexander Y., Roncarolo, Maria G., Battaglia, M., & Flavell, Richard A. (2011). Th17 Cells Express Interleukin-10 Receptor and Are Controlled by Foxp3- and Foxp3+ Regulatory CD4+ T Cells in an Interleukin-10-Dependent Manner. *Immunity*, ***34***(4), 554-565. https://doi.org/10.1016/j.immuni.2011.01.020

Huittinen, T., Hahn, D., Anttila, T., Wahlström, E., Saikku, P., & Leinonen, M. (2001). Host immune response to Chlamydia pneumoniae heat shock protein 60 is associated with asthma. *European Respiratory Journal*, ***17***(6), 1078-1082.

Hulse, L., Beagley, K., Larkin, R., Nicolson, V., Gosálvez, J., & Johnston, S. (2021). The effect of Chlamydia infection on koala (Phascolarctos cinereus) semen quality. *Theriogenology*, ***167***, 99-110. https://doi.org/10.1016/j.theriogenology.2021.03.016

Hulse, L., Palmieri, C., Beagley, K. W., Larkin, R., Keeley, T., Gosalvez, J., & Johnston, S. D. (2022). Investigation of pathology associated with Chlamydia pecorum infection in the male reproductive tract, and the effect on spermatogenesis and semen quality in the koala (Phascolarctos cinereus). *Theriogenology*, ***180***, 30-39. https://doi.org/10.1016/j.theriogenology.2021.12.011

Hulse, L. S., Beagley, K., Ellis, W., Fitzgibbon, S., Gillett, A., Barth, B., Robbins, A., Pyne, M., Larkin, R., & Johnston, S. D. (2019a). Epidemiology of chlamydia-induced reproductive disease in male koalas (Phascolarctos cinereus) from Southeast Queensland, Australia as assessed from penile urethral swabs and semen. *Journal of Wildlife Diseases*, ***56***(1), 82-92. https://doi.org/10.7589/2019-03-062

Hulse, L. S., Hickey, D., Mitchell, J. M., Beagley, K. W., Ellis, W., & Johnston, S. D. (2018). Development and application of two multiplex real-time PCR assays for detection and speciation of bacterial pathogens in the koala. *J Vet Diagn Invest*, ***30***(4), 523-529. https://doi.org/10.1177/1040638718770490

Hulse, L. S., McDonald, S., Johnston, S. D., & Beagley, K. W. (2019b). Rapid point-of-care diagnostics for the detection of Chlamydia pecorum in koalas (Phascolarctos

cinereus) using loop-mediated isothermal amplification without nucleic acid purification. *Microbiologyopen*, ***8***(12), e916. https://doi.org/10.1002/mbo3.916
Hulse, L. S., McDonald, S., Johnston, S. D., & Beagley, K. W. (2019c). Rapid point-of-care diagnostics for the detection of Chlamydia pecorum in koalas (Phascolarctos cinereus) using loop-mediated isothermal amplification without nucleic acid purification. *Microbiologyopen*, ***8***(12), e916.
Hussain, R., Talat, N., Shahid, F., & Dawood, G. (2007). Longitudinal Tracking of Cytokines after Acute Exposure to Tuberculosis: Association of Distinct Cytokine Patterns with Protection and Disease Development. *Clinical and Vaccine Immunology*, ***14***(12), 1578-1586. https://doi.org/doi:10.1128/CVI.00289-07
Husson, F., Josse, J., & Pages, J. (2010). Principal component methods-hierarchical clustering-partitional clustering: why would we need to choose for visualizing data. *Applied mathematics department*, ***17***.
Hyeon, J., Cho, S. Y., Hong, M. E., Kang, S. Y., Do, I., Im, Y. H., & Cho, E. Y. (2017). NanoString nCounter® approach in breast cancer: a comparative analysis with quantitative real-time polymerase chain reaction, in situ hybridization, and immunohistochemistry. *Journal of breast cancer*, ***20***(3), 286-296.
Hyman, I. T., Ahyong, S. T., Köhler, F., McEvey, S. F., Milledge, G. A., Reid, C. A., & Rowley, J. J. (2020). Impacts of the 2019–2020 bushfires on New South Wales biodiversity: a rapid assessment of distribution data for selected invertebrate taxa. *Technical Reports of the Australian Museum Online*, ***32***, 1-17.
Hyseni, B. (2021). *The Role of Immune Cells in Transport of Chlamydia muridarum from the Iliac Lymph Nodes to the Spleen and the Gastrointestinal Tract*. Southern Illinois University at Carbondale.
ICUN. (2022). *Red List* http://www.iucnredlist.org
Imanishi, T. (2023). Putative koala retrovirus-associated diseases in the Japanese captive koala (Phascolarctos cinereus) population. Proceedings of the Second Koala Retrovirus Workshop, ed. DE Alquezar-Planas, DP Higgins, CL Singleton, and AD Greenwood. Technical Reports of the Australian Museum Online,
Ingersoll, M. A., Spanbroek, R., Lottaz, C., Gautier, E. L., Frankenberger, M., Hoffmann, R., Lang, R., Haniffa, M., Collin, M., & Tacke, F. (2010). Comparison of gene expression profiles between human and mouse monocyte subsets. *Blood, The Journal of the American Society of Hematology*, ***115***(3), e10-e19.
Ishida, Y., Zhao, K., Greenwood, A. D., & Roca, A. L. (2015). Proliferation of endogenous retroviruses in the early stages of a host germ line invasion. *Molecular biology and evolution*, ***32***(1), 109-120.
Islam, M. M., Jelocnik, M., Anstey, S., Kaltenboeck, B., Borel, N., Timms, P., & Polkinghorne, A. (2019). In vitro analysis of genetically distinct Chlamydia pecorum isolates reveals key growth differences in mammalian epithelial and immune cells. *Veterinary microbiology*, ***232***, 22-29. https://doi.org/https://doi.org/10.1016/j.vetmic.2019.03.024
Islam, M. M., Jelocnik, M., Huston, W. M., Timms, P., & Polkinghorne, A. (2018). Characterization of the In Vitro Chlamydia pecorum Response to Gamma Interferon. *Infect Immun*, ***86***(4). https://doi.org/10.1128/iai.00714-17
Jackson, J. A., Begon, M., Birtles, R., Paterson, S., Friberg, I. M., Hall, A., Lowe, A., Ralli, C., Turner, A., Zawadzka, M., & Bradley, J. E. (2011). The analysis of immunological profiles in wild animals: a case study on immunodynamics in the field vole, Microtus

agrestis. *Molecular ecology*, ***20***(5), 893-909. https://doi.org/https://doi.org/10.1111/j.1365-294X.2010.04907.x

Jackson, M., White, N., Giffard, P., & Timms, P. (1999). Epizootiology of Chlamydia infections in two free-range koala populations. *Vet Microbiol*, ***65***(4), 255-264. https://doi.org/10.1016/s0378-1135(98)00302-2

James, T. Y., Toledo, L. F., Rödder, D., da Silva Leite, D., Belasen, A. M., Betancourt-Román, C. M., Jenkinson, T. S., Soto-Azat, C., Lambertini, C., Longo, A. V., Ruggeri, J., Collins, J. P., Burrowes, P. A., Lips, K. R., Zamudio, K. R., & Longcore, J. E. (2015). Disentangling host, pathogen, and environmental determinants of a recently emerged wildlife disease: lessons from the first 15 years of amphibian chytridiomycosis research. *Ecology and Evolution*, ***5***(18), 4079-4097. https://doi.org/https://doi.org/10.1002/ece3.1672

Jelocnik, M. (2019). Chlamydiae from down under: The curious cases of chlamydial infections in Australia. *Microorganisms*, ***7***(12), 602.

Jelocnik, M., Bachmann, N. L., Kaltenboeck, B., Waugh, C. A., Woolford, L., Speight, K. N., Gillett, A., Higgins, D. P., Flanagan, C., Myers, G. S., Timms, P., & Polkinghorne, A. (2015). Genetic diversity in the plasticity zone and the presence of the chlamydial plasmid differentiates Chlamydia pecorum strains from pigs, sheep, cattle, and koalas. *BMC Genomics*, ***16***, 893. https://doi.org/10.1186/s12864-015-2053-8

Jelocnik, M., Bachmann, N. L., Seth-Smith, H., Thomson, N. R., Timms, P., & Polkinghorne, A. M. (2016). Molecular characterisation of the Chlamydia pecorum plasmid from porcine, ovine, bovine, and koala strains indicates plasmid-strain co-evolution. *PeerJ*, ***4***, e1661. https://doi.org/10.7717/peerj.1661

Jelocnik, M., Islam, M. M., Madden, D., Jenkins, C., Branley, J., Carver, S., & Polkinghorne, A. (2017). Development and evaluation of rapid novel isothermal amplification assays for important veterinary pathogens: Chlamydia psittaci and Chlamydia pecorum. *PeerJ*, ***5***, e3799.

Jelocnik, M., Walker, E., Pannekoek, Y., Ellem, J., Timms, P., & Polkinghorne, A. (2014). Evaluation of the relationship between Chlamydia pecorum sequence types and disease using a species-specific multi-locus sequence typing scheme (MLST). *Veterinary microbiology*, ***174***(1-2), 214-222.

Jendro, M. C., Fingerle, F., Deutsch, T., Liese, A., Köhler, L., Kuipers, J. G., Raum, E., Martin, M., & Zeidler, H. (2004). Chlamydia trachomatis-infected macrophages induce apoptosis of activated T cells by secretion of tumor necrosis factor-alpha in vitro. *Med Microbiol Immunol*, ***193***(1), 45-52. https://doi.org/10.1007/s00430-003-0182-1

Jiang, J.-H., Wang, N., Li, A., Liao, W.-T., Pan, Z.-G., Mai, S.-J., Li, D.-J., Zeng, M.-S., Wen, J.-m., & Zeng, Y.-X. (2006). Hypoxia can contribute to the induction of the Epstein-Barr virus (EBV) lytic cycle. *Journal of clinical virology*, ***37***(2), 98-103.

Jiang, P., Zhang, Y., Ru, B., Yang, Y., Vu, T., Paul, R., Mirza, A., Altan-Bonnet, G., Liu, L., Ruppin, E., Wakefield, L., & Wucherpfennig, K. W. (2021). Systematic investigation of cytokine signaling activity at the tissue and single-cell levels. *Nature methods*, ***18***(10), 1181-1191. https://doi.org/10.1038/s41592-021-01274-5

Jobbins, S. E., Sanderson, C. E., Griffith, J. E., Krockenberger, M. B., Belov, K., & Higgins, D. P. (2012). Diversity of MHC class II DAB1 in the koala (Phascolarctos cinereus). *Australian journal of zoology*, ***60***(1), 1-9.

Johnson, B., Panek, P., Yu, A., Fischer, E., Koba, M., Mendoza Hermosillo, D., & Capaldo, C. T. (2022). Interferon gamma upregulates the cytokine receptors IFNGR1 and TNFRSF1A

in HT-29-MTX E12 cells. *Cytokine*, ***156***, 155892. https://doi.org/https://doi.org/10.1016/j.cyto.2022.155892
Johnson, R. M., Kerr, M. S., & Slaven, J. E. (2014). An atypical CD 8 T-cell response to Chlamydia muridarum genital tract infections includes T cells that produce interleukin-13. *Immunology*, ***142***(2), 248-257.
Johnson, R. M., Olivares-Strank, N., & Peng, G. (2020). A class II-restricted CD8γ13 T-cell clone protects during Chlamydia muridarum genital tract infection. *The Journal of infectious diseases*, ***221***(11), 1895-1906.
Johnson, R. N., O'Meally, D., Chen, Z., Etherington, G. J., Ho, S. Y. W., Nash, W. J., Grueber, C. E., Cheng, Y., Whittington, C. M., Dennison, S., Peel, E., Haerty, W., O'Neill, R. J., Colgan, D., Russell, T. L., Alquezar-Planas, D. E., Attenbrow, V., Bragg, J. G., Brandies, P. A., . . . Belov, K. (2018). Adaptation and conservation insights from the koala genome. *Nat Genet*, ***50***(8), 1102-1111. https://doi.org/10.1038/s41588-018-0153-5
Johnson, W. E. (2019). Origins and evolutionary consequences of ancient endogenous retroviruses. *Nature Reviews Microbiology*, ***17***(6), 355-370. https://doi.org/10.1038/s41579-019-0189-2
Johnston, C., & Corey, L. (2016). Current Concepts for Genital Herpes Simplex Virus Infection: Diagnostics and Pathogenesis of Genital Tract Shedding. *Clin Microbiol Rev*, ***29***(1), 149-161. https://doi.org/10.1128/cmr.00043-15
Johnston, S., Booth, R., Pyne, M., Keeley, T., Mackie, J., Hulse, L., & Ellis, W. (2013). Preliminary study of faecal cortisol and corticosterone as an index of acute cortisol secretion in the koala (Phascolarctos cinereus). *Australian veterinary journal*, ***91***(12), 534-537.
Johnston, S. D., Deif, H. H., McKinnon, A., Theilemann, P., Griffith, J. E., & Higgins, D. P. (2015). Orchitis and Epididymitis in Koalas (Phascolarctos cinereus) Infected With Chlamydia pecorum. *Vet Pathol*, ***52***(6), 1254-1257. https://doi.org/10.1177/0300985815570069
Jolliffe, I. T., & Cadima, J. (2016). Principal component analysis: a review and recent developments. *Philosophical transactions of the royal society A: Mathematical, Physical and Engineering Sciences*, ***374***(2065), 20150202.
Jones, E. A., Cheng, Y., O'Meally, D., & Belov, K. (2017). Characterization of the antimicrobial peptide family defensins in the Tasmanian devil (Sarcophilus harrisii), koala (Phascolarctos cinereus), and tammar wallaby (Macropus eugenii). *Immunogenetics*, ***69***, 133-143.
Jorgensen, I., Bednar, M. M., Amin, V., Davis, Beckley K., Ting, Jenny P. Y., McCafferty, D. G., & Valdivia, Raphael H. (2011). The Chlamydia Protease CPAF Regulates Host and Bacterial Proteins to Maintain Pathogen Vacuole Integrity and Promote Virulence. *Cell Host & Microbe*, ***10***(1), 21-32. https://doi.org/https://doi.org/10.1016/j.chom.2011.06.008
Joseph, M. B., Mihaljevic, J. R., Arellano, A. L., Kueneman, J. G., Preston, D. L., Cross, P. C., & Johnson, P. T. J. (2013). Taming wildlife disease: bridging the gap between science and management. *Journal of Applied Ecology*, ***50***(3), 702-712. https://doi.org/https://doi.org/10.1111/1365-2664.12084
Joyce, B. A., Blyton, M. D. J., Johnston, S. D., Meikle, W. D., Vinette Herrin, K., Madden, C., Young, P. R., & Chappell, K. J. (2022). Diversity and transmission of koala retrovirus: a case study in three captive koala populations. *Sci Rep*, ***12***(1), 15787. https://doi.org/10.1038/s41598-022-18939-6

Joyce, B. A., Blyton, M. D. J., Johnston, S. D., Young, P. R., & Chappell, K. J. (2021). Koala retrovirus genetic diversity and transmission dynamics within captive koala populations. *Proc Natl Acad Sci U S A*, ***118***(38). https://doi.org/10.1073/pnas.2024021118

Kaelin Emily, A., Skidmore Peter, T., Łaniewski, P., Holland LaRinda, A., Chase Dana, M., Herbst-Kralovetz Melissa, M., & Lim Efrem, S. (2022). Cervicovaginal DNA Virome Alterations Are Associated with Genital Inflammation and Microbiota Composition. *mSystems*, ***7***(2), e00064-00022. https://doi.org/10.1128/msystems.00064-22

Kajaia, D., Merabishvili, N., & Burkadze, G. (2006). Pap testing and direct immunofluorescence for Chlamydia trachomatis infection in pregnant women. *Georgian Med News*(131), 27-30.

Kalliolias, G. D., Gordon, R. A., & Ivashkiv, L. B. (2010). Suppression of TNF-α and IL-1 signaling identifies a mechanism of homeostatic regulation of macrophages by IL-27. *The Journal of Immunology*, ***185***(11), 7047-7056.

Kanowski, J. (2001). Effects of elevated CO2 on the foliar chemistry of seedlings of two rainforest trees from north-east Australia: implications for folivorous marsupials. *Austral Ecology*, ***26***(2), 165-172.

Karlsson, M., Zhang, C., Méar, L., Zhong, W., Digre, A., Katona, B., Sjöstedt, E., Butler, L., Odeberg, J., Dusart, P., Edfors, F., Oksvold, P., von Feilitzen, K., Zwahlen, M., Arif, M., Altay, O., Li, X., Ozcan, M., Mardinoglu, A., . . . Lindskog, C. (2021). A single–cell type transcriptomics map of human tissues. *Science Advances*, ***7***(31), eabh2169. https://doi.org/doi:10.1126/sciadv.abh2169

Kasimov, V., Stephenson, T., Speight, N., Chaber, A. L., Boardman, W., Easther, R., & Hemmatzadeh, F. (2020). Identification and Prevalence of Phascolarctid Gammaherpesvirus Types 1 and 2 in South Australian Koala Populations. *Viruses*, ***12***(9). https://doi.org/10.3390/v12090948

Kasozi, K. I., Zirintunda, G., Ssempijja, F., Buyinza, B., Alzahrani, K. J., Matama, K., Nakimbugwe, H. N., Alkazmi, L., Onanyang, D., Bogere, P., Ochieng, J. J., Islam, S., Matovu, W., Nalumenya, D. P., Batiha, G. E., Osuwat, L. O., Abdelhamid, M., Shen, T., Omadang, L., & Welburn, S. C. (2021). Epidemiology of Trypanosomiasis in Wildlife-Implications for Humans at the Wildlife Interface in Africa. *Front Vet Sci*, ***8***, 621699. https://doi.org/10.3389/fvets.2021.621699

Kato, M. (2020). New insights into IFN-γ in rheumatoid arthritis: role in the era of JAK inhibitors. *Immunological Medicine*, ***43***(2), 72-78. https://doi.org/10.1080/25785826.2020.1751908

Kayesh, M. E. H., Hashem, M. A., Maetani, F., Eiei, T., Mochizuki, K., Ochiai, S., Ito, A., Ito, N., Sakurai, H., Asai, T., & Tsukiyama-Kohara, K. (2020a). CD4, CD8b, and Cytokines Expression Profiles in Peripheral Blood Mononuclear Cells Infected with Different Subtypes of KoRV from Koalas (Phascolarctos cinereus) in a Japanese Zoo. *Viruses*, ***12***(12), 1415. https://www.mdpi.com/1999-4915/12/12/1415

Kayesh, M. E. H., Hashem, M. A., Maetani, F., Goto, A., Nagata, N., Kasori, A., Imanishi, T., & Tsukiyama-Kohara, K. (2022). Molecular Insights into Innate Immune Response in Captive Koala Peripheral Blood Mononuclear Cells Co-Infected with Multiple Koala Retrovirus Subtypes. *Pathogens*, ***11***(8), 911.

Kayesh, M. E. H., Hashem, M. A., & Tsukiyama-Kohara, K. (2020b). Koala retrovirus epidemiology, transmission mode, pathogenesis, and host immune response in

koalas (Phascolarctos cinereus): a review. *Arch Virol*, ***165***(11), 2409-2417. https://doi.org/10.1007/s00705-020-04770-9

Kayesh, M. E. H., Hashem, M. A., & Tsukiyama-Kohara, K. (2021a). Toll-Like Receptor and Cytokine Responses to Infection with Endogenous and Exogenous Koala Retrovirus, and Vaccination as a Control Strategy. *CIMB*, ***43***(1), 52-64.

Kayesh, M. E. H., Hashem, M. A., & Tsukiyama-Kohara, K. (2021b). Toll-like receptor expression profiles in koala (Phascolarctos cinereus) peripheral blood mononuclear cells infected with multiple KoRV subtypes. *Animals*, ***11***(4), 983.

Kayesh, M. E. H., Hashem, M. A., & Tsukiyama-Kohara, K. (2024). Epidemiology, Transmission Mode, and Pathogenesis of Chlamydia pecorum Infection in Koalas (Phascolarctos cinereus): An Overview. *Animals*, ***14***(18), 2686. https://www.mdpi.com/2076-2615/14/18/2686

Keller, M., Mazuch, J., Abraham, U., Eom, G. D., Herzog, E. D., Volk, H.-D., Kramer, A., & Maier, B. (2009). A circadian clock in macrophages controls inflammatory immune responses. *Proceedings of the National Academy of Sciences*, ***106***(50), 21407-21412.

Keller, R., Joller, P., Keist, R., Binz, H., & Van Der Meide, P. (1988). Modulation of Major Histocompatibility Complex (MHC) Expression by Interferons and Microbial Agents: Independent Regulation of MHC Class II Expression and Induction of Tumoricidal Activity in Bone Marrow-Derived Mononuclear Phagocytes. *Scandinavian Journal of Immunology*, ***28***(1), 113-121.

Kent, N., & Cristescu, R. (2020). Koala survey using detection dogs Beerburrum to Nambour Rail Upgrade.

Kerlin, D. H., Grogan, L. F., & McCallum, H. I. (2022). Insights and inferences on koala conservation from records of koalas arriving to care in South East Queensland. *Wildl Res*, ***50***(1), 57-67.

Khan, S. A., Desclozeaux, M., Waugh, C. A., Hanger, J., Loader, J., Gerdts, V., Potter, A., Polkinghorne, A., Beagley, K. W., & Timms, P. (2016). Antibody and Cytokine Responses of Koalas (Phascolarctos cinereus) Vaccinated with Recombinant Chlamydial Major Outer Membrane Protein (MOMP) with Two Different Adjuvants. *PLoS One*, ***11***(5), e0156094. https://doi.org/10.1371/journal.pone.0156094

Khan, S. A., Waugh, C., Rawlinson, G., Brumm, J., Nilsson, K., Gerdts, V., Potter, A., Polkinghorne, A., Beagley, K., & Timms, P. (2014). Vaccination of koalas (Phascolarctos cinereus) with a recombinant chlamydial major outer membrane protein adjuvanted with poly I: C, a host defense peptide and polyphosphazine, elicits strong and long lasting cellular and humoral immune responses. *Vaccine*, ***32***(44), 5781-5786.

Kidd, A., Casteriano, A., Krockenberger, M. B., Higgins, D. P., & Wright, B. R. (2024). Koala MHCII association with chlamydia infertility remains equivocal: a need for new research approaches. *Scientific reports*, ***14***(1), 31074. https://doi.org/10.1038/s41598-024-82217-w

Kim, H., de Jesus, A. A., Brooks, S. R., Liu, Y., Huang, Y., VanTries, R., Montealegre Sanchez, G. A., Rotman, Y., Gadina, M., & Goldbach-Mansky, R. (2018). Development of a Validated Interferon Score Using NanoString Technology. *Journal of Interferon & Cytokine Research*, ***38***(4), 171-185. https://doi.org/10.1089/jir.2017.0127

Kissick, H. T., Dunn, L. K., Ghosh, S., Nechama, M., Kobzik, L., & Arredouani, M. S. (2014). The Scavenger Receptor MARCO Modulates TLR-Induced Responses in Dendritic Cells. *PLoS One*, ***9***(8), e104148. https://doi.org/10.1371/journal.pone.0104148

Klase, Z., Kale, P., Winograd, R., Gupta, M. V., Heydarian, M., Berro, R., McCaffrey, T., & Kashanchi, F. (2007). HIV-1 TAR element is processed by Dicer to yield a viral micro-RNA involved in chromatin remodeling of the viral LTR. *BMC molecular biology*, ***8***, 1-19.

Klase, Z., Winograd, R., Davis, J., Carpio, L., Hildreth, R., Heydarian, M., Fu, S., McCaffrey, T., Meiri, E., & Ayash-Rashkovsky, M. (2009). HIV-1 TAR miRNA protects against apoptosis by altering cellular gene expression. *Retrovirology*, ***6***, 1-17.

Kleinerman, E. S., Lachman, L. B., Knowles, R. D., Snyderman, R., & Cianciolo, G. J. (1987). A synthetic peptide homologous to the envelope proteins of retroviruses inhibits monocyte-mediated killing by inactivating interleukin 1. *J Immunol*, ***139***(7), 2329-2337.

Klomp, J., & de Haan, J. (2010). Measuring Health: A Multivariate Approach. *Soc Indic Res*, ***96***(3), 433-457. https://doi.org/10.1007/s11205-009-9486-x

Koala Health Hub. (2019). *Sampling Protocols*. https://koalahealthhub.org.au/sampling-protocols/

Kock, R. A., Orynbayev, M., Robinson, S., Zuther, S., Singh, N. J., Beauvais, W., Morgan, E. R., Kerimbayev, A., Khomenko, S., Martineau, H. M., Rystaeva, R., Omarova, Z., Wolfs, S., Hawotte, F., Radoux, J., & Milner-Gulland, E. J. (2018). Saigas on the brink: Multidisciplinary analysis of the factors influencing mass mortality events. *Sci Adv*, ***4***(1), eaao2314. https://doi.org/10.1126/sciadv.aao2314

Koh, K.-Y., Ahmad, S., Lee, J.-i., Suh, G.-H., & Lee, C.-M. (2022). Hierarchical Clustering on Principal Components Analysis to Detect Clusters of Highly Pathogenic Avian Influenza Subtype H5N6 Epidemic across South Korean Poultry Farms. *Symmetry*, ***14***(3), 598. https://www.mdpi.com/2073-8994/14/3/598

Kollipara, A., George, C., Hanger, J., Loader, J., Polkinghorne, A., Beagley, K., & Timms, P. (2012). Vaccination of healthy and diseased koalas (Phascolarctos cinereus) with a Chlamydia pecorum multi-subunit vaccine: evaluation of immunity and pathology. *Vaccine*, ***30***(10), 1875-1885.

Kollipara, A., Polkinghorne, A., Wan, C., Kanyoka, P., Hanger, J., Loader, J., Callaghan, J., Bell, A., Ellis, W., & Fitzgibbon, S. (2013a). Genetic diversity of Chlamydia pecorum strains in wild koala locations across Australia and the implications for a recombinant C. pecorum major outer membrane protein based vaccine. *Veterinary microbiology*, ***167***(3-4), 513-522.

Kollipara, A., Wan, C., Rawlinson, G., Brumm, J., Nilsson, K., Polkinghorne, A., Beagley, K., & Timms, P. (2013b). Antigenic specificity of a monovalent versus polyvalent MOMP based Chlamydia pecorum vaccine in koalas (Phascolarctos cinereus). *Vaccine*, ***31***(8), 1217-1223.

Komori, C., Takahashi, T., Nakano, Y., & Ui-Tei, K. (2020). TRBP-Dicer interaction may enhance HIV-1 TAR RNA translation via TAR RNA processing, repressing host-cell apoptosis. *Biol Open*, ***9***(2). https://doi.org/10.1242/bio.050435

Kophamel, S., Illing, B., Ariel, E., Difalco, M., Skerratt, L. F., Hamann, M., Ward, L. C., Méndez, D., & Munns, S. L. (2022). Importance of health assessments for conservation in noncaptive wildlife. *Conserv Biol*, ***36***(1), e13724. https://doi.org/10.1111/cobi.13724

Koster, S., Gurumurthy, R. K., Kumar, N., Prakash, P. G., Dhanraj, J., Bayer, S., Berger, H., Kurian, S. M., Drabkina, M., Mollenkopf, H. J., Goosmann, C., Brinkmann, V., Nagel, Z., Mangler, M., Meyer, T. F., & Chumduri, C. (2022). Modelling Chlamydia and HPV

co-infection in patient-derived ectocervix organoids reveals distinct cellular reprogramming. *Nat Commun*, ***13***(1), 1030. https://doi.org/10.1038/s41467-022-28569-1

Kotani, N., Hashimoto, H., Sessler, D. I., Yasuda, T., Ebina, T., Muraoka, M., & Matsuki, A. (1999). Expression of Genes for Proinflammatory Cytokines in Alveolar Macrophages During Propofol and Isoflurane Anesthesia. *Anesthesia & Analgesia*, ***89***(5), 1250-1256. https://doi.org/10.1213/00000539-199911000-00032

Kraal, G., van der Laan, L. J. W., Elomaa, O., & Tryggvason, K. (2000). The macrophage receptor MARCO. *Microbes and Infection*, ***2***(3), 313-316. https://doi.org/https://doi.org/10.1016/S1286-4579(00)00296-3

Kretzschmar, G., Páez, L. P., Tan, Z., Wang, J., Gonzalez, L., Mugabo, C. H., Johnsson, A., Chen, Y., Mikeš, J., Lakshmikanth, T., James, A., Goldbach-Mansky, R., Fischer, M., Palmblad, K., Alehashemi, S., Horne, A., & Brodin, P. (2024). Normalized Interferon Signatures and Clinical Improvements by IFNAR1 Blocking Antibody (Anifrolumab) in Patients with Type I Interferonopathies. *Journal of Clinical Immunology*, ***45***(1), 31. https://doi.org/10.1007/s10875-024-01826-2

Krstanović, F., Mihalić, A., Rashidi, A. S., Sitnik, K. M., Ruzsics, Z., Čičin-Šain, L., Verjans, G. M. G. M., Jonjić, S., & Brizić, I. (2025). Neuron-restricted cytomegalovirus latency in the central nervous system regulated by CD4+ T-cells and IFN-γ. *Journal of Neuroinflammation*, ***22***(1), 95. https://doi.org/10.1186/s12974-025-03422-6

Kubo, S., & Tanaka, Y. (2024). The immune health metric as an indicator of health and disease. *Nature Reviews Rheumatology*, ***20***(12), 743-744. https://doi.org/10.1038/s41584-024-01162-0

Kulkarni, M. M. (2011). Digital multiplexed gene expression analysis using the NanoString nCounter system. *Current protocols in molecular biology*, ***94***(1), 25B. 10.21-25B. 10.17.

Kwon, J., Kim, Y. J., Choi, K., Seol, S., & Kang, H. J. (2019). Identification of stress resilience module by weighted gene co-expression network analysis in Fkbp5-deficient mice. *Molecular Brain*, ***12***, 1-4.

Lachish, S., McCALLUM, H., Mann, D., Pukk, C. E., & Jones, M. E. (2010). Evaluation of selective culling of infected individuals to control Tasmanian devil facial tumor disease. *Conservation Biology*, ***24***(3), 841-851.

Lane, A., Wallis, K., & Phillips, S. (2020). A review of the conservation status of New South Wales populations of the Koala (Phascolarctos cinereus) leading up to and including part of the 2019/20 fire event. *Report to International Fund for Animal Welfare (IFAW). Biolink Ecological Consultants, Uki NSW*.

Langmuir, A. D. (1963). The surveillance of communicable diseases of national importance. *New England journal of medicine*, ***268***(4), 182-192.

Langwig, K. E., Frick, W. F., Bried, J. T., Hicks, A. C., Kunz, T. H., & Marm Kilpatrick, A. (2012a). Sociality, density-dependence and microclimates determine the persistence of populations suffering from a novel fungal disease, white-nose syndrome. *Ecology Letters*, ***15***(9), 1050-1057. https://doi.org/https://doi.org/10.1111/j.1461-0248.2012.01829.x

Langwig, K. E., Frick, W. F., Bried, J. T., Hicks, A. C., Kunz, T. H., & Marm Kilpatrick, A. (2012b). Sociality, density-dependence and microclimates determine the persistence of populations suffering from a novel fungal disease, white-nose syndrome. *Ecology Letters*, ***15***(9), 1050-1057.

Lanyon, J. M., & Sanson, G. (1986). Koala (Phascolarctos cinereus) dentition and nutrition. II. Implications of tooth wear in nutrition. *Journal of Zoology*, ***209***(2), 169-181.
Latz, E., Xiao, T. S., & Stutz, A. (2013). Activation and regulation of the inflammasomes. *Nature Reviews Immunology*, ***13***(6), 397-411.
Lau, Q., Canfield, P. J., & Higgins, D. P. (2012). Expression and in vitro upregulation of MHCII in koala lymphocytes. *Vet Immunol Immunopathol*, ***147***(1-2), 35-43. https://doi.org/10.1016/j.vetimm.2012.04.010
Lau, Q., Griffith, J. E., & Higgins, D. P. (2014). Identification of MHCII variants associated with chlamydial disease in the koala (Phascolarctos cinereus). *PeerJ*, ***2***, e443.
Lau, Q., Jobbins, S. E., Belov, K., & Higgins, D. P. (2013). Characterisation of four major histocompatibility complex class II genes of the koala (Phascolarctos cinereus). *Immunogenetics*, ***65***(1), 37-46. https://doi.org/10.1007/s00251-012-0658-5
Laurell, H., Iacovoni, J. S., Abot, A., Svec, D., Maoret, J. J., Arnal, J. F., & Kubista, M. (2012). Correction of RT-qPCR data for genomic DNA-derived signals with ValidPrime. *Nucleic Acids Res*, ***40***(7), e51. https://doi.org/10.1093/nar/gkr1259
Lee, R. S. (2016). Glucocorticoid-dependent epigenetic regulation of Fkbp5. In *Epigenetics and Neuroendocrinology: Clinical Focus on Psychiatry, Volume 1* (pp. 97-114). Springer.
Legione, A. R., Patterson, J. L. S., Whiteley, P., Firestone, S. M., Curnick, M., Bodley, K., Lynch, M., Gilkerson, J. R., Sansom, F. M., & Devlin, J. M. (2017). Koala retrovirus genotyping analyses reveal a low prevalence of KoRV-A in Victorian koalas and an association with clinical disease. *J Med Microbiol*, ***66***(2), 236-244. https://doi.org/10.1099/jmm.0.000416
Legione, A. R., Patterson, J. L. S., Whiteley, P. L., Amery-Gale, J., Lynch, M., Haynes, L., Gilkerson, J. R., Polkinghorne, A., Devlin, J. M., & Sansom, F. M. (2016). Identification of unusual Chlamydia pecorum genotypes in Victorian koalas (Phascolarctos cinereus) and clinical variables associated with infection. *J Med Microbiol*, ***65***(5), 420-428. https://doi.org/https://doi.org/10.1099/jmm.0.000241
Lehman, B. M., Johnson, R. C., Adkison, M., Burgess, O. T., Connon, R. E., Fangue, N. A., Foott, J. S., Hallett, S. L., Martinez–López, B., & Miller, K. M. (2020). Disease in Central Valley Salmon: status and lessons from other systems. *San Francisco Estuary and Watershed Science*, ***18***(3).
Lehtinen, M., Lehtinen, T., Koivisto, V., Paavonen, J., & Leinikki, P. (1985). Serum antibodies to the major HSV-2-specified DNA-binding protein in patients with an acute HSV infection or cervical neoplasia. *J Med Virol*, ***16***(3), 245-256. https://doi.org/10.1002/jmv.1890160305
Leigh, K. A., Hofweber, L. N., Sloggett, B. K., Inman, V. L., Pettit, L. J., Sriram, A., & Haering, R. (2023). Outcomes for an arboreal folivore after rehabilitation and implications for management. *Scientific reports*, ***13***(1), 6542.
Leonard, C. A., Schoborg, R. V., & Borel, N. (2017). Productive and Penicillin-Stressed Chlamydia pecorum Infection Induces Nuclear Factor Kappa B Activation and Interleukin-6 Secretion In Vitro. *Front Cell Infect Microbiol*, ***7***, 180. https://doi.org/10.3389/fcimb.2017.00180
Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R., & Subgroup, G. P. D. P. (2009). The sequence alignment/map format and SAMtools. *bioinformatics*, ***25***(16), 2078-2079.

Li, J., Guo, W., Kaltenboeck, B., Sachse, K., Yang, Y., Lu, G., Zhang, J., Luan, L., You, J., Huang, K., Qiu, H., Wang, Y., Li, M., Yang, Z., & Wang, C. (2016a). Chlamydia pecorum is the endemic intestinal species in cattle while C. gallinacea, C. psittaci and C. pneumoniae associate with sporadic systemic infection. *Veterinary microbiology*, ***193***, 93-99. https://doi.org/https://doi.org/10.1016/j.vetmic.2016.08.008

Li, L., Feng, H., Da, Q., Jiang, H., Chen, L., Xie, L., Huang, Q., Xiong, H., Luo, F., & Kang, L. (2016b). Expression of HIV-encoded microRNA-TAR and its inhibitory effect on viral replication in human primary macrophages. *Archives of virology*, ***161***, 1115-1123.

Libert, N., Bigaillon, C., Chargari, C., Bensalah, M., Muller, V., Merat, S., & de Rudnicki, S. (2015). Epstein-Barr virus reactivation in critically ill immunocompetent patients. *Biomedical journal*, ***38***(1).

Lind, C. M., Agugliaro, J., Lorch, J. M., & Farrell, T. M. (2023). Ophidiomycosis is related to seasonal patterns of reproduction, ecdysis, and thermoregulatory behavior in a free-living snake species. *Journal of Zoology*, ***319***(1), 54-62. https://doi.org/https://doi.org/10.1111/jzo.13024

Lindenbaum, P. (2015). JVarkit: java-based utilities for Bioinformatics. *figshare*, ***10***, m9.

Littleford-Colquhoun, B. L., Weyrich, L. S., Hohwieler, K., Cristescu, R., & Frère, C. H. (2022). How microbiomes can help inform conservation: landscape characterisation of gut microbiota helps shed light on additional population structure in a specialist folivore. *Anim Microbiome*, ***4***(1), 12. https://doi.org/10.1186/s42523-021-00122-3

Lizárraga, D., Timms, P., Quigley, B. L., Hanger, J., & Carver, S. (2020a). Capturing complex vaccine-immune-disease relationships for free-ranging koalas: Higher chlamydial loads are associated with less IL17 expression and more chlamydial disease. *Frontiers in Veterinary Science*, ***7***, 530686.

Lizárraga, D., Timms, P., Quigley, B. L., Hanger, J., & Carver, S. (2020b). Capturing complex vaccine-immune-disease relationships for free-ranging koalas: Higher chlamydial loads are associated with less IL17 expression and more chlamydial disease. *Front Vet Sci*, ***7***, 530686.

Loftus, T. J., Shickel, B., Balch, J. A., Tighe, P. J., Abbott, K. L., Fazzone, B., Anderson, E. M., Rozowsky, J., Ozrazgat-Baslanti, T., Ren, Y., Berceli, S. A., Hogan, W. R., Efron, P. A., Moorman, J. R., Rashidi, P., Upchurch, G. R., & Bihorac, A. (2022). Phenotype clustering in health care: A narrative review for clinicians [Review]. *Frontiers in Artificial Intelligence*, ***5***. https://doi.org/10.3389/frai.2022.842306

López, G., López-Parra, M., Fernández, L., Martínez-Granados, C., Martínez, F., Meli, M. L., Gil-Sánchez, J. M., Viqueira, N., Díaz-Portero, M. A., Cadenas, R., Lutz, H., Vargas, A., & Simón, M. A. (2009). Management measures to control a feline leukemia virus outbreak in the endangered Iberian lynx. *Animal Conservation*, ***12***(3), 173-182. https://doi.org/https://doi.org/10.1111/j.1469-1795.2009.00241.x

Love, M., Anders, S., Huber, W., Love, M. M., BiocGenerics, I., Biobase, B., Rcpp, L., biocViews Sequencing, R., ChIPSeq, R., & SAGE, D. (2018). Package 'DESeq2'. In.

Love, M. I., Anders, S., & Huber, W. (2024, 04/30/2024). *Analyzing RNA-seq data with DESeq2*. Retrieved 9/3/2023 from https://www.bioconductor.org/packages/release/bioc/vignettes/DESeq2/inst/doc/DESeq2.html

Lozano, K. J. G., Gonçalves Santos, E., Vilas Boas, D. F., Oliveira, R. R. G., Diniz, L. F., Benedetti, M. D., Carneiro, C. M., C. Bandeira, L., Faria, G., Gonçalves, R. V., Novaes, R. D., Caldas, S., & Caldas, I. S. (2024). Schistosoma mansoni co-infection modulates

Chagas disease development but does not impair the effect of benznidazole-based chemotherapy. *International Immunopharmacology*, ***128***, 111467. https://doi.org/https://doi.org/10.1016/j.intimp.2023.111467

Lu, Y., Wu, Q., Wang, L., & Ji, L. (2024). Chlamydia trachomatis enhances HPV persistence through immune modulation. *BMC Infectious Diseases*, ***24***(1), 229.

Lubyayi, L., Mawa, P. A., Cose, S., Elliott, A. M., Levin, J., & Webb, E. L. (2021). Analysis of multivariate longitudinal immuno-epidemiological data using a pairwise joint modelling approach. *BMC Immunology*, ***22***(1), 63. https://doi.org/10.1186/s12865-021-00453-5

Lunney, D., Cope, H., Griffith, J., Orcheg, C., Bryant, J., & Haering, R. (2023). Trends in the rescue and fate of koalas in New South Wales (1973-2020), with a focus on disease and trauma. *Australian Zoologist*.

Lunney, D., Cope, H., Sonawane, I., Stalenberg, E., & Haering, R. (2022a). An analysis of the long-term trends in the records of Friends of the Koala in north-east New South Wales: I. Cause and fate of koalas admitted for rehabilitation (1989–2020). *Pacific Conservation Biology*, ***29***(3), 177-196.

Lunney, D., Cope, H., Sonawane, I., Stalenberg, E., & Haering, R. (2022b). An analysis of the long-term trends in the records of Friends of the Koala in north-east New South Wales: II. Post-release survival. *Pacific Conservation Biology*, ***29***(3), 197-222.

Lunney, D., Crowther, M. S., Shannon, I., & Bryant, J. V. (2009). Combining a map-based public survey with an estimation of site occupancy to determine the recent and changing distribution of the koala in New South Wales. *Wildlife Research*, ***36***(3), 262-273.

Lunney, D., Crowther, M. S., Wallis, I., Foley, W. J., Lemon, J., Wheeler, R., Madani, G., Orscheg, C., Griffith, J. E., & Krockenberger, M. (2012a). Koalas and climate change: a case study on the Liverpool Plains, north-west New South Wales.

Lunney, D., Lemon, J., Crowther, M., Stalenberg, E., Ross, K., & Wheeler, R. (2012b). An ecological approach to koala conservation in a mined landscape. Life-of-Mine Conference,

Lunney, D., Sonawane, I., Wheeler, R., Tasker, E., Ellis, M., Predavec, M., & Fleming, M. (2020). An ecological reading of the history of the koala population of Warrumbungle national park [Journal Article]. *Proceedings of the Linnean Society of New South Wales*, ***142***.

Lunney, D., Stalenberg, E., Santika, T., & Rhodes, J. R. (2014). Extinction in Eden: identifying the role of climate change in the decline of the koala in south-eastern NSW. *Wildlife Research*, ***41***(1), 22-34.

Mabbott, N. A. (2018). The influence of parasite infections on host immunity to co-infection with other pathogens. *Frontiers in Immunology*, ***9***, 2579.

Mackie, J. T., Gillett, A. K., Palmieri, C., Feng, T., & Higgins, D. P. (2016). Pneumonia due to Chlamydia pecorum in a Koala (Phascolarctos cinereus). *J Comp Pathol*, ***155***(4), 356-360. https://doi.org/10.1016/j.jcpa.2016.07.011

Madden, D., Whaite, A., Jones, E., Belov, K., Timms, P., & Polkinghorne, A. (2018). Koala immunology and infectious diseases: How much can the koala bear? *Dev Comp Immunol*, ***82***, 177-185. https://doi.org/10.1016/j.dci.2018.01.017

Maezawa, Y., Nakajima, H., Suzuki, K., Tamachi, T., Ikeda, K., Inoue, J.-i., Saito, Y., & Iwamoto, I. (2006). Involvement of TNF receptor-associated factor 6 in IL-25 receptor signaling. *The Journal of Immunology*, ***176***(2), 1013-1018.

Maher, I. E., Griffith, J. E., Lau, Q., Reeves, T., & Higgins, D. P. (2014). Expression profiles of the immune genes CD4, CD8β, IFNγ, IL-4, IL-6 and IL-10 in mitogen-stimulated koala lymphocytes (Phascolarctos cinereus) by qRT-PCR. *PeerJ*, ***2***, e280.

Maher, I. E., & Higgins, D. P. (2016). Altered immune cytokine expression associated with KoRV B infection and season in captive koalas. *PLoS One*, ***11***(10), e0163780.

Maher, I. E., Patterson, J., Curnick, M., Devlin, J., & Higgins, D. P. (2019). Altered immune parameters associated with Koala Retrovirus (KoRV) and Chlamydial infection in free ranging Victorian koalas (Phascolarctos cinereus). *Sci Rep*, ***9***(1), 11170.

Maidment, T. I., Bryan, E. R., Pyne, M., Barnes, M., Eccleston, S., Cunningham, S., Whitlock, E., Redman, K., Nicolson, V., & Beagley, K. W. (2023). Characterisation of the koala (Phascolarctos cinereus) pouch microbiota in a captive population reveals a dysbiotic compositional profile associated with neonatal mortality. *Microbiome*, ***11***(1), 75.

Mangar, C. (2018). *Characterisation of lymphocytes and cytokines in healthy and diseased koalas (Phascolarctos cinereus) using cell-type-specific monoclonal antibodies* Queensland University of Technology].

Mangar, C., Armitage, C. W., Timms, P., Corcoran, L. M., & Beagley, K. W. (2016). Characterisation of CD4 T cells in healthy and diseased koalas (Phascolarctos cinereus) using cell-type-specific monoclonal antibodies. *Dev Comp Immunol*, ***60***, 80-90.

Mangeney, M., & Heidmann, T. (1998). Tumor cells expressing a retroviral envelope escape immune rejection in vivo. *Proceedings of the National Academy of Sciences*, ***95***(25), 14920-14925.

Mangino, M., Roederer, M., Beddall, M. H., Nestle, F. O., & Spector, T. D. (2017). Innate and adaptive immune traits are differentially affected by genetic and environmental factors. *Nature communications*, ***8***(1), 13850. https://doi.org/10.1038/ncomms13850

Marcus, A. D., Higgins, D. P., & Gray, R. (2014). Epidemiology of hookworm (Uncinaria sanguinis) infection in free-ranging Australian sea lion (Neophoca cinerea) pups. *Parasitology research*, ***113***, 3341-3353.

Margalit, L., Strauss, C., Tal, A., & Schlesinger, S. (2020). Trim24 and Trim33 play a role in epigenetic silencing of retroviruses in embryonic stem cells. *Viruses*, ***12***(9), 1015.

Markey, B., Wan, C., Hanger, J., Phillips, C., & Timms, P. (2007). Use of quantitative real-time PCR to monitor the shedding and treatment of chlamydiae in the koala (Phascolarctos cinereus). *Veterinary microbiology*, ***120***(3), 334-342. https://doi.org/https://doi.org/10.1016/j.vetmic.2006.11.022

Marschner, C., Krockenberger, M. B., & Higgins, D. P. (2019a). Effects of eucalypt plant monoterpenes on koala (Phascolarctos Cinereus) cytokine expression in vitro. *Scientific reports*, ***9***(1), 16545.

Marschner, C., Krockenberger, M. B., Higgins, D. P., Mitchell, C., & Moore, B. D. (2019b). Ingestion and Absorption of Eucalypt Monoterpenes in the Specialist Feeder, the Koala (Phascolarctos cinereus). *Journal of Chemical Ecology*, ***45***(9), 798-807. https://doi.org/10.1007/s10886-019-01097-x

Marsh, J., Kollipara, A., Timms, P., & Polkinghorne, A. (2011). Novel molecular markers of Chlamydia pecorum genetic diversity in the koala (Phascolarctos cinereus). *BMC Microbiology*, ***11***(1), 77. https://doi.org/10.1186/1471-2180-11-77

Martini, F., & Champagne, E. (2021). The Contribution of Human Herpes Viruses to γδ T Cell Mobilisation in Co-Infections. *Viruses*, ***13***(12), 2372. https://www.mdpi.com/1999-4915/13/12/2372

Maslo, B., Gignoux-Wolfsohn, S. A., & Fefferman, N. H. (2017). Success of Wildlife Disease Treatment Depends on Host Immune Response [Original Research]. *Frontiers in Ecology and Evolution*, ***5***. https://doi.org/10.3389/fevo.2017.00028

Mathew, M. (2014). *Characterisation of cytokine response to Chlamydia pecorum infection in the koala, Phascolarctos cinereus* Queensland University of Technology].

Mathew, M., Beagley, K. W., Timms, P., & Polkinghorne, A. (2013a). Preliminary characterisation of tumor necrosis factor alpha and interleukin-10 responses to Chlamydia pecorum infection in the koala (Phascolarctos cinereus). *PLoS One*, ***8***(3), e59958.

Mathew, M., Pavasovic, A., Prentis, P. J., Beagley, K. W., Timms, P., & Polkinghorne, A. (2013b). Molecular characterisation and expression analysis of Interferon gamma in response to natural Chlamydia infection in the koala, Phascolarctos cinereus. *Gene*, ***527***(2), 570-577.

Mathew, M., Waugh, C. A., Beagley, K. W., Timms, P., & Polkinghorne, A. (2014). Interleukin 17A is an immune marker for chlamydial disease severity and pathogenesis in the koala (Phascolarctos cinereus). *Developmental & Comparative Immunology*, ***46***(2), 423-429.

Mathews, S., George, C., Flegg, C., Stenzel, D., & Timms, P. (2001). Differential expression of ompA, ompB, pyk, nlpD and Cpn0585 genes between normal and interferon-γ treated cultures of Chlamydia pneumoniae. *Microbial Pathogenesis*, ***30***(6), 337-345. https://doi.org/https://doi.org/10.1006/mpat.2000.0435

Matsumoto, A., & Manire, G. (1970). Electron microscopic observations on the effects of penicillin on the morphology of Chlamydia psittaci. *Journal of bacteriology*, ***101***(1), 278-285.

Maugeri, A., Barchitta, M., Basile, G., & Agodi, A. (2021). Applying a hierarchical clustering on principal components approach to identify different patterns of the SARS-CoV-2 epidemic across Italian regions. *Scientific reports*, ***11***(1), 7082.

Maugis, P.-A. G. (2018). Big data uncertainties. *Journal of forensic and legal medicine*, ***57***, 7-11.

Mazoti, M. A., Braz, M. G., de Assis Golim, M., Braz, L. G., Dias, N. H., Salvadori, D. M. F., Braz, J. R. C., & Fecchio, D. (2013). Comparison of inflammatory cytokine profiles in plasma of patients undergoing otorhinological surgery with propofol or isoflurane anesthesia. *Inflammation Research*, ***62***(10), 879-885. https://doi.org/10.1007/s00011-013-0643-y

Mazzamuto, M. V., Schilling, A. K., & Romeo, C. (2022). Wildlife Disease Monitoring: Methods and Perspectives. *Animals (Basel)*, ***12***(21). https://doi.org/10.3390/ani12213032

McAlpine, C., Brearley, G., Rhodes, J., Bradley, A., Baxter, G., Seabrook, L., Lunney, D., Liu, Y., Cottin, M., Smith, A. G., & Timms, P. (2017). Time-delayed influence of urban landscape change on the susceptibility of koalas to chlamydiosis. *Landscape Ecology*, ***32***(3), 663-679. https://doi.org/10.1007/s10980-016-0479-2

McAlpine, C., Lunney, D., Melzer, A., Menkhorst, P., Phillips, S., Phalen, D., Ellis, W., Foley, W., Baxter, G., de Villiers, D., Kavanagh, R., Adams-Hosking, C., Todd, C., Whisson, D., Molsher, R., Walter, M., Lawler, I., & Close, R. (2015). Conserving koalas: A review of

the contrasting regional trends, outlooks and policy challenges. *Biological conservation*, ***192***, 226-236. https://doi.org/https://doi.org/10.1016/j.biocon.2015.09.020

McCallum, H. (2012). Disease and the dynamics of extinction. *Philosophical Transactions of the Royal Society B: Biological Sciences*, ***367***(1604), 2828-2839.

McCallum, H., Foufopoulos, J., & Grogan, L. F. (2024). Infectious disease as a driver of declines and extinctions. *Cambridge Prisms: Extinction*, ***2***, e2, Article e2. https://doi.org/10.1017/ext.2024.1

McEwen, G. K., Alquezar-Planas, D. E., Dayaram, A., Gillett, A., Tarlinton, R., Mongan, N., Chappell, K. J., Henning, J., Tan, M., & Timms, P. (2021). Retroviral integrations contribute to elevated host cancer rates during germline invasion. *Nat Commun*, ***12***(1), 1316.

McInnes, L. M., Gillett, A., Hanger, J., Reid, S. A., & Ryan, U. M. (2011a). The potential impact of native Australian trypanosome infections on the health of koalas (Phascolarctos cinereus). *Parasitology*, ***138***(7), 873-883. https://doi.org/10.1017/s0031182011000369

McInnes, L. M., Gillett, A., Ryan, U. M., Austen, J., Campbell, R. S. F., Hanger, J., & Reid, S. A. (2009). Trypanosoma irwini n. sp (Sarcomastigophora: Trypanosomatidae) from the koala (Phascolarctos cinereus). *Parasitology*, ***136***(8), 875-885. https://doi.org/10.1017/S0031182009006313

McInnes, L. M., Hanger, J., Simmons, G., Reid, S. A., & Ryan, U. M. (2011b). Novel trypanosome Trypanosoma gilletti sp. (Euglenozoa: Trypanosomatidae) and the extension of the host range of Trypanosoma copemani to include the koala (Phascolarctos cinereus). *Parasitology*, ***138***(1), 59-70. https://doi.org/10.1017/S0031182010000971

McKay, P. A., & Jones, B. D. (2023). Incidence, trends, and significance of putative koala retrovirus-associated diseases in monitored wild koala populations in southeast Queensland. Proceedings of the Second Koala Retrovirus Workshop,

McLaren, G. W., Macdonald, D. W., Georgiou, C., Mathews, F., Newman, C., & Mian, R. (2003). Leukocyte coping capacity: a novel technique for measuring the stress response in vertebrates. *Exp Physiol*, ***88***(4), 541-546. https://doi.org/10.1113/eph8802571

McNew, G. L. (1960). The nature, origin, and evolution of parasitism. *Plant pathology: an advanced treatise*, ***2***, 19-69.

Medzhitov, R. (2007). Recognition of microorganisms and activation of the immune response. *Nature*, ***449***(7164), 819-826. https://doi.org/10.1038/nature06246

Mehta, S., Laudenslager, M., Stowe, R., Crucian, B., Sams, C., & Pierson, D. (2014). Multiple latent viruses reactivate in astronauts during Space Shuttle missions. *Brain, behavior, and immunity*, ***41***, 210-217.

Mella, V. S. A., Cooper, C. E., Karr, M., Krockenberger, A., Madani, G., Webb, E. B., & Krockenberger, M. B. (2024). Hot climate, hot koalas: the role of weather, behaviour and disease on thermoregulation. *Conservation Physiology*, ***12***(1). https://doi.org/10.1093/conphys/coae032

Meza Cerda, M. I., Gray, R., Thomson, P. C., Butcher, L., Simpson, K., Cameron, A., Marcus, A. D., & Higgins, D. P. (2022). Developing Immune Profiles of Endangered Australian Sea Lion (Neophoca cinerea) Pups Within the Context of Endemic Hookworm

(Uncinaria sanguinis) Infection. *Front Vet Sci*, ***9***, 824584. https://doi.org/10.3389/fvets.2022.824584

Meza, R., & Jeon, J. (2022). Invited Commentary: Mechanistic and Biologically Based Models in Epidemiology-A Powerful Underutilized Tool. *Am J Epidemiol*, ***191***(10), 1776-1780. https://doi.org/10.1093/aje/kwac099

Mezheyeuski, A., Backman, M., Mattsson, J., Martín-Bernabé, A., Larsson, C., Hrynchyk, I., Hammarström, K., Ström, S., Ekström, J., & Mauchanski, S. (2023). An immune score reflecting pro-and anti-tumoural balance of tumour microenvironment has major prognostic impact and predicts immunotherapy response in solid cancers. *EBioMedicine*, ***88***.

Miller, K. M., Günther, O. P., Li, S., Kaukinen, K. H., & Ming, T. J. (2017). Molecular indices of viral disease development in wild migrating salmon†. *Conservation Physiology*, ***5***(1). https://doi.org/10.1093/conphys/cox036

Miller, K. M., Teffer, A., Tucker, S., Li, S., Schulze, A. D., Trudel, M., Juanes, F., Tabata, A., Kaukinen, K. H., Ginther, N. G., Ming, T. J., Cooke, S. J., Hipfner, J. M., Patterson, D. A., & Hinch, S. G. (2014). Infectious disease, shifting climates, and opportunistic predators: cumulative factors potentially impacting wild salmon declines. *Evolutionary Applications*, ***7***(7), 812-855. https://doi.org/https://doi.org/10.1111/eva.12164

Mohamad, K. Y., Kaltenboeck, B., Rahman, K. S., Magnino, S., Sachse, K., & Rodolakis, A. (2014). Host Adaptation of Chlamydia pecorum towards Low Virulence Evident in Co-Evolution of the ompA, incA, and ORF663 Loci. *PLoS One*, ***9***(8), e103615. https://doi.org/10.1371/journal.pone.0103615

Mohamad, K. Y., Roche, S. M., Myers, G. S., Bavoil, P. M., Laroucau, K., Magnino, S., Laurent, S., Rasschaert, D., & Rodolakis, A. (2008). Preliminary phylogenetic identification of virulent Chlamydophila pecorum strains. *Infection, Genetics and Evolution*, ***8***(6), 764-771. https://doi.org/https://doi.org/10.1016/j.meegid.2008.06.009

Mordecai, G. J., Di Cicco, E., Günther, O. P., Schulze, A. D., Kaukinen, K. H., Li, S., Tabata, A., Ming, T. J., Ferguson, H. W., Suttle, C. A., & Miller, K. M. (2020). Emerging viruses in British Columbia salmon discovered via a viral immune response biomarker panel and metatranscriptomic sequencing. *BioRxiv*, 2020.2002.2013.948026. https://doi.org/10.1101/2020.02.13.948026

Morris, K., Prentis, P. J., O'Meally, D., Pavasovic, A., Brown, A. T., Timms, P., Belov, K., & Polkinghorne, A. (2014). The koala immunological toolkit: sequence identification and comparison of key markers of the koala *(Phascolarctos cinereus)* immune response. *Australian journal of zoology*, ***62***(3), 195-199. https://doi.org/https://doi.org/10.1071/ZO13105

Morris, K. M., Mathew, M., Waugh, C., Ujvari, B., Timms, P., Polkinghorne, A., & Belov, K. (2015a). Identification, characterisation and expression analysis of natural killer receptor genes in Chlamydia pecorum infected koalas (Phascolarctos cinereus). *BMC Genomics*, ***16***, 1-11.

Morris, K. M., Mathew, M., Waugh, C., Ujvari, B., Timms, P., Polkinghorne, A., & Belov, K. (2015b). Identification, characterisation and expression analysis of natural killer receptor genes in Chlamydia pecorum infected koalas (Phascolarctos cinereus). *BMC Genomics*, ***16***, 796. https://doi.org/10.1186/s12864-015-2035-x

Morris, K. M., O'Meally, D., Zaw, T., Song, X., Gillett, A., Molloy, M. P., Polkinghorne, A., & Belov, K. (2016). Characterisation of the immune compounds in koala milk using a combined transcriptomic and proteomic approach. *Scientific reports*, ***6***(1), 35011.

Morrison, W. I., Wells, P. W., Moloo, S. K., Paris, J., & Murray, M. (1982). Interference in the establishment of superinfections with Trypanosoma congolense in cattle. *J Parasitol*, ***68***(5), 755-764.

Mounsey, K., Harvey, R. J., Wilkinson, V., Takano, K., Old, J., Stannard, H., Wicker, L., Phalen, D., & Carver, S. (2022). Drug dose and animal welfare: important considerations in the treatment of wildlife. *Parasitology research*, ***121***(3), 1065-1071.

Mukherjee, S., Huda, S., & Sinha Babu, S. P. (2019). Toll-like receptor polymorphism in host immune response to infectious diseases: A review. *Scandinavian Journal of Immunology*, ***90***(1), e12771. https://doi.org/https://doi.org/10.1111/sji.12771

Muramatsu, M. K., Brothwell, J. A., Stein, B. D., Putman, T. E., Rockey, D. D., & Nelson, D. E. (2016). Beyond tryptophan synthase: identification of genes that contribute to Chlamydia trachomatis survival during gamma interferon-induced persistence and reactivation. *Infection and immunity*, ***84***(10), 2791-2801.

Murthy, A. K., Li, W., Chaganty, B. K., Kamalakaran, S., Guentzel, M. N., Seshu, J., Forsthuber, T. G., Zhong, G., & Arulanandam, B. P. (2011). Tumor necrosis factor alpha production from CD8+ T cells mediates oviduct pathological sequelae following primary genital Chlamydia muridarum infection. *Infection and immunity*, ***79***(7), 2928-2935.

Mysterud, A., Viljugrein, H., Hopp, P., Andersen, R., Bakka, H., Benestad, S. L., Madslien, K., Moldal, T., Rauset, G. R., Strand, O., Tran, L., Vikøren, T., Våge, J., & Rolandsen, C. M. (2023). Challenges and opportunities using hunters to monitor chronic wasting disease among wild reindeer in the digital era. *Ecological Solutions and Evidence*, ***4***(1), e12203. https://doi.org/https://doi.org/10.1002/2688-8319.12203

Nagao, I., Nakazawa, M., & Ambrosini, Y. M. (2024). Three-dimensional morphogenesis in canine gut-on-a-chip using intestinal organoids derived from inflammatory bowel disease patients. *Journal of visualized experiments: JoVE*(204), 10.3791/65720.

Nagarajan, U. M., Sikes, J. D., Yeruva, L., & Prantner, D. (2012). Significant Role of IL-1 Signaling, but Limited Role of Inflammasome Activation, in Oviduct Pathology during Chlamydia muridarum Genital Infection. *The Journal of Immunology*, ***188***(6), 2866-2875. https://doi.org/10.4049/jimmunol.1103461

Nakanishi, K. (2018). Unique Action of Interleukin-18 on T Cells and Other Immune Cells [Review]. *Frontiers in Immunology*, ***9***. https://doi.org/10.3389/fimmu.2018.00763

NanoString Technologies Inc. (2009). *Technoical Note: Reference Genes for Normalization of Expression Data*. https://nanostring.com/wp-content/uploads/TN_Normalization_of_Expression_Data.pdf

NanoString Technologies Inc. (2017). *MAN-C0011-04: Gene Expression Data Analysis Guidelines*. https://nanostring.com/wp-content/uploads/Gene_Expression_Data_Analysis_Guidelines.pdf

Narayan, E., & Vanderneut, T. (2019). Physiological Stress in Rescued Wild Koalas Are Influenced by Habitat Demographics, Environmental Stressors, and Clinical Intervention [Original Research]. *Frontiers in Endocrinology*, ***10***. https://doi.org/10.3389/fendo.2019.00018

Narayan, E. J., Webster, K., Nicolson, V., Mucci, A., & Hero, J.-M. (2013). Non-invasive evaluation of physiological stress in an iconic Australian marsupial: the Koala (Phascolarctos cinereus). *General and Comparative Endocrinology*, ***187***, 39-47.

Narayan, E. J., & Williams, M. (2016). Understanding the dynamics of physiological impacts of environmental stressors on Australian marsupials, focus on the koala (Phascolarctos cinereus). *BMC zoology*, ***1***, 1-13.

Natividad, A., Hanchard, N., Holland, M. J., Mahdi, O. S., Diakite, M., Rockett, K., Jallow, O., Joof, H. M., Kwiatkowski, D. P., Mabey, D. C., & Bailey, R. L. (2007). Genetic variation at the TNF locus and the risk of severe sequelae of ocular Chlamydia trachomatis infection in Gambians. *Genes Immun*, ***8***(4), 288-295. https://doi.org/10.1038/sj.gene.6364384

Ndoricyimpaye, E. L., Van Snick, J., Robert, R., Bikorimana, E., Majyambere, O., Mukantwari, E., Nshimiyimana, T., Mbonigaba, V., Coutelier, J. P., & Rujeni, N. (2023). Cytokine Kinetics during Progression of COVID-19 in Rwanda Patients: Could IL-9/IFNγ Ratio Predict Disease Severity? *Int J Mol Sci*, ***24***(15). https://doi.org/10.3390/ijms241512272

Neaves, L. E., Frankham, G. J., Dennison, S., FitzGibbon, S., Flannagan, C., Gillett, A., Hynes, E., Handasyde, K., Helgen, K. M., Tsangaras, K., Greenwood, A. D., Eldridge, M. D. B., & Johnson, R. N. (2016). Phylogeography of the Koala, (Phascolarctos cinereus), and Harmonising Data to Inform Conservation. *PLoS One*, ***11***(9), e0162207. https://doi.org/10.1371/journal.pone.0162207

Negash, M., Girma, T., Chanyalew, M., Alemayehu, D. H., Alcantara, D., Davey, G., Boyton, R. J., Altmann, D. M., Newport, M. J., & Howe, R. (2024). Differences in Cytokine Expression at Baseline and in Response to Mineral Stimulation by Peripheral Blood Mononuclear Cells from Podoconiosis Cases and Healthy Control Individuals. *Tropical Medicine and Infectious Disease*, ***9***(11), 252. https://www.mdpi.com/2414-6366/9/11/252

Nehar-Belaid, D., Sokolowski, M., Ravichandran, S., Banchereau, J., Chaussabel, D., & Ucar, D. (2023). Baseline immune states (BIS) associated with vaccine responsiveness and factors that shape the BIS. *Seminars in Immunology*, ***70***, 101842. https://doi.org/https://doi.org/10.1016/j.smim.2023.101842

Nguyen, K. D., Fentress, S. J., Qiu, Y., Yun, K., Cox, J. S., & Chawla, A. (2013). Circadian gene Bmal1 regulates diurnal oscillations of Ly6Chi inflammatory monocytes. *Science*, ***341***(6153), 1483-1488.

Nicolaides, N. C., Charmandari, E., Chrousos, G. P., & Kino, T. (2014). Recent advances in the molecular mechanisms determining tissue sensitivity to glucocorticoids: novel mutations, circadian rhythm and ligand-induced repression of the human glucocorticoid receptor. *BMC Endocr Disord*, ***14***, 71. https://doi.org/10.1186/1472-6823-14-71

Nieters, A., Beckmann, L., Deeg, E., & Becker, N. (2006). Gene polymorphisms in Toll-like receptors, interleukin-10, and interleukin-10 receptor alpha and lymphoma risk. *Genes & Immunity*, ***7***(8), 615-624. https://doi.org/10.1038/sj.gene.6364337

Norian, R., Delirezh, N., & Azadmehr, A. (2015). Evaluation of proliferation and cytokines production by mitogen-stimulated bovine peripheral blood mononuclear cells. *Vet Res Forum*, ***6***(4), 265-271.

Norment, A. M., Salter, R. D., Parham, P., Engelhard, V. H., & Littman, D. R. (1988). Cell-cell adhesion mediated by CD8 and MHC class I molecules. *Nature*, ***336***(6194), 79-81.

NSW Government. (2023). *Code of Practice for Injured Sick and Orphaned Koalas*. (EHG20230250). Retrieved from https://www.environment.nsw.gov.au/research-and-publications/publications-search/code-of-practice-for-injured-sick-and-orphaned-koalas

Nyari, S., Booth, R., Quigley, B. L., Waugh, C. A., & Timms, P. (2019). Therapeutic effect of a Chlamydia pecorum recombinant major outer membrane protein vaccine on ocular disease in koalas (Phascolarctos cinereus). *PLoS One*, ***14***(1), e0210245.

Nyari, S., Khan, S. A., Rawlinson, G., Waugh, C. A., Potter, A., Gerdts, V., & Timms, P. (2018). Vaccination of koalas (Phascolarctos cinereus) against Chlamydia pecorum using synthetic peptides derived from the major outer membrane protein. *PLoS One*, ***13***(6), e0200112.

Nyari, S., Waugh, C. A., Dong, J., Quigley, B. L., Hanger, J., Loader, J., Polkinghorne, A., & Timms, P. (2017). Epidemiology of chlamydial infection and disease in a free-ranging koala (Phascolarctos cinereus) population. *PLoS One*, ***12***(12), e0190114.

Obendorf, D. L. (1983). Causes of mortality and morbidity of wild koalas, *Phascolarctos cinereus (Goldfuss),* in Victoria, Australia. *Journal of Wildlife Diseases*, ***19***(2), 123-131. https://doi.org/10.7589/0090-3558-19.2.123

Oberholzer, A., Souza, S. M., Tschoeke, S. K., Oberholzer, C., Abouhamze, A., Pribble, J. P., & Moldawer, L. L. (2005). Plasma cytokine measurements augment prognostic scores as indicators of outcome in patients with severe sepsis. *Shock*, ***23***(6), 488-493. https://doi.org/10.1097/01.shk.0000163802.46355.59

Oelmann, E., Stein, H., Berdel, W. E., & Herbst, H. (2015). Expression of Interleukin-1 and Interleukin-1 Receptors Type 1 and Type 2 in Hodgkin Lymphoma. *PLoS One*, ***10***(9), e0138747. https://doi.org/10.1371/journal.pone.0138747

Ohmer, M. E. B., Costantini, D., Czirják, G. Á., Downs, C. J., Ferguson, L. V., Flies, A., Franklin, C. E., Kayigwe, A. N., Knutie, S., Richards-Zawacki, C. L., & Cramp, R. L. (2021). Applied ecoimmunology: using immunological tools to improve conservation efforts in a changing world. *Conservation Physiology*, ***9***(1). https://doi.org/10.1093/conphys/coab074

OIE. (2024). Terrestrial Animal Health Code. In. Paris: World Organisation for Animal Health.

Oishi, Y., Hayashi, S., Isagawa, T., Oshima, M., Iwama, A., Shimba, S., Okamura, H., & Manabe, I. (2017). Bmal1 regulates inflammatory responses in macrophages by modulating enhancer RNA transcription. *Scientific reports*, ***7***(1), 7086.

Olagoke, O., Miller, D., Hemmatzadeh, F., Stephenson, T., Fabijan, J., Hutt, P., Finch, S., Speight, N., & Timms, P. (2018). Induction of neutralizing antibody response against koala retrovirus (KoRV) and reduction in viral load in koalas following vaccination with recombinant KoRV envelope protein. *npj Vaccines*, ***3***, 30. https://doi.org/10.1038/s41541-018-0066-4

Olagoke, O., Quigley, B. L., Eiden, M. V., & Timms, P. (2019). Antibody response against koala retrovirus (KoRV) in koalas harboring KoRV-A in the presence or absence of KoRV-B. *Sci Rep*, ***9***(1), 12416.

Olagoke, O., Quigley, B. L., Hemmatzadeh, F., Tzipori, G., & Timms, P. (2020a). Therapeutic vaccination of koalas harbouring endogenous koala retrovirus (KoRV) improves antibody responses and reduces circulating viral load. *npj Vaccines*, ***5***(1), 60.

Olagoke, O., Quigley, B. L., & Timms, P. (2020b). Koalas vaccinated against Koala retrovirus respond by producing increased levels of interferon-gamma. *J Virol*, ***17***(1), 168.

Old, J. M. (2016). Haematopoiesis in Marsupials. *Developmental & Comparative Immunology*, ***58***, 40-46. https://doi.org/https://doi.org/10.1016/j.dci.2015.11.009
Oliveira, N. M., Farrell, K. B., & Eiden, M. V. (2006). In vitro characterization of a koala retrovirus. *Journal of Virology*, ***80***(6), 3104-3107.
Oliveira, N. M., Satija, H., Kouwenhoven, I. A., & Eiden, M. V. (2007). Changes in viral protein function that accompany retroviral endogenization. *Proc Natl Acad Sci U S A*, ***104***(44), 17506-17511. https://doi.org/10.1073/pnas.0704313104
Omosun, Y., McKeithen, D., Ryans, K., Kibakaya, C., Blas-Machado, U., Li, D., Singh, R., Inoue, K., Xiong, Z.-G., & Eko, F. (2015). Interleukin-10 modulates antigen presentation by dendritic cells through regulation of NLRP3 inflammasome assembly during Chlamydia infection. *Infection and immunity*, ***83***(12), 4662-4672.
Organization, W. H. (2000). Health impact assessment: main concepts and suggested aproach-Gothenburg consensus paper. https://www.healthedpartners.org/ceu/hia/hia01/01_02_gothenburg_paper_on_hia_1999.pdf
Ortiz-Baez, A. S., Cousins, K., Eden, J.-S., Chang, W.-S., Harvey, E., Pettersson, J. H. O., Carver, S., Polkinghorne, A., Šlapeta, J., Rose, K., & Holmes, E. C. (2020). Meta-transcriptomic identification of Trypanosoma spp. in native wildlife species from Australia. *Parasites & Vectors*, ***13***(1), 447. https://doi.org/10.1186/s13071-020-04325-6
Ouellette, S. P., Hatch, T. P., AbdelRahman, Y. M., Rose, L. A., Belland, R. J., & Byrne, G. I. (2006). Global transcriptional upregulation in the absence of increased translation in Chlamydia during IFNγ-mediated host cell tryptophan starvation. *Molecular microbiology*, ***62***(5), 1387-1401.
Pagliarani, S., Johnston, S. D., Beagley, K. W., Hulse, L., & Palmieri, C. (2022). Chlamydiosis and cystic dilatation of the ovarian bursa in the female koala (Phascolarctos cinereus): Novel insights into the pathogenesis and mechanisms of formation. *Theriogenology*, ***189***, 280-289. https://doi.org/10.1016/j.theriogenology.2022.06.022
Pagliarani, S., Johnston, S. D., Beagley, K. W., & Palmieri, C. (2024). Immunohistochemical characterization of the immune cell response during chlamydial infection in the male and female koala (Phascolarctos cinereus) reproductive tract. *Veterinary Pathology*, ***61***(4), 621-632. https://doi.org/10.1177/03009858231225499
Palmieri, C., Hulse, L., Pagliarani, S., Larkin, R., Higgins, D. P., Beagley, K., & Johnston, S. (2019). Chlamydia pecorum Infection in the Male Reproductive System of Koalas (Phascolarctos cinereus). *Vet Pathol*, ***56***(2), 300-306. https://doi.org/10.1177/0300985818806963
Panzetta, M. E., Valdivia, R. H., & Saka, H. A. (2018). Chlamydia Persistence: A Survival Strategy to Evade Antimicrobial Effects in-vitro and in-vivo. *Front Microbiol*, ***9***, 3101. https://doi.org/10.3389/fmicb.2018.03101
Park, J., Ke, W., Kaage, A., Feigin, C. Y., Griffing, A. H., Pritykin, Y., Donia, M. S., & Mallarino, R. (2025). Cathelicidin antimicrobial peptides mediate immune protection in marsupial neonates. *Science Advances*, ***11***(16), eads6359. https://doi.org/doi:10.1126/sciadv.ads6359
Parkkinen, V.-P., Wallmann, C., Wilde, M., Clarke, B., Illari, P., Kelly, M. P., Norell, C., Russo, F., Shaw, B., & Williamson, J. (2018). *Evaluating evidence of mechanisms in medicine: principles and procedures*. Springer Nature.

Patil, I. (2021). Visualizations with statistical details: The 'ggstatsplot' approach. *Journal of Open Source Software*, ***6***(61).

Patterson, J. L., Lynch, M., Anderson, G. A., Noormohammadi, A. H., Legione, A., Gilkerson, J. R., & Devlin, J. M. (2015). The prevalence and clinical significance of Chlamydia infection in island and mainland populations of Victorian koalas (Phascolarctos cinereus). *J Wildl Dis*, ***51***(2), 309-317.

Peacock, S. J., Mavrot, F., Tomaselli, M., Hanke, A., Fenton, H., Nathoo, R., Aleuy, O. A., Francesco, J. D., Aguilar, X. F., Jutha, N., Kafle, P., Mosbacher, J., Goose, A., Hunters, E., Organization, T., Association, K. A., Hunters, O., Committee, T., & Kutz, S. J. (2020). Linking co-monitoring to co-management: bringing together local, traditional, and scientific knowledge in a wildlife status assessment framework. *Arctic Science*, ***6***(3), 247-266. https://doi.org/10.1139/as-2019-0019

Peduzzi, P., Concato, J., Kemper, E., Holford, T. R., & Feinstein, A. R. (1996). A simulation study of the number of events per variable in logistic regression analysis. *Journal of Clinical Epidemiology*, ***49***(12), 1373-1379. https://doi.org/https://doi.org/10.1016/S0895-4356(96)00236-3

Peel, E., Cheng, Y., Djordjevic, J. T., O'Meally, D., Thomas, M., Kuhn, M., Sorrell, T. C., Huston, W. M., & Belov, K. (2021). Koala cathelicidin PhciCath5 has antimicrobial activity, including against Chlamydia pecorum. *PLoS One*, ***16***(4), e0249658. https://doi.org/10.1371/journal.pone.0249658

Peel, E., Gonsalvez, A., Hogg, C. J., & Belov, K. (2025). Marsupial cathelicidins: characterization, antimicrobial activity and evolution in this unique mammalian lineage [Original Research]. *Frontiers in Immunology*, ***Volume 16 - 2025***. https://doi.org/10.3389/fimmu.2025.1524092

Peel, E., Hogg, C., & Belov, K. (2024). Characterisation of defensins across the marsupial family tree. *Developmental & Comparative Immunology*, ***158***, 105207. https://doi.org/https://doi.org/10.1016/j.dci.2024.105207

Pei, H., Li, L., Fridley, B. L., Jenkins, G. D., Kalari, K. R., Lingle, W., Petersen, G., Lou, Z., & Wang, L. (2009). FKBP51 affects cancer cell response to chemotherapy by negatively regulating Akt. *Cancer cell*, ***16***(3), 259-266.

Perea, L., Cantó, E., Suarez-Cuartin, G., Aliberti, S., Chalmers, J. D., Sibila, O., & Vidal, S. (2021). A cluster analysis of bronchiectasis patients based on the airway immune profile. *Chest*, ***159***(5), 1758-1767.

Pérez-Soto, E., Fernández-Martínez, E., Oros-Pantoja, R., Medel-Flores, O., Miranda-Covarrubias, J. C., & Sánchez-Monroy, V. (2021). Proinflammatory and Oxidative Stress States Induced by Human Papillomavirus and Chlamydia trachomatis Coinfection Affect Sperm Quality in Asymptomatic Infertile Men. *Medicina (Kaunas)*, ***57***(9). https://doi.org/10.3390/medicina57090862

Perrin, A., Khimoun, A., Faivre, B., Ollivier, A., de Pracontal, N., Théron, F., Loubon, M., Leblond, G., Duron, O., & Garnier, S. (2021). Habitat fragmentation differentially shapes neutral and immune gene variation in a tropical bird species. *Heredity*, ***126***(1), 148-162. https://doi.org/10.1038/s41437-020-00366-w

Perucca, A., Llonín, A. G., Benach, O. M., Hallopeau, C., Rivas, E. I., Linares, J., Garrido, M., Sallent-Aragay, A., Golde, T., Colombelli, J., Dalaka, E., Linacero, J., Cazorla, M., Galan, T., Pastor Viel, J., Badenas, X., Recort-Bascuas, A., Comerma, L., Fernandez-Nogueira, P., . . . Labernadie, A. (2025). Micro Immune Response On-chip (MIRO)

models the tumour-stroma interface for immunotherapy testing. *Nature communications*, ***16***(1), 1279. https://doi.org/10.1038/s41467-025-56275-1

Pescarmona, R., Belot, A., Villard, M., Besson, L., Lopez, J., Mosnier, I., Mathieu, A.-L., Lombard, C., Garnier, L., & Frachette, C. (2019). Comparison of RT-qPCR and Nanostring in the measurement of blood interferon response for the diagnosis of type I interferonopathies. *Cytokine*, ***113***, 446-452.

Pestka, S., Krause, C. D., Sarkar, D., Walter, M. R., Shi, Y., & Fisher, P. B. (2004). Interleukin-10 and related cytokines and receptors. *Annu. Rev. Immunol.*, ***22***(1), 929-979.

Peters, A., Carver, S., Skerratt, L. F., Meredith, A., & Woods, R. (2019a). A Solutions-Focused Translational Research Framework for Wildlife Health. *BioScience*, ***69***(12), 1019-1027. https://doi.org/10.1093/biosci/biz125

Peters, A., Delhey, K., Nakagawa, S., Aulsebrook, A., & Verhulst, S. (2019b). Immunosenescence in wild animals: meta-analysis and outlook. *Ecology Letters*, ***22***(10), 1709-1722. https://doi.org/https://doi.org/10.1111/ele.13343

Pfenning-Butterworth, A., Buckley, L. B., Drake, J. M., Farner, J. E., Farrell, M. J., Gehman, A.-L. M., Mordecai, E. A., Stephens, P. R., Gittleman, J. L., & Davies, T. J. (2024). Interconnecting global threats: climate change, biodiversity loss, and infectious diseases. *The Lancet Planetary Health*, ***8***(4), e270-e283. https://doi.org/https://doi.org/10.1016/S2542-5196(24)00021-4

Phillips, S., Hanger, J., Grosmaire, J., Mehdi, A., Jelocnik, M., Wong, J., & Timms, P. (2024a). Immunisation of koalas against Chlamydia pecorum results in significant protection against chlamydial disease and mortality. *npj Vaccines*, ***9***(1), 139. https://doi.org/10.1038/s41541-024-00938-5

Phillips, S., O'Meally, D., Bommana, S., Jelocnik, M., Quigley, B. L., & Timms, P. (2024b). Koala ocular disease grades are defined by chlamydial load changes and increases in Th2 immune responses [Original Research]. *Frontiers in Cellular and Infection Microbiology*, ***14***. https://doi.org/10.3389/fcimb.2024.1447119

Phillips, S., Quigley, B. L., Aziz, A., Bergen, W., Booth, R., Pyne, M., & Timms, P. (2019). Antibiotic treatment of Chlamydia-induced cystitis in the koala is linked to expression of key inflammatory genes in reactive oxygen pathways. *PLoS One*, ***14***(8), e0221109. https://doi.org/10.1371/journal.pone.0221109

Phillips, S., Quigley, B. L., Olagoke, O., Booth, R., Pyne, M., & Timms, P. (2020). Vaccination of koalas during antibiotic treatment for Chlamydia-induced cystitis induces an improved antibody response to Chlamydia pecorum. *Scientific reports*, ***10***(1), 10152.

Phillips, S., Robbins, A., Loader, J., Hanger, J., Booth, R., Jelocnik, M., Polkinghorne, A., & Timms, P. (2018). Chlamydia pecorum gastrointestinal tract infection associations with urogenital tract infections in the koala (Phascolarctos cinereus). *PLoS One*, ***13***(11), e0206471. https://doi.org/10.1371/journal.pone.0206471

Phillips, S., Timms, P., & Jelocnik, M. (2021). Is Chlamydia to Blame for Koala Reproductive Cysts? *Pathogens*, ***10***(9). https://doi.org/10.3390/pathogens10091140

Pina, A., Macedo, M. P., & Henriques, R. (2020). Clustering clinical data in R. *Mass Spectrometry Data Analysis in Proteomics*, 309-343.

Plank, M. J., Hendy, S. C., Binny, R. N., Vattiato, G., Lustig, A., & Maclaren, O. J. (2022). Using mechanistic model-based inference to understand and project epidemic dynamics with time-varying contact and vaccination rates. *Scientific reports*, ***12***(1), 20451. https://doi.org/10.1038/s41598-022-25018-3

Plumb, G., Babiuk, L., Mazet, J., Olsen, S., Rupprecht, C., Pastoret, P.-P., & Slate, D. (2007). Vaccination in conservation medicine. *Revue scientifique et technique (International Office of Epizootics)*, ***26***(1), 229-241.

Polkinghorne, A., Hanger, J., & Timms, P. (2013). Recent advances in understanding the biology, epidemiology and control of chlamydial infections in koalas. *Vet Microbiol*, ***165***(3-4), 214-223.

Porcella, S. F., Carlson, J. H., Sturdevant, D. E., Sturdevant, G. L., Kanakabandi, K., Virtaneva, K., Wilder, H., Whitmire, W. M., Song, L., & Caldwell, H. D. (2015). Transcriptional profiling of human epithelial cells infected with plasmid-bearing and plasmid-deficient Chlamydia trachomatis. *Infection and immunity*, ***83***(2), 534-543.

Porta, M. S., Greenland, S., Hernán, M., dos Santos Silva, I., & Last, J. M. (2014). *A dictionary of epidemiology*. Oxford university press.

Poudel, A., Elsasser, T. H., Rahman, K. S., Chowdhury, E. U., & Kaltenboeck, B. (2012). Asymptomatic endemic Chlamydia pecorum infections reduce growth rates in calves by up to 48 percent.

Prager, K. C., Buhnerkempe, M. G., Greig, D. J., Orr, A. J., Jensen, E. D., Gomez, F., Galloway, R. L., Wu, Q., Gulland, F. M. D., & Lloyd-Smith, J. O. (2020). Linking longitudinal and cross-sectional biomarker data to understand host-pathogen dynamics: Leptospira in California sea lions (Zalophus californianus) as a case study. *PLOS Neglected Tropical Diseases*, ***14***(6), e0008407. https://doi.org/10.1371/journal.pntd.0008407

Prantner, D., Darville, T., Sikes, J. D., Andrews Jr, C. W., Brade, H., Rank, R. G., & Nagarajan, U. M. (2009). Critical role for interleukin-1β (IL-1β) during Chlamydia muridarum genital infection and bacterial replication-independent secretion of IL-1β in mouse macrophages. *Infection and immunity*, ***77***(12), 5334-5346.

Premachandra, H., Piza-Roca, C., Casteriano, A., Higgins, D. P., Hohwieler, K., Powell, D., & Cristescu, R. H. (2024). Advancements in noninvasive koala monitoring through combining Chlamydia detection with a targeted koala genotyping assay. *Scientific reports*, ***14***(1), 1-8.

Premachandre, C. K., Quah, P. S., Tran, B. M., Vincan, E., Deliyannis, G., Wong, C. Y., Diaz-Méndez, A., Jackson, D. C., Reading, P. C., & Browning, G. F. (2025). Bovine tracheal organoids for studying Mycoplasma bovis respiratory infections. *Veterinary microbiology*, ***300***, 110340.

Prentice, J. C., Fox, N. J., Hutchings, M. R., White, P. C., Davidson, R. S., & Marion, G. (2019). When to kill a cull: factors affecting the success of culling wildlife for disease control. *Journal of the royal society interface*, ***16***(152), 20180901.

Prentice, J. C., Marion, G., White, P. C., Davidson, R. S., & Hutchings, M. R. (2014). Demographic processes drive increases in wildlife disease following population reduction. *PLoS One*, ***9***(5), e86563.

Prusty, B. K., Böhme, L., Bergmann, B., Siegl, C., Krause, E., Mehlitz, A., & Rudel, T. (2012). Imbalanced oxidative stress causes chlamydial persistence during non-productive human herpes virus co-infection. *PLoS One*, ***7***(10), e47427. https://doi.org/10.1371/journal.pone.0047427

Prusty, B. K., Siegl, C., Hauck, P., Hain, J., Korhonen, S. J., Hiltunen-Back, E., Puolakkainen, M., & Rudel, T. (2013). Chlamydia trachomatis infection induces replication of latent HHV-6. *PLoS One*, ***8***(4), e61400. https://doi.org/10.1371/journal.pone.0061400

Pye, G. W., Johnson, R. N., & Greenwood, A. D. (2014). The Koala and its Retroviruses: Implications for Sustainability and Survival [Online]. *Tech Rep Aust Mus*, ***24***, 1-105. https://doi.org/10.3853/j.1835-4211.24.2014.1629

Quatrini, L., Ricci, B., Ciancaglini, C., Tumino, N., & Moretta, L. (2021). Regulation of the Immune System Development by Glucocorticoids and Sex Hormones [Mini Review]. *Frontiers in Immunology*, ***Volume 12 - 2021***. https://doi.org/10.3389/fimmu.2021.672853

Queensland Government. (2021). *KoalaBase* https://www.koalabase.com.au

Quigley, B. L., Carver, S., Hanger, J., Vidgen, M. E., & Timms, P. (2018a). The relative contribution of causal factors in the transition from infection to clinical chlamydial disease. *Sci Rep*, ***8***(1), 8893. https://doi.org/10.1038/s41598-018-27253-z

Quigley, B. L., Ong, V. A., Hanger, J., & Timms, P. (2018b). Molecular dynamics and mode of transmission of koala retrovirus as it invades and spreads through a wild Queensland koala population. *J Virol*, ***92***(5), 10.1128/jvi. 01871-01817.

Quigley, B. L., Phillips, S., Olagoke, O., Robbins, A., Hanger, J., & Timms, P. (2019). Changes in endogenous and exogenous koala retrovirus subtype expression over time reflect koala health outcomes. *J Virol*, ***93***(18), 10.1128/jvi. 00849-00819.

Quigley, B. L., & Timms, P. (2020). Helping koalas battle disease–Recent advances in Chlamydia and koala retrovirus (KoRV) disease understanding and treatment in koalas. *FEMS Microbiol Rev*, ***44***(5), 583-605.

Quigley, B. L., & Timms, P. (2021). The koala immune response to chlamydial infection and vaccine development—advancing our immunological understanding. *Animals*, ***11***(2), 380.

Quigley, B. L., Timms, P., Nyari, S., McKay, P., Hanger, J., & Phillips, S. (2023). Reduction of Chlamydia pecorum and Koala Retrovirus subtype B expression in wild koalas vaccinated with novel peptide and peptide/recombinant protein formulations. *Vaccine: X*, ***14***, 100329. https://doi.org/https://doi.org/10.1016/j.jvacx.2023.100329

Quigley, B. L., Tzipori, G., Nilsson, K., & Timms, P. (2020). High-throughput immunogenetic typing of koalas suggests possible link between MHC alleles and cancers. *Immunogenetics*, ***72***(9), 499-506.

R Development Core Team. (2024). *R: A language and environment for statistical computing.* In R Foundation for Statistical Computing.

Raimondi, S., Candeliere, F., Amaretti, A., Foschi, C., Morselli, S., Gaspari, V., Rossi, M., & Marangoni, A. (2021). Vaginal and Anal Microbiome during Chlamydia trachomatis Infections. *Pathogens*, ***10***(10), 1347. https://www.mdpi.com/2076-0817/10/10/1347

Rajaram, K., & Nelson David, E. (2015). Chlamydia muridarum Infection of Macrophages Elicits Bactericidal Nitric Oxide Production via Reactive Oxygen Species and Cathepsin B. *Infection and immunity*, ***83***(8), 3164-3175. https://doi.org/10.1128/iai.00382-15

Rajeeve, K., Vollmuth, N., Janaki-Raman, S., Wulff, T. F., Baluapuri, A., Dejure, F. R., Huber, C., Fink, J., Schmalhofer, M., & Schmitz, W. (2020). Reprogramming of host glutamine metabolism during Chlamydia trachomatis infection and its key role in peptidoglycan synthesis. *Nature microbiology*, ***5***(11), 1390-1402.

Rangel, S. C., da Silva, M. D., da Silva, A. L., Dos Santos, J. M. B., Neves, L. M., Pedrosa, A., Rodrigues, F. M., Trettel, C. D. S., Furtado, G. E., de Barros, M. P., Bachi, A. L. L., Romano, C. M., & Nali, L. (2022). Human endogenous retroviruses and the

inflammatory response: A vicious circle associated with health and illness. *Front Immunol*, ***13***, 1057791. https://doi.org/10.3389/fimmu.2022.1057791

Rank, R. G., Lacy, H. M., Goodwin, A., Sikes, J., Whittimore, J., Wyrick, P. B., & Nagarajan, U. M. (2010). Host Chemokine and Cytokine Response in the Endocervix within the First Developmental Cycle of *Chlamydia muridarum*. *Infection and immunity*, ***78***(1), 536-544. https://doi.org/doi:10.1128/iai.00772-09

Rascón-García, K., Martínez-López, B., Cecchi, G., Scoglio, C., Matovu, E., & Muhanguzi, D. (2023). Prevalence of African animal trypanosomiasis among livestock and domestic animals in Uganda: a systematic review and meta-regression analysis from 1980 to 2022. *Scientific reports*, ***13***(1), 20337. https://doi.org/10.1038/s41598-023-47141-5

Ravindra, N. G., Alfajaro, M. M., Gasque, V., Huston, N. C., Wan, H., Szigeti-Buck, K., Yasumoto, Y., Greaney, A. M., Habet, V., Chow, R. D., Chen, J. S., Wei, J., Filler, R. B., Wang, B., Wang, G., Niklason, L. E., Montgomery, R. R., Eisenbarth, S. C., Chen, S., . . . Wilen, C. B. (2021). Single-cell longitudinal analysis of SARS-CoV-2 infection in human airway epithelium identifies target cells, alterations in gene expression, and cell state changes. *PLOS Biology*, ***19***(3), e3001143. https://doi.org/10.1371/journal.pbio.3001143

Rawre, J., Dhawan, B., Khanna, N., Sreenivas, V., Broor, S., & Chaudhry, R. (2019). Distribution of Chlamydia trachomatis ompA genotypes in patients attending a sexually transmitted disease outpatient clinic in New Delhi, India. *Indian Journal of Medical Research*, ***149***(5), 662-670.

Ray, P., Reddy, S. S., & Banerjee, T. (2021). Various dimension reduction techniques for high dimensional data analysis: a review. *Artificial Intelligence Review*, ***54***(5), 3473-3515. https://doi.org/10.1007/s10462-020-09928-0

Read, A. F., Baigent, S. J., Powers, C., Kgosana, L. B., Blackwell, L., Smith, L. P., Kennedy, D. A., Walkden-Brown, S. W., & Nair, V. K. (2015). Imperfect vaccination can enhance the transmission of highly virulent pathogens. *PLOS Biology*, ***13***(7), e1002198.

Reddehase, M. J., Simon, C. O., Seckert, C. K., Lemmermann, N., & Grzimek, N. K. A. (2008). Murine Model of Cytomegalovirus Latency and Reactivation. In T. E. Shenk & M. F. Stinski (Eds.), *Human Cytomegalovirus* (pp. 315-331). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-540-77349-8_18

Redgrove, K. A., & McLaughlin, E. A. (2014). The Role of the Immune Response in Chlamydia trachomatis Infection of the Male Genital Tract: A Double-Edged Sword [Review]. *Frontiers in Immunology*, ***5***. https://doi.org/10.3389/fimmu.2014.00534

Reiff, D. D., & Cron, R. Q. (2021). Performance of Cytokine Storm Syndrome Scoring Systems in Pediatric COVID-19 and Multisystem Inflammatory Syndrome in Children. *ACR Open Rheumatology*, ***3***(12), 820-826. https://doi.org/https://doi.org/10.1002/acr2.11331

Reinhold, P., Jaeger, J., Liebler-Tenorio, E., Berndt, A., Bachmann, R., Schubert, E., Melzer, F., Elschner, M., & Sachse, K. (2008). Impact of latent infections with Chlamydophila species in young cattle. *The veterinary journal*, ***175***(2), 202-211.

Rhodes, J. R., Beyer, H., Preece, H., & McAlpine, C. (2015). *South East Queensland Koala Population Modelling Study*.

Rhodes, J. R., Ng, C. F., de Villiers, D. L., Preece, H. J., McAlpine, C. A., & Possingham, H. P. (2011). Using integrated population modelling to quantify the implications of multiple threatening processes for a rapidly declining population. *Biol Conserv*, ***144***(3), 1081-1088.

Ribas, M. P., García-Ulloa, M., Espunyes, J., & Cabezón, O. (2023). Improving the assessment of ecosystem and wildlife health: microbiome as an early indicator. *Current Opinion in Biotechnology*, ***81***, 102923. https://doi.org/https://doi.org/10.1016/j.copbio.2023.102923

Rixon, J. A., Depew, C. E., & McSorley, S. J. (2022). Th1 cells are dispensable for primary clearance of Chlamydia from the female reproductive tract of mice. *PLOS Pathogens*, ***18***(2), e1010333. https://doi.org/10.1371/journal.ppat.1010333

Robbins, A. (2020). *Chlamydial epidemiology and therapeutics in south east Queensland koalas* University of the Sunshine Coast, Queensland].

Robbins, A., Hanger, J., Jelocnik, M., Quigley, B. L., & Timms, P. (2019). Longitudinal study of wild koalas (Phascolarctos cinereus) reveals chlamydial disease progression in two thirds of infected animals. *Sci Rep*, ***9***(1), 13194.

Robbins, A., Hanger, J., Jelocnik, M., Quigley, B. L., & Timms, P. (2020). Koala immunogenetics and chlamydial strain type are more directly involved in chlamydial disease progression in koalas from two south east Queensland koala populations than koala retrovirus subtypes. *Sci Rep*, ***10***(1), 15013.

Robbins, A., Loader, J., Timms, P., & Hanger, J. (2018). Optimising the short and long-term clinical outcomes for koalas (Phascolarctos cinereus) during treatment for chlamydial infection and disease. *PLoS One*, ***13***(12), e0209679.

Robinson, G. A., Peng, J., Dönnes, P., Coelewij, L., Naja, M., Radziszewska, A., Wincup, C., Peckham, H., Isenberg, D. A., & Ioannou, Y. (2020). Disease-associated and patient-specific immune cell signatures in juvenile-onset systemic lupus erythematosus: patient stratification using a machine-learning approach. *The Lancet Rheumatology*, ***2***(8), e485-e496.

Rooney, B. V., Crucian, B. E., Pierson, D. L., Laudenslager, M. L., & Mehta, S. K. (2019). Herpes virus reactivation in astronauts during spaceflight and its application on earth. *Frontiers in Microbiology*, ***10***, 432964.

Ruiz-Ojeda, F. J., Olza, J., Gil, A., & Aguilera, C. M. (2018). Oxidative Stress and Inflammation in Obesity and Metabolic Syndrome. In A. M. d. M. a. C. M. A. García (Ed.), *Obesity* (pp. 1-15). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-812504-5.00001-5

Ruiz-Rodriguez, C. T., Ishida, Y., Murray, N. D., O'Brien, S. J., Graves, J. A., Greenwood, A. D., & Roca, A. L. (2016). Koalas (Phascolarctos cinereus) from Queensland are genetically distinct from 2 populations in Victoria. *Journal of Heredity*, ***107***(7), 573-580.

Rusinova, I., Forster, S., Yu, S., Kannan, A., Masse, M., Cumming, H., Chapman, R., & Hertzog, P. J. (2012). Interferome v2. 0: an updated database of annotated interferon-regulated genes. *Nucleic acids research*, ***41***(D1), D1040-D1046.

Russ, E., & Iordanskiy, S. (2023). Endogenous Retroviruses as Modulators of Innate Immunity. *Pathogens*, ***12***(2), 162. https://www.mdpi.com/2076-0817/12/2/162

Ryan, M. T., Martinez, C., Jahns, H., Mooney, C. T., Browne, J. A., O'Neill, E. J., & Shiel, R. E. (2023). The comparative performance of a custom Canine NanoString® panel on FFPE and snap frozen liver biopsies. *Research in Veterinary Science*, ***159***, 225-231. https://doi.org/https://doi.org/10.1016/j.rvsc.2023.04.023

Rycaj, K., Plummer, J. B., Yin, B., Li, M., Garza, J., Radvanyi, L., Ramondetta, L. M., Lin, K., Johanning, G. L., Tang, D. G., & Wang-Johanning, F. (2015). Cytotoxicity of Human Endogenous Retrovirus K–Specific T Cells toward Autologous Ovarian Cancer Cells.

Clinical Cancer Research, ***21***(2), 471-483. https://doi.org/10.1158/1078-0432.Ccr-14-0388

Sachse, K., & Borel, N. (2020). Recent advances in epidemiology, pathology and immunology of veterinary Chlamydiae. *Chlamydia Biology: From Genome to Disease*, 403-428.

Saeidi, A., Chong, Y. K., Yong, Y. K., Tan, H. Y., Barathan, M., Rajarajeswaran, J., Sabet, N. S., Sekaran, S. D., Ponnampalavanar, S., Che, K. F., Velu, V., Kamarulzaman, A., Larsson, M., & Shankar, E. M. (2015). Concurrent loss of co-stimulatory molecules and functional cytokine secretion attributes leads to proliferative senescence of CD8(+) T cells in HIV/TB co-infection. *Cell Immunol*, ***297***(1), 19-32. https://doi.org/10.1016/j.cellimm.2015.05.005

Sagerström, C. G., Kerr, E. M., Allison, J. P., & Davis, M. M. (1993). Activation and differentiation requirements of primary T cells in vitro. *Proceedings of the National Academy of Sciences*, ***90***(19), 8987-8991. https://doi.org/doi:10.1073/pnas.90.19.8987

Saidulu, D., & Sasikala, R. (2017). Machine learning and statistical approaches for big data: issues, challenges and research directions. *International Journal of Applied Engineering Research*, ***12***(21), 11691-11699.

Sait, M., Livingstone, M., Clark, E. M., Wheelhouse, N., Spalding, L., Markey, B., Magnino, S., Lainson, F. A., Myers, G. S. A., & Longbottom, D. (2014). Genome sequencing and comparative analysis of three Chlamydia pecorum strains associated with different pathogenic outcomes. *BMC Genomics*, ***15***(1), 23. https://doi.org/10.1186/1471-2164-15-23

Sakata, N., Yoshimatsu, G., Kawakami, R., Nakano, K., Yamada, T., Yamamura, A., Nagashima, H., & Kodama, S. (2024). The porcine islet-derived organoid showed the characteristics as pancreatic duct. *Scientific reports*, ***14***(1), 6401.

Sanches-Vaz, M., Temporão, A., Luis, R., Nunes-Cabaço, H., Mendes, A. M., Goellner, S., Carvalho, T., Figueiredo, L. M., & Prudêncio, M. (2019). Trypanosoma brucei infection protects mice against malaria. *PLoS Pathog*, ***15***(11), e1008145. https://doi.org/10.1371/journal.ppat.1008145

Santamaria, F., Barlow, C. K., Schlagloth, R., Schittenhelm, R. B., Palme, R., & Henning, J. (2021a). Identification of koala (Phascolarctos cinereus) faecal cortisol metabolites using liquid chromatography-mass spectrometry and enzyme immunoassays. *Metabolites*, ***11***(6), 393.

Santamaria, F., Palme, R., Schlagloth, R., Klobetz-Rassam, E., & Henning, J. (2021b). Seasonal Variations of Faecal Cortisol Metabolites in Koalas in South East Queensland. *Animals*, ***11***(6), 1622. https://www.mdpi.com/2076-2615/11/6/1622

Santamaria, F., Schlagloth, R., Valenza, L., Palme, R., de Villiers, D., & Henning, J. (2023). The Effect of Disease and Injury on Faecal Cortisol Metabolites, as an Indicator of Stress in Wild Hospitalised Koalas, Endangered Australian Marsupials. *Vet Sci*, ***10***(1). https://doi.org/10.3390/vetsci10010065

Santos, F. A. A. d., Monteiro, M., Pinto, A., Carvalho, C. L., Peleteiro, M. C., Carvalho, P., Mendonça, P., Carvalho, T., & Duarte, M. D. (2020). First description of a herpesvirus infection in genus Lepus. *PLoS One*, ***15***(4), e0231795. https://doi.org/10.1371/journal.pone.0231795

Sarker, N., Fabijan, J., Emes, R. D., Hemmatzadeh, F., Meers, J., Moreton, J., Owen, H., Seddon, J. M., Simmons, G., Speight, N., Trott, D., Woolford, L., & Tarlinton, R. E.

(2018). Identification of stable reference genes for quantitative PCR in koalas. *Sci Rep*, ***8***(1), 3364. https://doi.org/10.1038/s41598-018-21723-0

Sarker, N., Fabijan, J., Owen, H., Seddon, J., Simmons, G., Speight, N., Kaler, J., Woolford, L., Emes, R. D., Hemmatzadeh, F., Trott, D. J., Meers, J., & Tarlinton, R. E. (2020a). Koala retrovirus viral load and disease burden in distinct northern and southern koala populations. *Sci Rep*, ***10***(1), 263. https://doi.org/10.1038/s41598-019-56546-0

Sarker, N., Fabijan, J., Seddon, J., Tarlinton, R., Owen, H., Simmons, G., Thia, J., Blanchard, A. M., Speight, N., Kaler, J., Emes, R. D., Woolford, L., Trott, D., Hemmatzadeh, F., & Meers, J. (2019). Genetic diversity of Koala retrovirus env gene subtypes: insights into northern and southern koala populations. *J Gen Virol*, ***100***(9), 1328-1339. https://doi.org/10.1099/jgv.0.001304

Sarker, N., Tarlinton, R., Owen, H., Emes, R. D., Seddon, J., Simmons, G., & Meers, J. (2020b). Novel insights into viral infection and oncogenesis from koala retrovirus (KoRV) infection of HEK293T cells. *Gene*, ***733***, 144366.

Sayed, S., Idriss, N., Sayyed, H., Ashry, A., Rafatt, D., Mohamed, A., & Blann, A. (2015). Effects of propofol and isoflurane on haemodynamics and the inflammatory response in cardiopulmonary bypass surgery. *British journal of biomedical science*, ***72***(3), 93-101.

Sayyad, Z., Acharya, D., & Gack, M. U. (2024). TRIM Proteins: Key Regulators of Immunity to Herpesvirus Infection. *Viruses*, ***16***(11). https://doi.org/10.3390/v16111738

Schachtschneider, K. M., Yeoman, C. J., Isaacson, R. E., White, B. A., Schook, L. B., & Pieters, M. (2013). Modulation of Systemic Immune Responses through Commensal Gastrointestinal Microbiota. *PLoS One*, ***8***(1), e53969. https://doi.org/10.1371/journal.pone.0053969

Scheele, B. C., Legge, S., Blanchard, W., Garnett, S., Geyle, H., Gillespie, G., Harrison, P., Lindenmayer, D., Lintermans, M., & Robinson, N. (2019). Continental-scale assessment reveals inadequate monitoring for threatened vertebrates in a megadiverse country. *Biological conservation*, ***235***, 273-278.

Schlamp, F., Delbare, S. Y. N., Early, A. M., Wells, M. T., Basu, S., & Clark, A. G. (2021). Dense time-course gene expression profiling of the Drosophila melanogaster innate immune response. *BMC Genomics*, ***22***(1), 304. https://doi.org/10.1186/s12864-021-07593-3

Schnyder, M., Vanzetti, T., Stärk, K. D. C., Schleiss, W., Salman, M. D., Thur, B., & Griot, C. (2002). Epidemiology and control of an outbreak of classical swine fever in wild boar in Switzerland. *Veterinary Record*, ***150***(4), 102-109.

Schoborg, R. (2011). Chlamydia persistence–a tool to dissect chlamydia–host interactions. *Microbes and Infection*, ***13***(7), 649-662.

Scholthof, K.-B. G. (2007). The disease triangle: pathogens, the environment and society. *Nature Reviews Microbiology*, ***5***(2), 152-156. https://doi.org/10.1038/nrmicro1596

Schrader, S., Klos, A., Hess, S., Zeidler, H., Kuipers, J. G., & Rihl, M. (2007). Expression of inflammatory host genes in Chlamydia trachomatis-infected human monocytes. *Arthritis Res Ther*, ***9***(3), R54. https://doi.org/10.1186/ar2209

Schreiner, C. L., Nuismer, S. L., & Basinski, A. J. (2020). When to vaccinate a fluctuating wildlife population: Is timing everything? *Journal of Applied Ecology*, ***57***(2), 307-319. https://doi.org/https://doi.org/10.1111/1365-2664.13539

Schuchardt, L., & Rupp, J. (2018). Chlamydia trachomatis as the Cause of Infectious Infertility: Acute, Repetitive or Persistent Long-Term Infection? *Curr Top Microbiol Immunol*, ***412***, 159-182. https://doi.org/10.1007/82_2016_15

Schust, D. J., Ibana, J. A., Buckner, L. R., Ficarra, M., Sugimoto, J., Amedee, A. M., & Quayle, A. J. (2012). Potential mechanisms for increased HIV-1 transmission across the endocervical epithelium during C. trachomatis infection. *Current HIV research*, ***10***(3), 218-227.

Schütte, K., Springer, A., Brandes, F., Reuschel, M., Fehr, M., & Strube, C. (2025). Myiasis in European hedgehogs (Erinaceus europaeus). *Veterinary Quarterly*, ***45***(1), 15-24. https://doi.org/10.1080/01652176.2025.2463328

Scidmore, M. A., Rockey, D. D., Fischer, E. R., Heinzen, R. A., & Hackstadt, T. (1996). Vesicular interactions of the Chlamydia trachomatis inclusion are determined by chlamydial early protein synthesis rather than route of entry. *Infection and immunity*, ***64***(12), 5366-5372. https://doi.org/10.1128/iai.64.12.5366-5372.1996

Seabrook, L., McAlpine, C., Baxter, G., Rhodes, J., Bradley, A., & Lunney, D. (2011). Drought-driven change in wildlife distribution and numbers: a case study of koalas in south west Queensland. *Wildlife Research*, ***38***(6), 509-524.

Segner, H., Rehberger, K., Bailey, C., & Bo, J. (2022). Assessing Fish Immunotoxicity by Means of In Vitro Assays: Are We There Yet? *Front Immunol*, ***13***, 835767. https://doi.org/10.3389/fimmu.2022.835767

Semenza, J. C., Rocklöv, J., & Ebi, K. L. (2022). Climate Change and Cascading Risks from Infectious Disease. *Infect Dis Ther*, ***11***(4), 1371-1390. https://doi.org/10.1007/s40121-022-00647-3

Sharma, V., Mobeen, F., & Prakash, T. (2016). Comparative genomics of herpesviridae family to look for potential signatures of human infecting strains. *International journal of genomics*, ***2016***(1), 9543274.

Sheriff, M. J., Dantzer, B., Delehanty, B., Palme, R., & Boonstra, R. (2011). Measuring stress in wildlife: techniques for quantifying glucocorticoids. *Oecologia*, ***166***(4), 869-887. https://doi.org/10.1007/s00442-011-1943-y

Shibata, Y., Foster, L. A., Kurimoto, M., Okamura, H., Nakamura, R. M., Kawajiri, K., Justice, J. P., Van Scott, M. R., Myrvik, Q. N., & Metzger, W. J. (1998). Immunoregulatory Roles of IL-10 in Innate Immunity: IL-10 Inhibits Macrophage Production of IFN-γ-Inducing Factors but Enhances NK Cell Production of IFN-γ1. *The Journal of Immunology*, ***161***(8), 4283-4288. https://doi.org/10.4049/jimmunol.161.8.4283

Shimada, K., Crother, T. R., Karlin, J., Chen, S., Chiba, N., Ramanujan, V. K., Vergnes, L., Ojcius, D. M., & Arditi, M. (2011). Caspase-1 dependent IL-1β secretion is critical for host defense in a mouse model of Chlamydia pneumoniae lung infection. *PLoS One*, ***6***(6), e21477.

Shimazui, T., Matsumura, Y., Nakada, T. A., & Oda, S. (2017). Serum levels of interleukin-6 may predict organ dysfunction earlier than SOFA score. *Acute Med Surg*, ***4***(3), 255-261. https://doi.org/10.1002/ams2.263

Shulgina, S. M., Osetsky, N. Y., Rykova, M. P., Antropova, E. N., Zhuravleva, T. V., Shmarov, V. A., Kutko, O. V., Vlasova, D. D., Kotikova, A. A., Orlova, K. D., Zhirova, E. A., & Ponomarev, S. A. (2025). Reactivation of latent human intracellular infections during a months-long expedition at the Antarctic Vostok station. *Scientific reports*, ***15***(1), 9980. https://doi.org/10.1038/s41598-025-94539-4

Sieber, S., Wirth, L., Cavak, N., Koenigsmark, M., Marx, U., Lauster, R., & Rosowski, M. (2018). Bone marrow-on-a-chip: long-term culture of human haematopoietic stem cells in a three-dimensional microfluidic environment. *Journal of tissue engineering and regenerative medicine*, ***12***(2), 479-489.

Silswal, N., Singh, A. K., Aruna, B., Mukhopadhyay, S., Ghosh, S., & Ehtesham, N. Z. (2005). Human resistin stimulates the pro-inflammatory cytokines TNF-alpha and IL-12 in macrophages by NF-kappaB-dependent pathway. *Biochem Biophys Res Commun*, ***334***(4), 1092-1101. https://doi.org/10.1016/j.bbrc.2005.06.202

Silver, A. C., Buckley, S. M., Hughes, M. E., Hastings, A. K., Nitabach, M. N., & Fikrig, E. (2018). Daily oscillations in expression and responsiveness of Toll-like receptors in splenic immune cells. *Heliyon*, ***4***(3).

Silver, L. W., Cheng, Y., Quigley, B. L., Robbins, A., Timms, P., Hogg, C. J., & Belov, K. (2022). A targeted approach to investigating immune genes of an iconic Australian marsupial. *Molecular ecology*, ***31***(12), 3286-3303.

Silver, L. W., Hogg, C. J., & Belov, K. (2024). Plethora of New Marsupial Genomes Informs Our Knowledge of Marsupial MHC Class II. *Genome Biology and Evolution*, ***16***(8). https://doi.org/10.1093/gbe/evae156

Silverman, J. D., Roche, K., Mukherjee, S., & David, L. A. (2020). Naught all zeros in sequence count data are the same. *Computational and Structural Biotechnology Journal*, ***18***, 2789-2798.

Simmons, G., Clarke, D., McKee, J., Young, P., & Meers, J. (2014). Discovery of a novel retrovirus sequence in an Australian native rodent (Melomys burtoni): a putative link between gibbon ape leukemia virus and koala retrovirus. *PLoS One*, ***9***(9), e106954.

Simmons, G., Young, P., Hanger, J., Jones, K., Clarke, D., McKee, J., & Meers, J. (2012). Prevalence of koala retrovirus in geographically diverse populations in Australia. *Australian veterinary journal*, ***90***(10), 404-409.

Simonetto, C., Heier, M., Peters, A., Kaiser, J. C., & Rospleszcz, S. (2022). From Atherosclerosis to Myocardial Infarction: A Process-Oriented Model Investigating the Role of Risk Factors. *Am J Epidemiol*, ***191***(10), 1766-1775. https://doi.org/10.1093/aje/kwac038

Simonnet, A., Engelmann, I., Moreau, A.-S., Garcia, B., Six, S., El Kalioubie, A., Robriquet, L., Hober, D., & Jourdain, M. (2021). High incidence of Epstein–Barr virus, cytomegalovirus, and human-herpes virus-6 reactivations in critically ill patients with COVID-19. *Infectious Diseases Now*, ***51***(3), 296-299.

Simpson, S. J., Higgins, D. P., Timms, P., Mella, V. S. A., Crowther, M. S., Fernandez, C. M., McArthur, C., Phillips, S., & Krockenberger, M. B. (2023). Efficacy of a synthetic peptide Chlamydia pecorum major outer membrane protein vaccine in a wild koala (Phascolarctos cinereus) population. *Scientific reports*, ***13***(1), 15087. https://doi.org/10.1038/s41598-023-42296-7

Singleton, C. L., & Hamlin-Andrus, C. (2023). Koala retrovirus status and putative koala retrovirus-associated diseases in koalas (Phascolarctos cinereus) in North American Zoos. Proceedings of the Second Koala Retrovirus Workshop,

Slade, J., Hall, J. V., Kintner, J., & Schoborg, R. V. (2016). Chlamydial Pre-Infection Protects from Subsequent Herpes Simplex Virus-2 Challenge in a Murine Vaginal Super-Infection Model. *PLoS One*, ***11***(1), e0146186. https://doi.org/10.1371/journal.pone.0146186

Slade, J. A. (2016). *In Vitro and In Vivo Characterization of Chlamydia and HSV Co-infection* East Tennessee State Universtiy].

Slade, J. A., Brockett, M., Singh, R., Liechti, G. W., & Maurelli, A. T. (2019). Fosmidomycin, an inhibitor of isoprenoid synthesis, induces persistence in Chlamydia by inhibiting peptidoglycan assembly. *PLoS Pathog*, ***15***(10), e1008078. https://doi.org/10.1371/journal.ppat.1008078

Smith, D. G. M., & Williams, S. J. (2016). Immune sensing of microbial glycolipids and related conjugates by T cells and the pattern recognition receptors MCL and Mincle. *Carbohydrate Research*, ***420***, 32-45. https://doi.org/https://doi.org/10.1016/j.carres.2015.11.009

Snyderman, R., & Cianciolo, G. J. (1984). Immunosuppressive activity of the retroviral envelope protein P 15E and its possible relationship to neoplasia. *Immunology Today*, ***5***(8), 240-244.

Somboonna, N., Ziklo, N., Ferrin, T., Suh, J., Dean, D., Nacy, C., Ayiar, A., & Hammerschlag, M. (2019). Clinical persistence of Chlamydia trachomatis sexually transmitted strains involves novel mutations in the functional abba tetramer of the tryptophan synthase operon. mBio 10: e01464-19. In.

Song, L., Carlson, J. H., Whitmire, W. M., Kari, L., Virtaneva, K., Sturdevant, D. E., Watkins, H., Zhou, B., Sturdevant, G. L., & Porcella, S. F. (2013). Chlamydia trachomatis plasmid-encoded Pgp4 is a transcriptional regulator of virulence-associated genes. *Infection and immunity*, ***81***(3), 636-644.

Sparks, R., Rachmaninoff, N., Lau, W. W., Hirsch, D. C., Bansal, N., Martins, A. J., Chen, J., Liu, C. C., Cheung, F., Failla, L. E., Biancotto, A., Fantoni, G., Sellers, B. A., Chawla, D. G., Howe, K. N., Mostaghimi, D., Farmer, R., Kotliarov, Y., Calvo, K. R., . . . Tsang, J. S. (2024). A unified metric of human immune health. *Nature Medicine*, ***30***(9), 2461-2472. https://doi.org/10.1038/s41591-024-03092-6

Spector, W. G., & Willoughby, D. A. (1963). THE INFLAMMATORY RESPONSE. *Bacteriological Reviews*, ***27***(2), 117-154. https://doi.org/10.1128/br.27.2.117-154.1963

Speight, K. N., Polkinghorne, A., Penn, R., Boardman, W., Timms, P., Fraser, T., Johnson, K., Faull, R., Bate, S., & Woolford, L. (2016). Prevalence and Pathalogic Features of *Chlamydia pecorum* Infections in South Australian Koalas *(Phascolarctos cinereus)*. *J Wildl Dis*, ***52***(2), 301-306. https://doi.org/10.7589/2015-05-120

Speight, N. (2023). Koala retrovirus infection and disease in South Australian koala (Phascolarctos cinereus) populations. Proceedings of the Second Koala Retrovirus Workshop,

Spengler, M. L., Kuropatwinski, K. K., Comas, M., Gasparian, A. V., Fedtsova, N., Gleiberman, A. S., Gitlin, I. I., Artemicheva, N. M., Deluca, K. A., & Gudkov, A. V. (2012). Core circadian protein CLOCK is a positive regulator of NF-κB–mediated transcription. *Proceedings of the National Academy of Sciences*, ***109***(37), E2457-E2465.

Srinivasachar Badarinarayan, S., & Sauter, D. (2021). Switching sides: how endogenous retroviruses protect us from viral infections. *Journal of Virology*, ***95***(12), 10.1128/jvi. 02299-02220.

Staibano, S., Mascolo, M., Ilardi, G., Siano, M., & De Rosa, G. (2011). Immunohistochemical analysis of FKBP51 in human cancers. *Current opinion in pharmacology*, ***11***(4), 338-347.

Stalder, K., Vaz, P. K., Gilkerson, J. R., Baker, R., Whiteley, P., Ficorilli, N., Tatarczuch, L., Portas, T., Skogvold, K., & Anderson, G. A. (2015). Prevalence and clinical significance

of herpesvirus infection in populations of Australian marsupials. *PLoS One*, ***10***(7), e0133807.

Stein, C. M., Zalwango, S., Chiunda, A. B., Millard, C., Leontiev, D. V., Horvath, A. L., Cartier, K. C., Chervenak, K., Boom, W. H., Elston, R. C., Mugerwa, R. D., Whalen, C. C., & Iyengar, S. K. (2007). Linkage and association analysis of candidate genes for TB and TNFα cytokine expression: evidence for association with IFNGR1, IL-10, and TNF receptor 1 genes. *Human Genetics*, ***121***(6), 663-673. https://doi.org/10.1007/s00439-007-0357-8

Stelzner, K., Vollmuth, N., & Rudel, T. (2023). Intracellular lifestyle of Chlamydia trachomatis and host–pathogen interactions. *Nature Reviews Microbiology*, ***21***(7), 448-462. https://doi.org/10.1038/s41579-023-00860-y

Stepanenko, E., Bondareva, N., Sheremet, A., Fedina, E., Tikhomirov, A., Gerasimova, T., Poberezhniy, D., Makarova, I., Tarantul, V., & Zigangirova, N. (2023). Identification of key TRIM genes involved in response to Pseudomonas aeruginosa or Chlamydia spp. infections in human cell lines and in mouse organs. *International Journal of Molecular Sciences*, ***24***(17), 13290.

Stephens, M. (2017). False discovery rates: a new deal. *Biostatistics*, ***18***(2), 275-294.

Stephenson, T. (2021). *Pathology, coinfections and oncogenesis in South Australian koalas (Phascolarctos cinereus) and their association with koala retrovirus (KoRV)*

Stijlemans, B., Choi, B., Álvarez-Rodríguez, A., Jin, B.-k., Radwanska, M., & Magez, S. (2024). Chapter 4 - Trypanosomiasis. In T. K. Bhatt (Ed.), *The Diagnosis and Treatment of Protozoan Diseases* (pp. 95-148). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-443-19161-9.00004-8

Stoye, J. P. (2006). Koala retrovirus: a genome invasion in real time. *Genome biology*, ***7***, 1-3.

Sullivan, B., Baxter, G., Lisle, A., Pahl, L., & Norris, W. (2004). Low-density koala (Phascolarctos cinereus) populations in the mulgalands of south-west Queensland. IV. Abundance and conservation status. *Wildlife Research*, ***31***(1), 19-29.

Sullivan, K. E., Cutilli, J., Piliero, L. M., Ghavimi-Alagha, D., Starr, S. E., Campbell, D. E., & Douglas, S. D. (2000). Measurement of Cytokine Secretion, Intracellular Protein Expression, and mRNA in Resting and Stimulated Peripheral Blood Mononuclear Cells. *Clinical Diagnostic Laboratory Immunology*, ***7***(6), 920-924. https://doi.org/doi:10.1128/cdli.7.6.920-924.2000

Sun, H. S., Eng, E. W., Jeganathan, S., Sin, A. T., Patel, P. C., Gracey, E., Inman, R. D., Terebiznik, M. R., & Harrison, R. E. (2012). Chlamydia trachomatis vacuole maturation in infected macrophages. *J Leukoc Biol*, ***92***(4), 815-827. https://doi.org/10.1189/jlb.0711336

Sun, N.-K., Huang, S.-L., Chang, P.-Y., Lu, H.-P., & Chao, C. C. (2014). Transcriptomic profiling of taxol-resistant ovarian cancer cells identifies FKBP5 and the androgen receptor as critical markers of chemotherapeutic response. *Oncotarget*, ***5***(23), 11939.

Sun, Z., Guo, X., Chen, H., Ling, J., Zhao, H., Chang, A., & Zhuo, X. (2023). MYO1B as a prognostic biomarker and a therapeutic target in Arecoline-associated oral carcinoma. *Mol Carcinog*, ***62***(7), 920-939. https://doi.org/10.1002/mc.23535

Supino, D., Minute, L., Mariancini, A., Riva, F., Magrini, E., & Garlanda, C. (2022). Negative Regulation of the IL-1 System by IL-1R2 and IL-1R8: Relevance in Pathophysiology and Disease [Review]. *Frontiers in Immunology*, ***13***. https://doi.org/10.3389/fimmu.2022.804641

Symington, J. (1898). The thymus gland in the marsupialia. *Journal of Anatomy and Physiology*, ***32***(Pt 2), 278.

Szabo, K. V., O'Neill, C. E., & Clarke, I. N. (2020). Diversity in Chlamydial plasmids. *PLoS One*, ***15***(5), e0233298.

Takenouchi, N., Yoshihisa, Y., Koichiro, U., Mitsuhiro, O., & and Izumo, S. (2003). Usefulness of Proviral Load Measurement for Monitoring of Disease Activity in Individual Patients with Human T-Lymphotropic Virus Type I-Associated Myelopathy/Tropical Spastic Paraparesis. *Journal of Neurovirology*, ***9***(1), 29-35. https://doi.org/10.1080/13550280390173418

Tan, S. Y., Foo, C. N., Ng, F. L., Tan, C. H., & Lim, Y. M. (2025). Gene Expression Profiling of Maslinic Acid-treated MCF-7 Breast Cancer Cells Using Nanostring nCounter Pancancer Pathway Panel. *Gene*, ***935***, 149043. https://doi.org/https://doi.org/10.1016/j.gene.2024.149043

Tang, L., Chen, J., Zhou, Z., Yu, P., Yang, Z., & Zhong, G. (2015). Chlamydia-secreted protease CPAF degrades host antimicrobial peptides. *Microbes and Infection*, ***17***(6), 402-408. https://doi.org/https://doi.org/10.1016/j.micinf.2015.02.005

Tarlinton, R., & Greenwood, A. (2024). Koala retrovirus and neoplasia: correlation and underlying mechanisms. *Current Opinion in Virology*, ***67***, 101427. https://doi.org/10.1016/j.coviro.2024.101427

Tarlinton, R., Meers, J., Hanger, J., & Young, P. (2005). Real-time reverse transcriptase PCR for the endogenous koala retrovirus reveals an association between plasma viral load and neoplastic disease in koalas. *J Gen Virol*, ***86***(Pt 3), 783-787. https://doi.org/10.1099/vir.0.80547-0

Tarlinton, R., Meers, J., & Young, P. (2008a). Biology and evolution of the endogenous koala retrovirus. *Cell Mol Life Sci*, ***65***(21), 3413-3421. https://doi.org/10.1007/s00018-008-8499-y

Tarlinton, R., Meers, J., & Young, P. (2008b). Endogenous retroviruses. *Cellular and Molecular Life Sciences*, ***65***(21), 3413-3421. https://doi.org/10.1007/s00018-008-8499-y

Tarlinton, R., Tanasescu, R., Shannon-Lowe, C., & Gran, B. (2024). Ocrelizumab B cell depletion has no effect on HERV RNA expression in PBMC in MS patients. *Multiple Sclerosis and Related Disorders*, ***86***, 105597. https://doi.org/https://doi.org/10.1016/j.msard.2024.105597

Tarlinton, R. E., Fabijan, J., Hemmatzadeh, F., Meers, J., Owen, H., Sarker, N., Seddon, J. M., Simmons, G., Speight, N., Trott, D. J., Woolford, L., & Emes, R. D. (2021). Transcriptomic and genomic variants between koala populations reveals underlying genetic components to disorders in a bottlenecked population. *Conservation Genetics*, ***22***(3), 329-340. https://doi.org/10.1007/s10592-021-01340-7

Tarlinton, R. E., Legione, A. R., Sarker, N., Fabijan, J., Meers, J., McMichael, L., Simmons, G., Owen, H., Seddon, J. M., & Dick, G. (2022a). Differential and defective transcription of koala retrovirus indicates the complexity of host and virus evolution. *J Gen Virol*, ***103***(6), 001749.

Tarlinton, R. E., Legione, A. R., Sarker, N., Fabijan, J., Meers, J., McMichael, L., Simmons, G., Owen, H., Seddon, J. M., & Dick, G. (2022b). Differential and defective transcription of koala retrovirus indicates the complexity of host and virus evolution. *J Gen Virol*, 211466.

Tarlinton, R. E., Legione, A. R., Sarker, N., Fabijan, J., Meers, J., McMichael, L., Simmons, G., Owen, H., Seddon, J. M., Dick, G., Ryder, J. S., Hemmatzedah, F., Trott, D. J., Speight, N., Holmes, N., Loose, M., & Emes, R. D. (2022c). Differential and defective transcription of koala retrovirus indicates the complexity of host and virus evolution. *J Gen Virol*, ***103***(6). https://doi.org/10.1099/jgv.0.001749

Tarlinton, R. E., Meers, J., & Young, P. R. (2006). Retroviral invasion of the koala genome. *Nature*, ***442***(7098), 79-81. https://doi.org/10.1038/nature04841

Taylor, R. A., Pare, J. R., Venkatesh, A. K., Mowafi, H., Melnick, E. R., Fleischman, W., & Hall, M. K. (2016). Prediction of In-hospital Mortality in Emergency Department Patients With Sepsis: A Local Big Data–Driven, Machine Learning Approach. *Academic Emergency Medicine*, ***23***(3), 269-278. https://doi.org/https://doi.org/10.1111/acem.12876

Taylor-Brown, A., Booth, R., Gillett, A., Mealy, E., Ogbourne, S. M., Polkinghorne, A., & Conroy, G. C. (2019). The impact of human activities on Australian wildlife. *PLoS One*, ***14***(1), e0206958.

Teffer, A. K., Hinch, S., Miller, K., Jeffries, K., Patterson, D., Cooke, S., Farrell, A., Kaukinen, K. H., Li, S., & Juanes, F. (2019). Cumulative Effects of Thermal and Fisheries Stressors Reveal Sex-Specific Effects on Infection Development and Early Mortality of Adult Coho Salmon (Oncorhynchus kisutch). *Physiological and Biochemical Zoology*, ***92***(5), 505-529. https://doi.org/10.1086/705125

Teffer, A. K., Hinch, S. G., Miller, K. M., Patterson, D. A., Bass, A. L., Cooke, S. J., Farrell, A. P., Beacham, T. D., Chapman, J. M., & Juanes, F. (2022). Host-pathogen-environment interactions predict survival outcomes of adult sockeye salmon (Oncorhynchus nerka) released from fisheries. *Molecular ecology*, ***31***(1), 134-160. https://doi.org/https://doi.org/10.1111/mec.16214

Tempesta, M., Buonavoglia, D., Sagazio, P., Pratelli, A., & Buonavoglia, C. (1998). Natural reactivation of caprine herpesvirus 1 in latently infected goats. *Veterinary Record*, ***143***, 200-200.

Terraube, J., Gardiner, R., Hohwieler, K., Frère, C., & Cristescu, R. (2023). Protected area coverage has a positive effect on koala occurrence in eastern Australia. *Biodiversity and Conservation*, ***32***(7), 2495-2511.

Thacker, S. B., & Berkelman, R. L. (1988). Public health surveillance in the United States. *Epidemiologic reviews*, ***10***(1), 164-190.

Thompson, C. K., Godfrey, S. S., & Thompson, R. C. A. (2014). Trypanosomes of Australian mammals: A review. *International Journal for Parasitology: Parasites and Wildlife*, ***3***(2), 57-66. https://doi.org/https://doi.org/10.1016/j.ijppaw.2014.02.002

Thorolfsdottir, R. B., Jonsdottir, A. B., Sveinbjornsson, G., Aegisdottir, H. M., Oddsson, A., Stefansson, O. A., Halldorsson, G. H., Saevarsdottir, S., Thorleifsson, G., & Stefansdottir, L. (2024). Variants at the interleukin 1 gene locus and pericarditis. *JAMA cardiology*, ***9***(2), 165-172.

Thurman, L. L., Alger, K., LeDee, O., Thompson, L. M., Hofmeister, E., Hudson, J. M., Martin, A. M., Melvin, T. A., Olson, S. H., Pruvot, M., Rohr, J. R., Szymanksi, J. A., Aleuy, O. A., & Zuckerberg, B. (2024). Disease-smart climate adaptation for wildlife management and conservation. *Frontiers in Ecology and the Environment*, ***22***(4), e2716. https://doi.org/https://doi.org/10.1002/fee.2716

Tian, C., Plenge, R. M., Ransom, M., Lee, A., Villoslada, P., Selmi, C., Klareskog, L., Pulver, A. E., Qi, L., & Gregersen, P. K. (2008). Analysis and application of European genetic substructure using 300 K SNP information. *PLoS genetics*, ***4***(1), e4.

Tietzel, I., Quayle, A. J., & Carabeo, R. A. (2019). Alternatively Activated Macrophages Are Host Cells for Chlamydia trachomatis and Reverse Anti-chlamydial Classically Activated Macrophages [Original Research]. *Frontiers in Microbiology*, ***10***. https://doi.org/10.3389/fmicb.2019.00919

Tkaczynski, A., & Rundle-Thiele, S. (2023). Koala conservation in South East Queensland: A grey literature review analysis. *Conservation Science and Practice*, ***5***(3), e12874.

Tokuyama, M., Kong, Y., Song, E., Jayewickreme, T., Kang, I., & Iwasaki, A. (2018). ERVmap analysis reveals genome-wide transcription of human endogenous retroviruses. *Proceedings of the National Academy of Sciences*, ***115***(50), 12565-12572.

Touma, C., Gassen, N. C., Herrmann, L., Cheung-Flynn, J., Büll, D. R., Ionescu, I. A., Heinzmann, J.-M., Knapman, A., Siebertz, A., & Depping, A.-M. (2011). FK506 binding protein 5 shapes stress responsiveness: modulation of neuroendocrine reactivity and coping behavior. *Biological psychiatry*, ***70***(10), 928-936.

Travis, D. A., Sriramarao, P., Cardona, C., Steer, C. J., Kennedy, S., Sreevatsan, S., & Murtaugh, M. P. (2014). One Medicine One Science: a framework for exploring challenges at the intersection of animals, humans, and the environment. *Annals of the New York Academy of Sciences*, ***1334***(1), 26-44. https://doi.org/https://doi.org/10.1111/nyas.12601

Tribe, A., & Orr, B. (2019). Wildlife rehabilitation practices in Australia. *Current Therapy in Medicine of Australian Mammals; CSIRO Publishing: Clayton, Australia*, 51-62.

Tseng, C.-T. K., & Rank, R. G. (1998). Role of NK cells in early host response to chlamydial genital infection. *Infection and immunity*, ***66***(12), 5867-5875.

Turman, B. J., Alzhanov, D., Nagarajan, U. M., Darville, T., & O'Connell, C. M. (2023). Virulence protein Pgp3 is insufficient to mediate plasmid-dependent infectivity of Chlamydia trachomatis. *Infection and immunity*, ***91***(2), e00392-00322.

Ueda, S., Uchiyama, S., Azzi, T., Gysin, C., Berger, C., Bernasconi, M., Harabuchi, Y., Zinkernagel, A. S., & Nadal, D. (2014). Oropharyngeal group A streptococcal colonization disrupts latent Epstein-Barr virus infection. *The Journal of infectious diseases*, ***209***(2), 255-264.

Valente, G., Ozmen, L., Novelli, F., Geuna, M., Palestro, G., Forni, G., & Garotta, G. (1992). Distribution of interferon-γ receptor in human tissues. *European Journal of Immunology*, ***22***(9), 2403-2412. https://doi.org/https://doi.org/10.1002/eji.1830220933

Van Dyk, L. F., Virgin IV, H. W., & Speck, S. H. (2003). Maintenance of gammaherpesvirus latency requires viral cyclin in the absence of B lymphocytes. *Journal of Virology*, ***77***(9), 5118-5126.

Van Eeden, L. M., Nimmo, D., Mahony, M., Herman, K., Ehmke, G., Driessen, J., O'Connor, J., Bino, G., Taylor, M., & Dickman, C. (2020). Impacts of the unprecedented 2019-2020 bushfires on Australian animals.

Vanover, J., Kintner, J., Whittimore, J., & Schoborg, R. V. (2010). Interaction of herpes simplex virus type 2 (HSV-2) glycoprotein D with the host cell surface is sufficient to induce Chlamydia trachomatis persistence. *Microbiology (Reading)*, ***156***(Pt 5), 1294-1302. https://doi.org/10.1099/mic.0.036566-0

Vats, V., Agrawal, T., Salhan, S., & Mittal, A. (2007). Primary and secondary immune responses of mucosal and peripheral lymphocytes during Chlamydia trachomatis infection. *FEMS Immunology & Medical Microbiology*, ***49***(2), 280-287. https://doi.org/10.1111/j.1574-695X.2006.00196.x

Vaz, P. K., Hartley, C. A., Lee, S., Sansom, F. M., Adams, T. E., Stalder, K., Pearce, L., Lovrecz, G., Browning, G. F., & Müller, C. E. (2019a). Koala and wombat gammaherpesviruses encode the first known viral NTPDase homologs and are phylogenetically divergent from all known gammaherpesviruses. *J Virol*, ***93***(6), 10.1128/jvi. 01404-01418.

Vaz, P. K., Legione, A. R., Hartley, C. A., & Devlin, J. M. (2019b). Detection and Differentiation of Two Koala Gammaherpesviruses by Use of High-Resolution Melt (HRM) Analysis Reveals Differences in Viral Prevalence and Clinical Associations in a Large Study of Free-Ranging Koalas. *J Clin Microbiol*, ***57***(3). https://doi.org/10.1128/jcm.01478-18

Vaz, P. K., Whiteley, P. L., Wilks, C. R., Browning, G. F., Gilkerson, J. R., Ficorilli, N., & Devlin, J. M. (2012). Detection of a second novel gammaherpesvirus in a free-ranging koala (Phascolarctos cinereus). *Journal of Wildlife Diseases*, ***48***(1), 226-229.

Vaz, P. K., Whiteley, P. L., Wilks, C. R., Duignan, P. J., Ficorilli, N., Gilkerson, J. R., Browning, G. F., & Devlin, J. M. (2011). Detection of a novel gammaherpesvirus in koalas (Phascolarctos cinereus). *Journal of Wildlife Diseases*, ***47***(3), 787-791.

Veenbergen, S., Li, P., Raatgeep, H. C., Lindenbergh-Kortleve, D. J., Simons-Oosterhuis, Y., Farrel, A., Costes, L. M. M., Joosse, M. E., van Berkel, L. A., de Ruiter, L. F., van Leeuwen, M. A., Winter, D., Holland, S. M., Freeman, A. F., Wakabayashi, Y., Zhu, J., de Ridder, L., Driessen, G. J., Escher, J. C., . . . Samsom, J. N. (2019). IL-10 signaling in dendritic cells controls IL-1β-mediated IFNγ secretion by human CD4+ T cells: relevance to inflammatory bowel disease. *Mucosal Immunology*, ***12***(5), 1201-1211. https://doi.org/10.1038/s41385-019-0194-9

Veldman-Jones, M. H., Brant, R., Rooney, C., Geh, C., Emery, H., Harbron, C. G., Wappett, M., Sharpe, A., Dymond, M., Barrett, J. C., Harrington, E. A., & Marshall, G. (2015). Evaluating Robustness and Sensitivity of the NanoString Technologies nCounter Platform to Enable Multiplexed Gene Expression Analysis of Clinical Samples. *Cancer Research*, ***75***(13), 2587-2593. https://doi.org/10.1158/0008-5472.Can-15-0262

Versteeg, B., Bruisten, S. M., Pannekoek, Y., Jolley, K. A., Maiden, M. C., van der Ende, A., & Harrison, O. B. (2018). Genomic analyses of the Chlamydia trachomatis core genome show an association between chromosomal genome, plasmid type and disease. *BMC Genomics*, ***19***, 1-13.

Vetter, K. M., Barnes, R. C., Oberle, M. W., Rosero-Bixby, L., & Schachter, J. (1990). Seroepidemiology of chlamydia in Costa Rica. *Genitourin Med*, ***66***(3), 182-188. https://doi.org/10.1136/sti.66.3.182

Vicente-Santos, A., Willink, B., Nowak, K., Civitello, D. J., & Gillespie, T. R. (2023). Host–pathogen interactions under pressure: A review and meta-analysis of stress-mediated effects on disease dynamics. *Ecology Letters*, ***26***(11), 2003-2020. https://doi.org/https://doi.org/10.1111/ele.14319

Vinuesa, C. G., He, Y., & Cook, M. C. (2024). A global metric of immune health. *Nature Medicine*, ***30***(9), 2411-2412. https://doi.org/10.1038/s41591-024-03210-4

Vitali, S. D., Reiss, A. E., Jakob-Hoff, R. M., Stephenson, T. L., Holz, P. H., & Higgins, D. P. (2023). National Koala Disease Risk Analysis Report V 1.2. In *National Koala Disease Risk Analysis Report V 1.2*.

Vittinghoff, E., & McCulloch, C. E. (2006). Relaxing the Rule of Ten Events per Variable in Logistic and Cox Regression. *American Journal of Epidemiology*, ***165***(6), 710-718. https://doi.org/10.1093/aje/kwk052

Vlcek, K. R., Li, W., Manam, S., Zanotti, B., Nicholson, B. J., Ramsey, K. H., & Murthy, A. K. (2016). The contribution of Chlamydia-specific CD8+ T cells to upper genital tract pathology. *Immunology and Cell Biology*, ***94***(2), 208-212.

Vogelnest, L., & Portas, T. (2019). Current therapy in medicine of Australian mammals. In. Csiro Publishing.

Vollmuth, N., Schlicker, L., Guo, Y., Hovhannisyan, P., Janaki-Raman, S., Kurmasheva, N., Schmitz, W., Schulze, A., Stelzner, K., Rajeeve, K., & Rudel, T. (2022). c-Myc plays a key role in IFN-γ-induced persistence of Chlamydia trachomatis. *Elife*, ***11***. https://doi.org/10.7554/eLife.76721

Walton, L., Marion, G., Davidson, R. S., White, P. C. L., Smith, L. A., Gavier-Widen, D., Yon, L., Hannant, D., & Hutchings, M. R. (2016). The ecology of wildlife disease surveillance: demographic and prevalence fluctuations undermine surveillance. *Journal of Applied Ecology*, ***53***(5), 1460-1469. https://doi.org/https://doi.org/10.1111/1365-2664.12671

Wan, C., Loader, J., Hanger, J., Beagley, K., Timms, P., & Polkinghorne, A. (2011). Using quantitative polymerase chain reaction to correlate Chlamydia pecorum infectious load with ocular, urinary and reproductive tract disease in the koala (Phascolarctos cinereus). *Australian veterinary journal*, ***89***(10), 409-412.

Wang, C., Zhang, K., Meng, L., Zhang, X., Song, Y., Zhang, Y., Gai, Y., Zhang, Y., Yu, B., Wu, J., Wang, S., & Yu, X. (2020). The C-terminal domain of feline and bovine SAMHD1 proteins has a crucial role in lentiviral restriction. *J Biol Chem*, ***295***(13), 4252-4264. https://doi.org/10.1074/jbc.RA120.012767

Wang, L., Wang, S., & Li, W. (2012). RSeQC: quality control of RNA-seq experiments. *bioinformatics*, ***28***(16), 2184-2185.

Ward, M., Tulloch, A. I., Radford, J. Q., Williams, B. A., Reside, A. E., Macdonald, S. L., Mayfield, H. J., Maron, M., Possingham, H. P., & Vine, S. J. (2020). Impact of 2019–2020 mega-fires on Australian fauna habitat. *Nature Ecology & Evolution*, ***4***(10), 1321-1326.

Wassenaar, T. M., & Gaastra, W. (2001). Bacterial virulence: can we draw the line? *FEMS Microbiology Letters*, ***201***(1), 1-7. https://doi.org/10.1111/j.1574-6968.2001.tb10724.x

Waugh, C. A., Hanger, J., Loader, J., King, A., Hobbs, M., Johnson, R., & Timms, P. (2017). Infection with koala retrovirus subgroup B (KoRV-B), but not KoRV-A, is associated with chlamydial disease in free-ranging koalas (Phascolarctos cinereus). *Sci Rep*, ***7***(1), 134. https://doi.org/10.1038/s41598-017-00137-4

Waugh, C. A., Khan, S. A., Carver, S., Hanger, J., Loader, J., Polkinghorne, A., Beagley, K., & Timms, P. (2016). A prototype recombinant-protein based Chlamydia pecorum vaccine results in reduced chlamydial burden and less clinical disease in free-ranging koalas (Phascolarctos cinereus). *PLoS One*, ***11***(1), e0146934.

Waugh, C. A., Timms, P., Andrew, D., Rawlinson, G., Brumm, J., Nilsson, K., & Beagley, K. W. (2015). Comparison of subcutaneous versus intranasal immunization of male koalas (Phascolarctos cinereus) for induction of mucosal and systemic immunity against Chlamydia pecorum. *Vaccine*, ***33***(7), 855-860. https://doi.org/https://doi.org/10.1016/j.vaccine.2014.12.052

Webster, K., Narayan, E., & De Vos, N. (2017). Fecal glucocorticoid metabolite response of captive koalas (Phascolarctos cinereus) to visitor encounters. *General and Comparative Endocrinology*, ***244***, 157-163.

Wedrowicz, F., Saxton, T., Mosse, J., Wright, W., & Hogan, F. E. (2016). A non-invasive tool for assessing pathogen prevalence in koala (Phascolarctos cinereus) populations: detection of Chlamydia pecorum and koala retrovirus (KoRV) DNA in genetic material sourced from scats. *Conserv Genet Resour*, ***8***, 511-521.

Weiskopf, S. R., Rubenstein, M. A., Crozier, L. G., Gaichas, S., Griffis, R., Halofsky, J. E., Hyde, K. J. W., Morelli, T. L., Morisette, J. T., Muñoz, R. C., Pershing, A. J., Peterson, D. L., Poudel, R., Staudinger, M. D., Sutton-Grier, A. E., Thompson, L., Vose, J., Weltzin, J. F., & Whyte, K. P. (2020). Climate change effects on biodiversity, ecosystems, ecosystem services, and natural resource management in the United States. *Science of The Total Environment*, ***733***, 137782. https://doi.org/https://doi.org/10.1016/j.scitotenv.2020.137782

Whisson, D. A., Dixon, V., Taylor, M. L., & Melzer, A. (2016). Failure to respond to food resource decline has catastrophic consequences for koalas in a high-density population in southern Australia. *PLoS One*, ***11***(1), e0144348.

Whisson, D. A., Zylinski, S., Ferrari, A., Yokochi, K., & Ashman, K. R. (2020). Patchy resources and multiple threats: How do koalas navigate an urban landscape? *Landscape and Urban Planning*, ***201***, 103854.

White, D. W., Suzanne Beard, R., & Barton, E. S. (2012). Immune modulation during latent herpesvirus infection. *Immunological Reviews*, ***245***(1), 189-208. https://doi.org/https://doi.org/10.1111/j.1600-065X.2011.01074.x

White, R. T., Legione, A. R., Taylor-Brown, A., Fernandez, C. M., Higgins, D. P., Timms, P., & Jelocnik, M. (2021). Completing the Genome Sequence of Chlamydia pecorum Strains MC/MarsBar and DBDeUG: New Insights into This Enigmatic Koala (Phascolarctos cinereus) Pathogen. *Pathogens*, ***10***(12), 1543. https://www.mdpi.com/2076-0817/10/12/1543

Wiertsema, S. P., van Bergenhenegouwen, J., Garssen, J., & Knippels, L. M. J. (2021). The Interplay between the Gut Microbiome and the Immune System in the Context of Infectious Diseases throughout Life and the Role of Nutrition in Optimizing Treatment Strategies. *Nutrients*, ***13***(3), 886. https://www.mdpi.com/2072-6643/13/3/886

Wilkinson, R., Allanson, M., Kolega, V., Lawrence, D., & Neville, S. (1991). Purification and initial characterisation of koala immunoglobulins. *Veterinary Immunology and Immunopathology*, ***29***(1-2), 189-195.

Wilkinson, R., Barton, M., & Kotlarski, I. (1995). Identification of koala T lymphocytes using an anti-human CD3 antibody. *Dev Comp Immunol*, ***19***(6), 537-545.

Wilkinson, R., Kotlarski, I., & Barton, M. (1992a). Koala lymphoid cells: analysis of antigen-specific responses. *Vet Immunol Immunopathol*, ***33***(3), 237-247.

Wilkinson, R., Kotlarski, I., & Barton, M. (1994). Further characterisation of the immune response of the koala. *Vet Immunol Immunopathol*, ***40***(4), 325-339.

Wilkinson, R., Kotlarski, I., Barton, M., & Phillips, P. (1992b). Isolation of koala lymphoid cells and their in vitro responses to mitogens. *Vet Immunol Immunopathol*, ***31***(1-2), 21-33.

Wilkinson, V., Richards, S. A., Burgess, L., Næsborg-Nielsen, C., Gutwein, K., Vermaak, Y., Mounsey, K., & Carver, S. (2024). Adaptive interventions for advancing in situ wildlife

disease management. *Ecological Applications*, ***34***(6), e3019. https://doi.org/https://doi.org/10.1002/eap.3019

Williams, N., Walling, D., & Leeks, G. (2008). An analysis of the factors contributing to the settling potential of fine fluvial sediment. *Hydrological Processes: An International Journal*, ***22***(20), 4153-4162.

Wilson, D. P., Craig, A. P., Hanger, J., & Timms, P. (2015). The Paradox of Euthanizing Koalas *(Phascolarctos cinereus)* to Save Populations from Elimination. *J Wildl Dis*, ***51***(4), 833-842. https://doi.org/10.7589/2014-12-278

Wilton, Z. E., Jamus, A. N., Core, S. B., & Frietze, K. M. (2025). Pathogenic and Protective Roles of Neutrophils in Chlamydia trachomatis Infection. *Pathogens*, ***14***(2), 112.

Witt, R. R., Beranek, C. T., Howell, L. G., Ryan, S. A., Clulow, J., Jordan, N. R., Denholm, B., & Roff, A. (2020). Real-time drone derived thermal imagery outperforms traditional survey methods for an arboreal forest mammal. *PLoS One*, ***15***(11), e0242204.

Woods, C. W., McClain, M. T., Chen, M., Zaas, A. K., Nicholson, B. P., Varkey, J., Veldman, T., Kingsmore, S. F., Huang, Y., Lambkin-Williams, R., Gilbert, A. G., Hero, A. O., III, Ramsburg, E., Glickman, S., Lucas, J. E., Carin, L., & Ginsburg, G. S. (2013). A Host Transcriptional Signature for Presymptomatic Detection of Infection in Humans Exposed to Influenza H1N1 or H3N2. *PLoS One*, ***8***(1), e52198. https://doi.org/10.1371/journal.pone.0052198

Wright, B. R., Casteriano, A., Muir, Y. S. S., Hulse, L., Simpson, S. J., Legione, A. R., Vaz, P. K., Devlin, J. M., Krockenberger, M. B., & Higgins, D. P. (2024). Expanding the known distribution of phascolartid gammaherpesvirus 1 in koalas to populations across Queensland and New South Wales. *Sci Rep*, ***14***(1), 1223. https://doi.org/10.1038/s41598-023-50496-4

Wright, B. R., Jelocnik, M., Casteriano, A., Muir, Y. S. S., Legione, A. R., Vaz, P. K., Devlin, J. M., & Higgins, D. P. (2023). Development of diagnostic and point of care assays for a gammaherpesvirus infecting koalas. *PLoS One*, ***18***(6), e0286407. https://doi.org/10.1371/journal.pone.0286407

WTOB. (2020). *Code of Practice: Care of Sick, Injured or Orphaned Protected Animals in Queensland, Nature Conservation Act 1992*. Brisbane: Queensland Government, Department of Environment and Science. Wildlife and Threatened Species Operations Branch

Wu, X., Lu, Y., Dong, Y., Zhang, G., Zhang, Y., Xu, Z., Culley, D. J., Crosby, G., Marcantonio, E. R., Tanzi, R. E., & Xie, Z. (2012). The inhalation anesthetic isoflurane increases levels of proinflammatory TNF-α, IL-6, and IL-1β. *Neurobiology of Aging*, ***33***(7), 1364-1378. https://doi.org/https://doi.org/10.1016/j.neurobiolaging.2010.11.002

Xiang, W., Yu, N., Lei, A., Li, X., Tan, S., Huang, L., & Zhou, Z. (2021). Insights Into Host Cell Cytokines in Chlamydia Infection [Review]. *Frontiers in Immunology*, ***12***. https://doi.org/10.3389/fimmu.2021.639834

Xiong, H., Pandey, G., Steinbach, M., & Kumar, V. (2006). Enhancing data analysis with noise removal. *IEEE transactions on knowledge and data engineering*, ***18***(3), 304-319.

Xu, W., Anwaier, A., Ma, C., Liu, W., Tian, X., Su, J., Zhu, W., Shi, G., Wei, S., & Xu, H. (2021). Prognostic immunophenotyping clusters of clear cell renal cell carcinoma defined by the unique tumor immune microenvironment. *Frontiers in Cell and Developmental Biology*, ***9***, 785410.

Xu, W., Gorman, K., Santiago, J. C., Kluska, K., & Eiden, M. V. (2015). Genetic diversity of koala retroviral envelopes. *Viruses*, ***7***(3), 1258-1270.

Xu, W., Stadler, C. K., Gorman, K., Jensen, N., Kim, D., Zheng, H., Tang, S., Switzer, W. M., Pye, G. W., & Eiden, M. V. (2013). An exogenous retrovirus isolated from koalas with malignant neoplasias in a US zoo. *Proceedings of the National Academy of Sciences*, ***110***(28), 11547-11552.

Yabsley, M. J. (2019). The Role of Wildlife Rehabilitation in Wildlife Disease Research and Surveillance. In *Medical Management of Wildlife Species* (pp. 159-165). https://doi.org/https://doi.org/10.1002/9781119036708.ch13

Yang, C., Kari, L., Lei, L., Carlson, J. H., Ma, L., Couch, C. E., Whitmire, W. M., Bock, K., Moore, I., Bonner, C., McClarty, G., & Caldwell, H. D. (2020). Chlamydia trachomatis Plasmid Gene Protein 3 Is Essential for the Establishment of Persistent Infection and Associated Immunopathology. *mBio*, ***11***(4). https://doi.org/10.1128/mBio.01902-20

Yang, R., Jacobson, C., Gardner, G., Carmichael, I., Campbell, A. J., & Ryan, U. (2014). Longitudinal prevalence and faecal shedding of Chlamydia pecorum in sheep. *The veterinary journal*, ***201***(3), 322-326.

Yang, X., Gartner, J., Zhu, L., Wang, S., & Brunham, R. C. (1999). IL-10 gene knockout mice show enhanced Th1-like protective immunity and absent granuloma formation following Chlamydia trachomatis lung infection. *The Journal of Immunology*, ***162***(2), 1010-1017.

Yap, S. H., Abdullah, N. K., McStea, M., Takayama, K., Chong, M. L., Crisci, E., Larsson, M., Azwa, I., Kamarulzaman, A., Leong, K. H., Woo, Y. L., & Rajasuriar, R. (2017). HIV/Human herpesvirus co-infections: Impact on tryptophan-kynurenine pathway and immune reconstitution. *PLoS One*, ***12***(10), e0186000. https://doi.org/10.1371/journal.pone.0186000

Ye, F., Lei, X., & Gao, S.-J. (2011a). Mechanisms of Kaposi' s sarcoma-associated herpesvirus latency and reactivation. *Advances in virology*, ***2011***(1), 193860.

Ye, F., Zhou, F., Bedolla, R. G., Jones, T., Lei, X., Kang, T., Guadalupe, M., & Gao, S.-J. (2011b). Reactive oxygen species hydrogen peroxide mediates Kaposi's sarcoma-associated herpesvirus reactivation from latency. *PLOS Pathogens*, ***7***(5), e1002054.

Yeung, A. T. Y., Hale, C., Lee, A. H., Gill, E. E., Bushell, W., Parry-Smith, D., Goulding, D., Pickard, D., Roumeliotis, T., Choudhary, J., Thomson, N., Skarnes, W. C., Dougan, G., & Hancock, R. E. W. (2017). Exploiting induced pluripotent stem cell-derived macrophages to unravel host factors influencing Chlamydia trachomatis pathogenesis. *Nat Commun*, ***8***, 15013. https://doi.org/10.1038/ncomms15013

Yin, Q., Yang, Z., Chong, S. W., Li, J., Liu, X., Vigolo, D., Li, J. J., Sheehy, P. A., & Yong, K. T. (2025). Application of microfluidic technologies in veterinary science with a view toward development of animal-on-a-chip models. *View*, ***6***(1), 20240073.

Young, G. R., Stoye, J. P., & Kassiotis, G. (2013). Are human endogenous retroviruses pathogenic? An approach to testing the hypothesis. *BioEssays*, ***35***(9), 794-803. https://doi.org/https://doi.org/10.1002/bies.201300049

Youngentob, K. N., Lindenmayer, D. B., Marsh, K. J., Krockenberger, A. K., & Foley, W. J. (2021). Food intake: an overlooked driver of climate change casualties? *Trends in Ecology & Evolution*, ***36***(8), 676-678. https://doi.org/https://doi.org/10.1016/j.tree.2021.04.003

Yu, M.-W., Yeh, S.-H., Chen, P.-J., Liaw, Y.-F., Lin, C.-L., Liu, C.-J., Shih, W.-L., Kao, J.-H., Chen, D.-S., & Chen, C.-J. (2005). Hepatitis B virus genotype and DNA level and hepatocellular carcinoma: a prospective study in men. *Journal of the National Cancer Institute*, ***97***(4), 265-272.

Zadka, Ł., Kulus, M. J., Kurnol, K., Piotrowska, A., Glatzel-Plucińska, N., Jurek, T., Czuba, M., Nowak, A., Chabowski, M., Janczak, D., & Dzięgiel, P. (2018). The expression of IL10RA in colorectal cancer and its correlation with the proliferation index and the clinical stage of the disease. *Cytokine*, ***110***, 116-125. https://doi.org/https://doi.org/10.1016/j.cyto.2018.04.030

Zannas, A. S., Jia, M., Hafner, K., Baumert, J., Wiechmann, T., Pape, J. C., Arloth, J., Ködel, M., Martinelli, S., & Roitman, M. (2019). Epigenetic upregulation of FKBP5 by aging and stress contributes to NF-κB–driven inflammation and cardiovascular risk. *Proceedings of the National Academy of Sciences*, ***116***(23), 11370-11379.

Zarinsefat, A., Dobi, D., Kelly, Y. M., Szabo, G., Henrich, T., Laszik, Z. G., & Stock, P. G. (2025). An Enhanced Role of Innate Immunity in the Immune Response After Kidney Transplant in People Living With HIV: A Transcriptomic Analysis. *Transplantation*, ***109***(1), 153-160. https://doi.org/10.1097/tp.0000000000005096

Zdyrski, C., Gabriel, V., Gessler, T. B., Ralston, A., Sifuentes-Romero, I., Kundu, D., Honold, S., Wickham, H., Topping, N. E., & Sahoo, D. K. (2024). Establishment and characterization of turtle liver organoids provides a potential model to decode their unique adaptations. *Communications biology*, ***7***(1), 218.

Zgajnar, N. R., De Leo, S. A., Lotufo, C. M., Erlejman, A. G., Piwien-Pilipuk, G., & Galigniana, M. D. (2019). Biological Actions of the Hsp90-binding Immunophilins FKBP51 and FKBP52. *Biomolecules*, ***9***(2). https://doi.org/10.3390/biom9020052

Zhang, D. J., X. Yang, C. Shen, and R. C. Brunham. (1999). Characterization of immune responses following intramuscular DNA immunization with the MOMP gene of Chlamydia trachomatis mouse pneumonitis strain. *Immunology*, ***96***(2), 314-321. https://doi.org/https://doi.org/10.1046/j.1365-2567.1999.00682.x

Zhang, H. R., Lai, S. Y., Huang, L. J., Zhang, Z. F., Liu, J., Zheng, S. R., Ding, K., Bai, X., & Zhou, J. Y. (2018). Myosin 1b promotes cell proliferation, migration, and invasion in cervical cancer. *Gynecol Oncol*, ***149***(1), 188-197. https://doi.org/10.1016/j.ygyno.2018.01.024

Zhang, R., Bloch, N., Nguyen, L. A., Kim, B., & Landau, N. R. (2014). SAMHD1 Restricts HIV-1 Replication and Regulates Interferon Production in Mouse Myeloid Cells. *PLoS One*, ***9***(2), e89558. https://doi.org/10.1371/journal.pone.0089558

Zhao, L., Lundy, S. R., Eko, F. O., Igietseme, J. U., & Omosun, Y. O. (2023a). Genital tract microbiome dynamics are associated with time of Chlamydia infection in mice. *Scientific reports*, ***13***(1), 9006. https://doi.org/10.1038/s41598-023-36130-3

Zhao, X., Li, Z., Guo, Y., Liu, Q., Qing, M., Sheng, Y., Chen, Y., Xie, L., & Zhou, Z. (2023b). Alfaxalone Alleviates Neurotoxicity and Cognitive Impairment Induced by Isoflurane Anesthesia in Offspring Rats. *Anesthesia & Analgesia*, ***136***(6). https://journals.lww.com/anesthesia-analgesia/fulltext/2023/06000/alfaxalone_alleviates_neurotoxicity_and_cognitive.25.aspx

Zheng, D., Liwinski, T., & Elinav, E. (2020a). Inflammasome activation and regulation: toward a better understanding of complex mechanisms. *Cell Discovery*, ***6***(1), 36. https://doi.org/10.1038/s41421-020-0167-x

Zheng, H., Pan, Y., Tang, S., Pye, G. W., Stadler, C. K., Vogelnest, L., Herrin, K. V., Rideout, B. A., & Switzer, W. M. (2020b). Koala retrovirus diversity, transmissibility, and disease associations. *Retrovirology*, ***17***, 1-23.

Zheng, Y., Zhao, W.-M., Wang, H., Zhou, Y.-B., Luan, Y., Qi, M., Cheng, Y.-Z., Tang, W., Liu, J., Yu, H., Yu, X.-P., Fan, Y.-J., & Yang, X. (2007). Codon usage bias in Chlamydia trachomatis and the effect of codon modification in the MOMP gene on immune responses to vaccination. *Biochemistry and Cell Biology*, ***85***(2), 218-226. https://doi.org/10.1139/o06-211 %M 17534403

Zhong, G. (2017). Chlamydial plasmid-dependent pathogenicity. *Trends in microbiology*, ***25***(2), 141-152.

Zhou, L., Lopez Rodas, A., Llangarí, L. M., Romero Sandoval, N., Cooper, P., & Sadiq, S. T. (2022). Single gene targeted nanopore sequencing enables simultaneous identification and antimicrobial resistance detection of sexually transmitted infections. *PLoS One*, ***17***(1), e0262242.

Zhou, Z., Tian, Q., Wang, L., Xue, M., Xu, D., & Zhong, G. (2021). Chlamydia Spreads to the Large Intestine Lumen via Multiple Pathways. *Infect Immun*, ***89***(10), e0025421. https://doi.org/10.1128/iai.00254-21

Zimmer, C., Jimeno, B., & Martin, L. B. (2024). HPA flexibility and FKBP5: promising physiological targets for conservation. *Philos Trans R Soc Lond B Biol Sci*, ***379***(1898), 20220512. https://doi.org/10.1098/rstb.2022.0512

Zimmerman, J. E., Kramer, A. A., McNair, D. S., & Malila, F. M. (2006). Acute Physiology and Chronic Health Evaluation (APACHE) IV: Hospital mortality assessment for today's critically ill patients*. *Critical Care Medicine*, ***34***, 1297-1310.

Chapter 7 Supplementary Materials

Chapter 1 Supplementary materials:

Table 7.1: Summary of koala admission outcomes and trends in chlamydiosis admissions from retrospective studies

	Study 1[1]	Study 2[2]	Study 3[3]	Study 4[4]	Study 5[5]	Study 6[6]	Study 7[7]	Study 8[8]	Study 9[9]	NSW DPEI[5]
Study clinics	AZWH, CWSH, RSPCA, MKH	AZWH, CWSH, MKH	AZWH, CWSH, RSPCA, MKH	FOK	PMKH, PSK, FOK	AKWC	PMKH	11 sources	PMKH	40 sources
State	Qld	Qld	Qld	NSW	NSW	SA	NSW	NSW	NSW	NSW
Time period	1997-2013	1997-2019	2009-2014	1988-2020	1989-2018	2014-2021	1975-2004	1973-2020	2014-2022	2014-2022
Total koala admissions	29,442	24,024	10,139	5,051	12,543	214	3,781	18,039	1227	9010
No observations with outcomes	24,379	20,250	10,139	5,017	12,543	214	NA	18,039	1227	9010
Died*	67.3%	81.9%	66.5%	63.8%	35.7%	32.7%	NA	41.9%	45.7%	45%
Treated/Released	22.5%	17.2%	27%	29.2%	20.7%	59.8%	NA	51.6%	54.3%	29%
Not treated & released	8.7%	NA	NA	NA	17.30%	NA	NA	NA	NA	NA
Other fates	NA	NA	NA	6.7%	NA	NA	NA	NA	NA	26%
Total chlamydial disease related koala admissions (%)	48%	52%	22%	43%	52%	24%	20%	43%	21.8%	18.7%
Died*	78.5%	61%	NA	73%	NA	NA	NA	NA	53.1%	70.6%
Released	21.5%	39%	NA	27%	NA	NA	NA	NA	46.9%	24.7%
Other fates	NA	NA	NA	NA	NA	NA	NA	NA	NA	4.7%
Greater frequency of female disease	P < 0.001	NA	NA	P < 0.001	NA	NA	P < 0.001	NA	NA	NA
Greater frequency of female mortality	P < 0.001	P < 0.001	NA	P < 0.001	NA	NA	NA	NA	NA	NA

*Died: NSW data groups all cases that have 'died', whereas other studies specify those that 'died on arrival' and those which were euthanised. Here these are combined for simplicity.
1:(Lunney *et al.*, 2022), 2:(Charalambous & Narayan, 2020), 3:(Pahuja & Narayan, 2023), 4:(Griffith & Higgins, 2012), 5:(Lunney *et al.*, 2023), 6:(Dutton-Regester, 2024), 7:(Casadevall & Pirofski, 2003), 8:(DCCEEW, 2022), 9:(ICUN, 2022), NSW DPEI: Rehabilitation Dashboard Data. Last updated 2022.

Table 7.2: Summary of associations between immune genes, clinical signs, and infectious statuses in koalas derived from the literature

Biomarker Category	Immune gene	Study Cohort	Sample	Association	Study
Chemokine	GRO	Human donated PBMCs	KoRV stimulated PBMCs	- Upregulated expression when exposing human PBMCs to KoRV *in vitro*	(Fiebig et al., 2006)
Chemokine	IL-8	69 wild-caught northern (SE-Qld) koalas vaccinated with a synthetic peptide MOMP vaccine (Chlamydia vaccine, N = 23), or a Chlamydia & KoRV Vaccine (N = 22), or not vaccinated (N = 23)	Urogenital mucosa swabs mRNA transcription (NanoString)	- Not differentially expressed between study groups - Chlamydia vaccine, Chlamydia & KoRV Vaccine, or Unvaccinated - throughout the study period	(Quigley et al., 2023)
Chemokine	MCP-1	Human donated PBMCs	KoRV stimulated PBMCs	- Upregulated expression when exposing human PBMCs to KoRV *in vitro*	(Fiebig et al., 2006)
Cytokine	IFN-γ	11-13 captive northern koalas with no history of chlamydial disease	PMAio PBMCs (5 hour incubation)	- Strongly up-regulated in August	(Maher & Higgins, 2016)
			PMAio/PMAPHA stimulated PBMCs	- Strongly up-regulated in August - Upregulated in KoRV A and KoRV B positive koalas compared to those which were only KoRV A positive - Association between KoRV B infection and increased up-regulation of IFNy dependent on sampling month - Differential gene expression not associated with KoRV A or KoRV B viral loads	
			Unstimulated PBMCs	- Greatest expression in April and December and lowest in February and August	
Cytokine	IFN-γ	74 wild southern koalas (Victoria)	Buffy coat cDNA RT-qPCR	- Low expression in Victorian koalas that were KoRV A positive compared to those which were KoRV A negative - Expression not associated with the Chlamydia infection status or any additional effect of dual infection with KoRV and Chlamydia infection, presence of pouch or back young, abnormal health status or age	(Maher et al., 2019)
Cytokine	IFN-γ	69 wild-caught northern (SE-Qld) koalas vaccinated with a synthetic peptide MOMP vaccine (Chlamydia vaccine, N = 23), or a Chlamydia & KoRV Vaccine (N = 22), or not vaccinated (N = 23)	Urogenital mucosa swabs mRNA transcription (NanoString)	- Not differentially expressed between study groups - Chlamydia vaccine, Chlamydia & KoRV Vaccine, or Unvaccinated - throughout the study period	(Quigley et al., 2023)
Cytokine	IFN-γ	43 wild-caught koalas from SE-Qld (Old Hidden Vale and Moreton Bay) that acquired a Chlamydial infection at some point during the monitoring period	Whole genome and RRS sequencing of DNA extracted from koala whole blood	- Associated with the resolution of *Chlamydia* infection	(Silver et al., 2022)

Biomarker Category	Immune gene	Study Cohort	Sample	Association	Study
Cytokine	IFN-γ	63 chlamydia free wild-caught northern koalas (SE-Qld) vaccinated with 3MOMP TriAdj vaccine (N = 21), PmpG TriAdj vaccine (N = 21), and PmpG vaccine (N = 15)	UV-inactivated *C. pecorum* G stimulated PBMCs prior to immunisation and then 5-7 months post-vaccination	- 3MOMP TriAdj vaccine and PmpG TriAdj vaccine induced a significant increase in IFN-γ - 86.6% of the PmpG vaccinated koalas exhibited an increase in IFN-γ post-vaccination	(Desclozeaux et al., 2017)
Cytokine	IFN-γ	15 wild-caught clinically healthy and qPCR *C. pecorum* negative northern koalas (SE-Qld) vaccinated with rMOMP + ISC adjuvant (N = 5) or the rMOMP + Tri-adjuvant (N = 10)	UV-inactivated C. pecorum G stimulated or PMAio stimulated PBMCs (pre-stimulated, 12, and 24 hours post-stimulation) prior to immunisation and then 2 and 6 months post-vaccination	- 6/10 for Tri-Adj and 3/5 for ISC adjuvant produced IFN-γ at 2 or 6 months post vaccination in response to stimulation of PBMCs with UV-inactivated EBs	(Khan et al., 2016)
Cytokine	IFN-γ	40 wild-caught northern koalas (SE-Qld) MOMP vaccinated from Waugh et al., 2016 and Desclozeaux et al., 2017	UV-inactivated C. pecorum G stimulated PBMCs prior to immunisation and then 5-7 months post-vaccination	- No association between IFN-γ expression and urogenital chlamydial load or urogenital disease	(Lizárraga et al., 2020)
Cytokine	IFN-γ	4 male and 3 female koalas from a captive, chlamydia-free, KoRV-A positive collection, with no history of disease within the past 6 months	Unstimulated PBMCs	- Greatest upregulation using PMA-ionomycin - Con A and PMA-PHA also induced expression	(Maher et al., 2014)
Cytokine	IFN-γ	10 wild northern koalas admitted to AZWH (N = 4 with clinical chlamydiosis)	UV-inactivated *C. pecorum* G stimulated PBMCs (unstimulated, 12, 24, and 48 h post-stimulation)	- Greater expression in PBMCs from animals in koalas with clinical signs of chlamydiosis compared to koalas without clinical signs of chlamydiosis (*C. pecorum* qPCR negative and +/- anti-MOMP IgG) - Expression comparable between koalas with clinical signs of chlamydiosis which were PCR negative by *C. pecorum* 16S rRNA PCR and PCR negative koalas in other groups (no clinical signs +/- anti-MOMP IgG). - Expression did not significantly differ in koalas without clinical chlamydiosis but with and without anti-MOMP IgG	(Mathew et al., 2013b)
Cytokine	IFN-γ	41 wild-caught northern koalas (SE-Qld), 30% affected by chlamydial disease	UV-inactivated *C. pecorum* G stimulated PBMCs (0, 12 and 24 h post-stimulation)	- Expression did not significantly differ between koalas with and without chlamydial disease	(Mathew et al., 2014)
Cytokine	IFN-γ	7 wild northern koalas admitted to AZWH for ocular disease	rMOMP G protein stimulated PBMCs	- Expression did not differ, regardless of natural infection and diseased state, at pre-vaccination and at six-weeks post-vaccination	(Nyari et al., 2019)

Biomarker Category	Immune gene	Study Cohort	Sample	Association	Study
Cytokine	IFN-γ	52 wild-caught northern (central NSW) koalas 50% vaccinated with a synthetic peptide MOMP vaccine	rMOMP genotype G stimulated koala buffy-coat samples taken at 0, 6, 12 and 18 months following vaccination	- Expression was not associated with treatment, sex, or the interaction of time and treatment - Expression was higher 12 months post vaccination	(Simpson et al., 2023a)
Cytokine	IL-10	Human donated PBMCs	KoRV stimulated PBMCs	- Upregulated expression when exposing human PBMCs to KoRV *in vitro*	(Fiebig et al., 2006)
Cytokine	IL-10	9 captive koalas (Japan)	Con-A stimulated PBMCs	- No significant differences between subtype profiles in gene expression after stimulation	(Kayesh et al., 2020)
			Unstimulated PBMCs	- Expression was markedly higher in one KoRV-B-positive, KoRV-C-negative individual (Koala also showed lymphoma) than in koalas with endogenous infection only (KoRV-A)	
Cytokine	IL-10	15 wild-caught clinically healthy and qPCR *C. pecorum* negative northern koalas (SE-Qld) vaccinated with rMOMP + ISC adjuvant (N = 5) or the rMOMP + Tri-adjuvant (N = 10)	UV-inactivated C. pecorum G stimulated or PMAio stimulated PBMCs (pre-stimulated, 12, and 24 hours post-stimulation) prior to immunisation and then 2 and 6 months post-vaccination	- No measurable expression following PBMCS stimulation from any koala's post vaccination	(Khan et al., 2016)
Cytokine	IL-10	11-13 captive northern koalas with no history of chlamydial disease	PMAio PBMCs (5 hour incubation)	- Up-regulated in August	(Maher & Higgins, 2016)
			PMAio/PMAPHA stimulated and unstimulated PBMCs	- Significantly greater up-regulation in 3/4 sampling periods in the KoRV B positive group but dependent on sampling month - Up-regulation in August - Baseline gene expression significantly higher in the KoRV B negative group in two sampling periods (April and December) - Differential gene expression not associated with KoRV A or KoRV B viral loads - Baseline gene expression greatest in April and December and lowest in February and August	
Cytokine	IL-10	4 male and 3 female koalas from a captive, chlamydia-free, KoRV-A positive collection, with no history of disease within the past 6 months	Unstimulated PBMCs	- Expression induced by PMA-ionomycin - PMA–PHA and Con A did not measurably increase its expression	(Maher et al., 2014)

Biomarker Category	Immune gene	Study Cohort	Sample	Association	Study
Cytokine	IL-10	74 wild southern koalas (Victoria)	Buffy coat cDNA RT-qPCR	- Differential expression not associated with KoRV A infection status - Male baseline expression significantly higher females. - Expression not associated with *C. pecorum* infection status or any additional effect of dual infection with KoRV and *C. pecorum* infection, the presence of pouch or back young, abnormal health status or age on expression	(Maher et al., 2019)
Cytokine	IL-10	10 wild northern koalas admitted to AZWH (N = 4 with clinical chlamydiosis)	UV-inactivated *C. pecorum* G stimulated PBMCs (unstimulated, 12, 24, and 48 h post-stimulation)	- Koalas with clinical signs of chlamydiosis had significantly higher expression levels compared to koalas without clinical signs of chlamydiosis (*C. pecorum* qPCR negative and +/- anti-MOMP IgG) - In koalas with clinical signs of chlamydiosis on average, IL10 mRNA expression levels were significantly higher than TNFα mRNA expression levels - In koalas without clinical signs of chlamydiosis +/- anti-MOMP IgG, no significant difference could be observed between TNFα and IL-10 mRNA expression	(Mathew et al., 2013a)
Cytokine	IL-10	41 wild-caught northern koalas (SE-Qld), 30% affected by chlamydial disease	UV-inactivated *C. pecorum* G stimulated PBMCs (0, 12 and 24 h post-stimulation)	- Expression did not significantly differ between koalas with and without chlamydial disease	(Mathew et al., 2014)
Cytokine	IL-10	69 wild-caught northern (SE-Qld) koalas vaccinated with a synthetic peptide MOMP vaccine (Chlamydia vaccine, N = 23), or a Chlamydia & KoRV Vaccine (N = 22), or not vaccinated (N = 23)	Urogenital mucosa swabs mRNA transcription (NanoString)	- Not differentially expressed between study groups - Chlamydia vaccine, Chlamydia & KoRV Vaccine, or Unvaccinated - throughout the study period	(Quigley et al., 2023)
Cytokine	IL-17A	9 captive koalas (Japan)	Con-A stimulated PBMCs Unstimulated PBMCs	- No significant differences between subtype profiles in gene expression before after stimulation	(Kayesh et al., 2020)
Cytokine	IL-17A	36 wild-caught northern koalas (SE-Qld) MOMP vaccinated from Waugh et al., 2016 and Desclozeaux et al., 2017	UV-inactivated *C. pecorum* G stimulated PBMCs prior to immunisation and then 5-7 months post-vaccination	- Decreased expression in koalas with greater chlamydial loads and clinical disease - MOMP vaccination increased expression - Urogenital and ocular chlamydial load negatively predicted expression - Expression was not associated with urogenital disease	(Lizárraga et al., 2020)
Cytokine	IL-17A	11-13 captive northern koalas with no history of chlamydial disease	PMAio PBMCs (5 hour incubation)	- Upregulated in December	(Maher & Higgins, 2016)

Biomarker Category	Immune gene	Study Cohort	Sample	Association	Study
			PMAio/PMAPHA stimulated PBMCs	- Expression greater in KoRV A and KoRV B positive koalas compared to only KoRV A positive koalas - Upregulated gene expression greatest in April and December and lowest in February and August - Association between KoRV B infection and increased up-regulation dependent on sampling month - Differential gene expression not associated with KoRV A or KoRV B viral loads	
			Unstimulated PBMCs	- Baseline gene expression greatest in April and December and lowest in February and August	
Cytokine	IL-17A	74 wild southern koalas (Victoria)	Buffy coat cDNA RT-qPCR	- Gene expression lower in KoRV A positive koalas compared to KoRV A negative koalas - Males had significantly higher resting expression - No effect of Chlamydia infection status or any additional effect of dual infection with KoRV and Chlamydia infection, the presence of pouch or back young, abnormal health status or age on expression	(Maher et al., 2019)
Cytokine	IL-17A	69 wild-caught northern (SE-Qld) koalas vaccinated with a synthetic peptide MOMP vaccine (Chlamydia vaccine, N = 23), or a Chlamydia & KoRV Vaccine (N = 22), or not vaccinated (N = 23)	Urogenital mucosa swabs mRNA transcription (NanoString)	- Expression significantly decreased in both the 'Chlamydia only' and 'Chlamydia and KoRV' vaccine groups 3–6 months post-vaccination and more so > 6 months post-vaccination	(Quigley et al., 2023)
Cytokine	IL-17A	52 wild-caught northern (central NSW) koalas 50% vaccinated with a synthetic peptide MOMP vaccine	rMOMP genotype G stimulated koala buffy-coat samples taken at 0, 6, 12 and 18 months following vaccination	- Expression was not associated with vaccination treatment, time, sex, or the interaction of time and treatment	(Simpson et al., 2023a)
Cytokine	IL-17A	63 chlamydia free wild-caught northern koalas (SE-Qld) vaccinated with 3MOMP TriAdj vaccine (N = 21), PmpG TriAdj vaccine (N = 21), and PmpG vaccine (N = 15)	UV-inactivated *C. pecorum* G stimulated PBMCs prior to immunisation and then 5-7 months post-vaccination	- 3MOMP TriAdj vaccine and PmpG TriAdj vaccine induced a significant increase in IL-17 - 100% of the PmpG vaccinated koalas exhibited an increase in IL-17 post-vaccination	(Desclozeaux et al., 2017)
Cytokine	IL-17A	15 wild-caught clinically healthy and qPCR *C. pecorum* negative northern koalas (SE-Qld) vaccinated with rMOMP + ISC adjuvant (N = 5) or the rMOMP + Tri-adjuvant (N = 10)	UV-inactivated *C. pecorum* G stimulated or PMAio stimulated PBMCs (pre-stimulated, 12, and 24 hours post-stimulation) prior to immunisation and then 2 and 6 months post-vaccination	- IL-17A responses were lower than IFN-γ, and only 40% of animals (4/10 Tri-Adj; 2/5 ISC) produced IL-17A responses to stimulation above 1.0 fold	(Khan et al., 2016)
Cytokine	IL-17A	41 wild-caught northern koalas (SE-Qld), 30% affected by chlamydial disease		- Differential expression between koalas with and without chlamydial disease	(Mathew et al., 2014)

Biomarker Category	Immune gene	Study Cohort	Sample	Association	Study
			UV-inactivated *C. pecorum* G stimulated PBMCs (0, 12 and 24 h post-stimulation)	- Higher IL17A gene expression observed in koalas with active chlamydial urogenital disease than in animals with inactive disease	
Cytokine	IL-17A	7 wild northern koalas admitted to AZWH for ocular disease	rMOMP G protein stimulated PBMCs	- Expression did not differ, regardless of natural infection and diseased state, at pre-vaccination and at six-weeks post-vaccination	(Nyari et al., 2019)
Cytokine	IL-1β	69 wild-caught northern (SE-Qld) koalas vaccinated with a synthetic peptide MOMP vaccine (Chlamydia vaccine, N = 23), or a Chlamydia & KoRV Vaccine (N = 22), or not vaccinated (N = 23)	Urogenital mucosa swabs mRNA transcription (NanoString)	- Not differentially expressed between study groups - Chlamydia vaccine, Chlamydia & KoRV Vaccine, or Unvaccinated - throughout the study period	(Quigley et al., 2023)
Cytokine	IL-4	11-13 captive northern koalas with no history of chlamydial disease	PMAio PBMCs (5 hour incubation)	- Upregulated in December	(Maher & Higgins, 2016)
			PMAio/PMAPHA stimulated PBMCs	- Upregulated in koalas with KoRV B infection compared to no KoRV B infection - Upregulated gene expression in stimulated koala PBMCs greatest in April and December and lowest in February and August - Association between KoRV B infection and increased up-regulation on sampling month - Differential gene expression not associated with KoRV A or KoRV B viral loads	
			Unstimulated PBMCs	- Baseline gene expression greatest in April and December and lowest in February and August	
Cytokine	IL-4	4 male and 3 female koalas from a captive, chlamydia-free, KoRV-A positive collection, with no history of disease within the past 6 months	Unstimulated PBMCs	- Most upregulated by PMA-ionomycin followed by Con A - PMA-PHA did not induce expression.	(Maher et al., 2014)
Cytokine	IL-4	69 wild-caught northern (SE-Qld) koalas vaccinated with a synthetic peptide MOMP vaccine (Chlamydia vaccine, N = 23), or a Chlamydia & KoRV Vaccine (N = 22), or not vaccinated (N = 23)	Urogenital mucosa swabs mRNA transcription (NanoString)	- Not differentially expressed between study groups - Chlamydia vaccine, Chlamydia & KoRV Vaccine, or Unvaccinated - throughout the study period	(Quigley et al., 2023)
Cytokine	IL-6	Human donated PBMCs	KoRV stimulated PBMCs	- Upregulated expression when exposing human PBMCs to KoRV *in vitro*	(Fiebig et al., 2006)
Cytokine	IL-6	9 captive koalas (Japan)	Con-A stimulated PBMCs	- No significant differences between subtype profiles in gene expression after stimulation	(Kayesh et al., 2020)
			Unstimulated PBMCs	- Significantly higher in koalas positive for both exogenous subtypes (KoRV-B and KoRV-C) than in those with endogenous infection only	

Biomarker Category	Immune gene	Study Cohort	Sample	Association	Study
Cytokine	IL-6	11-13 captive northern koalas with no history of chlamydial disease	PMAio PBMCs (5 hour incubation)	- Upregulated in August	(Maher & Higgins, 2016)
			PMAio/PMAPHA stimulated PBMCs	- Upregulation greatest in August - Up-regulation in stimulated PBMCs of KoRV B infected captive northern koalas compared to KoRV B negative Association between KoRV B infection and increased up-regulation of IL-6 dependent on sampling month - Differential gene expression not associated with KoRV A or KoRV B viral loads	
			Unstimulated PBMCs	- Baseline gene expression greatest in December	
Cytokine	IL-6	4 male and 3 female koalas from a captive, chlamydia-free, KoRV-A positive collection, with no history of disease within the past 6 months	Unstimulated PBMCs	- No consistent or significant change in expression with any protocol (PMA-ionomycin, Con A, or PMA-PHA)	(Maher et al., 2014)
Cytokine	IL-6	7 wild northern koalas admitted to AZWH for ocular disease	rMOMP G protein stimulated PBMCs	- Expression did not differ, regardless of natural infection and diseased state, at pre-vaccination and at six-weeks post-vaccination	(Nyari et al., 2019)
Cytokine	IL-6	69 wild-caught northern (SE-Qld) koalas vaccinated with a synthetic peptide MOMP vaccine (Chlamydia vaccine, N = 23), or a Chlamydia & KoRV Vaccine (N = 22), or not vaccinated (N = 23)	Urogenital mucosa swabs mRNA transcription (NanoString)	- Not differentially expressed between study groups - Chlamydia vaccine, Chlamydia & KoRV Vaccine, or Unvaccinated - throughout the study period	(Quigley et al., 2023)
Cytokine	TNF-α	15 wild-caught clinically healthy and qPCR *C. pecorum* negative northern koalas (SE-Qld) vaccinated with rMOMP + ISC adjuvant (N = 5) or the rMOMP + Tri-adjuvant (N = 10)	UV-inactivated C. pecorum G stimulated or PMAio stimulated PBMCs (pre-stimulated, 12, and 24 hours post-stimulation) prior to immunisation and then 2 and 6 months post-vaccination	- No measurable expression following PBMCS stimulation from any koala's post vaccination	(Khan et al., 2016)
Cytokine	TNF-α	11-13 captive northern koalas with no history of chlamydial disease	PMAio PBMCs (5 hour incubation)	- Upregulation in December	(Maher & Higgins, 2016)
			PMAio/PMAPHA stimulated PBMCs	- Upregulated in koalas with KoRV B infection compared to no KoRV B infection - Upregulated gene expression greatest in April and December and lowest in February and August - Association between KoRV B infection and increased up-regulation dependent on sampling month - Differential gene expression not associated with KoRV A or KoRV B viral loads	
			Unstimulated PBMCs	- Baseline gene expression greatest in April and December and lowest in February and August	

Biomarker Category	Immune gene	Study Cohort	Sample	Association	Study
Cytokine	TNF-α	10 wild northern koalas admitted to AZWH (N = 4 with clinical chlamydiosis)	UV-inactivated *C. pecorum* G stimulated PBMCs (unstimulated, 12, 24, and 48 h post-stimulation)	- PBMCs from animals in koalas with clinical signs of chlamydiosis had significantly higher expression levels when compared to koalas without clinical signs of chlamydiosis (*C. pecorum* qPCR negative and detectable anti-MOMP IgG) - Expression in koalas with clinical signs of chlamydiosis was comparable to that from koalas without clinical signs of chlamydiosis (*C. pecorum* qPCR negative and no anti-MOMP IgG)	(Mathew et al., 2013a)
Cytokine	TNF-α	41 wild-caught northern koalas (SE-Qld), 30% affected by chlamydial disease	UV-inactivated *C. pecorum* G stimulated PBMCs (0, 12 and 24 h post-stimulation)	- No statistically significant difference was observed for expression between koalas with and without chlamydial disease	(Mathew et al., 2014)
Cytokine	TNF-α	69 wild-caught northern (SE-Qld) koalas vaccinated with a synthetic peptide MOMP vaccine (Chlamydia vaccine, N = 23), or a Chlamydia & KoRV Vaccine (N = 22), or not vaccinated (N = 23), a total of 3 (one in each group) had clinical chlamydiosis	Urogenital mucosa swabs mRNA transcription (NanoString)	- Not differentially expressed between study groups - Chlamydia vaccine, Chlamydia & KoRV Vaccine, or Unvaccinated - throughout the study period	(Quigley et al., 2023)
Surface marker	CD3	79 hospital admitted northern koalas (SE-Qld)	Immunohistochemistry of 424 tissue samples	- Positively correlated with *C. pecorum* PCR loads within reproductive tract tissue of females and males - Negative correlation with the female genital tract gross pathology score	(Pagliarani et al., 2024)
Surface marker	CD4	9 captive koalas (Japan)	Con-A stimulated PBMCs Unstimulated PBMCs	- No significant differences between subtype profiles in gene expression before or after stimulation	(Kayesh et al., 2020)
Surface marker	CD4	4 male and 3 female koalas from a captive, chlamydia-free, KoRV-A positive collection, with no history of disease within the past 6 months	Unstimulated PBMCs	- Expression was significantly down-regulated with PMA–PHA stimulation	(Maher et al., 2014)
Surface marker	CD4	79 hospital admitted northern koalas (SE-Qld)	Immunohistochemistry of 424 tissue samples	- Positive correlation between *C. pecorum* PCR loads and CD4 scores in females and males - Statistically significant negative correlation with the female genital tract gross pathology score	(Pagliarani et al., 2024)
Surface marker	CD4	69 wild-caught northern (SE-Qld) koalas vaccinated with a synthetic peptide MOMP vaccine (Chlamydia vaccine, N = 23), or a Chlamydia & KoRV Vaccine (N = 22), or not vaccinated (N = 23), a total of 3 (one in each group) had clinical chlamydiosis	Urogenital mucosa swabs mRNA transcription (NanoString)	- Expression had decreasing trends, with > 6 months post-vaccination showing a statistically significant reduction in expression compared to baseline but not in the unvaccinated group	(Quigley et al., 2023)
Surface marker	CD4:CD8 ratio	9 captive koalas (Japan)	Con-A stimulated PBMCs	- Fold change in CD4:CD8b ratio (vs. unstimulated PBMCs) was markedly increased in one koala with	(Kayesh et al., 2020)

Biomarker Category	Immune gene	Study Cohort	Sample	Association	Study
				endogenous infection only (H6; KoRV-A positive) and one koala positive for both exogenous subtypes (H7; KoRV-A, -B, and -C positive)	
			Unstimulated PBMCs	- CD4:CD8b ratio in unstimulated koala PBMCs was markedly higher in the KoRV-B-positive, KoRV-C-negative individual (KM, which showed lymphoma) than in koalas with endogenous infection only (KoRV-A); however, this ratio showed no other significant differences between KoRV subtype infection profiles	
Surface marker	CD4:CD8 ratio	11-13 captive northern koalas with no history of chlamydial disease	PMAio/PMAPHA stimulated PBMCs	- Differential gene expression ratio not associated with KoRV A or KoRV B viral loads	(Maher & Higgins, 2016)
			Unstimulated PBMCs	- Indifferent between KoRV subtype profiles - Baseline gene expression ratio highest in August and lowest in April - No differences detected between sexes	
Surface marker	CD4:CD8 ratio	4 male and 3 female koalas from a captive, chlamydia-free, KoRV-A positive collection, with no history of disease within the past 6 months	Unstimulated PBMCs	- Expression levels of CD4 and CD8β are much higher than cytokine levels	(Maher et al., 2014)
Surface marker	CD4:CD8 ratio	74 wild southern koalas (Victoria)	Buffy coat cDNA RT-qPCR	- Lower in KoRV A positive compared to those which were KoRV A negative - No apparent effect of Chlamydia infection status or any additional effect of dual infection with KoRV and Chlamydia infection, the presence of pouch or back young, abnormal health status or age on expression	(Maher et al., 2019)
Surface marker	CD79b	79 hospital admitted northern koalas (SE-Qld)	Immunohistochemistry of 424 tissue samples	- Positively correlated with *C. pecorum* PCR loads in females and males - Negative correlation with the female genital tract gross pathology score	(Pagliarani et al., 2024)
Surface marker	CD8b	9 captive koalas (Japan)	Con-A stimulated PBMCs Unstimulated PBMCs	- No significant differences between subtype profiles in gene expression before or after stimulation	(Kayesh et al., 2020)
Surface marker	CD8b	4 male and 3 female koalas from a captive, chlamydia-free, KoRV-A positive collection, with no history of disease within the past 6 months	PMA-ionomycin, Con A, & PMA-PHA stimulated PBMCs	- Expression down-regulated with all mitogen stimulation protocols	(Maher et al., 2014)
Surface marker	CD8b	79 hospital admitted northern koalas (SE-Qld)	Immunohistochemistry of 424 tissue samples	- Positive correlations between *C. pecorum* PCR loads and CD8 scores in females and males - Negative correlation with the female genital tract gross pathology score	(Pagliarani et al., 2024)

Biomarker Category	Immune gene	Study Cohort	Sample	Association	Study
Surface marker	CD8b	69 wild-caught northern (SE-Qld) koalas vaccinated with a synthetic peptide MOMP vaccine (Chlamydia vaccine, N = 23), or a Chlamydia & KoRV Vaccine (N = 22), or not vaccinated (N = 23), a total of 3 (one in each group) had clinical chlamydiosis	Urogenital mucosa swabs mRNA transcription (NanoString)	- Expression had decreasing trends, with > 6 months post-vaccination showing a statistically significant reduction in expression compared to baseline but not in the unvaccinated group	(Quigley et al., 2023)
Surface marker	MHC I	101 wild-caught northern koalas (SE-Qld): 60 from Moreton Bay (30 *Chlamydia*-affected koalas and 30 additional healthy koalas) and 20 koalas from Hidden Vale (sexually mature koalas undergoing monitoring)	PCR amplification of DNA extracted from koala serum	- UC*01:01 identified in koalas with *C. pecorum infection* but no clinical disease (asymptomatic cases)	(Robbins et al. 2020)
Surface marker	MHC I	43 wild-caught koalas from SE-Qld (Old Hidden Vale and Moreton Bay) that acquired a Chlamydial infection at some point during the monitoring period	Whole genome and RRS sequencing of DNA extracted from koala whole blood	- UA*6 more prevalent in koalas that resolved Chlamydia infection (25%, 3/12) than those that did not resolve an infection (0%, 0/31) - Koalas that were heterozygous at UC were more likely to resolve a Chlamydia infection (50%, 6/12) than those that did not resolve an infection (9.68%, 3/31)	(Silver et al., 2022)
Surface marker	MHC II	79 hospital admitted northern koalas (SE-Qld)	Immunohistochemistry of 424 tissue samples	- *C. pecorum* PCR loads and HLA-DR scores were positively correlated in the female and male genital tracts. - Negative correlation with the female genital tract gross pathology score	(Pagliarani et al., 2024)
Surface marker	MHC II	101 wild-caught northern koalas (SE-Qld): 60 from Moreton Bay (30 *Chlamydia*-affected koalas and 30 additional healthy koalas) and 20 koalas from Hidden Vale (sexually mature koalas undergoing monitoring)	PCR amplification of DNA extracted from koala serum	- DAB*10 and UC*01:01 alleles associated with greater protection against chlamydial infection and disease - DBB*04 and DCB*03 alleles associated with increased susceptibility to chlamydiosis infection and disease	(Robbins et al. 2020)
Surface marker	MHC II	94 wild female koalas admitted to Port Macquarie Koala Hospital and originating from Port Macquarie and Hastings River districts on the mid-north coast of New South Wales, Australia	Isolation and sequencing of koala-specific DAB and DBB amplified through PCR of DNA samples that were extracted from ocular and/or urogenital swabs, separated blood cells, or liver	- Koalas with DBB*04 allele had higher levels of Chlamydia heat shock protein 60 (c-hsp60) antibody levels - DAB*10 frequently found in Chlamydia-infected koalas relative to non-infected koalas - DAB*10 associated with *C. pecorum* infection, past-infection, and persistence - DAB*15 associated with greater protection against chlamydial infection and disease	(Lau et al. 2014)
Surface marker	MHC II	57 wild-caught koalas from SE-Qld (Moreton Bay) with the most ocular microbiome reads maintained	Isolation and sequencing of Dab and DBb PCR amplicons from whole blood extracted DNA	- Absence of DBB*03 linked with chlamydial disease at any site	(Quigley et al. 2018)

Biomarker Category	Immune gene	Study Cohort	Sample	Association		Study
		a comparable representation of chlamydia disease states and KoRV-B results to the overall population				
Surface marker	MHC II	43 wild-caught koalas from SE-Qld (Old Hidden Vale and Moreton Bay) that acquired a Chlamydial infection at some point during the monitoring period	Whole genome and RRS sequencing of DNA extracted from koala whole blood	-	No associations with MHC Class II genes	(Silver et al., 2022)
Surface marker	MHC II	69 wild-caught northern (SE-Qld) koalas vaccinated with a synthetic peptide MOMP vaccine (Chlamydia vaccine, N = 23), or a Chlamydia & KoRV Vaccine (N = 22), or not vaccinated (N = 23), a total of 3 (one in each group) had clinical chlamydiosis	Urogenital mucosa swabs mRNA transcription (NanoString)	-	No consistent or significant trends in DAA or DBA allele diversity or detection frequency observed between study groups - Chlamydia vaccine, Chlamydia & KoRV Vaccine, or Unvaccinated - throughout the study period	(Quigley et al., 2023)

Chapter 1 Supplementary material references:

Casadevall, A., & Pirofski, L. A. (2003). The damage-response framework of microbial pathogenesis. *Nat Rev Microbiol*, ***1***(1), 17-24. https://doi.org/10.1038/nrmicro732

Charalambous, R., & Narayan, E. (2020). A 29-year retrospective analysis of koala rescues in New South Wales, Australia. *PLoS One*, ***15***(10), e0239182.

DCCEEW. (2022). *Conservation Advice for Phascolarctos cinereus (Koala) combined populations of Queensland, New South Wales and the Australian Capital Territory. In effect under the Environment Protection and Biodiversity Conservation Act 1999 from 12 February 2022.*: Australian Government Retrieved from http://www.environment.gov.au/biodiversity/threatened/species/pubs/85104-conservation-advice-12022022.pdf

Dutton-Regester, K. J. (2024). Koala admissions to a wildlife hospital in coastal New South Wales, Australia, over a nine-year period, 2014–2022. *Australian journal of zoology*, ***71***(6).

Griffith, J. E., & Higgins, D. P. (2012). Diagnosis, treatment and outcomes for koala chlamydiosis at a rehabilitation facility (1995-2005). *Aust Vet J*, ***90***(11), 457-463. https://doi.org/10.1111/j.1751-0813.2012.00963.x

ICUN. (2022). *Red List* http://www.iucnredlist.org

Lunney, D., Cope, H., Griffith, J., Orcheg, C., Bryant, J., & Haering, R. (2023). Trends in the rescue and fate of koalas in New South Wales (1973-2020), with a focus on disease and trauma. *Australian Zoologist*.

Lunney, D., Cope, H., Sonawane, I., Stalenberg, E., & Haering, R. (2022). An analysis of the long-term trends in the records of Friends of the Koala in north-east New South Wales: I. Cause and fate of koalas admitted for rehabilitation (1989–2020). *Pacific Conservation Biology*, ***29***(3), 177-196.

Pahuja, H. P., & Narayan, E. J. (2023). An 8-year long retrospective analysis identifies the major causes of morbidity and mortality in rescued koala joeys. *Wildlife Research*, ***50***(12), 1141-1153.

Chapter 2 Supplementary materials:

Table 7.3: Inclusion criteria for allocation of koala syndromes on admission

Clinical Criteria		Analytical Groups		
Admission Syndromes	**Clinical Signs**	**Chlamydiosis**	**Euthanasia?**	**Female Reproductive disease**
Chlamydiosis	1. Reproductive Disease (incl. ovarian bursal cyst(s), pyometra, uterine oedema, and endometriosis) 2. Cystitis 3. Renal Disease (incl. hydronephrosis, hydroureter) 4. Conjunctivitis +/- Wet bottom (incl. rump ulceration) +/- Candidiasis +/- *C. pecorum* LAMP positive +/- Fibrotic (irreversible) change to urogenital structures +/- Infection associated anaemia +/- Poor body condition / emaciation +/- Cataracts +/- Other (Pulmonary infection, growths, alopecia, pustules)	Chlamydiosis	Yes = Untreatable Chlamydiosis No = Treatable Chlamydiosis	Female = Yes
Chlamydiosis & Trauma	Any one or combination of clinical signs listed in 'Chlamydiosis' with at least one or more clinical signs listed in 'Trauma'			
Trauma	1. Superficial wounds 2. Abdominal and/or visceral trauma (incl. haemoabdomen, peritonitis, metabolic alkalosis) 3. Single or multiple fractures (incl. comminated, compound, and pathogenic fractures) 4. Wound associated septicaemia 5. Vestibular & neurological trauma 6. Trauma associated anaemia			Female = No
Other Disease	1. Hepatitis 2. Myelodysplasia 3. Mammary adenocarcinoma 4. Pulmonary Disease 5. Congenital renal disease +/- elevated SDMA 6. Congenital enophthalmos & Horners syndrome 7. Emaciation without evidence of chlamydiosis 8. Dysbiosis / Caecal Dysbiosis Typhlocolitis Syndrome	No clinical signs of chlamydiosis	NA	
No Disease	1. Nothing abnormal detected using any of the following diagnostic tools; visual assessment, ultrasonography, C. pecorum LAMP negative 2. Mended musculoskeletal abnormalities			

Table 7.4: NanoString Pathogen and House-Keeping Gene Target Summary

Gene	Full Name	Accession	Position	Target Sequence
PhaHV1 dpol	Herpesvirus 1 dpol	JN585829.1	71-170	GCTCCAAGGCCTTCATGGAATCCCTATCCACCTCAGATCTAGAATCAATAGTGGGACACACAGTCCCAAGC GAAAAGGACTCATCCCTTCGTGTCATCTA
PhaHV2 dpol	Herpesvirus 2 dpol	JQ996387.1	108-207	TCGCTCCTGTCCAAGCTCCTGACCACGTGGCTTGCCCGGCGCAAGGAGATCCGACGCCAACACGCGGCC TGCAACGATCCAGCTCTGCGCACTATTCTGG
CpecG_0573	Cpecorum MC/Marsbar strain	NZ_CM002310.1	209-308	TGGGAGTCTTCCTTACAACAGCCCTGCTTACGCTTTTTTGCCTCTGTGCCATCTTGAAGCCCAAAGCAATC TCAGGTAAGTCTGTAACCAAACCAGAGGT
pGP3	Cpecorum L1 strain	KT223773.1	4625-4724	CAATATTCGTTTGGGACAAATGACCAATGAATTGCAAGACAAGCAAATTATAATAGGCACACCAACTAC TCCTACTGTAACAAGCCTCTCTTCTTCTAAT
ompA	Cpecorum L17 strain ompA	GQ228181.1	19-118	TCGGCGTTTTTATCCGCCGCATTTTTTGCTGGTGACGCCTCCTTACACGCTTTGCCTGTAGGGAACCCAGC AGAGCCAAGTTTATTAATTGATGGGACGA
Cpec_hsp60	CPE1_RS04735, chaperonin GroEL	Cpec_hsp60.1	1084-1183	CCCTGGGGTTCTAGACGAGTAATTGTATCGGCTGGAGAAGAACCATAATAGCCGTGTTCTAACGTACAC AAGCGCACCAAAGAACAGAGAGATCGCTTTG
KoRVpol	KoRV pol	NC_039228.1	2901-3000	GGAATACCGATTACACGAAAAGCCGGTCCCTCCTTCTATCGACCCGTCATGGCTCCAACTCTTTCCCATG GTTTGGGCCGAGAAGGCAGGTATGGGACTG
KoRVAenvRBD	KoRV-A env	AF151794.2	6219-6318	CCCCTGATTCAAACTATGAACATGCTTATAATCAGATCACTTGGGGAACCCTGGGATGTAGCTATCCCCG GGCCAGAACAAGAATTGCTAGGTCCCAGTT
KoRVBenvRBD	KoRV-B env	AB822553.1	315-414	GGTGCTTTATAGTCAGGTCGGCAGGGGGTTAGTCAGGCAATCGAGCTATGGAACCTTGGGGTGTCGCT GTCCCCGAGACCGGAACAGACTGGCTCAATCT
KoRVDenvRBD	KoRV-D env	AB828004.1	121-220	AACCCTCACCAACCCATGACTCTCACCTGGCAGGTACTGTCCCAGACGGGAAGTGTCGTTTGGGAAAAG AAAGCAGTCGAGCCACCCTGGACGTGGTGGC
KoRVenvCKS17	KoRV env p15E	AF151794.2	7503-7602	TCCAGAATAGGAGAGGCCTTGATCTGCTATTTTTGAAGGAAGGGGGCCTTTGTGCAGCCCTAAAAGAG GAATGCTGTTTCTATGTTGACCACTCAGGCGC
Tcop18S	T. copemani 18s rRNA	GU966588.1	595-694	GTTGGTATTTAAAAGTCCATTGGAGATTATGGGGTCGTGTGACAAGCGGTCGGGCGTGTTGCTTTTTTG GTCTTCACGGCCAGGGCGCCGCCCGTCGCCC
Tirwini18S	T. irwini 18s rRNA	FJ649479.1	539-638	CAAGTCTGGTGCCAGCACCCGCGGTAATTCCAGCTCCAAAAGCGTATATTAATGCTGTTGCTGTTAAAG GGTTCGTAGTTGAACTGTGGGCCTTCAAGGC
Tgil18S	T. giletti 18s rRNA	GU966589.1	715-814	CGTGCACGGTGTGGTGCGAGGCACTTTGTGTGTTCCCGTCACCCACGCGCACGCCTCTTTCGGCTCGCG GCGCCCAGGAATGAAGGAGGGTAGTTCGGGG
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	XM_020994140.1	1136-1235	CACCACTGGGGGAGCCCACATTCCTAACTTAACGTTCCTGTACTGGGGATCTCATGTCCCCATTCGCATC CTTGTCCCAAAGCACCCCTGTAGTCTGGAG
ACTB	Beta-actin	XM_021008442.1	1805-1904	TTTACAGTCTCCCTGGGAGTTTTACGAGATTGGTGCCAGTACTTGGGGGAGGGGAGGAGCTTTACCTGT ACACTGACTTAAGACCAGTTCAAATAAAAGT
Stx12	Syntaxin 12	XM_020983277.1	674-773	AGTTGGCCATTACAGAGCAAGACCTTGAACTTATCAAGGAGCGAGAAACTGCGATCAGGCAACTGGAG GCCGACATTTTGGACGTCAATCAGATATTTAA
Nckap1l	Nck-associated protein 1-like	XM_020966159.1	2922-3021	CTGCCCATTCCTTATGGGCCCTATTGAGTGCCTGAAGGATTTCGTCACTCCAGATACAGATATAAAGGTG ACCATGAGTGTCTTTGAGCTGGCCTCTGCT

Table 7.5: qPCR Primer/Probe Set Information for Chlamydia Multiplex qPCR, PhaHV-1 & -2 qPCR, and KoRV pol qPCR

qPCR Assay	Gene	Amplicon size (bp)	End	Sequence	Reference
Chlamydia multiplex Probe qPCR	*Chlamydia* (23S rRNA)	*137*	Forward	5'-GCTCACCAATCGAGAATC-3'	(Hulse *et al.*, 2018)
		137	Reverse	5-'CCAACACTCCTTTCGGTA-3'	
		137	Probe	ROX-CTGAATACTACGCTCTCCTACCGC-BHQ2	
	C. pecorum (*ompB* gene)	*141*	Forward	5'–CCAAGCATAATCGTAACAA-3'	
		141	Reverse	5'-CGAAGCAAGATTCTTGTC-3'	
		141	Probe	FAM-ACTTGTTGGCAATTCTTCTCTTCACA-BHQ1	
	Koala *β-actin* mRNA	*145*	Forward	5'-CTCAGATTATGTTTGAGACCTTC-3'	
		145	Reverse	5'-CCTTCATAGATGGGCACA-3'	
		145	Probe	HEX-ACCATCACCAGAGTCCATCACAAT-BHQ1	
Phascolarctid herpesvirus 1 & 2 SYBR qPCR	PhaHV-1 *dpol*	*22*	Forward	5'-GGGAAGAACTATGTTGGAACGC-3'	(Wright *et al.*, 2023)
		20	Reverse	5'-TGAGTCCTTTTCGCTTGGGA-3'	
	PhaHV-2 *dpol*	*20*	Forward	5'-GGTGACGTGCAATTCAGTGT-3'	(Church *et al.*, 2025; Kasimov *et al.*, 2020)
		20	Reverse	5'-TTTCGAGCATCATGCGTCCT-3'	
	Koala *β-actin* mRNA	*145*	Forward	5'-CTCAGATTATGTTTGAGACCTTC-3'	(Hulse *et al.*, 2018)
		145	Reverse	5'-CCTTCATAGATGGGCACA-3'	
KoRV pol Probe qPCR	KoRV *pol*	*110*	Forward	5'-TTGGAGGAGGAATACCGATTACAC-3'	(Hulse *et al.*, 2018; Tarlinton *et al.*, 2005)
		110	Reverse	5'-GCCAGTCCCATACCTGCCTT-3'	
		110	Probe	FAM-TCGACCCGTCATGGC-BHQ1	
	Koala *β-actin* mRNA	*145*	Forward	5'-CTCAGATTATGTTTGAGACCTTC -3'	(Hulse *et al.*, 2018)
		145	Reverse	5'-CCTTCATAGATGGGCACA-3'	
		145	Probe	HEX-ACCATCACCAGAGTCCATCACAAT-BHQ1	

Chapter 2 Supplementary material references:

Church, C., Casteriano, A., Muir, Y. S. S., Krockenberger, M. B., Vaz, P. K., Higgins, D. P., & Wright, B. R. (2025). New insights into the range and transmission dynamics of a koala gammaherpesvirus, phascolarctid gammaherpesvirus 2. *Sci Rep*, ***In press.***

Hulse, L. S., Hickey, D., Mitchell, J. M., Beagley, K. W., Ellis, W., & Johnston, S. D. (2018). Development and application of two multiplex real-time PCR assays for detection and speciation of bacterial pathogens in the koala. *J Vet Diagn Invest*, ***30***(4), 523-529. https://doi.org/10.1177/1040638718770490

Kasimov, V., Stephenson, T., Speight, N., Chaber, A. L., Boardman, W., Easther, R., & Hemmatzadeh, F. (2020). Identification and Prevalence of Phascolarctid Gammaherpesvirus Types 1 and 2 in South Australian Koala Populations. *Viruses*, ***12***(9). https://doi.org/10.3390/v12090948

Tarlinton, R., Meers, J., Hanger, J., & Young, P. (2005). Real-time reverse transcriptase PCR for the endogenous koala retrovirus reveals an association between plasma viral load and neoplastic disease in koalas. *J Gen Virol*, ***86***(Pt 3), 783-787. https://doi.org/10.1099/vir.0.80547-0

Wright, B. R., Jelocnik, M., Casteriano, A., Muir, Y. S. S., Legione, A. R., Vaz, P. K., Devlin, J. M., & Higgins, D. P. (2023). Development of diagnostic and point of care assays for a gammaherpesvirus infecting koalas. *PLoS One*, ***18***(6), e0286407. https://doi.org/10.1371/journal.pone.0286407

Chapter 3 Supplementary materials:

Table 7.6: NanoString nCounter Probe Design

Well Position	Gene Target	Probe A Name	Probe A Sequence	Probe B Name	Probe B Sequence
A01	GAPDH	XM_020994140.1:1135_T001	ATCCCCAGTACAGGAACGTTAAGTTAGGAATGTGGGCTCCCCCA GTGGTGCCTCAAGACCTAAGCGACAGCGTGACCTTGTTTCA	XM_020994140.1:1135_ProbeB	CGAAAGCCATGACCTCCGATCACTCCTCCAGACTACAGGG GTGCTTTGGGACAAGGATGCGAATGGGGACATGAG
A02	ACTB	XM_021008442.1:1804_T002	TCCCCCAAGTACTGGCACCAATCTCGTAAAACTCCCAGGGAGAC TGTAAACATCCTCTTCTTTTCTTGGTGTTGAGAAGATGCTC	XM_021008442.1:1804_ProbeB	CGAAAGCCATGACCTCCGATCACTCACTTTTATTTGAACTG GTCTTAAGTCAGTGTACAGGTAAAGCTCCTCCCC
A03	Tmem97	XM_020963254.1:244_T003	AATGCCTGCACATACCACTTGAGCATGTCTGTCACCCACAATTCT GCGGGTTAGCAGGAAGGTTAGGGAAC	XM_020963254.1:244_ProbeB	CGAAAGCCATGACCTCCGATCACTCAAAGAAAGGACTTGA ACCAGCTTGGAGGGTTCAGCATCAGAGGGTCTTTA
A04	Stx12	XM_020983277.1:673_T004	AGTTTCTCGCTCCTTGATAAGTTCAAGGTCTTGCTCTGTAATGGCT GTTGAGATTATTGAGCTTCATCATGACCAGAAG	XM_020983277.1:673_ProbeB	CGAAAGCCATGACCTCCGATCACTCTTAAATATCTGATTGA CGTCCAAAATGTCGGCCTCCAGTTGCCTGATCGC
A05	Nckap1l	XM_020966159.1:2921_T005	GAGTGACGAAATCCTTCAGGCACTCAATAGGGCCCATAAGGAAT GGGCAGCAAAGACGCCTATCTTCCAGTTTGATCGGGAAACT	XM_020966159.1:2921_ProbeB	CGAAAGCCATGACCTCCGATCACTCAGAGGCCAGCTCAAA GACACTCATGGTCACCTTTATATCTGTATCTG
A06	IL1beta	XM_020963753.1:1051_T006	CCAAACTATTACCGTGATTTTCCCAGATTCTGCTCAGCCAGGCACT GAAACGAACCTAACTCCTCGCTACATTCCTATTGTTTTC	XM_020963753.1:1051_ProbeB	CGAAAGCCATGACCTCCGATCACTCCCTGCCTATCAGCTAT CTAGTGCTCAAACCTCCATCTTGAATA
A07	IL4	XM_020991403.1:166_T007	CTCAAAGATGTCCGGCACTTCCATCTCAAAGCAGGGAAACTTTTT CATAGCCAATTTGGTTTTACTCCCCTCGATTATGCGGAGT	XM_020991403.1:166_ProbeB	CGAAAGCCATGACCTCCGATCACTCCATGATGACTCCTCAG GAAACAGCTTCGGAGTACTGGAATTCTCTGTATC
A08	IL6	XM_020993366.1:449_T008	CTCACAATCCTTGGCAAGCATGTCTCCTCATTGAATCCAGTAGCTT TCGGGTTATATCTATCATTTACTTGACACCCT	XM_020993366.1:449_ProbeB	CGAAAGCCATGACCTCCGATCACTCTCATCTCTGTTTCCATG TACCGAAGATACGTGTCAAACTCCTGAAGACCA
A09	IL8	XM_020988483.1:279_T009	GCTCCCTGTTATCTTGAAGAGTGACAATGATTTCTGTGTTCGGGCA ACAGCCACTTTTTTTCCAAATTTTGCAAGAGCC	XM_020988483.1:279_ProbeB	CGAAAGCCATGACCTCCGATCACTCAAACACATGAAGAATT TTCTGCACCCACTTTGCTTGAGGGTCCAGGCAGA
A10	IL10	XM_021002936.1:518_T010	CTCATAGGTGCTCTTGACTTGCTTGACAACTCTGCTCCACCGTGTG GACGGCAACTCAGAGATAACGCATAT	XM_021002936.1:518_ProbeB	CGAAAGCCATGACCTCCGATCACTCAATATATCAAAATCCCC CATTGCTTTGTAGACTCCTCGTTCCTGGAGCTT
A11	IL12A	XM_020988856.1:865_T011	CTAAAGTCATCGGGTGAGGAGCCCTTCACGAAGAAGCGGTCAGG AAGCCTGGAGTTTATGTATTGCCAACGAGTTTGTCTTT	XM_020988856.1:865_ProbeB	CGAAAGCCATGACCTCCGATCACTCCCTGATGGTGACAATC TTTGAGATCCAGGCTTTACTGTTATCCCGAAGAT
A12	IL17A	XM_020993976.1:292_T012	CCTCGGCATTGATGCAGCCTGAATGACGGCACCTAGCAGATAAG GTTGTTATTGTGGAGGATGTTACTACA	XM_020993976.1:292_ProbeB	CGAAAGCCATGACCTCCGATCACTCGATTTCGACGGAGTTC ACGTGGTGGTCCACTTTCC
B01	IL18	XM_020989757.1:308_T013	TTTGGGGTGCATTTGCTTGAATCTCACAATCAGTCATATCCTCGAG TACACTTCCTTCCTGTGTTCCAGCTACAAACTTAGAAAC	XM_020989757.1:308_ProbeB	CGAAAGCCATGACCTCCGATCACTCTGCCAAGCCTTTGGGT ACAGTCTCTTTATAGGCTTGCATTATAAATACAG
B02	IL22	XM_020990310.1:159_T014	TTATCGAACAAACTGCCCTGTTGGGCCAGCGACATGATTCGGTGG CTAATCATAAAATTGGTTTTGCCTTTCAGCAATTCAACTT	XM_020990310.1:159_ProbeB	CGAAAGCCATGACCTCCGATCACTCCACATCACTGAACATGG AACTCTCGAGGAGTCGAGTGCTGGTTTCA
B03	INFgamma	XM_020990282.1:359_T015	GATATTCTCCACACTTTTTTTGATGACGGTGTTGTTCTTGAAGATTT CAACTGGTCAAGACTTGCATGAGGACCCGCAAATTCCT	XM_020990282.1:359_ProbeB	CGAAAGCCATGACCTCCGATCACTCACTTTGCTGGCAGTGTT GTTAGTGAAGAATTTCATGATCATATCCTCTTT
B04	TNFalpha	XM_020974214.1:440_T016	ATCTGACTCTCTGGGTCAGAGGTTCCATGCCAAGAAAATCTGTGG CTTTCGTTGGGACGCTTGAAGCGCAAGTAGAAAAC	XM_020974214.1:440_ProbeB	CGAAAGCCATGACCTCCGATCACTCATCAGCTACAACATGA GCTACAGGCTTGTTACTTTCAGTCTGACAGG
B05	PhciDAB	XR_002323139.1:731_T017	CAGCACGAGGCCCCCGACTCCACTCAGCATCTTACTCTGGCCAGC AGACCTGCAATATCAAAGTTATAAGCGCGT	XR_002323139.1:731_ProbeB	CGAAAGCCATGACCTCCGATCACTCTTCTGACTCCTCT TGTAGACAAAGAGGCCAACCCCAAAGAAGATCAGGCC
B06	PhciDBB	XM_020974227.1:1563_T018	CTAAGCTCTCCCCATTTATGGCATCCTCTCAATTCCACAGAATAAC AAGGCCTGCCAATGCACTCGATCTTGTCATTTTTTTGCG	XM_020974227.1:1563_ProbeB	CGAAAGCCATGACCTCCGATCACTCTCAGGAGAAAGA AACATTGTAGATTAACTTGGGTTTACTCAGCCCTCCCA
B07	MHCIUA	XM_020974926.1:289_T019	ACTCGGTAAGTCTGTGTGACCGCCTTCATGTTCCGCGTCTCCTGGC AAACTGGAGAGAGAAGTGAAGACGATTTAACCCA	XM_020974926.1:289_ProbeB	CGAAAGCCATGACCTCCGATCACTCCCTTCGCTCTGGT TGAAGTAGCCGCGGAGGTTCTGCAGGCTC

Well Position	Gene Target	Probe A Name	Probe A Sequence	Probe B Name	Probe B Sequence
B08	RETN	XM_020979876.1:0_T020	TGCCAGACTCTGGGATGACAGCACCTCTTGGTCCCCAATGTAGCG ATTGCTGCATTCCGCTCAACGCTTGAGGAAGTA	XM_020979876.1:0_ProbeB	CGAAAGCCATGACCTCCGATCACTCTCCCACTGGCCCC TTTGAGCAGAGTTGAGAAGAAGGTGCTGATAAGAATC
B09	LEP	XM_021002262.1:114_T021	ATGGTCTTGGTGAGGGTTTTAGTGTCGTCTTGGACCTGAGGCTGT TAAAGCTGTAGCAACTCTTCCACGA	XM_021002262.1:114_ProbeB	CGAAAGCCATGACCTCCGATCACTCGTTTGGCAGAGA TTGAGTACATGTGTGAGATGTCATTGATCCTGGTGATG
B10	FOXP3	XM_020976440.1:719_T022	TGGGCAACCACTCAAGGTTGGCAAGGTTGATCCCAGCTAGGACG CAAATCACTTGAAGAAGTGAAAGCGAG	XM_020976440.1:719_ProbeB	CGAAAGCCATGACCTCCGATCACTCACACAGATAAGT TGTCAGCATGTTGGCTGGCTCCT
B11	TLR2	XM_020994004.1:2643_T023	GAGAAAGAATGCACTGTGTTTTCACTGATGTTCATTGCCACCAAG TTGGGCCACGCGATGACGTTCGTCAAGAGTCGCATAATCT	XM_020994004.1:2643_ProbeB	CGAAAGCCATGACCTCCGATCACTCCAGCTGCCAAACT TCCCATTGTCTGAAAGGACTCAAGTTGTGCCTTA
B12	TLR4	XM_020990604.1:933_T024	CTCCAAGGACCAGCTTATCAACTTGTAAGCCAGTCATTCCTTTAAG ACAACATTTGGAATGATGTGTACTGGGAATAAGACGACG	XM_020990604.1:933_ProbeB	CGAAAGCCATGACCTCCGATCACTCGTGTTGCTTTCAAA GCTGCCCAAGCGGATTTCATTTTTGTAGT
C01	TLR7	XM_021007481.1:1701_T025	ATAATGCATGGAGTCATATACTTGGGAAGCACGGAACTCTGTCGA AGTCACACAAGAATCCCTGCTAGCTGAAGGAGGGTCAAAC	XM_021007481.1:1701_ProbeB	CGAAAGCCATGACCTCCGATCACTCTTCTCTTTGGTTTTG AACATGCAAGTTCGGGCAGACTCGTCGTATTTGAA
C02	PhciCATH5	XM_020999124.1:314_T026	AGGCATTCTTTCACCACCCCATTTTCCTTGAAGTCACATTCATCCGG ATTCTTGACGTAGATTGCTATCAGGTTACGATGACTGC	XM_020999124.1:314_ProbeB	CGAAAGCCATGACCTCCGATCACTCCACAGGAAATATCA GCAGAGGGCTTAGGAGAATCCAGGGCAATGGCTCCA
C03	CCR4	XM_020987510.1:1095_T027	TCATGATCCATAGTAGACTGAGTGTGAGATGAACTGGGTGTTTCC GAAGCCTTACAGATCGTGTGCTCATGACTTCCACAGACGT	XM_020987510.1:1095_ProbeB	CGAAAGCCATGACCTCCGATCACTCCTCTGGTGGAAGGG AGTCTGCTGTCGTTTACAAAGCATCATGGATT
C04	NCR3	XM_020974295.1:474_T028	AGCCAAGCTGAAAATGTAGAGGGCCGCCCGAAGGAAGAGTGGGA ACACTTCTTGGAGGAGTTGATAGTGGTAAAACAACATTAGC	XM_020974295.1:474_ProbeB	CGAAAGCCATGACCTCCGATCACTCGTGATTGCGACTTG TCTCTTGTAATACAGAGTGGTCCCTATCACCACAAA
C05	CLEC4E	XM_020990221.1:659_T029	CACTCCCAATGACCATCAACCACTTGGTCTGTCAGCCCTACGTATAT ATCCAAGTGGTTATGTCCGACGGC	XM_020990221.1:659_ProbeB	CGAAAGCCATGACCTCCGATCACTCCCCCTATATCCCAGA AGCTCAAATTTTTGTTGAATGGCGTACCATCTACC
C06	CD4	XM_020994251.1:1137_T030	AAATTTGGAGCCCCCTTTTCTAGAGTCAAAGAGTCACCCTTCCATGA AAACAGCAAGAAGGAGTATGGAACTTATAGCAAGAGAG	XM_020994251.1:1137_ProbeB	CGAAAGCCATGACCTCCGATCACTCGCAAGACAGGATGA AGAGTGATGGTGAGTGGGAACTTAGGATCACGACTG
C07	CD8beta	XM_020969485.1:855_T031	ACATACTTATGCTATGAACCCAACTCACAAGAGGGACAGGCAATGG TGCACACCCCTCCAAACGCATTCTTATTGGCAAATGGAA	XM_020969485.1:855_ProbeB	CGAAAGCCATGACCTCCGATCACTCCGGAAAGTGCTCGA GGTTTTAGAGAATGTTATGCTACTGATGACCGCCAA
C08	CD3G	XM_020984783.1:271_T032	GGTTTTTTCCCTTGATCTTCCTGGGCCACAATACCCCCGAAGCAATA CTGTCGTCACTCTGTATGTCCGT	XM_020984783.1:271_ProbeB	CGAAAGCCATGACCTCCGATCACTCTACAGTTCCATCTTG TCGATTGTCATCCAAGGCCACT
C09	CD79b	XM_020997546.1:415_T033	GATGCCATTGTCCTCAAATTGTATCTTTGAGATGATGAGAGTGGCG ATGGCCGGGAATCGGCATTTCGCATTCTTAGGATCTAAA	XM_020997546.1:415_ProbeB	CGAAAGCCATGACCTCCGATCACTCCAGCCCCGGGAGAC ATTATGACCCATTCCACAATCTTGTTGACAGTAATA
C10	CYP2E1	XM_020994684.1:502_T034	AAGGGCTGGGAATTTGTATTCTTGAGTATCTCGAGCAGGAACTGAG ATTCCCGATCTTCATAACGGACAAACTGAACGGGCCATT	XM_020994684.1:502_ProbeB	CGAAAGCCATGACCTCCGATCACTCTGTCGTGGATGACAT TTAAGGGAGCACCACCGAGGACAAAGGTTGGATCA
C11	CYP3A4	XM_020977781.1:1546_T035	CCTGCAGGACAATAGGTACAGTTGGTGAAAGAAGTGGCTTATTGCT CAGCCGCTATGCAGACGAGCTGGCAGAGGAGAGAAATCA	XM_020977781.1:1546_ProbeB	CGAAAGCCATGACCTCCGATCACTCTGAGAAGAAGAACTT CTAAGGTTGGTTTGCCTTCTTGGACCTTGGTACAG
C12	CYP4A15	XM_020987668.1:1170_T036	AATGGATGGGACAGGAGGATAGAGACGAAGGGCTTCCTTGATGC ACATAGCATTCGCAACCATGTGAAGTAATGTGAGCGTACTT	XM_020987668.1:1170_ProbeB	CGAAAGCCATGACCTCCGATCACTCCCATCTGGAAAGG TGATGGTCTCTTTGATATCCCTGCC
D01	CYP3A78	HQ595724.1:443_T037	GCATGAGAACATCTCCATGGTGTTTGATAATGGGAAACATCTCCTT CAGTCACCAGTTAGCGTGGCGTATACCATGTTGTTAACA	HQ595724.1:443_ProbeB	CGAAAGCCATGACCTCCGATCACTCATCCTTCATATTCAG AGACTCATCTTTAGCCATTCTCTTCTCAATATTTT
D02	POMC	XM_020983954.1:298_T038	ATTTGATCCATAGAGTGCATGGTCCAGATCACAGGATTTGAGGCA TGCCTGAATCAATAGAACAATATCAGTTATGGCGGTG	XM_020983954.1:298_ProbeB	CGAAAGCCATGACCTCCGATCACTCTATCATCATGGATA TCTCTTTGCTTTGTGCTACGTTTCAGCTC
D03	CRH	XM_021002119.1:59_T039	AATCACGCAGTACTTGCCTAATAAGCTGACGCTTTCTTGACAGCTA GATTCGGTTGTTAATATGACAGGCCGCTAAAGACGTTCT	XM_021002119.1:59_ProbeB	CGAAAGCCATGACCTCCGATCACTCTCTAATCAGAGCTT GGTTGCGGGATTTTTATAGCTTAGACCCTCTTATGG
D04	MC2R	XM_020985612.1:989_T040	AGGTGATCTTCTTGGCATGGGAATGAGCCAGTAGGAACATATGCA CATAGCCGTCTCAGATGAGTGGGTTAATCAATCAAGTATG	XM_020985612.1:989_ProbeB	CGAAAGCCATGACCTCCGATCACTCCTCCTTTCATGTTGA CTCTTGGGTGCACTCTATTTGAGGGTAAAG
D05	AVPR1A	XM_020990181.1:1847_T041	TCTTCAACTTACTCCTGTGCCAACAGAGAGGACATTCTCAGTTGCTG	XM_020990181.1:1847_ProbeB	CGAAAGCCATGACCTCCGATCACTCAACCTTCTCCATTTT

Well Position	Gene Target	Probe A Name	Probe A Sequence	Probe B Name	Probe B Sequence
			ACACATTAGTAACGTCGGCAAGCACTTAGTCG		TGCATAAGGCTGATAGGAGGGACCTAGGATATCTG
D06	FKBP5	XM_020999591.1:2852_T042	CTGCTCCAGACAAAATTTCTCCTTGTGTCGAGCTAACTAGATTGCAT GGACGTGAACCAGATTATGTATGGACGCGCAATAGATA	XM_020999591.1:2852_ProbeB	CGAAAGCCATGACCTCCGATCACTCTGTGACTAGAAGC ACAGTTTGGGAAACAGTTGGCTGGAGTTGGTC
D07	NR3C2	XM_020994026.1:1490_T043	GAGAATTGGGAATCAGAATTGACTTTGCTATTGGCTCTGAGACATG AGCTCATACGAAATTTGAGCAAGCAATTGAAGGCTTAGA	XM_020994026.1:1490_ProbeB	CGAAAGCCATGACCTCCGATCACTCAAGAAGTACCTGA GCATGAATGCTTGGCTGACTCTTGTTTTACTGGTACT
D08	AR	XM_020967225.1:4408_T044	AGAAGAAGTGAGGCAAAAAGGAGGCTGCGGAAGAGAGGATGGCA GTGAGACTATCAGCTAATAGGGTCGGCTCAACAGTGTATCC	XM_020967225.1:4408_ProbeB	CGAAAGCCATGACCTCCGATCACTCCACCATTCAGACCA AGCAGAGTCAGAAGGTGCTAAGGTAGATGGGAATTT
D09	ESR1	XM_020986619.1:3088_T045	GAAATGTAAGGGATGATGAACTTGCTCTCTGGTGACTGGGTAACCA TAGTCTATCAATTCGTGACCCCGATCATCCAGTCCAGAA	XM_020986619.1:3088_ProbeB	CGAAAGCCATGACCTCCGATCACTCAGACAAGACCATT AAGCCCCAAGCTGAGCCTGCCTAAGGAAAATGTTGAA
D10	PGR	XM_020996182.1:1576_T046	TCCCAGCTTCCTTTTGTACATCAAGTCCTGGTTCGAGTCAAAACCTTG AGCTCTAGGCCCAAAACGACCTTAATGGTCA	XM_020996182.1:1576_ProbeB	CGAAAGCCATGACCTCCGATCACTCGCCCATCCAAACCC AAGCCACTGAACCTAAAATAGATCTTGGTCACTTTC
D11	OXT	XM_021005530.1:118_T047	CCCCCTCACTTAGCCAGGTCAGGCTGGAGCACAAGCTAGCCCAGAT CCTACGAGATGAGCTACGTAACTA	XM_021005530.1:118_ProbeB	CGAAAGCCATGACCTCCGATCACTCTTGGAAGCTCACGC CCGGTCAGCGGGAAGTGAGTGCCACCAGTG
D12	DRD1	XM_020969184.1:2262_T048	GGCTCATGATGGCTTGAAAACACGGCCCCATTATTGTTGATGCTAA CTGTCAAATGCACTCTATATGGAGGGAGAGTAGCTGGAT	XM_020969184.1:2262_ProbeB	CGAAAGCCATGACCTCCGATCACTCCTTGTGGAATCAGG TAAACCAGATTGCACTCCTTGGAAATGGACCCTCTG
E01	DRD3	XM_021007165.1:1424_T049	TGCCCCACCCTATACAGTTTCAATCGTCATTGTCCCCTGGTCTAGGTA TCTAATTCGTGGGTCGGGTACT	XM_021007165.1:1424_ProbeB	CGAAAGCCATGACCTCCGATCACTCATGTCAAGCATTCT GGCAGGTGAAAGCCTAGAATGTAGGAGGCTTCTAAC
E02	HTR1A	XM_021007256.1:767_T050	GACCTGGTAGAGAGCAGCCATGGGCAAAACAAGCACGGAAACCAT CAGGTCATTAGCTCGGATGCTATCAGCTTGCGCCTATTAT	XM_021007256.1:767_ProbeB	CGAAAGCCATGACCTCCGATCACTCATAAAAATGTCGCA GGTTACCTGGCCCAGGGTCCACTTGTTGAG
E03	HTR2A	XM_020993783.1:786_T051	CATTTGTATCCGTACAAGATGGTTAATGTTGACACTGGCATCACCAG GAACACGATCTGTATTTTGCACCTTTCGCTATGCTGAG	XM_020993783.1:786_ProbeB	CGAAAGCCATGACCTCCGATCACTCAAAGCACATCCAGA TAAATCCAGACCGCACAGAGTTTTGTGGGCAAGGGC
E04	HTR3A	XM_020989830.1:836_T052	CAGAGTAGCCCAAGAGGAGAGTGATCTTGAAGGAGACTCTTTCACC TGTGTCCGTCTATACGCATACTGGTCCACATATA	XM_020989830.1:836_ProbeB	CGAAAGCCATGACCTCCGATCACTCGGTGCCAATAGCTG TGGCTGGAAGTGTATCAGACACGATGATAAGGAAGA
E05	CARD9	XM_020981897.1:1153_T053	AGCTTCAATGCGTTCTTTGTACATCTTGGAATCCTTACGCAGGGCATG TTGGAGTTAACGGAGACCCGCCATCGTTTAC	XM_020981897.1:1153_ProbeB	CGAAAGCCATGACCTCCGATCACTCCCTTTCTATAGCTAC TTCCTCCATCTGCTGCAAGAT
E06	SLC29A1	XM_020993889.1:912_T054	CATCTTTGCTTGCTGGTAATACTGGTAGAATTTCAGACTAGGCAGGA CCACGCTCATTTTGAACATACGATTGCGATTACGGAAA	XM_020993889.1:912_ProbeB	CGAAAGCCATGACCTCCGATCACTCATTCCTCTTCGAATC AAATCCATTTTGGTTTCCTGCTCCCCAAAAGCTTC
E07	TRIM24	XM_020997959.1:1828_T055	TGGTAGGACTAGAAGGAGTAGATGTGGTACTACTGGCAGTTGAAGG AACACCTATGCATCATGTGCCTCACTAGGACATCATGCT	XM_020997959.1:1828_ProbeB	CGAAAGCCATGACCTCCGATCACTCAATCATAGGTGGTC CAAAAGACTTAGCATCATATCCAGCTGCGCTAGTAA
E08	SAMHD1	XM_020998818.1:653_T056	TCTGAACACAAAGCACATCCCGCTCAGTTATCTTTAGTTCAGGCTG TTTTCCTAAATTGGGAAAAAAGGTTTTAGCTATTGATGG	XM_020998818.1:653_ProbeB	CGAAAGCCATGACCTCCGATCACTCAAACATGTGTGAAA ATGGCCCATGACCTAGATCATGACAGAGCCCAGCAA
E09	LOC110217150	XM_020999329.1:491_T057	TCAGTCTCAGAAAGGAATTGAAAAGACCAGTGGGACCTCAAATGG AAGTTCTTCAGTTAAAGGCTATCTTGCTCCGCTCGTTCTC	XM_020999329.1:491_ProbeB	CGAAAGCCATGACCTCCGATCACTCAATCTGAGGAGCAC AAGGCCGAGCGCAGGGCCTTAAACAGAAACTGAGGC
E10	DICER1	XM_021005138.1:4783_T058	ATAGCAGCCCAATAGAGCTTCTACACAGTCTGCAATGCTTTTATCAG CAACTTAAAGCTATCCACGAATGTCAAAAATGTGGTTT	XM_021005138.1:4783_ProbeB	CGAAAGCCATGACCTCCGATCACTCCCCAAAGAACAGAG GAAAAGTTGAGCAGCTCTCTCACCACAGCTGGTTAA
E11	PhaHV1 dpol	JN585829.1:70_T059	TATTGATTCTAGATCTGAGGTGGATAGGGATTCCATGAAGGCCTTG GAGCCCCGAATGTATAATGCTGACGTTCTTGCTTTTGGC	JN585829.1:70_ProbeB	CGAAAGCCATGACCTCCGATCACTCTAGATGACACGAAG GGATGAGTCCTTTTCGCTTGGGACTGTGTGTCCCAC
E12	PhaHV2 dpol	JQ996387.1:107_T060	ATCTCCTTGCGCCGGGCAAGCCACGTGGTCAGGAGCCTATTGAAG CAATCCTCTCCCCAATACTTAAAAA	JQ996387.1:107_ProbeB	CGAAAGCCATGACCTCCGATCACTCCAGAATAGTGCGCA GAGCTGGATCGTTGCAGGCCGCGTGTTGGCGTCGG
F01	CpecG_0573	NZ_CM002310.1:208_T061	GGCACAGAGGCAAAAAAGCGTAAGCAGGGCTGTTGTAAGGAAGA CTCCCACTACGGTTACCGTCTTTATAAGTGAACAAAACCGG	NZ_CM002310.1:208_ProbeB	CGAAAGCCATGACCTCCGATCACTCACCTCTGGTTTGGTT ACAGACTTACCTGAGATTGCTTTGGGCTTCAAGAT
F02	pGP3	KT223773.1:4624_T062	TAATTTGCTTGTCTTGCAATTCATTGGTCATTTGTCCCAAACGAATA TTGCTCTGTGAACTGTCATCGGTCCGATCAATTAGTCT	KT223773.1:4624_ProbeB	CGAAAGCCATGACCTCCGATCACTCATTAGAAGAAGAGA GGCTTGTTACAGTAGGAGTAGTTGGTGTGCCTATTA

Well Position	Gene Target	Probe A Name	Probe A Sequence	Probe B Name	Probe B Sequence
F03	ompA	GQ228181.1:18_T063	GCGTGTAAGGAGGCGTCACCAGCAAAAAATGCGGCGGATAAAAAC GCCGACTCCCCTTTCCCAAGTAAATGTACGGGAATTATCG	GQ228181.1:18_ProbeB	CGAAAGCCATGACCTCCGATCACTCTCGTCCCATCAATTA ATAAACTTGGCTCTGCTGGGTTCCCTACAGGCAAA
F04	Cpec_hsp60	Cpec_hsp60.1:1083_T064	TATTATGGTTCTTCTCCAGCCGATACAATTACTCGTCTAGAACCCCA GGGCGCTTTATTATGTGTTCGTCTAACTCTGTTTCTGT	Cpec_hsp60.1:1083_ProbeB	CGAAAGCCATGACCTCCGATCACTCCAAAGCGATCTCTCT GTTCTTTGGTGCGCTTGTGTACGTTAGAACACGGC
F05	KoRVpol	NC_039228.1:2900_T065	ATGACGGGTCGATAGAAGGAGGGACCGGCTTTTCGTGTAATCGGT ATTCCCCGAGTGCATGAGCTGTCTTTCACATGATACATCG	NC_039228.1:2900_ProbeB	CGAAAGCCATGACCTCCGATCACTCCAGTCCCATACCTGC CTTCTCGGCCCAAACCATGGGAAAGAGTTGGAGCC
F06	KoRVAenvRBD	AF151794.2:6218_T066	GGTTCCCCAAGTGATCTGATTATAAGCATGTTCATAGTTTGAATCAG GGGCTATTTCTGTTCACGGATGAAGGCCTATATCAATG	AF151794.2:6218_ProbeB	CGAAAGCCATGACCTCCGATCACTCAACTGGGACCTAGC AATTCTTGTTCTGGCCCGGGGATAGCTACATCCCAG
F07	KoRVBenvRBD	AB822553.1:314_T067	CATAGCTCGATTGCCTGACTAACCCCCTGCCGACCTGACTATAAAG CACCCCATCCACTTTCATGGAAACAATAAGAGCAGGGAA	AB822553.1:314_ProbeB	CGAAAGCCATGACCTCCGATCACTCTCTGTTCCGGTCTCG GGGACAGCGACACCCCAAGGTTC
F08	KoRVDenvRBD	AB828004.1:120_T068	CCCGTCTGGGACAGTACCTGCCAGGTGAGAGTCATGGGTTGGCACA AACTCACTACTACCAACAACCTCACCAAAAA	AB828004.1:120_ProbeB	CGAAAGCCATGACCTCCGATCACTCGCCACCACGTCCAG GGTGGCTCGACTGCTTTCTTTTCCCAAACGACACTT
F09	KoRVenvCKS17	AF151794.2:7502_T069	AAGGCCCCCTTCCTTCAAAAATAGCAGATCAAGGCCTCTCCTATTCTG GACTCATGTCCTCTGTTAATCCAGCCTGAATATGCCA	AF151794.2:7502_ProbeB	CGAAAGCCATGACCTCCGATCACTCGCGCCTGAGTGGTC AACATAGAAACAGCATTCCTCTTTTAGGGCTGCACA
F10	Tcop18S	GU966588.1:594_T070	ACCGCTTGTCACACGACCCCATAATCTCCAATGGACTTTTAAATACCA ACCAGAAATGTCACTCCCATGGTGGCTGATATAGAAA	GU966588.1:594_ProbeB	CGAAAGCCATGACCTCCGATCACTCGGGCGACGGGCGGC GCCCTGGCCGTGAAGACCAAAAAAGCAACACGCCCG
F11	Tirwini18S	FJ649479.1:538_T071	AATATACGCTTTTGGAGCTGGAATTACCGCGGGTGCTGGCACCAGAC TTGCATGTCGAACCTTGGATAGGAGCGACCGATTACGT	FJ649479.1:538_ProbeB	CGAAAGCCATGACCTCCGATCACTCGCCTTGAAGGCCCA CAGTTCAACTACGAACCCTTTAACAGCAACAGCATT
F12	Tgil18S	GU966589.1:714_T072	GCGCGTGGGTGACGGGAACACACAAAGTGCCTCGCACCACACCGTG CACGCTCAGGTTGTTACTTGAAGGGTTCAACACGAGCTC	GU966589.1:714_ProbeB	CGAAAGCCATGACCTCCGATCACTCCCCGAACTACCCTCC TTCATTCCTGGGCGCCGCGAGCCGAAAGAGGCGT

Table 7.7: NanoString Gene Target Performance

Gene	Full Name	Accession	System/Role	% of samples with Gene Counts Above 20	# of samples with Gene Counts Above 20	Retained for Analysis?
FKBP5	FKBP Prolyl Isomerase 5	XM_020999591.1	Endocrine	95	100	YES
IL1beta	Interleukin 1 beta	XM_020963753.1	Immune	82	86.3	YES
IL6	Interleukin 6	XM_020993366.1	Immune	91	95.7	YES
IL8	Interleukin 8-like	XM_020988483.1	Immune	95	100	YES
IL18	Interleukin 18	XM_020989757.1	Immune	84	88.4	YES
TNFalpha	Tumour necrosis factor alpha	XM_020974214.1	Immune	46	48.4	YES
PhciDAB	MHC classII DAB	XR_002323139.1	Immune	95	100	YES
PhciDBB	MHC classII DBB	XM_020974227.1	Immune	94	98.9	YES
RETN	Resistin	XM_020979876.1	Immune	95	100	YES
TLR2	Toll-like receptor 2	XM_020994004.1	Immune	94	98.9	YES
TLR4	Toll-like receptor 4	XM_020990604.1	Immune	95	100	YES
TLR7	Toll-like receptor 7	XM_021007481.1	Immune	73	76.8	YES
CLEC4E	C-type lectin domain family 4 member E	XM_020990221.1	Immune	95	100	YES
CD4	T cell receptor	XM_020994251.1	Immune	84	88.4	YES
CD8beta	Cytotoxic T cell receptor	XM_020969485.1	Immune	92	96.8	YES
CD3G	T cell receptor	XM_020984783.1	Immune	95	100	YES
CD79b	B cell receptor	XM_020997546.1	Immune	94	98.9	YES
CARD9	Caspase recruitment domain-containing protein 9	XM_020981897.1	Immune	95	100	YES
SAMHD1	SAM domain & HD domain-containing protein 1	XM_020998818.1	Immune	95	100	YES
LOC110217150	Cathelicidin	XM_020999329.1	Immune	95	100	YES
PhaHV1 dpol	Herpesvirus 1 dpol	JN585829.1	Infectious Agent	2	2.1	YES
PhaHV2 dpol	Herpesvirus 2 dpol	JQ996387.1	Infectious Agent	4	4.2	YES
CpecG_0573	Cpecorum MC/Marsbar strain	NZ_CM002310.1	Infectious Agent	2	2.1	YES

Gene	Full Name	Accession	System/Role	% of samples with Gene Counts Above 20	# of samples with Gene Counts Above 20	Retained for Analysis?
pGP3	Cpecorum L1 strain	KT223773.1	Infectious Agent	1	1.1	YES
ompA	Cpecorum L17 strain ompA	GQ228181.1	Infectious Agent	1	1.1	YES
Cpec_hsp60	CPE1_RS04735, chaperonin GroEL	WP_024010594.1	Infectious Agent	4	4.2	YES
KoRVpol	KoRV pol	NC_039228.1	Infectious Agent	95	100	YES
KoRVAenvRBD	KoRV-A env	AF151794.2	Infectious Agent	95	100	YES
KoRVBenvRBD	KoRV-B env	AB822553.1	Infectious Agent	33	34.7	YES
KoRVDenvRBD	KoRV-D env	AB828004.1	Infectious Agent	95	100	YES
KoRVenvCKS17	KoRV env p15E	AF151794.2	Infectious Agent	95	100	YES
Tcop18S	*T. copemani* 18s rRNA	GU966588.1	Infectious Agent	25	26.3	YES
Tirwini18S	*T. irwini* 18s rRNA	FJ649479.1	Infectious Agent	55	57.9	YES
Tgil18S	*T. giletti* 18s rRNA	GU966589.1	Infectious Agent	3	3.2	YES
SLC29A1	ENT1, adenosine transporter	XM_020993889.1	KoRV IS associated – Immune*	93	97.9	YES
TRIM24	Tripartite Motif Containing 24	XM_020997959.1	KoRV IS associated – Immune*	86	90.5	YES
DICER1	Gene expression regulation & microRNA production	XM_021005138.1	KoRV IS associated – Immune*	48	50.5	YES
HTR1A	5-hydroxytryptamine receptor 1A	XM_021007256.1	Behavioural/immune regulation	1	1.1	NO
HTR2A	5-hydroxytryptamine receptor 2A	XM_020993783.1	Behavioural/immune regulation	1	1.1	NO
HTR3A	5-hydroxytryptamine receptor 3A	XM_020989830.1	Behavioural/immune regulation	4	4.2	NO
LEP	Leptin	XM_021002262.1	Endocrine	8	8.4	NO
POMC	Proopiomelanocortin	XM_020983954.1	Endocrine	2	2.1	NO
CRH	Corticotropin releasing hormone	XM_021002119.1	Endocrine	8	8.4	NO
MC2R	Melanocortin Receptor 2	XM_020985612.1	Endocrine	6	6.3	NO
AVPR1A	Vasopressin receptor 1A	XM_020990181.1	Endocrine	1	1.1	NO
NR3C2	Mineralocorticoid receptor	XM_020994026.1	Endocrine	9	9.5	NO
AR	Androgen receptor- testosterone mediation	XM_020967225.1	Endocrine	18	18.9	NO

Gene	Full Name	Accession	System/Role	% of samples with Gene Counts Above 20	# of samples with Gene Counts Above 20	Retained for Analysis?
ESR1	Estrogen receptor 1	XM_020986619.1	Endocrine	54	56.8	NO
PGR	Progesterone receptor	XM_020996182.1	Endocrine	1	1.1	NO
OXT	Oxytocin/neurophysin I prepropeptide	XM_021005530.1	Endocrine	3	3.2	NO
DRD1	Dopamine receptor D1	XM_020969184.1	Endocrine	8	8.4	NO
DRD3	Dopamine receptor D3	XM_021007165.1	Endocrine	4	4.2	NO
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	XM_020994140.1	Housekeeping	95	100	NO
ACTB	Beta-actin	XM_021008442.1	Housekeeping	95	100	NO
Tmem97	Transmembrane protein 97	XM_020963254.1	Housekeeping	33	34.7	NO
Stx12	Syntaxin 12	XM_020983277.1	Housekeeping	95	100	NO
Nckap1l	Nck-associated protein 1-like	XM_020966159.1	Housekeeping	94	98.9	NO
IL4	Inerleukin 4	XM_020991403.1	Immune	3	3.2	NO
IL10	Interleukin 10	XM_021002936.1	Immune	14	14.7	NO
IL12A	Interleukin 12 A	XM_020988856.1	Immune	3	3.2	NO
IL17A	Interleukin 17 A	XM_020993976.1	Immune	2	2.1	NO
IL22	Interleukin 22	XM_020990310.1	Immune	1	1.1	NO
INFgamma	Interferon gamma	XM_020990282.1	Immune	11	11.6	NO
MHCIUA	MHC class I A-11 alpha chain-like	XM_020974926.1	Immune	14	14.7	NO
FOXP3	Forkhead box P3, T-reg cell marker	XM_020976440.1	Immune	2	2.1	NO
PhciCATH5	Cathelicidin	XM_020999124.1	Immune	20	21.1	NO
CCR4	C-C Motif Chemokine Receptor 4	XM_020987510.1	Immune	44	46.3	NO
NCR3	Natural cytotoxicity triggering receptor 3	XM_020974295.1	Immune	30	31.6	NO
CYP2E1	Cytochrome P450 2E1	XM_020994684.1	Metabolism/detoxification	2	2.1	NO
CYP3A4	Cytochrome oxidase P450 3a4	XM_020977781.1	Metabolism/detoxification	2	2.1	NO
CYP4A15	Cytochrome oxidase P450 4a11/15	XM_020987668.1	Metabolism/detoxification	5	5.3	NO
CYP3A78	Cytochrome oxidase P450 3A78	HQ595724.1	Metabolism/detoxification	50	52.6	NO

Table 7.8: qPCR Primer/Probe Set Information for Chlamydia Multiplex qPCR, PhaHV-1 & -2 qPCR, and KoRV pol qPCR

qPCR Assay	Gene	Amplicon size (bp)	End	Sequence	Reference
Chlamydia multiplex Probe qPCR	*Chlamydia* (23S rRNA)	*137*	Forward	5'-GCTCACCAATCGAGAATC-3'	(Hulse *et al.*, 2018)
		137	Reverse	5-'CCAACACTCCTTTCGGTA-3'	
		137	Probe	ROX-CTGAATACTACGCTCTCCTACCGC-BHQ2	
	C. pecorum (*ompB* gene)	*141*	Forward	5'–CCAAGCATAATCGTAACAA-3'	
		141	Reverse	5'-CGAAGCAAGATTCTTGTC-3'	
		141	Probe	FAM-ACTTGTTGGCAATTCTTCTCTTCACA-BHQ1	
	Koala *β-actin* mRNA	*145*	Forward	5'-CTCAGATTATGTTTGAGACCTTC-3'	
		145	Reverse	5'-CCTTCATAGATGGGCACA-3'	
		145	Probe	HEX-ACCATCACCAGAGTCCATCACAAT-BHQ1	
Phascolarctid herpesvirus 1 & 2 SYBR qPCR	PhaHV-1 *dpol*	*22*	Forward	5'-GGGAAGAACTATGTTGGAACGC-3'	(Wright *et al.*, 2023)
		20	Reverse	5'-TGAGTCCTTTTCGCTTGGGA-3'	
	PhaHV-2 *dpol*	*20*	Forward	5'-GGTGACGTGCAATTCAGTGT-3'	(Church *et al.*, 2025; Kasimov *et al.*, 2020)
		20	Reverse	5'-TTTCGAGCATCATGCGTCCT-3'	
	Koala *β-actin* mRNA	*145*	Forward	5'-CTCAGATTATGTTTGAGACCTTC-3'	(Hulse *et al.*, 2018)
		145	Reverse	5'-CCTTCATAGATGGGCACA-3'	
KoRV pol Probe qPCR	KoRV *pol*	*110*	Forward	5'-TTGGAGGAGGAATACCGATTACAC-3'	(Hulse *et al.*, 2018; Tarlinton *et al.*, 2005)
		110	Reverse	5'-GCCAGTCCCATACCTGCCTT-3'	
		110	Probe	FAM-TCGACCCGTCATGGC-BHQ1	
	Koala *β-actin* mRNA	*145*	Forward	5'-CTCAGATTATGTTTGAGACCTTC -3'	(Hulse *et al.*, 2018)
		145	Reverse	5'-CCTTCATAGATGGGCACA-3'	
		145	Probe	HEX-ACCATCACCAGAGTCCATCACAAT-BHQ1	

Table 7.9: Principal Component Analysis (PCA) eigenvalues

PC	Eigenvalue	Variance %	Cumulative Variance %
Dim.1	8.0	29.6	29.6
Dim.2	4.2	15.7	45.3
Dim.3	3.0	11.1	56.4
Dim.4	1.6	6.0	62.5
Dim.5	1.3	4.6	67.1

Table 7.10: Correlation coefficients (cor) between genes and PCA dimensions (1-5)

	Dim.1	Dim.2	Dim.3	Dim.4	Dim.5
CARD9	-0.32	0.47	0.42	0.01	-0.54
CD3G	0.84	0.21	0.07	0.29	-0.01
CD4	0.86	0.20	0.10	0.16	-0.07
CD79b	0.80	0.09	-0.33	0.09	0.07
CD8beta	0.86	0.18	0.01	0.09	-0.05
CLEC4E	-0.32	0.59	0.48	0.07	-0.10
DICER1	0.74	-0.15	0.21	-0.02	-0.05
FKBP5	-0.43	-0.29	0.43	0.25	0.41
IL18	-0.35	-0.20	0.54	-0.30	0.10
IL1beta	0.18	0.16	0.57	-0.40	0.15
IL6	0.02	0.26	0.53	-0.19	0.23
IL8	0.08	0.26	0.33	0.06	0.60
KoRVAenvRBD	0.41	-0.55	0.24	-0.06	-0.14
KoRVDenvRBD	0.10	-0.73	0.40	0.25	-0.27
KoRVenvCKS17	0.13	-0.78	0.36	0.16	-0.13
KoRVpol	0.05	-0.75	0.34	0.31	-0.09
Cathelicidin	-0.53	-0.41	0.20	0.19	0.20
PhciDAB	0.47	0.51	0.30	0.51	0.02
PhciDBB	0.56	0.35	0.17	0.38	0.22
RETN	-0.58	0.50	0.08	0.11	-0.07
SAMHD1	0.58	-0.24	0.32	0.00	0.05
SLC29A1	0.77	-0.10	0.00	-0.19	0.13
TLR2	0.37	0.33	0.44	-0.24	-0.11
TLR4	-0.45	0.41	0.47	0.02	-0.21
TLR7	0.89	0.12	-0.12	0.03	-0.09
TNFalpha	0.57	0.03	0.28	-0.37	-0.16
TRIM24	0.58	-0.26	-0.02	-0.53	0.10

Table 7.11: Cosine squared values (cos2) for genes according to PCA dimensions (1-5)

	Dim.1	Dim.2	Dim.3	Dim.4	Dim.5
CARD9	0.10	0.23	0.17	1.92E-04	0.29
CD3G	0.71	0.04	4.82E-03	0.08	1.82E-04
CD4	0.74	0.04	0.01	0.03	4.73E-03
CD79b	0.64	0.01	0.11	0.01	4.26E-03
CD8beta	0.74	0.03	4.36E-05	0.01	2.73E-03
CLEC4E	0.10	0.35	0.23	4.84E-03	0.01
DICER1	0.55	0.02	0.05	3.81E-04	2.85E-03
FKBP5	0.18	0.08	0.18	0.06	0.17
IL18	0.12	0.04	0.29	0.09	0.01
IL1beta	0.03	0.03	0.33	0.16	0.02
IL6	4.54E-04	0.07	0.28	0.03	0.05
IL8	0.01	0.07	0.11	3.51E-03	0.36
KoRVAenvRBD	0.17	0.31	0.06	4.03E-03	0.02
KoRVDenvRBD	0.01	0.54	0.16	0.06	0.07
KoRVenvCKS17	0.02	0.61	0.13	0.03	0.02
KoRVpol	2.06E-03	0.57	0.12	0.09	0.01
Cathelicidin	0.28	0.16	0.04	0.04	0.04
PhciDAB	0.22	0.26	0.09	0.26	2.74E-04
PhciDBB	0.32	0.12	0.03	0.14	0.05
RETN	0.33	0.25	0.01	0.01	4.43E-03
SAMHD1	0.33	0.06	0.10	1.20E-07	2.21E-03
SLC29A1	0.59	0.01	5.75E-06	0.04	0.02
TLR2	0.14	0.11	0.20	0.06	0.01
TLR4	0.20	0.17	0.22	2.60E-04	0.05
TLR7	0.79	0.01	0.01	9.42E-04	0.01
TNFalpha	0.33	8.72E-04	0.08	0.13	0.03
TRIM24	0.33	0.07	2.58E-04	0.28	0.01

Table 7.12: Clustering Determination Tests - Optimal Clustering Summary

Method	Optimal # Clusters
Total within sum of squares	4-5
Gap Statistics	1
Average silhouette	3
Consensus-based algorithm	1 or 3
Dendrogram	5-8
K-means	3 or 5
Final Cluster Decision	**5**

Table 7.13: Characteristics of clusters according to health assessments and infection status

Characteristics		Cluster 1, n = 20	Cluster 2, n = 29	Cluster 3, n = 6	Cluster 4, n = 29	Cluster 5, n = 11	P value[1]
Age group, n (%)	*Young adult*	1 (5)	8 (28)	0 (0)	12 (42)	6 (55)	**0.04**
	Adult	16 (80)	19 (65)	5 (83)	14 (48)	3 (37)	
	Mature	3 (15)	2 (7)	1 (17)	3 (10)	2 (18)	
Sex, n (%)	*Male*	*7 (35)*	*9 (31)*	*1 (17)*	*13 (45)*	*9 (82)*	**0.03**
	Female	*13 (65)*	*20 (69)*	*5 (83)*	*16 (55)*	*2 (18)*	
Body Condition Group, n (%)	*≤ BCS 2*	*4 (20)*	*3 (10)*	*2 (30)*	*2 (7)*	*1 (9)*	0.349
	> BCS 2	*16 (80)*	*26 (90)*	*4 (70)*	*27 (93)*	*10 (91)*	
Pathology, n (%)	*No*	*0 (0)*	*7 (24)*	*1 (16)*	*6 (21)*	*4 (36)*	0.114
	Yes	*20 (100)*	*22 (76)*	*5 (84)*	*23 (79)*	*7 (64)*	
Chlamydiosis, n (%)	*No*	*7 (35)*	*13 (45)*	*2 (30)*	*15 (52)*	*8 (72)*	0.305
	Yes	*13 (65)*	*16 (55)*	*4 (70)*	*14 (48)*	*3 (28)*	
Reproductive Disease, n (%)	*No*	*12 (60)*	*21 (72)*	*2 (30)*	*21 (72)*	*9 (81)*	0.247
	Yes	*8 (40)*	*8 (28)*	*4 (70)*	*8 (28)*	*2 (19)*	
Other disease, n (%)	*No*	*13 (65)*	*23 (79)*	*5 (84)*	*20 (69)*	*7 (64)*	0.713
	Yes	*7 (35)*	*6 (21)*	*1 (16)*	*9 (31)*	*4 (36)*	
Trauma, n (%)	*No*	*11 (55)*	*21 (72)*	*5 (84)*	*23 (79)*	*7 (64)*	0.383
	Yes	*9 (45)*	*8 (38)*	*1 (16)*	*6 (21)*	*4 (37)*	
Mucosal *C. pecorum* (Aggregated), n (%)	*No*	*8 (40)*	*17 (57)*	*2 (33)*	*14 (48)*	*7 (64)*	0.614
	Yes	*12 (60)*	*12 (43)*	*3 (50)*	*15 (52)*	*4 (37)*	
Circulating *C. pecorum,* n (%)	*No*	*20 (100)*	*27 (93)*	*3 (50)*	*28 (97)*	*9 (82)*	**0.001**
	Yes	*0 (0)*	*2 (7)*	*3 (50)*	*1 (3)*	*2 (18)*	
Mucosal PhaHV-1, n (%)	*No*	*8 (40)*	*11 (38)*	*0 (0)*	*12 (63)*	*8 (72)*	0.064
	Yes	*12 (60)*	*18 (62)*	*6 (100)*	*17 (37)*	*3 (28)*	
Mucosal PhaHV-2, n (%)	*No*	*15 (75)*	*24 (83)*	*3 (50)*	*25 (86)*	*9 (82)*	0.337
	Yes	*5 (25)*	*5 (17)*	*3 (50)*	*4 (14)*	*2 (18)*	
Circulating PhaHV-1, n (%)	*No*	*20 (100)*	*29 (100)*	*2 (33)*	*29 (100)*	*11 (100)*	0
	Yes	*0 (0)*	*0 (0)*	*4 (64)*	*0 (0)*	*0 (0)*	
Circulating PhaHV-2, n (%)	*No*	*18 (90)*	*28 (97)*	*5 (83)*	*29 (100)*	*11 (100)*	0.217
	Yes	*2 (10)*	*1 (3)*	*1 (17)*	*0 (0)*	*0 (0)*	
Circulating KoRV-B *env,* n (%)	*No*	*11 (55)*	*21 (72)*	*3 (50)*	*21 (72)*	*6 (55)*	0.491
	Yes	*9 (45)*	*8 (28)*	*3 (50)*	*8 (28)*	*5 (45)*	
Circulating trypanosomes (Aggregated), n (%)	*No*	*7 (35)*	*10 (35)*	*3 (50)*	*11 (38)*	*6 (55)*	0.768
	Yes	*13 (65)*	*19 (65)*	*3 (50)*	*18 (62)*	*5 (45)*	
Triage Outcomes, n (%)	*Euthanised*	*15 (75)*	*12 (43)*	*4 (70)*	*10 (35)*	*1 (9)*	**0.004**
	Survived	*5 (25)*	*17 (57)*	*2 (30)*	*19 (65)*	*10 (91)*	

1. Kruskal Wallis rank sum test used for continuous variables and chi-squared tests of dependence for categorical variables between clusters.

Table 7.14: Circulating and mucosal target quantities for KoRV, C. pecorum, PhaHV, and trypanosomes on admission between clusters.

Infectious agent parameter	Cluster 1, n = 20	Cluster 2, n = 29	Cluster 3, n = 6	Cluster 4, n = 29	Cluster 5, n = 11	P value[1]
KoRV *pol* DNA copies/mL	2063 (1078)*	2091 (420)	3592 (1415)	2125 (2354)	2234 (2435)	0.123
KoRV-B *env* mRNA counts	2474 (1262)	820 (1707)	1062 (2740)	922 (1875)	705 (3319)	0.290
C.pecorum DNA loads (mucosal)	0.08 (0.35)	0.09 (0.17)	0.005 (0.36)	0.002 (0.02)	0.05 (0.5)	0.223
C.pecorum G-0573 mRNA counts (circulating)	NA	4.81 (0)	NA	42.0 (0)	NA	0.317
C.pecorum Hsp_60 mRNA counts (circulating)	NA	32.7 (0)	48.3 (0)	NA	72.7 (19.8)	0.259
C.pecorum ompA mRNA counts (circulating)	NA	NA	47.9 (0)	NA	NA	NA
C.pecorum pGP3 mRNA counts (circulating)	NA	NA	18.4 (0)	NA	NA	NA
Normalised PhaHV-1 DNA copies/mL (mucosal)	2.95 (6.9)	1.21 (2.09)	3.33 (3.56)	2.74 (4.5)	0.445 (0.472)	0.169
Normalised PhaHV-2 DNA copies/mL (mucosal)	0.05 (0.136)	0.427 (1.04)	0.0356 (0.03)	0.0443 (0.133)	0.0062 (0.003)	0.052
PhaHV-1 mRNA counts (circulating)	NA	NA	86.9 (77.1)	NA	NA	NA
PhaHV-2 mRNA counts (circulating)	10.1 (1.39)	9.89 (0)	38.1 (0)	NA	NA	0.388
T. copemani 16s mRNA counts (circulating)	24.3 (19.0)	32.7 (30.1)	45.3 (27.6)	90.9 (183)	98.9 (36430)	0.224
T. irwini 16s mRNA counts (circulating)	77.1 (555)	193 (414)	3157 (2386)	173 (341)	50.3 (537)	0.254
T. gilletti 16s mRNA counts (circulating)	7.1 (0)	NA	14.3 (0)	NA	39.8 (0)	0.368

NA denotes no cases with detectable loads

*Median (Inter-quartile range: IQR)

1. Mann-Whitney-Wilcoxson's Test

Table 7.15: HCPC V.test determination of significant PC contributions to Clusters (1-5)

Cluster	PC/Dimension	V.test	Cluster Mean	Overall mean	Cluster SD	Overall SD	P-value
1	Dim.2	-2.9	-1.2	-4.11E-15	1.7	2.1	0.004
1	Dim.1	-6.4	-3.6	-1.16E-14	1.4	2.8	1.17E-10
2	Dim.2	3.9	1.3	-4.11E-15	1.2	2.1	9.02E-05
2	Dim.3	-2.7	-0.7	-2.81E-14	1.2	1.7	0.007
2	Dim.1	-2.9	-1.3	-1.16E-14	1.0	2.8	0.003
3	Dim.4	-2.1	-1.1	2.04E-15	1.0	1.3	0.04
3	Dim.2	-5.7	-4.7	-4.11E-15	2.0	2.1	9.22E-09
4	Dim.1	4.3	1.9	-1.16E-14	1.2	2.8	1.39E-05
4	Dim.3	3.6	1.0	-2.81E-14	1.6	1.7	2.91E-04
4	Dim.2	2.2	0.7	-4.11E-15	1.2	2.1	0.03
4	Dim.5	-2.1	-0.4	1.80E-14	1.2	1.1	0.03
5	Dim.1	5.2	4.2	-1.16E-14	1.1	2.8	2.24E-07
5	Dim.4	2.5	0.9	2.04E-15	1.0	1.3	0.01
5	Dim.3	-2.9	-1.4	-2.81E-14	1.4	1.7	0.004

Table 7.16: Post-hoc pairwise comparison Dunn's test with Bonferroni correction

Dimension/PC	Cluster	Cluster	Z	P.unadj	P.adj
PC1	1	3	-4.009429	6.09E-05	0.0006
PC1	1	4	-6.827551	8.64E-12	8.64E-11
PC1	2	4	-4.639147	3.50E-06	3.50E-05
PC1	1	5	-7.137197	9.53E-13	9.53E-12
PC1	2	5	-5.402208	6.58E-08	6.58E-07
PC2	1	2	-4.53169	5.85E-06	5.85E-05
PC2	2	3	4.882002	1.05E-06	1.05E-05
PC2	1	4	-3.339649	8.39E-04	0.008
PC2	3	4	-4.109471	3.97E-05	0.0004
PC2	2	5	3.08693	2.02E-03	2.02E-02
PC3	2	4	-4.077115	4.56E-05	0.0005
PC3	4	5	4.216953	2.48E-05	0.0002
PC4	3	5	-2.940131	0.003	0.03

Table 7.17: HCPC V.test determination of significant gene contributions to Clusters (1-5)

Cluster	Gene	V.test	Cluster Mean	Overall mean	Cluster SD	Overall SD	P-value
1	TLR7	-6.02	3.50	4.90	0.64	1.16	1.72E-09
1	CD8beta	-5.62	5.42	7.37	1.09	1.74	1.96E-08
1	CD3G	-5.56	11.42	15.77	2.64	3.91	2.72E-08
1	CD4	-5.49	4.29	5.86	0.88	1.44	4.00E-08
1	CD79b	-5.35	4.92	6.17	0.70	1.17	8.66E-08
1	SLC29A1	-5.12	2.92	3.16	0.17	0.24	2.99E-07
1	DICER1	-4.94	1.79	1.95	0.11	0.16	8.00E-07
1	PhciDBB	-4.51	7.70	9.46	1.53	1.95	6.42E-06
1	TNFalpha	-4.48	1.91	2.23	0.23	0.36	7.49E-06
1	SAMHD1	-4.11	1.77	1.78	0.01	0.01	3.90E-05
1	PhciDAB	-3.89	3.06	3.08	0.03	0.03	1.01E-04
1	TRIM24	-3.33	2.83	3.06	0.30	0.35	8.72E-04
1	TLR2	-2.38	63.68	79.68	35.55	33.69	0.02
1	IL1beta	-2.30	2.35	2.48	0.28	0.28	0.02
1	TLR4	2.17	1.53	1.53	0.01	0.01	0.03
1	IL18	2.23	2.12	2.05	0.15	0.14	0.03
1	KoRVpol	2.84	2.27	2.25	0.04	0.04	0.005
1	FKBP5	4.10	2.11	2.06	0.05	0.07	4.13E-05
1	Cathelicidin	4.57	1.32	1.31	0.007	0.01	4.87E-06
2	KoRVpol	-4.75	2.21	2.25	0.03	0.04	2.07E-06
2	KoRVDenvRBD	-4.66	3.38	3.43	0.06	0.08	3.13E-06

Cluster	Gene	V.test	Cluster Mean	Overall mean	Cluster SD	Overall SD	P-value
2	KoRVenvCKS17	-4.43	2.29	2.30	0.02	0.02	9.22E-06
2	KoRVAenvRBD	-3.88	3.15	3.21	0.06	0.09	1.04E-04
2	DICER1	-3.43	1.86	1.95	0.10	0.16	6.13E-04
2	CD3G	-2.30	14.37	15.77	2.04	3.91	0.02
2	CD4	-2.29	5.35	5.86	0.68	1.44	0.02
2	SLC29A1	-2.28	3.08	3.16	0.16	0.24	0.02
2	SAMHD1	-2.17	1.77	1.78	0.009	0.01	0.03
2	TNFalpha	-1.99	2.12	2.23	0.32	0.36	0.05
2	CLEC4E	2.06	23.00	22.14	2.27	2.67	0.04
2	RETN	4.18	1115.41	924.54	185.84	293.71	2.96E-05
3	RETN	-3.40	527.84	924.54	272.51	293.71	6.74E-04
3	CARD9	-3.37	3.11	3.28	0.17	0.13	7.44E-04
3	CLEC4E	-3.20	18.75	22.14	2.91	2.67	0.001
3	TLR4	-3.08	1.51	1.53	0.02	0.01	0.002
3	PhciDAB	-2.30	3.05	3.08	0.04	0.03	0.02
3	SLC29A1	2.09	3.36	3.16	0.14	0.24	0.04
3	IL18	2.42	2.19	2.05	0.12	0.14	0.02
3	TNFalpha	2.50	2.59	2.23	0.34	0.36	0.01
3	DICER1	2.85	2.13	1.95	0.10	0.16	0.004
3	TRIM24	3.06	3.48	3.06	0.16	0.35	0.002
3	KoRVpol	3.79	2.31	2.25	0.04	0.04	1.49E-04
3	SAMHD1	4.01	1.80	1.78	0.01	0.01	6.09E-05
3	KoRVAenvRBD	4.08	3.36	3.21	0.10	0.09	4.51E-05
3	KoRVDenvRBD	4.11	3.56	3.43	0.08	0.08	4.01E-05
3	KoRVenvCKS17	4.77	2.34	2.30	0.01	0.02	1.86E-06
4	Cathelicidin	-2.84	1.31	1.31	0.008	0.01	0.005
4	FKBP5	-2.75	2.03	2.06	0.06	0.07	0.006
4	TRIM24	2.12	3.18	3.06	0.30	0.35	0.03
4	CARD9	2.17	3.33	3.28	0.12	0.13	0.03
4	PhciDBB	2.39	10.19	9.46	1.76	1.95	0.02
4	SLC29A1	2.56	3.26	3.16	0.16	0.24	0.01
4	SAMHD1	2.70	1.78	1.78	0.007	0.01	0.007
4	IL6	2.70	1.13	1.12	0.008	0.01	0.007
4	CD79b	3.07	6.73	6.17	0.95	1.17	0.002
4	IL1beta	3.35	2.62	2.48	0.19	0.28	8.20E-04
4	PhciDAB	3.36	3.10	3.08	0.03	0.03	7.87E-04
4	TLR7	3.76	5.58	4.90	0.72	1.16	1.67E-04
4	TNFalpha	4.07	2.46	2.23	0.27	0.36	4.71E-05
4	DICER1	4.15	2.05	1.95	0.11	0.16	3.30E-05
4	CD8beta	4.36	8.56	7.37	1.19	1.74	1.28E-05
4	CD3G	4.37	18.43	15.77	2.01	3.91	1.22E-05
4	TLR2	4.49	103.23	79.68	26.47	33.69	7.04E-06
4	CD4	4.66	6.90	5.86	0.84	1.44	3.19E-06
5	IL18	-4.21	1.88	2.05	0.09	0.14	2.57E-05
5	RETN	-4.20	572.85	924.54	146.47	293.71	2.66E-05
5	TLR4	-4.17	1.51	1.53	0.008	0.01	2.99E-05
5	CARD9	-3.83	3.14	3.28	0.08	0.13	1.29E-04
5	CLEC4E	-3.47	19.51	22.14	1.22	2.67	5.27E-04
5	Cathelicidin	-2.51	1.31	1.31	0.004	0.01	0.01
5	IL6	-2.26	1.11	1.12	0.008	0.01	0.02
5	PhciDAB	2.49	3.11	3.08	0.02	0.03	0.01
5	DICER1	3.08	2.09	1.95	0.07	0.16	0.002
5	PhciDBB	3.45	11.38	9.46	1.87	1.95	5.63E-04
5	CD8beta	4.31	9.51	7.37	0.88	1.74	1.67E-05
5	CD4	4.46	7.69	5.86	0.66	1.44	8.28E-06
5	SLC29A1	4.53	3.47	3.16	0.15	0.24	5.88E-06
5	TLR7	4.59	6.42	4.90	0.57	1.16	4.54E-06
5	CD3G	4.83	21.16	15.77	1.82	3.91	1.37E-06
5	CD79b	5.12	7.88	6.17	0.49	1.17	3.01E-07

Chapter 3 Supplementary material references:

Church, C., Casteriano, A., Muir, Y. S. S., Krockenberger, M. B., Vaz, P. K., Higgins, D. P., & Wright, B. R. (2025). New insights into the range and transmission dynamics of a koala gammaherpesvirus, phascolarctid gammaherpesvirus 2. *Sci Rep*, ***In press.***

Hulse, L. S., Hickey, D., Mitchell, J. M., Beagley, K. W., Ellis, W., & Johnston, S. D. (2018). Development and application of two multiplex real-time PCR assays for detection and speciation of bacterial pathogens in the koala. *J Vet Diagn Invest*, ***30***(4), 523-529. https://doi.org/10.1177/1040638718770490

Kasimov, V., Stephenson, T., Speight, N., Chaber, A. L., Boardman, W., Easther, R., & Hemmatzadeh, F. (2020). Identification and Prevalence of Phascolarctid Gammaherpesvirus Types 1 and 2 in South Australian Koala Populations. *Viruses*, ***12***(9). https://doi.org/10.3390/v12090948

Tarlinton, R., Meers, J., Hanger, J., & Young, P. (2005). Real-time reverse transcriptase PCR for the endogenous koala retrovirus reveals an association between plasma viral load and neoplastic disease in koalas. *J Gen Virol*, ***86***(Pt 3), 783-787. https://doi.org/10.1099/vir.0.80547-0

Wright, B. R., Jelocnik, M., Casteriano, A., Muir, Y. S. S., Legione, A. R., Vaz, P. K., Devlin, J. M., & Higgins, D. P. (2023). Development of diagnostic and point of care assays for a gammaherpesvirus infecting koalas. *PLoS One*, ***18***(6), e0286407. https://doi.org/10.1371/journal.pone.0286407

Chapter 4 Supplementary materials:

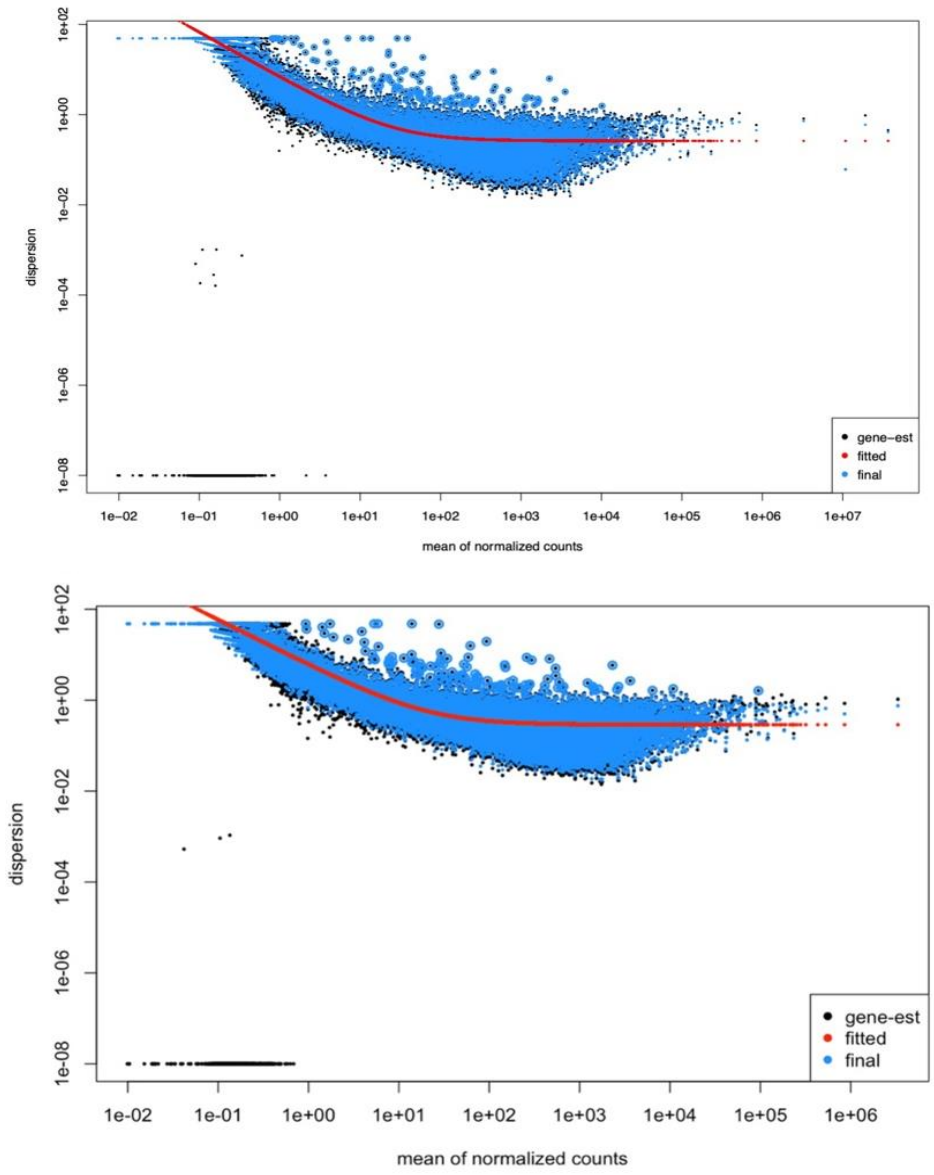

Figure 7.1: Dispersion plots of three DESeq experiments assessing differential gene expression in koalas according to their chlamydiosis status and outcome.

Figure 7.1 presents the dispersion plots for the two comparisons: chlamydiosis status (top) and survival outcome (bottom). The red fitted line plots the estimate for the expected dispersion value for genes of a given expression strength. Gene mean expression and maximum likelihood of the dispersion is plotted as black points (gene-est). Final dispersion estimates are plotted in blue, with those slightly above the curve shrunken towards the fitted line to improve the dispersion estimation. Genes with high dispersion are not shrunken and are highlighted with a blue border to indicate that the model assumptions are not followed, and the higher variability is associated with biological or technical differences, i.e. differentially expressed genes.

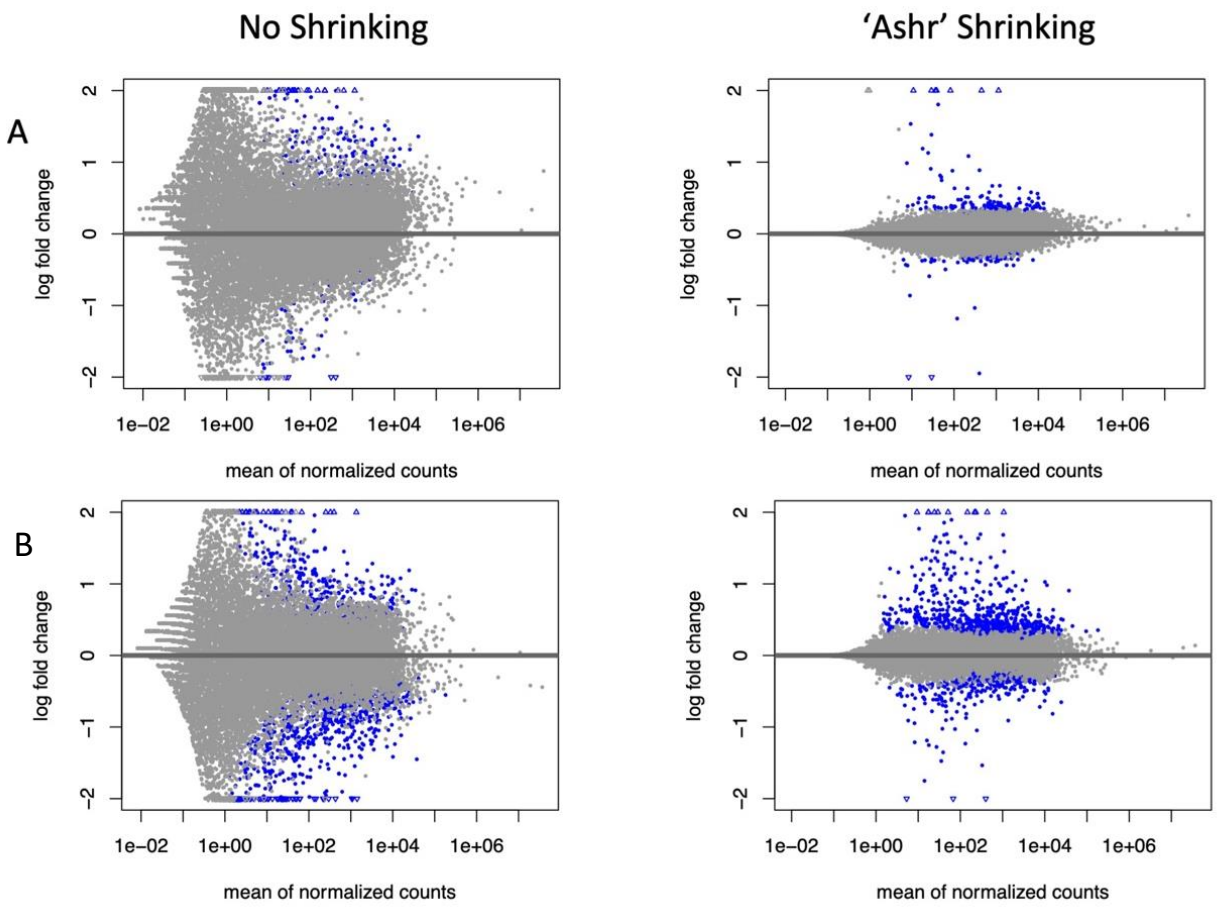

Figure 7.2: MA-plots displaying pre- and post-shrinking fold-change for the normalised read counts of koala mRNA for each comparison (chlamydiosis status and outcome) using no shrinking (left) and ashr shrinking (right).

The two comparisons were generated using the three DESeq designs as follows: Experiment 1 (SYNDROME) comparing (A) koalas with chlamydiosis and koalas without signs of disease, and (B) experiment 2 (Outcome) comparing koalas euthanised with koalas that survived.

Table 7.18: Frequency of detection of infectious agents according to cohort among the three comparisons

Values are reported as counts with the associated percentage of the respective group in brackets.

		Sample population	Comparison 1 (N = 36)		Comparison 2 (N = 49)	
		N = 49	Chlamydiosis (N = 22)	None (N = 14)	Survived (N = 26)	Euthanised (N = 23)
Mucosal *C. pecorum* shedding	*Detected*	22 (45)	18 (82)	1 (7)	5 (20)	17 (74)
	Not detected	27 (55)	4 (18)	13 (93)	21 (80)	6 (26)
Circulating *C. pecorum* transcription	*Detected*	4 (8)	1 (4)	2 (14)	2 (8)	2 (9)
	Not detected	45 (92)	21 (96)	12 (86)	24 (92)	21 (91)
Mucosal PhaHV-1 shedding	*Detected*	28 (57)	15 (68)	5 (36)	11 (42)	17 (74)
	Not detected	21 (43)	7 (32)	9 (64)	15 (58)	6 (26)
Mucosal PhaHV-2 shedding	*Detected*	12 (25)	5 (23)	2 (14)	5 (19)	7 (30)
	Not detected	37 (75)	17 (77)	12 (86)	21 (81)	16 (70)
Circulating PhaHV-1 transcription	*Detected*	1 (2)	1 (4)	0 (0)	0 (0)	1 (4)
	Not detected	48 (98)	21 (96)	14 (100)	26 (100)	22 (96)
Circulating KoRV B transcription	*Detected*	17 (35)	10 (45)	3 (21)	4 (15)	13 (56)
	Not detected	32 (65)	12 (55)	11 (79)	22 (85)	10 (44)
Circulating trypanosome transcription	*Detected*	27 (55)	10 (45)	6 (43)	14 (54)	13 (56)
	Not detected	22 (45)	12 (55)	8 (57)	12 (46)	10 (44)

*No samples had detectable PhaHV-2 transcription in circulating cell samples

Table 7.19: Differentially expressed genes between koalas with clinical signs of chlamydiosis compared to koalas without chlamydiosis

Gene	Full/Alternative Name	Base Mean	log^2 Fold Change	LFC SE[1]	p-value	P.adj[2]	Fold Change	% Change	Context[3]
IL1R2	Interleukin 1 receptor 2	1134.3	3.9	0.8	5.80E-09	8.69E-05	14.82	1381.7	Upregulated
MYO1B	Myosin IB	82.2	3.7	0.8	1.47E-08	0.0001	13.37	1236.8	Upregulated
LOC110218592	Uncharacterized	8.3	-5.6	1.4	2.44E-08	0.0001	0.02	97.9	Downregulated
LOC110212645	Uncharacterized	29.4	-2.2	0.7	3.33E-07	0.001	0.21	78.7	Downregulated
BDNF	Brain derived neurotrophic factor	29.0	15.9	5.7	4.25E-07	0.001	62807.30	6280630.5	Upregulated
FAM19A2	TAFA chemokine like family member 2	398.0	-1.9	0.9	2.12E-06	0.003	0.27	73.2	Downregulated
RARRES1	Retinoic acid receptor responder 1	449.9	3.4	1.5	1.43E-06	0.003	10.21	921.3	Upregulated
PDSS2	Decaprenyl diphosphate synthase subunit 2	118.7	-1.1	0.7	1.86E-06	0.003	0.47	52.8	Downregulated
LOC110219949	Kallikrein-11-like	36.0	4.6	3.6	2.82E-06	0.003	24.21	2320.8	Upregulated
LOC110192234	Monocyte chemotactic protein 1B-like	42.0	1.7	1.0	3.08E-06	0.003	3.33	233.5	Upregulated
SOCS1	Suppressor of cytokine signalling 1	806.3	0.6	0.4	5.95E-06	0.006	1.53	53.0	Upregulated
CDKN1A	Cyclin dependent kinase inhibitor 1A	3527.1	0.6	0.4	9.42E-06	0.008	1.50	50.2	Upregulated
MIA	MIA SH3 domain containing	9.3	1.4	1.1	9.98E-06	0.008	2.73	172.6	Upregulated
LOC110197580	Uncharacterized	10.8	5.6	7.5	1.15E-05	0.008	48.16	4716.0	Upregulated
FAM177B	Family with sequence similarity 177 member B	29.1	1.3	1.1	1.54E-05	0.009	2.45	145.3	Upregulated
LOC110219845	Sialic acid-binding Ig-like lectin 9	208.9	0.6	0.4	1.63E-05	0.009	1.50	49.6	Upregulated
AKAP4	A-kinase anchoring protein 4	219.9	1.0	0.9	2.31E-05	0.01	1.98	98.4	Upregulated
LOC110206414	WAP four-disulfide core domain protein 12-like	28.0	0.8	0.7	2.30E-05	0.01	1.76	75.8	Upregulated
CSRNP1	Cysteine and serine rich nuclear protein 1	1214.3	0.6	0.5	2.40E-05	0.01	1.53	52.5	Upregulated
SKOR2	SKI family transcriptional corepressor 2	38.6	3.2	6.1	2.62E-05	0.01	9.19	818.6	Upregulated
ARMC12	Armadillo repeat containing 12	46.7	0.7	0.7	2.70E-05	0.01	1.66	65.9	Upregulated
GPR84	G protein-coupled receptor 84	401.3	0.8	0.8	2.77E-05	0.01	1.74	73.8	Upregulated
LOC110205351	Uncharacterized	310.0	-1.0	1.0	3.57E-05	0.01	0.52	48.4	Downregulated
IL18	Interleukin 18	91.6	0.8	0.8	4.18E-05	0.01	1.74	74.0	Upregulated
MARCO	Macrophage receptor with collagenous structure	18.0	1.2	1.5	4.34E-05	0.01	2.28	127.9	Upregulated
LOC110211842	Uncharacterized	24.3	1.1	1.3	4.65E-05	0.01	2.16	115.6	Upregulated
NR4A3	Nuclear receptor subfamily 4 group A member 3	50.8	0.7	0.7	5.16E-05	0.01	1.60	59.8	Upregulated

[1]Standard error of the log^2 fold change (FC). [2]DESeq2 adjusted wald test p-values using Benjamini and Hochberg method (BH-adjusted p values). [3] Context refers to gene expression up- or downregulation in koalas with clinical chlamydiosis compared to koalas without chlamydiosis.

Table 7.20: Differentially expressed genes in koalas that were euthanised compared to koalas that survived triage

Gene	Full/Alternative Name	Base Mean	log[2] Fold Change	LFC SE[1]	p-value	P.adj[2]	Fold Change	% Change	Context[3]
IL1R2	Interleukin 1 receptor type 2	1064.0	3.8	0.6	2.30E-13	3.91E-09	14.07	1306.8	Upregulated
MARCO	Macrophage receptor with collagenous structure	29.4	4.4	0.7	1.82E-12	1.54E-08	21.40	2040.2	Upregulated
RARRES1	Retinoic acid receptor responder 1	431.8	4.6	0.7	5.89E-12	3.34E-08	24.18	2317.9	Upregulated
RENBP	Renin binding protein	218.8	2.1	0.4	6.42E-11	2.42E-07	4.41	341.3	Upregulated
SCUBE3	Signal peptide, CUB domain and EGF like domain containing 3	9.4	3.0	0.6	7.14E-11	2.42E-07	8.21	720.6	Upregulated
LOC110197639	Cathepsin L1-like	231.5	2.3	0.4	3.69E-10	1.05E-06	4.92	392.2	Upregulated
LOC110194680	60S ribosomal protein L36a	1209.2	0.7	0.2	5.92E-09	1.44E-05	1.66	66.0	Upregulated
LOC110206415	WAP four-disulfide core domain protein 5-like	41.1	1.9	0.4	1.08E-08	2.29E-05	3.62	261.6	Upregulated
LOC110215329	Zinc finger protein 345-like	39.0	-1.4	0.3	1.71E-08	3.24E-05	0.39	60.9	Downregulated
LOC110193772	Uncharacterized	263.7	1.6	0.3	3.21E-08	5.45E-05	3.11	210.6	Upregulated
ATP5F1	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit b	990.2	0.6	0.1	3.86E-08	5.46E-05	1.50	50.4	Upregulated
MYO1B	Myosin IB	51.3	2.4	0.6	4.22E-08	5.51E-05	5.11	410.7	Upregulated
AKAP4	A-kinase anchoring protein 4	219.9	2.1	0.5	5.30E-08	6.43E-05	4.18	318.0	Upregulated
CREG2	Cellular repressor of E1A stimulated genes 2	17.5	2.1	0.5	6.73E-08	7.62E-05	4.31	330.6	Upregulated
VSIG4	V-set and immunoglobulin domain containing 4	1032.6	1.7	0.4	7.90E-08	7.89E-05	3.21	221.4	Upregulated
KIAA1324L	ELAPOR2 endosome-lysosome associated apoptosis and autophagy regulator family member 2	67.5	-6.1	1.8	8.55E-08	8.07E-05	0.01	98.5	Downregulated
MARK1	Microtubule affinity regulating kinase 1	102.8	1.7	0.4	1.41E-07	0.0001	3.16	215.5	Upregulated
NECTIN3	Nectin cell adhesion molecule 3	10.5	1.8	0.4	1.50E-07	0.0001	3.41	241.1	Upregulated
PEX7	Peroxisomal biogenesis factor 7	341.4	0.7	0.2	1.35E-07	0.0001	1.58	57.9	Upregulated
SH3PXD2B	SH3 and PX domains 2B	47.4	1.7	0.4	1.46E-07	0.0001	3.24	223.6	Upregulated
FAM19A2	TAFA2 - TAFA chemokine like family member 2	398.0	-2.0	0.5	1.60E-07	0.0001	0.24	75.7	Downregulated
KLF9	KLF transcription factor 9	181.9	1.3	0.3	2.38E-07	0.0002	2.53	153.2	Upregulated
SOCS1	Suppressor of cytokine signaling 1	806.3	1.0	0.3	2.66E-07	0.0002	2.01	101.1	Upregulated
LOC110211842	Uncharacterized LOC110211842	24.3	2.6	0.8	2.79E-07	0.0002	6.27	527.0	Upregulated
AEN	Apoptosis enhancing nuclease	498.3	0.9	0.3	3.53E-07	0.0002	1.85	84.6	Upregulated
FLT1	Fms related receptor tyrosine kinase 1	41.2	1.7	0.4	3.43E-07	0.0002	3.21	221.2	Upregulated
LOC110210342	Cytochrome C oxidase subunit 4 isoform 1, mitochondrial	2367.0	0.7	0.2	4.09E-07	0.0002	1.59	58.5	Upregulated
DDIT4	DNA damage inducible transcript 4	691.8	1.2	0.3	4.42E-07	0.0002	2.36	135.5	Upregulated
NR4A3	Nuclear receptor subfamily 4 group A member 3	50.8	1.6	0.4	4.39E-07	0.0002	2.99	199.1	Upregulated
MAFF	MAF bZIP transcription factor F	297.4	1.7	0.4	4.91E-07	0.0003	3.23	223.3	Upregulated
ARMC12	Armadillo repeat containing 12	46.7	1.4	0.4	6.87E-07	0.0003	2.65	165.4	Upregulated
LOC110219949	Kallikrein-11-like	147.2	4.8	2.2	7.43E-07	0.0003	28.76	2775.6	Upregulated
LOC110221111	C-type lectin domain family 4 member K-like - CLEC4K	61.5	1.9	0.6	7.91E-07	0.0004	3.72	271.8	Upregulated
PFDN4	Prefoldin subunit 4	244.9	0.8	0.3	8.85E-07	0.0004	1.78	78.2	Upregulated
PPP1R15A	Protein phosphatase 1 regulatory subunit 15A	520.5	1.4	0.4	9.03E-07	0.0004	2.58	158.3	Upregulated
CHD9	Chromodomain helicase DNA binding protein 9	9896.0	-0.6	0.2	9.37E-07	0.0004	0.64	35.6	Downregulated
CMBL	Carboxymethylenebutenolidase homolog	43.7	0.8	0.3	1.09E-06	0.0004	1.79	79.1	Upregulated
LOC110194448	Histone H2B type 1-B-like	683.1	1.1	0.3	1.22E-06	0.0004	2.10	110.3	Upregulated
LRP12	LDL receptor related protein 12	50.1	1.2	0.4	1.45E-06	0.0005	2.31	130.5	Upregulated
ENTPD2	Ectonucleoside triphosphate diphosphohydrolase 2	72.9	1.4	0.4	1.50E-06	0.0005	2.71	171.1	Upregulated

Gene	Full/Alternative Name	Base Mean	log^2 Fold Change	LFC SE[1]	p-value	P.adj[2]	Fold Change	% Change	Context[3]
LOC110196119	Cytochrome C oxidase subunit 6C	734.0	0.7	0.2	1.72E-06	0.0006	1.58	58.1	Upregulated
LOC110217150	Cathelicidin antimicrobial peptide-like	295.0	1.3	0.4	1.75E-06	0.0006	2.45	145.3	Upregulated
WSCD2	WSC domain containing 2	5.3	-2.8	1.2	1.76E-06	0.0006	0.15	85.3	Downregulated
TMED3	Transmembrane p24 trafficking protein 3	138.0	1.1	0.4	2.03E-06	0.0006	2.22	121.6	Upregulated
MTHFD2	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2	1766.7	1.2	0.4	2.23E-06	0.0007	2.24	123.9	Upregulated
KIAA1549L	KIAA1549 like - integral component of membrane	35.2	-1.5	0.5	2.46E-06	0.0007	0.36	64.0	Downregulated
C2	Complement C2	133.6	1.6	0.5	2.86E-06	0.0008	2.96	196.2	Upregulated
LOC110219482	Probable hydrolase PNKD	108.0	1.4	0.4	2.93E-06	0.0008	2.55	155.2	Upregulated
NEFM	Neurofilament medium chain	20.2	1.3	0.4	3.04E-06	0.0009	2.50	150.0	Upregulated
MRPS18C	Mitochondrial ribosomal protein S18C	99.7	0.7	0.2	3.19E-06	0.0009	1.58	57.8	Upregulated
CD207	CLEC4K	17.5	2.4	1.3	4.15E-06	0.001	5.36	436.0	Upregulated
CDKN1A	Cyclin dependent kinase inhibitor 1A	3527.1	1.0	0.4	3.99E-06	0.001	2.07	106.5	Upregulated
CENPM	Centromere protein M	74.1	1.1	0.4	4.19E-06	0.001	2.21	121.0	Upregulated
CUNH9orf152	Chromosome unknown C9orf152 homolo	33.1	1.6	0.5	3.76E-06	0.001	2.99	198.6	Upregulated
DDAH2	DDAH family member 2, ADMA-independent	221.1	0.8	0.3	4.18E-06	0.001	1.70	69.8	Upregulated
GGH	Gamma-glutamyl hydrolase	125.8	0.9	0.3	4.15E-06	0.001	1.90	90.1	Upregulated
RPS17	Ribosomal protein S17	11225.9	1.0	0.3	4.15E-06	0.001	1.97	97.4	Upregulated
LOC110207566	Uncharacterized	327.6	-1.5	0.5	4.37E-06	0.001	0.35	65.5	Downregulated
LOC110219478	Tubulin alpha-1D chain-like	25.4	1.4	0.5	4.34E-06	0.001	2.57	156.5	Upregulated
LAMTOR4	Late endosomal/lysosomal adaptor, MAPK and MTOR activator 4	597.1	0.7	0.2	4.98E-06	0.001	1.63	63.2	Upregulated
VIPR2	Vasoactive intestinal peptide receptor 2	4.9	2.0	0.9	5.09E-06	0.001	3.87	286.9	Upregulated
JAG1	Jagged canonical Notch ligand 1	15.3	1.4	0.5	5.26E-06	0.001	2.57	156.6	Upregulated
SEC11C	SEC11 homolog C, signal peptidase complex subunit	610.6	0.6	0.2	5.36E-06	0.001	1.52	52.1	Upregulated
LOC110208071	Cytochrome C oxidase copper chaperone	451.0	0.7	0.2	5.73E-06	0.001	1.59	58.6	Upregulated
PSME2	Proteasome activator subunit 2	2844.2	0.7	0.2	5.89E-06	0.001	1.62	61.7	Upregulated
ZWINT	ZW10 interacting kinetochore protein	355.5	0.9	0.3	5.88E-06	0.001	1.85	85.2	Upregulated
CDK5RAP3	CDK5 regulatory subunit associated protein 3	2027.2	1.0	0.4	6.27E-06	0.001	1.96	96.2	Upregulated
NPC2	NPC intracellular cholesterol transporter 2	2235.0	0.6	0.2	6.21E-06	0.001	1.52	51.8	Upregulated
MMP14	Matrix metallopeptidase 14	41.7	1.3	0.5	6.42E-06	0.001	2.42	142.1	Upregulated
AHCY	Adenosylhomocysteinase	1661.3	0.6	0.2	6.68E-06	0.001	1.54	53.8	Upregulated
LOC110199719	T-cell immunoglobulin and mucin domain-containing protein 4-like	21.7	1.5	0.5	6.62E-06	0.001	2.75	175.4	Upregulated
ADGRG7	Adhesion G protein-coupled receptor G7	27.2	-1.3	0.5	7.06E-06	0.001	0.41	58.8	Downregulated
FKBP5	FKBP prolyl isomerase 5	2958.5	0.9	0.3	7.17E-06	0.001	1.88	88.3	Upregulated
FGL2	Fibrinogen like 2	15319.5	-0.8	0.3	7.94E-06	0.001	0.57	43.2	Downregulated
EPSTI1	Epithelial stromal interaction 1	103.3	0.9	0.4	8.54E-06	0.002	1.92	92.4	Upregulated
LOC110195293	Uncharacterized	23.7	1.6	0.7	8.76E-06	0.002	3.05	204.5	Upregulated
CH25H	Cholesterol 25-hydroxylase	26.2	1.7	0.7	8.92E-06	0.002	3.15	214.7	Upregulated
SHMT2	Serine hydroxymethyltransferase 2	1044.8	1.1	0.4	9.24E-06	0.002	2.10	110.4	Upregulated
LOC110222717	40S ribosomal protein S23	530.2	0.6	0.2	9.40E-06	0.002	1.52	52.0	Upregulated
LOC110201055	Uncharacterized	8.4	1.1	0.4	1.03E-05	0.002	2.17	116.9	Upregulated
LOC110205308	Neutrophil gelatinase-associated lipocalin-like	731.1	1.2	0.5	1.04E-05	0.002	2.38	137.7	Upregulated
PM20D1	Peptidase M20 domain containing 1	1089.2	1.5	0.6	1.07E-05	0.002	2.74	173.5	Upregulated
MRC1	Mannose receptor C-type 1	888.5	1.0	0.4	1.20E-05	0.002	1.96	96.3	Upregulated

Gene	Full/Alternative Name	Base Mean	log[2] Fold Change	LFC SE[1]	p-value	P.adj[2]	Fold Change	% Change	Context[3]
BBC3	BCL2 binding component 3	438.7	0.7	0.2	1.23E-05	0.002	1.59	58.9	Upregulated
EGR2	Early growth response 2	56.2	1.1	0.4	1.22E-05	0.002	2.10	109.7	Upregulated
SLC45A3	Solute carrier family 45 member 3	70.3	1.2	0.5	1.23E-05	0.002	2.35	134.7	Upregulated
LOC110216652	Uncharacterized	891.6	0.7	0.3	1.30E-05	0.002	1.63	62.9	Upregulated
FBLIM1	Filamin binding LIM protein 1	12.2	1.3	0.5	1.33E-05	0.002	2.54	153.7	Upregulated
DYDC1	DPY30 domain containing 1	60.4	1.4	0.6	1.39E-05	0.002	2.73	173.1	Upregulated
LOC110204197	Histone H3	1288.6	0.9	0.4	1.44E-05	0.002	1.85	85.4	Upregulated
SHANK1	SH3 and multiple ankyrin repeat domains 1	131.1	-1.2	0.5	1.86E-05	0.003	0.43	57.4	Downregulated
LOC110214205	Lymphocyte antigen 6H-like	40.6	1.2	0.5	1.89E-05	0.003	2.33	133.5	Upregulated
CAPNS1	Calpain small subunit 1	4280.3	0.6	0.2	1.93E-05	0.003	1.51	51.0	Upregulated
LOC110220763	Uncharacterized	74.7	-1.0	0.4	1.96E-05	0.003	0.49	51.0	Downregulated
CD36	CD36 molecule	28.3	1.4	0.7	2.06E-05	0.003	2.72	172.4	Upregulated
G6PD	Glucose-6-phosphate dehydrogenase	10083.0	1.0	0.4	2.07E-05	0.003	2.04	103.7	Upregulated
UPP1	Uridine phosphorylase 1	3898.2	0.7	0.3	2.22E-05	0.003	1.66	66.4	Upregulated
IGF2BP1	Insulin like growth factor 2 mRNA binding protein 1	14.1	-1.8	1.3	2.29E-05	0.003	0.30	70.3	Downregulated
LOC110202533	Glutathione S-transferase theta-2-like	13.0	1.1	0.5	2.49E-05	0.003	2.19	119.0	Upregulated
TMEM102	Transmembrane protein 102	262.6	0.7	0.3	2.54E-05	0.003	1.62	61.6	Upregulated
CHAC1	ChaC glutathione specific gamma-glutamylcyclotransferase 1	129.8	0.9	0.4	2.70E-05	0.003	1.85	84.7	Upregulated
EGR1	Early growth response 1	1242.2	1.1	0.5	2.72E-05	0.003	2.12	112.4	Upregulated
MSRA	Methionine sulfoxide reductase A	1059.0	0.7	0.3	2.84E-05	0.003	1.60	60.0	Upregulated
FAM177B	Family with sequence similarity 177 member B	29.1	1.3	0.7	3.04E-05	0.003	2.49	149.5	Upregulated
MTCH1	Mitochondrial carrier 1	23.3	0.7	0.3	3.03E-05	0.003	1.67	66.6	Upregulated
CFAP206	Cilia and flagella associated protein 206	245.1	-1.0	0.5	3.08E-05	0.003	0.48	51.6	Downregulated
PROCR	Protein C receptor	31.5	0.7	0.3	3.10E-05	0.003	1.67	67.1	Upregulated
LOC110197056	Uncharacterized	46.5	1.1	0.5	3.17E-05	0.004	2.20	119.8	Upregulated
PER1	Period circadian regulator 1	3734.0	0.7	0.3	3.22E-05	0.004	1.66	66.2	Upregulated
CKS1B	CDC28 protein kinase regulatory subunit 1B	58.8	1.1	0.5	3.63E-05	0.004	2.08	108.1	Upregulated
LOC110214748	Fatty acid-binding protein, epidermal-like	59.7	0.9	0.4	3.73E-05	0.004	1.86	86.4	Upregulated
ALPL	Alkaline phosphatase, biomineralization associated	62.1	1.1	0.5	3.94E-05	0.004	2.15	115.5	Upregulated
CCDC146	Coiled-coil domain containing 146	45.6	-0.9	0.4	4.25E-05	0.004	0.53	46.6	Downregulated
MLLT11	MLLT11 transcription factor 7 cofactor	34.3	0.7	0.3	4.34E-05	0.004	1.64	64.0	Upregulated
NR4A2	Nuclear receptor subfamily 4 group A member 2	278.8	1.0	0.5	4.44E-05	0.004	2.03	103.1	Upregulated
LOC110213587	Cysteine-rich secretory protein 3-like	47.3	1.2	0.6	4.58E-05	0.005	2.22	122.4	Upregulated
LOC110203919	Uncharacterized	520.7	0.9	0.4	4.79E-05	0.005	1.81	81.4	Upregulated
VGLL3	Vestigial like family member 3	76.0	0.8	0.4	4.92E-05	0.005	1.78	77.6	Upregulated
LOC110219719	Carcinoembryonic antigen-related cell adhesion molecule 3-like	137.9	-0.8	0.4	5.09E-05	0.005	0.57	43.2	Downregulated
WNT8B	Wnt family member 8B	19.0	1.2	0.7	5.78E-05	0.005	2.31	131.3	Upregulated
LOC110220060	CD177 antigen-like	256.4	1.2	0.7	5.91E-05	0.005	2.26	125.7	Upregulated
LOC110206301	Uncharacterized	8.2	-0.9	0.5	5.95E-05	0.005	0.52	48.1	Downregulated
ARSA	Arylsulfatase A	325.7	0.8	0.4	6.13E-05	0.006	1.71	71.4	Upregulated
STOX2	Storkhead box 2	62.7	-0.9	0.4	6.30E-05	0.006	0.54	45.8	Downregulated
SLIT3	Slit guidance ligand 3	17.8	-1.3	0.8	6.60E-05	0.006	0.42	58.1	Downregulated
TIMP1	TIMP metallopeptidase inhibitor 1	998.4	0.6	0.3	6.73E-05	0.006	1.56	56.1	Upregulated

Gene	Full/Alternative Name	Base Mean	log^2 Fold Change	LFC SE[1]	p-value	P.adj[2]	Fold Change	% Change	Context[3]
APOE	Apolipoprotein E	26.1	0.9	0.5	6.85E-05	0.006	1.91	90.6	Upregulated
STRIP2	Striatin interacting protein 2	37.4	-1.0	0.5	6.88E-05	0.006	0.49	50.8	Downregulated
TSGA10	Testis specific 10	90.7	-0.7	0.3	6.99E-05	0.006	0.63	37.5	Downregulated
TUBB6	Tubulin beta 6 class V	109.1	0.7	0.4	7.00E-05	0.006	1.68	68.1	Upregulated
TSPO	Translocator protein	1385.8	0.6	0.3	7.40E-05	0.006	1.54	53.6	Upregulated
ASB2	Ankyrin repeat and SOCS box containing 2	32.7	1.1	0.6	7.52E-05	0.006	2.08	107.5	Upregulated
BCOR	BCL6 corepressor	7596.1	-0.7	0.3	7.51E-05	0.006	0.61	39.3	Downregulated
PYCR1	Pyrroline-5-carboxylate reductase 1	1643.6	0.9	0.5	7.67E-05	0.006	1.87	86.6	Upregulated
AIF1	Allograft inflammatory factor 1	1327.5	0.6	0.3	7.84E-05	0.006	1.50	50.1	Upregulated
LOC110192486	Uncharacterized	82.3	-0.8	0.4	7.84E-05	0.006	0.58	42.1	Downregulated
LOC110218567	Uncharacterized	7.1	1.2	1.0	7.83E-05	0.006	2.36	135.6	Upregulated
LOC110209790	Permeability factor 2-like	195.7	0.9	0.5	8.22E-05	0.006	1.91	90.9	Upregulated
NXN	Nucleoredoxin	2.1	1.2	0.9	8.26E-05	0.006	2.32	131.8	Upregulated
TENM4	Teneurin transmembrane protein 4	16.1	-1.2	0.8	8.30E-05	0.006	0.44	56.1	Downregulated
LOC110204914	Netrin-4-like	8.2	-1.2	1.0	8.46E-05	0.006	0.43	56.9	Downregulated
DOCK1	Dedicator of cytokinesis 1	11.4	1.1	0.6	8.79E-05	0.006	2.08	108.0	Upregulated
SLC7A11	Solute carrier family 7 member 11	305.1	0.8	0.4	8.88E-05	0.006	1.73	73.1	Upregulated
GPX1	Glutathione peroxidase 1	37361.7	0.9	0.5	9.19E-05	0.007	1.87	87.3	Upregulated
LOC110213814	Uncharacterized	218.5	-0.7	0.3	9.28E-05	0.007	0.62	37.9	Downregulated
SFRP4	Secreted frizzled related protein 4	146.6	0.7	0.4	9.60E-05	0.007	1.66	66.5	Upregulated
GPR1	CMKLR2 chemerin chemokine-like receptor 2	3.4	1.2	1.1	9.59E-05	0.007	2.24	123.6	Upregulated
ISYNA1	Inositol-3-phosphate synthase 1	71.6	0.6	0.3	9.46E-05	0.007	1.57	56.7	Upregulated
KLK9	Kallikrein related peptidase 9	9.0	1.0	0.5	9.56E-05	0.007	1.96	95.8	Upregulated
PLCH1	Phospholipase C eta 1	522.5	1.0	0.6	9.83E-05	0.007	1.98	98.3	Upregulated
ACTL6B	Actin like 6B	52.8	0.7	0.3	0.0001	0.007	1.60	60.2	Upregulated
LOC110200073	Uncharacterized	97.7	0.8	0.4	0.0001	0.007	1.69	69.1	Upregulated
SEP10	MED31 mediator complex subunit 31	13.0	0.9	0.5	0.0001	0.007	1.84	83.8	Upregulated
SLC16A14	Solute carrier family 16 member 14	49.3	0.8	0.4	0.0001	0.007	1.73	73.2	Upregulated
GPR3	G protein-coupled receptor 3	23.2	1.1	0.9	0.0001	0.007	2.19	118.7	Upregulated
GJD3	Gap junction protein delta 3	241.3	1.0	0.7	0.0001	0.007	2.06	106.1	Upregulated
LOC110206414	WAP four-disulfide core domain protein 12-like	28.0	0.9	0.5	0.0001	0.007	1.83	82.9	Upregulated
IRS1	Insulin receptor substrate 1	64.6	0.7	0.4	0.0001	0.008	1.68	67.6	Upregulated
UBE2M	Ubiquitin conjugating enzyme E2 M	1004.1	0.7	0.4	0.0001	0.008	1.63	63.5	Upregulated
LOC110202552	Vomeronasal type-2 receptor 26-like	22.6	-0.6	1.5	0.0001	0.008	0.65	34.8	Downregulated
LOC110202416	Patr class I histocompatibility antigen, A-5 alpha chain-like	1842.1	0.8	0.4	0.0001	0.008	1.72	72.5	Upregulated
NR4A1	Nuclear receptor subfamily 4 group A member 1	265.6	0.7	0.4	0.0001	0.009	1.63	63.3	Upregulated
LOC110201454	Phospholipid-transporting ATPase FetA-like	34.0	-0.6	0.3	0.0001	0.009	0.66	34.2	Downregulated
GSR	Glutathione-disulfide reductase	1802.7	0.6	0.3	0.0001	0.009	1.50	49.6	Upregulated
PLK2	Polo like kinase 2	123.2	-0.7	0.4	0.0001	0.009	0.61	39.1	Downregulated
EPB41L4B	Erythrocyte membrane protein band 4.1 like 4B	320.1	-0.6	0.3	0.0002	0.009	0.64	35.9	Downregulated
LOC110222268	Uncharacterized	11.1	0.9	0.5	0.0002	0.009	1.81	81.4	Upregulated
LOC110201193	Cyclin-dependent kinase inhibitor 2A-like	48.7	0.6	0.3	0.0002	0.009	1.55	54.7	Upregulated
FKBPL	FKBP prolyl isomerase like	93.3	0.6	0.3	0.0002	0.009	1.55	55.0	Upregulated

Gene	Full/Alternative Name	Base Mean	log^2 Fold Change	LFC SE[1]	p-value	P.adj[2]	Fold Change	% Change	Context[3]
KLK8	Kallikrein related peptidase 8	125.6	0.7	0.4	0.0002	0.009	1.64	64.0	Upregulated
LOC110192889	Uncharacterized	14.4	-0.7	0.4	0.0002	0.01	0.63	37.2	Downregulated
LOC110217723	Zinc-alpha-2-glycoprotein-like	23.8	1.0	0.7	0.0002	0.01	1.96	96.1	Upregulated
MISP	Mitotic spindle positioning	21.5	1.0	0.7	0.0002	0.01	1.95	95.2	Upregulated
CDC20B	Cell division cycle 20B	3.9	0.8	1.2	0.0002	0.01	1.73	73.4	Upregulated
C1QA	Complement C1q A chain	3907.5	0.7	0.4	0.0002	0.01	1.59	58.6	Upregulated
LOC110217725	Zinc-alpha-2-glycoprotein-like	9.0	0.9	0.9	0.0002	0.01	1.92	92.2	Upregulated
LOC110207421	Uncharacterized	21.2	-0.8	0.5	0.0002	0.01	0.58	41.8	Downregulated
LOC110215351	Galactosylceramide sulfotransferase-like	5.8	-0.9	0.9	0.0002	0.01	0.53	46.8	Downregulated
GUCY2C	Guanylate cyclase 2C	44.7	-0.7	0.4	0.0002	0.01	0.61	38.8	Downregulated
EPAS1	Endothelial PAS domain protein 1	532.7	0.6	0.4	0.0002	0.01	1.55	54.5	Upregulated
CRISPLD2	Cysteine rich secretory protein LCCL domain containing 2	1414.1	0.9	0.7	0.0002	0.01	1.89	89.5	Upregulated
LOC110203729	Rho GTPase-activating protein 20-like	135.9	-0.9	0.6	0.0002	0.01	0.55	45.4	Downregulated
LOC110195805	Uncharacterized	1.6	0.8	1.1	0.0002	0.01	1.77	76.6	Upregulated
LOC110192487	Uncharacterized	61.5	-0.6	0.3	0.0002	0.01	0.65	35.1	Downregulated
CFB	Complement factor B	8.4	0.9	0.7	0.0002	0.01	1.86	86.3	Upregulated
LOC110203396	Uncharacterized	1358.1	-0.9	0.8	0.0003	0.01	0.53	46.8	Downregulated
HBEGF	Heparin binding EGF like growth factor	21.7	0.9	0.7	0.0003	0.01	1.87	87.0	Upregulated
SYT14	Synaptotagmin 14	30.8	-0.7	0.4	0.0003	0.01	0.63	37.5	Downregulated
INA	Internexin neuronal intermediate filament protein alpha	46.8	-0.8	0.5	0.0003	0.01	0.58	42.0	Downregulated
PSME1	Proteasome activator subunit 1	15825.7	0.6	0.4	0.0003	0.01	1.56	55.6	Upregulated
LOC110218422	OX-2 membrane glycoprotein-like	17.6	0.8	0.5	0.0003	0.01	1.70	69.8	Upregulated
C1QB	Complement C1q B chain	2266.2	0.6	0.4	0.0003	0.01	1.56	55.6	Upregulated
NECTIN2	Nectin cell adhesion molecule 2	1042.8	0.6	0.3	0.0003	0.01	1.50	49.7	Upregulated
RFTN2	Raftlin family member 2	35.8	-0.6	0.3	0.0003	0.01	0.66	34.0	Downregulated
HS6ST1	Heparan sulfate 6-O-sulfotransferase 1	441.8	0.6	0.3	0.0003	0.01	1.53	52.6	Upregulated
LOC110204450	Cytochrome P450 2C42-like	23.5	0.8	0.9	0.0003	0.01	1.80	79.5	Upregulated
ZGLP1	Zinc finger GATA like protein 1	11.9	0.7	0.4	0.0003	0.01	1.60	59.6	Upregulated
CDYL	Chromodomain Y like	24586.6	-0.7	0.4	0.0003	0.01	0.64	36.4	Downregulated
GBGT1	Globoside alpha-1,3-N-acetylgalactosaminyltransferase 1 (FORS blood group)	23.5	0.6	0.4	0.0003	0.01	1.57	56.6	Upregulated
PROS1	Protein S	48.5	0.7	0.5	0.0003	0.01	1.66	65.8	Upregulated
AWAT2	Acyl-CoA wax alcohol acyltransferase 2	12.1	0.8	0.6	0.0004	0.01	1.72	72.3	Upregulated
CHI3L2	Chitinase 3 like 2	850.2	0.7	0.4	0.0004	0.01	1.59	59.0	Upregulated
TEX15	Testis expressed 15, meiosis and synapsis associated	173.8	-0.6	0.3	0.0004	0.01	0.67	33.3	Downregulated
LOC110193741	Uncharacterized	532.8	0.7	0.4	0.0004	0.01	1.59	59.1	Upregulated
QPCT	Glutaminyl-peptide cyclotransferase	3309.2	0.6	0.4	0.0004	0.01	1.56	56.4	Upregulated
LOC110194733	Heat shock factor protein 3-like	80.1	0.6	0.4	0.0004	0.01	1.55	54.7	Upregulated
CD109	CD109 molecule	282.3	0.6	0.4	0.0004	0.01	1.56	56.2	Upregulated
SAPCD2	Suppressor APC domain containing 2	9.6	0.6	0.4	0.0004	0.01	1.54	53.5	Upregulated
CDC20	Cell division cycle 20	259.0	0.6	0.4	0.0004	0.01	1.56	56.3	Upregulated
VWA3B	Von Willebrand factor A domain containing 3B	61.6	-0.6	0.4	0.0004	0.01	0.66	34.3	Downregulated

[1]Standard error of the log^2 fold change (FC). [2]DESeq2 adjusted wald test p-values using Benjamini and Hochberg method (BH-adjusted p values). [3] Context refers to gene expression up- or downregulation in koalas with that were euthanised compared to koalas that survived triage.

Table 7.21: Comparison of differential expression (DE) analysis results for genes that were significant DE genes across the two comparisons.

	Chlamydiosis vs Normal						Euthanised vs Survived					
Gene	**Base Mean**	**log² Fold Change**	**LFC SE[1]**	**P.adj[2]**	**Fold Change**	**% Change**	**Base Mean**	**log² Fold Change**	**LFC SE[1]**	**P.adj[2]**	**Fold Change**	**% Change**
AKAP4	219.88	0.99	0.93	0.01	1.98	+98.4	219.88	2.06	0.49	6.43E-05	4.18	+318
ARMC12	46.75	0.73	0.67	0.01	1.66	+65.9	46.75	1.41	0.38	0.0003	2.65	+165.4
CDKN1A	3527.06	0.59	0.36	0.008	1.5	+50.2	3527.06	1.05	0.36	0.001	2.07	+106.5
FAM177B	29.09	1.29	1.1	0.009	2.45	+145.3	29.09	1.32	0.65	0.003	2.49	+149.5
FAM19A2	398.04	-1.9	0.94	0.003	0.27	-73.2	398.04	-2.04	0.53	0.0001	0.24	-75.7
IL1R2	1064.01	3.89	0.75	8.69E-05	14.82	+1381.7	1064.01	3.81	0.61	3.91E-09	14.07	+1306.8
KIAA1324L	67.51	-0.64	1.72	0.02	0.64	-35.8	67.51	-6.08	1.8	8.07E-05	0.01	-98.5
LOC110206414	28.01	0.81	0.75	0.01	1.76	+75.8	28.01	0.87	0.49	0.007	1.83	+82.9
LOC110211842	24.29	1.11	1.34	0.01	2.16	+115.6	24.29	2.65	0.85	0.0002	6.27	+527
LOC110219949	147.19	4.6	3.57	0.003	24.21	+2320.8	147.19	4.85	2.24	0.0003	28.76	+2775.6
MARCO	29.36	1.19	1.46	0.01	2.28	+127.9	29.36	4.42	0.7	1.54E-08	21.4	+2040.2
MYO1B	51.27	3.74	0.76	0.0001	13.37	+1236.8	51.27	2.35	0.59	5.51E-05	5.11	+410.7
NR4A3	50.8	0.68	0.71	0.01	1.6	+59.8	50.80	1.58	0.41	0.0002	2.99	+199.1
RARRES1	431.79	3.35	1.48	0.003	10.21	+921.3	431.79	4.6	0.75	3.34E-08	24.18	+2317.9
SOCS1	806.26	0.61	0.36	0.006	1.53	+53	806.26	1.01	0.28	0.0002	2.01	+101.1
CREG2	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	17.46	2.11	0.51	7.62E-05	4.31	+330.5
DYDC1	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	60.40	1.45	0.62	0.002	2.73	+173.1
LOC110194733	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	80.08	0.63	0.39	0.01	1.55	+54.6
LOC110197639	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	231.54	2.30	0.45	1.05E-06	4.92	+392.2
LOC110204450	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	23.50	0.84	0.88	0.01	1.80	+79.5
LOC110213587	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	47.29	1.15	0.59	0.005	2.22	+122.4
LOC110217725	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	8.95	0.94	0.89	0.01	1.92	+92.2
PM20D1	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	1089.25	1.45	0.58	0.002	2.74	+173.5
PYCR1	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	1643.58	0.90	0.47	0.006	1.87	+86.6
QPCT	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	3309.18	0.65	0.41	0.01	1.56	+56.4
RENBP	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	218.80	2.14	0.38	2.42E-07	4.41	+341.3

[1]Standard error of the log² fold change (FC). [2]DESeq2 adjusted wald test p-values using Benjamini and Hochberg method (BH-adjusted p values). Cell colour for percentage (%) change indicates genes that are upregulated (red) and downregulated (blue) in the subject group (stated first) compared to the comparison group (stated second).

Chapter 5 Supplementary materials:

Figure 7.3: **Chlamydia pecorum *mucosal infection status and outcomes for admission and treatments for 221 koalas sampled from sites of low (N = 91) and high (N = 130) morbidity.***

C. pecorum *status was classified according to combined data obtained from point of care clinical examinations, LAMP and laboratory qPCR of mucosal swabs to detect shedding at the point of sampling and post-treatment (where applicable): no clinical signs of chlamydiosis or evidence of mucosal* C. pecorum *shedding (green), and clinical signs of chlamydiosis and/or evidence of mucosal* C. pecorum *shedding (orange). Clinical signs of chlamydiosis and detection of mucosal* C. pecorum *shedding were considered together as both form inclusion criteria for treatment; 71% (90/126) displayed clinical chlamydiosis and 98% (124/126) had detectable shedding. Detection of circulating* C. pecorum *in the whole sample population through laboratory gene expression analysis is demonstrated in pink. Admission outcome results, admitted (red border) or not admitted (pink border) for anti-chlamydia treatment, are described in the blue ovals. Koalas not admitted for treatment at T0 are further described by records of previous, future or no treatment throughout the monitoring period. Survival outcomes are described in the short-term post-admission (peach) and for the long-term at the end of the monitoring period (yellow). LTFU refers to cases that were lost to follow up due to dropped radio collars, lost radio signals, or removal from monitoring program. Koalas admitted for anti-chlamydial treatment are further described by their post-treatment* C. pecorum *LAMP results to provide an indication of treatment success. Koala released post-treatment are further categorised according to long-term follow-up* C. pecorum *detection results: 'remained* C. pecorum *negative', 'future* C. pecorum *positive', or 'NA' for those without follow-up LAMP data. The small, numbered, blue circles indicate sample cohort for respective statistical analyses (1-5) of factors associated with mortality.*

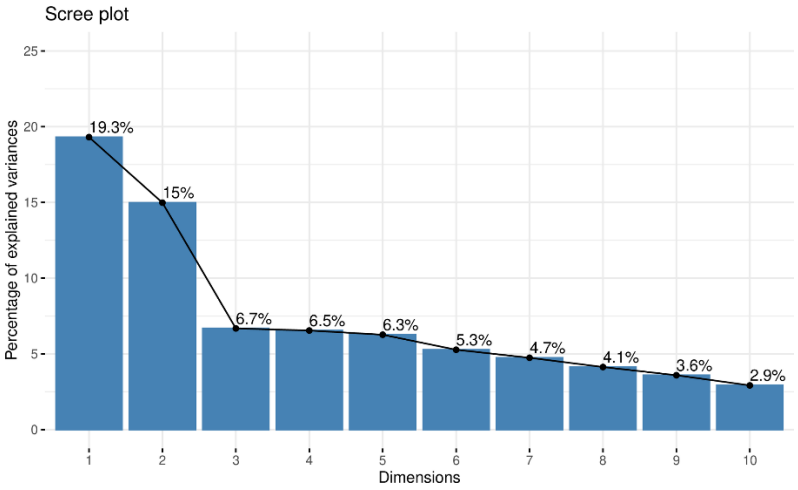

Figure 7.4: Principal components analysis scree-plot.

The scree-plot helps to identify which dimensions to retained for further analysis by demonstrating where the percentage of explained variance (y-axis) drops off, otherwise called the 'elbow'. Here we can see that the elbow appears at PC3, with a small percentage of variation explained with each following dimension. Dimensions 1-8 were retained to account for 68% of the cumulative variation within the dataset.

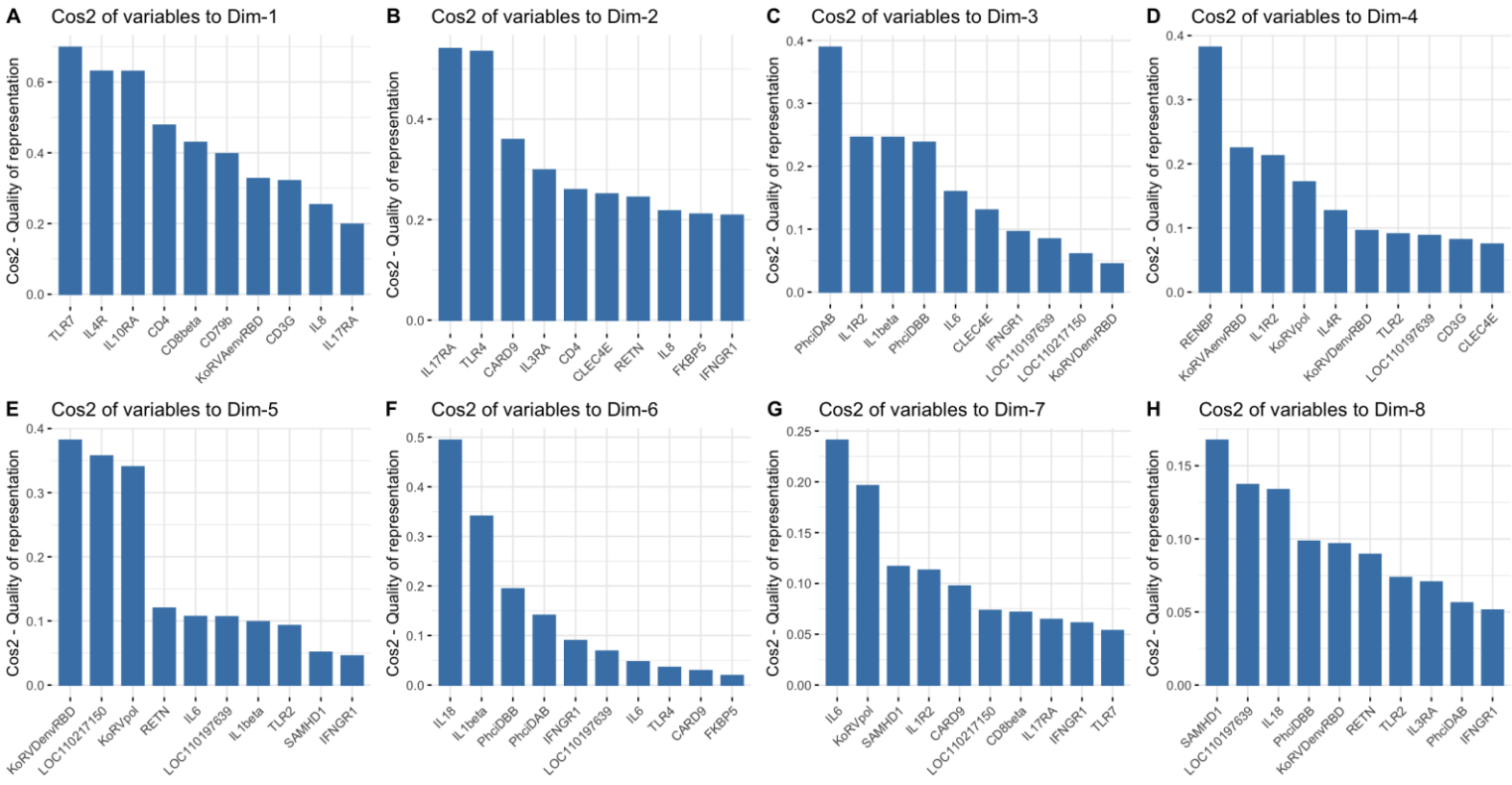

Figure 7.5: Bar-charts demonstrating the top 10 genes represented by each dimension based on cos² values (A-H, PC1-8).

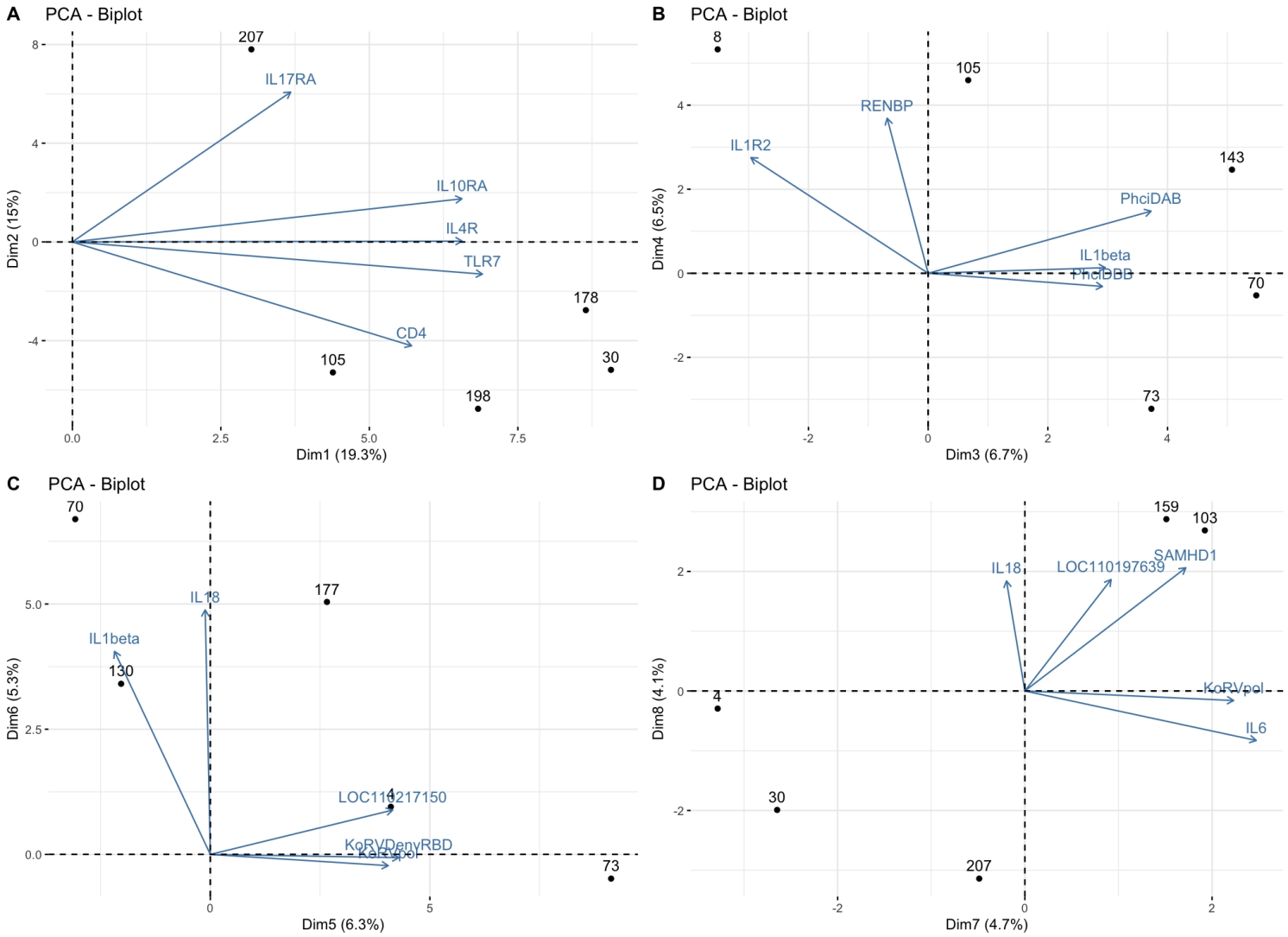

Figure 7.6: PCA bi-plots demonstrating the five most represented individuals and their associated top five genes to dimensions 1 and 2 (plot A), dimensions 3 and 4 (plot B), dimensions 4 and 5 (plot C), and dimensions 7 and 8 (plot D).

Table 7.22: Detection results for infectious agents that were not ubiquitously detected in samples obtained from 221 koalas originating from high morbidity and low morbidity sites

Koala infectious agent detection status		*High morbidity (N = 130)*	*Low morbidity (N = 91)*	*Total (N = 221)*
Chlamydia pecorum				
Samples with complete results*	Mucosal *C. pecorum* only	52/85 (61%)	22/68 (32%)	74/153 (48%)
	Circulating *C. pecorum* only	4/85 (5%)	4/68 (6%)	8/153 (5%)
	Mucosal & circulating *C. pecorum*	5/85 (6%)	0/68 (0%)	5/153 (3%)
	None	24/85 (28%)	42/68 (62%)	66/153 (43%)
Samples with results missing for one site**	Circulating *C. pecorum* (Mucosal NA)	0/130 (0%)	0/91 (2%)	0/221 (0%)
	No circulating *C. pecorum* (Mucosal NA)	10/130 (8%)	6/91 (7%)	16/221 (7%)
	Mucosal *C. pecorum* (Circulating NA)	25/130 (19%)	3/91 (3%)	28/221 (13%)
	No mucosal *C. pecorum* (Circulating NA)	10/130 (8%)	14/91 (15%)	24/221 (11%)
All results***	Mucosal *C. pecorum*	82/120 (68%)	25/85 (29%)	107/205 (52%)
	Circulating *C. pecorum*	9/95 (9%)	4/74 (5%)	13/169 (8%)
	C. pecorum	86/110 (78%)	2/73 (3%)	115/183 (63%)
Gammaherpesviruses				
Samples with complete results	Mucosal HV1 only	28/83 (33%)	30/66 (38%)	58/149 (39%)
	Mucosal HV2 only	9/83 (10%)	2/66 (3%)	11/149 (7%)
	Circulating HV2 only	1/83 (1%)	0/66 (8%)	1/149 (1%)
	Mucosal HV1 & HV2	25/83 (30%)	6/66 (6%)	31/149 (21%)
	Mucosal HV1 & circulating HV2	4/83 (2%)	0/66 (8%)	4/149 (3%)
	Mucosal HV2 & circulating HV2	0/83 (1%)	0/66 (0%)	0/149 (0%)
	Mucosal HV1, HV2 & circulating HV2	4/83 (5%)	0/66 (3%)	4/149 (3%)
	None	15/83 (18%)	28/66 (35%)	43/149 (15%)
Samples with results missing for one site	Mucosal HV2 only (Mucosal HV1 NA)	1/130 (%)	0/91 (0%)	1/221 (1%)
	No mucosal or circulating HV2 (Mucosal HV1 NA)	2/130 (2%)	1/91 (1%)	3/221 (1%)
	Mucosal HV1 (Mucosal HV2 NA)	0/130 (0%)	1/91 (1%)	1/221 (1%)
	Circulating HV2 (Mucosal HV2 NA)	0/130 (0%)	0/91 (2%)	0/221 (0%)
	No circulating HV2 (Mucosal HV1 & HV2 NA)	9/130 (7%)	6/91 (4%)	15/221 (7%)
	Mucosal HV1 (Circulating HV2 NA)	11/130 (9%)	0/91 (0%)	11/221 (5%)
	Mucosal HV2 (Circulating HV2 NA)	2/130 (2%)	2/91 (2%)	4/221 (2%)
	Mucosal HV1 & HV2 (Circulating HV2 NA)	11/130 (9%)	0/91 (0%)	11/221 (5%)
	No mucosal HV1 or HV2 (Circulating HV2 NA)	11/130 (9%)	15/91 (17%)	26/221 (12%)
All results	Mucosal HV1	80/118 (68%)	37/84 (44%)	117/202 (58%)
	Mucosal HV2	52/121 (43%)	10/84 (12%)	62/205 (30%)
	Circulating HV2	6/95 (8%)	0/74 (19%)	6/169 (4%)
KoRV B				
All results	Circulating KoRV B	13/95 (14%)	8/74 (11%)	21/169 (12%)

*Detection status frequency for samples with results for all targets only.

**Detection status frequency for samples that had results missing for one other target, which is indicated in the brackets.

***Detection status frequency for target regardless of result for any other target. Not including NA samples.

NA: Sample not obtained or sample failed quality control measures for pathogen detection technique

Table 7.23: PCA Dimension Metrics

Dimensions	Eigenvalue	Variance (%)	Cumulative variance (%)
Dim.1	5.8	19.3	19.3
Dim.2	4.5	15.0	34.3
Dim.3	2.0	6.7	41.0
Dim.4	2.0	6.5	47.5
Dim.5	1.9	6.3	53.8
Dim.6	1.6	5.3	59.0
Dim.7	1.4	4.7	63.8
Dim.8	1.2	4.1	67.9
Dim.9	1.1	3.6	71.5
Dim.10	0.9	2.9	74.4
Dim.11	0.8	2.7	77.1
Dim.12	0.8	2.6	79.8
Dim.13	0.7	2.4	82.2
Dim.14	0.7	2.2	84.4
Dim.15	0.6	1.8	86.2
Dim.16	0.5	1.7	87.9
Dim.17	0.5	1.6	89.5
Dim.18	0.4	1.5	91.0
Dim.19	0.4	1.3	92.3
Dim.20	0.3	1.1	93.5
Dim.21	0.3	1.0	94.5
Dim.22	0.3	1.0	95.5
Dim.23	0.3	0.9	96.4
Dim.24	0.2	0.8	97.2
Dim.25	0.2	0.6	97.9
Dim.26	0.2	0.6	98.5
Dim.27	0.1	0.5	98.9
Dim.28	0.1	0.4	99.3
Dim.29	0.1	0.4	99.7
Dim.30	0.1	0.3	100

Table 7.24: Quality (cos2) of representation of each gene within the principal components

Gene	Dim.1	Dim.2	Dim.3	Dim.4	Dim.5	Dim.6	Dim.7	Dim.8
CARD9	0.19	0.36	0.01	0.06	0.0004	0.03	0.10	0.02
CD3G	0.32	0.21	0.03	0.08	0.03	0.0001	0.01	0.01
CD4	0.48	0.26	0.01	0.02	0.01	0.01	0.02	0.00
CD79b	0.40	0.21	0.00	0.01	0.00	0.01	0.00	0.04
CD8beta	0.43	0.12	0.02	0.03	0.02	0.02	0.07	0.02
CLEC4E	0.00	0.25	0.13	0.07	0.00	0.01	0.03	0.03
FKBP5	0.20	0.21	0.01	0.06	0.01	0.02	0.01	0.01
IFNGR1	0.10	0.21	0.10	0.01	0.05	0.09	0.06	0.05
IL10RA	0.63	0.04	0.0001	0.00	0.00	0.02	0.04	0.02
IL17RA	0.20	0.54	0.0003	0.03	0.0004	0.00001	0.06	0.00
IL18	0.01	0.03	0.001	0.02	0.0003	0.49	0.00	0.13
IL1R2	0.03	0.08	0.25	0.21	0.00	0.001	0.11	0.00
IL1beta	0.02	0.03	0.25	0.001	0.10	0.34	0.02	0.001
IL3RA	0.03	0.30	0.04	0.03	0.04	0.01	0.01	0.07
IL4R	0.63	0.00002	0.03	0.13	0.02	0.00	0.00	0.02
IL6	0.08	0.04	0.16	0.001	0.11	0.05	0.24	0.03
IL8	0.25	0.22	0.04	0.02	0.01	0.01	0.02	0.01
KoRVAenvRBD	0.33	0.01	0.0001	0.22	0.00	0.0003	0.04	0.01
KoRVDenvRBD	0.04	0.05	0.04	0.10	0.38	0.0001	0.02	0.10
KoRVpol	0.01	0.01	0.02	0.17	0.34	0.00	0.20	0.00
LOC110197639	0.16	0.001	0.08	0.09	0.11	0.07	0.03	0.14
LOC110217150	0.0005	0.09	0.06	0.00	0.36	0.02	0.07	0.00
PhciDAB	0.04	0.02	0.39	0.06	0.00	0.14	0.00	0.06
PhciDBB	0.01	0.01	0.24	0.00	0.001	0.19	0.01	0.10
RENBP	0.10	0.06	0.01	0.38	0.02	0.00	0.02	0.00003
RETN	0.13	0.24	0.00	0.0003	0.12	0.0002	0.04	0.09
SAMHD1	0.05	0.15	0.02	0.07	0.05	0.00	0.12	0.17
TLR2	0.16	0.19	0.02	0.09	0.09	0.00	0.01	0.07
TLR4	0.07	0.53	0.04	0.00	0.00	0.04	0.00001	0.03
TLR7	0.70	0.02	0.01	0.0001	0.001	0.01	0.05	0.0001

Table 7.25: Loadings (correlation) of each gene within the principal components

Gene	Dim.1	Dim.2	Dim.3	Dim.4	Dim.5	Dim.6	Dim.7	Dim.8
CARD9	0.44	0.60	-0.11	-0.24	-0.02	0.17	-0.31	0.15
CD3G	0.57	-0.46	0.17	0.29	0.18	0.01	-0.11	-0.09
CD4	0.69	-0.51	0.09	0.13	0.07	0.11	-0.14	-0.06
CD79b	0.63	-0.45	-0.06	0.08	-0.03	-0.10	0.06	0.20
CD8beta	0.66	-0.35	0.15	0.17	0.15	0.13	-0.27	-0.14
CLEC4E	-0.04	0.50	0.36	0.27	0.04	0.10	0.17	0.17
FKBP5	0.44	0.46	-0.11	0.24	0.10	-0.14	0.10	-0.12
IFNGR1	0.31	0.46	-0.31	-0.12	-0.21	-0.30	0.25	0.23
IL10RA	0.79	0.21	0.01	-0.04	-0.07	-0.12	-0.19	0.13
IL17RA	0.44	0.74	0.02	-0.16	-0.02	-0.002	-0.25	0.06
IL18	-0.09	0.16	0.03	-0.14	-0.02	0.70	-0.04	0.37
IL1beta	0.16	0.28	-0.50	0.46	0.04	0.03	0.34	-0.05
IL1R2	0.15	0.18	0.50	0.02	-0.31	0.58	0.13	0.03
IL3RA	0.18	0.55	-0.19	0.16	0.19	0.12	0.10	-0.27
IL4R	0.79	0.004	-0.19	-0.36	-0.13	0.04	-0.06	0.13
IL6	0.28	0.19	0.40	-0.02	-0.33	0.22	0.49	-0.16
IL8	0.50	-0.47	0.19	0.14	-0.11	0.11	0.14	-0.09
KoRVAenvRBD	0.57	-0.11	-0.01	-0.47	0.05	0.02	0.19	-0.11
KoRVDenvRBD	0.19	0.21	0.21	-0.31	0.62	-0.01	0.15	-0.31
KoRVpol	0.11	-0.11	0.12	-0.41	0.58	-0.03	0.44	-0.03
LOC110197639	0.40	0.02	-0.29	0.30	0.33	0.26	0.18	0.37
LOC110217150	-0.02	0.30	0.25	0.04	0.60	0.13	-0.27	-0.06
PhciDAB	0.19	0.14	0.62	0.25	-0.06	-0.37	0.05	0.24
PhciDBB	0.11	0.10	0.49	-0.05	-0.03	-0.44	0.08	0.31
RENBP	0.32	0.24	-0.11	0.62	0.14	0.04	0.16	0.01
RETN	-0.36	0.49	0.05	-0.02	0.35	-0.01	-0.20	0.30
SAMHD1	0.22	-0.38	-0.13	-0.26	0.23	-0.04	0.34	0.41
TLR2	0.40	0.43	-0.13	-0.30	-0.30	0.03	0.09	-0.27
TLR4	0.27	0.73	0.19	0.06	-0.06	-0.19	-0.002	-0.18
TLR7	0.84	-0.16	-0.08	-0.01	-0.03	-0.09	-0.23	-0.01

Table 7.26: The relative contribution (%) of gene expression to each principal component (dimension)

Gene	Dim.1	Dim.2	Dim.3	Dim.4	Dim.5	Dim.6	Dim.7	Dim.8
CARD9	3.31	8.00	0.56	2.86	0.02	1.83	6.85	1.90
CD3G	5.54	4.64	1.49	4.16	1.70	0.01	0.90	0.69
CD4	8.25	5.78	0.45	0.91	0.29	0.75	1.29	0.30
CD79b	6.86	4.59	0.17	0.34	0.06	0.65	0.29	3.33
CD8beta	7.41	2.76	1.15	1.52	1.26	1.07	5.03	1.64
CLEC4E	0.03	5.59	6.51	3.81	0.09	0.63	2.04	2.41
FKBP5	3.37	4.69	0.57	3.00	0.56	1.21	0.73	1.11
IFNGR1	1.65	4.65	4.79	0.68	2.42	5.67	4.29	4.14
IL10RA	10.88	1.00	0.004	0.08	0.23	0.98	2.56	1.31
IL17RA	3.42	12.02	0.01	1.28	0.02	0.0004	4.54	0.31
IL18	0.14	0.57	0.03	1.05	0.02	31.24	0.11	10.79
IL1R2	0.45	1.78	12.28	10.83	0.10	0.06	7.95	0.18
IL1beta	0.40	0.71	12.27	0.03	5.25	21.54	1.17	0.07
IL3RA	0.57	6.65	1.90	1.37	1.90	0.93	0.68	5.69
IL4R	10.88	0.0004	1.74	6.46	0.96	0.09	0.23	1.44
IL6	1.36	0.84	7.97	0.03	5.69	2.96	16.94	2.19
IL8	4.37	4.84	1.86	1.02	0.69	0.76	1.29	0.63
KoRVAenvRBD	5.64	0.26	0.004	11.45	0.15	0.02	2.60	0.95
KoRVDenvRBD	0.62	1.01	2.23	4.88	20.32	0.01	1.50	7.80
KoRVpol	0.19	0.28	0.76	8.75	18.11	0.07	13.80	0.08
LOC110197639	2.80	0.01	4.21	4.49	5.66	4.33	2.35	11.07
LOC110217150	0.01	1.94	3.02	0.08	19.02	1.03	5.16	0.26
PhciDAB	0.64	0.46	19.43	3.12	0.19	8.90	0.18	4.54
PhciDBB	0.21	0.23	11.88	0.14	0.04	12.27	0.42	7.95
RENBP	1.81	1.33	0.66	19.46	1.00	0.08	1.74	0.002
RETN	2.29	5.44	0.13	0.01	6.38	0.01	2.75	7.22
SAMHD1	0.81	3.28	0.90	3.36	2.72	0.09	8.20	13.52
TLR2	2.75	4.20	0.87	4.62	4.93	0.07	0.63	5.94
TLR4	1.28	11.90	1.80	0.19	0.17	2.25	0.0004	2.54
TLR7	12.05	0.56	0.34	0.01	0.04	0.51	3.77	0.01

Table 7.27: NanoString Gene Target Information, Performance, and Inclusion Result for T0 analysis

Gene Target	Full Name/Classification	Gene type	Count > 20	% > 20	Inclusion
PhaHV2 dpol	Phascolarctid herpesvirus 2 DNA polymerase gene	Non-ubiquitous pathogen	17	12	EXCLUDED
Cpec_hsp60	*Chlamydia pecorum* heat shock protein 60 gene	Non-ubiquitous pathogen	19	14	EXCLUDED
CpecG_0573	*Chlamydia pecorum* G_0573 locus of Marsbar strain	Non-ubiquitous pathogen	26	19	EXCLUDED
KoRVBenvRBD	Koala Retrovirus subtype B envelope gene	Non-ubiquitous pathogen	30	22	EXCLUDED
KoRVAenvRBD	Koala Retrovirus subtype A envelope gene	Ubiquitous pathogen	139	100	RETAINED
KoRVDenvRBD	Koala Retrovirus subtype D envelope gene	Ubiquitous pathogen	139	100	RETAINED
KoRVpol	Koala Retrovirus polymerase gene	Ubiquitous pathogen	139	100	RETAINED
MHCIUA	Major histocompatibility complex class I UA gene	Host – Immune response	32	25	EXCLUDED
PhciDBB	Major histocompatibility complex class II DBB gene	Host – Immune response	138	99	RETAINED
PhciDAB	Major histocompatibility complex class II DAB gene	Host – Immune response	139	100	RETAINED
IL12RB2	Interleukin-12 receptor subunit beta	Host – Immune response	59	46	EXCLUDED
IL1R2	Interleukin-1 receptor 2	Host – Immune response	116	83	RETAINED
IL4R	Interleukin-4 receptor	Host – Immune response	136	98	RETAINED
IFNGR1	Interferon gamma receptor 1	Host – Immune response	139	100	RETAINED
IL10RA	Interleukin-10 receptor subunit alpha	Host – Immune response	139	100	RETAINED
IL17RA	Interleukin-17 receptor A	Host – Immune response	139	100	RETAINED
IL3RA	Interleukin-3 receptor subunit alpha	Host – Immune response	139	100	RETAINED
TNFalpha	Tumour necrosis factor alpha	Host – Immune response	38	29	EXCLUDED
IL18	Interleukin-18	Host – Immune response	114	82	RETAINED
IL1beta	Interleukin-1 beta	Host – Immune response	127	91	RETAINED
IL6	Interleukin-6	Host – Immune response	137	99	RETAINED
IL8	Interleukin-8 / CXC motif chemokine ligand 8	Host – Immune response	139	100	RETAINED
CD4	Cluster of differentiation 4 (T lymphocyte)	Host – Immune response	78	56	RETAINED
CD8beta	Cluster of differentiation 8 beta (cytotoxic T lymphocyte)	Host – Immune response	99	63	RETAINED
CD3G	Cluster of differentiation 3 gamma polypeptide (T lymphocyte receptor complex)	Host – Immune response	132	95	RETAINED
CD79b	Cluster of differentiation 79 immunoglobulin-associated beta (B lymphocyte)	Host – Immune response	139	100	RETAINED
TLR7	Toll-like receptor 7	Host – Immune response	107	77	RETAINED
TLR2	Toll-like receptor 2	Host – Immune response	138	99	RETAINED
TLR4	Toll-like receptor 4	Host – Immune response	139	100	RETAINED
CCR4	CC Motif Chemokine Receptor 4	Host – Immune response	41	32	EXCLUDED
FAM19A2	TAFA chemokine-like family member 2	Host – Immune response	47	37	EXCLUDED
CLEC4E	C-type lectin domain family 4 member E / Mincle receptor	Host – Immune response	139	100	RETAINED
PhciCATH5	Koala cathelicidin	Host – Immune response	8	6	EXCLUDED
LOC110217150	Cathelicidin-like gene	Host – Immune response	137	99	RETAINED
LOC110197639	Cathepsin-like gene	Host – Immune response	92	66	RETAINED
CARD9	Caspase recruitment domain family member 9	Host – Immune response	139	100	RETAINED
MARCO	Macrophage receptor with collagenous structure	Host – Immune response	9	7	EXCLUDED

Gene Target	Full Name/Classification	Gene type	Count > 20	% > 20	Inclusion
NCR3	Natural cytotoxicity triggering receptor 3	Host – Immune response	17	13	EXCLUDED
FKBP5	FK506 (immunosuppressant) binding protein 5	Host – stress response	137	99	RETAINED
RETN	Resistin	Host – metabolic	139	100	RETAINED
SAMHD1	SAM and HD domain containing deoxynucleoside triphosphate triphosphohydrolase 1	Host – anti-viral response	139	100	RETAINED
RENBP	Renin binding protein	Host – kidney function	78	56	RETAINED
SLCO2A1	Solute carrier organic anion transporter family member 2A1	Host – pain response	9	7	EXCLUDED
AR	Androgen receptor	Host – endocrine	29	23	EXCLUDED
ACTB	*B-actin*	Host - Housekeeping	139	100	EXCLUDED
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Host - Housekeeping	139	100	EXCLUDED
Nckap1l	Nck-associated protein 1-like	Host - Housekeeping	139	100	EXCLUDED
Stx12	Syntaxin-12	Host - Housekeeping	139	100	EXCLUDED

Table 7.28: Variables and inclusion criteria utilised to define target groups and perform modelling

Variable	Factor Levels	Inclusion Criteria / Calculation	Variable Type	Tests for indicators of mortality in: high morbidity site	low morbidity site	chlamydiosis and/or mucosal *C. pecorum* shedding	treated for chlamydiosis
PC1	NA	Host gene expressed principal components derived from principal components analyses.	Continuous	Assessed	Assessed	Assessed	Assessed
PC2	NA		Continuous	Assessed	Assessed	Assessed	Assessed
PC3	NA		Continuous	Assessed	Assessed	Assessed	Assessed
PC4	NA		Continuous	Assessed	Assessed	Assessed	Assessed
PC5	NA		Continuous	Assessed	Assessed	Assessed	Assessed
PC6	NA		Continuous	Assessed	Assessed	Assessed	Assessed
PC7	NA		Continuous	Assessed	Assessed	Assessed	Assessed
PC8	NA		Continuous	Assessed	Assessed	Assessed	Assessed
Age	NA	Estimated at the first veterinary examination through the assessment of dentition and the amount of wear on the upper premolar (Gordon, 1991) and then calculated at follow-up, based on time elapsed	Continuous	Assessed	Assessed	Assessed	Assessed
Sex	Male or Female	Determined through visual, physical and ultrasonographical examination of morphology and reproductive organs.	Categorical	Assessed	Assessed	Assessed	Assessed
Body Condition Score (BCS) ≤ 2	Yes or no	Determined by grading the mass of muscle tissue around the scapula (Ellis & Carrick, 1992). In Queensland, scores range from 1 (emaciated) to 10 (excellent). To keep consistent with previous chapters, which used the 1-5 scale, scores were halved. Body condition scores equal to or below 2 were identified and categorised as 'yes'. Scores of 3-5 were categorised as 'no'.	Categorical	Assessed	Assessed	Assessed	Assessed
Diagnosis	Chlamydiosis	Reproductive Disease (incl. female uterine/ovarian/bursal cyst(s), pyometra/pyometron, metritis, and vaginal prolapse/hyperproliferative vaginal mucosa or male prostatomegaly, prostatic cyst(s), prostatitis, and/or testicular fibrosis); Cystitis (bladder wall thickness, and inflammation on doppler, cytological pyuria and/or haematuria, and/or wet-bottom or rump-ulceration); Conjunctivitis (mild-severe conjunctival proliferation, chemosis, and/or discharge in one or both eyes); +/- Any other clinical abnormality.	Categorical	Assessed	Assessed	Not assessed	Assessed
	Other: *Trauma or Disease*	*Trauma:* Superficial wounds; Abdominal and/or visceral trauma (incl. haemoabdomen, peritonitis, metabolic alkalosis); Single or multiple fractures (incl. comminuted, compound, and pathogenic fractures); Wound associated septicaemia; Vestibular & neurological trauma; Trauma associated anaemia; Chronic musculoskeletal injury (incl. hip dysplasia, osteoarthritis/arthritis).					

		Disease: Growths/masses/cysts (Keratinised mass, mammary mass, subcutaneous mass, dermal cysts); Neoplasia (Myelodysplasia, Mammary adenocarcinoma, papilloma); Lymphadenopathy/lymphomegaly; Pulmonary Disease and/or laryngeal/pharyngeal occlusion; Oxalate nephrosis; Ocular abnormalities (nystagmus, corneal scarring/ulceration, microphthalmia, cataract/glaucoma, epistaxis); Emaciation without evidence of chlamydiosis; Dysbiosis / Caecal Dysbiosis Typhlocolitis Syndrome; Candidiasis; Periodontal disease/gingivitis; Haematological abnormalities (anaemia, hypoproteinaemia, neutrophilia, eosinophilia); Other conditions (Otitis, dermatopathy, ascites).					
	Healthy	Nothing abnormal detected using any of the following diagnostic tools; visual assessment, ultrasonography, cytology; Mended musculoskeletal abnormalities (e.g. scars or healed fractures).					
Vaccination status	Yes or No	Evidence of vaccination administration prior to sampling using any of the following: three implemented by Phillips *et al.* (2024) MOMP (2022), Tri-adjuvant (2023), and ISC (2023), and one currently under trial at Currumbin Wildlife Sanctuary referred to as the "Currumbin vaccine"(Currumbin Wildlife Sanctuary, 2022).	Categorical	Assessed	Assessed	Assessed	Assessed
Ovariohysterec-tomy status	Yes or No	Recorded as 'yes' for OHE if they had undergone OHE as part of treatment for female reproductive disease previously, or underwent it during the monitoring period.	Categorical	Assessed	Assessed	Assessed	Assessed
Site	High morbidity or low morbidity	Koala management areas (KMA) situated north of the Coomera River (Supplementary materials: Figure 12) were identified as the 'high morbidity' site (TKMA – 1, KMA12b, KMA13a, and KMA13b) and those south of the river as the 'low morbidity' site (KMA4, KMA5, KMA6, KMA7a, KMA8, KMA9, KMA10a, KMA11a, and KMA11b).	Categorical	Not assessed	Not assessed	Assessed	Assessed
Released at site of capture	Yes or No	Recorded as 'yes' if they were released at the exact or within 50 meters of point of capture. Recorded as 'no' if the koala was relocated (< 5 km) or translocated (> 5km) from point of capture.	Categorical	Assessed	Assessed	Assessed	Assessed
Treatment complications	Yes or No	Recorded as 'yes' for adverse effects if during their anti-chlamydiosis treatment they developed (not identified at first exam) candidiasis, dysbiosis, and/or oxalate nephrosis.	Categorical	Not assessed	Not assessed	Not assessed	Assessed
Chlamydiosis treatment during the monitoring period	Current	Koalas that were retained in care following the first veterinary examination and treated with Chloramphenicol (Chloramphenicol 150, Ceva, Glenorie, NSW; 60mg/kg once a day, subcutaneously for 14 days), an antibiotic that targets *C. pecorum*.	Categorical	Assessed	Assessed	Assessed	Not assessed
	Future	Koalas that were treated as described above at another veterinary examination following time-zero sampling.					
	Previous	Koalas that were treated as described above at a veterinary examination prior to time-zero sampling.					
	None	Koalas that were not treated as described above at any veterinary examination throughout the monitoring period.					

Chloramphenicol Treatment Duration (days)	NA	The number of days Chloramphenicol 150, Ceva, Glenorie, NSW; was administered at 60mg/kg for chlamydiosis treatment	Continuous	Not assessed	Not assessed	Not assessed	Assessed
Chlamydiosis Treatment Outcome	LAMP negative	*C. pecorum* not detected using point-of-care LAMP testing post-treatment prior to release.	Categorical	Not assessed	Not assessed	Not assessed	Assessed
	LAMP positive	*C. pecorum* detected using point-of-care LAMP testing post-treatment prior to release.					
Mucosal *C. pecorum* shedding	Yes or No	Koalas with detectable mucosal shedding (qPCR amplification of either *C. pecorum ompB* or 23s genus and *β-actin* in duplicate samples) in ocular or urogenital swab samples taken at T0.	Categorical	Assessed	Assessed	Not assessed	Assessed
Mucosal PhaHV-1 shedding	Yes or No	Koalas with detectable mucosal shedding (qPCR amplification of PhaHV-1 *dpol* and *β-actin* in duplicate samples) in oropharyngeal swab samples taken at T0.	Categorical	Assessed	Assessed	Assessed	Assessed
Mucosal PhaHV-2 shedding	Yes or No	Koalas with detectable mucosal shedding (qPCR amplification of PhaHV-2 *dpol* and *β-actin* in duplicate samples) in oropharyngeal swab samples taken at T0.	Categorical	Assessed	Assessed	Assessed	Assessed
Circulating PhaHV-2 transcription	Yes or No	Koalas transcribing the PhaHV-2 dpol gene at mRNA counts above 20 in buffy coat sample RNA extracts taken at T0.	Categorical	Assessed	Not assessed	Assessed	Assessed
Circulating *C. pecorum* transcription	Yes or No	Koalas transcribing either *C. pecorum* G_0573 or Hsp60 genes at mRNA counts above 20 in buffy coat sample RNA extracts taken at T0.	Categorical	Assessed	Assessed	Assessed	Assessed
Circulating KoRV B transcription	Yes or No	Koalas transcribing the KoRVBenvRBD gene at mRNA counts above 20 in buffy coat sample RNA extracts taken at T0.	Categorical	Assessed	Assessed	Assessed	Assessed

Table 7.29: qPCR Primer Set Information for Chlamydia Multiplex qPCR, PhaHV-1 & -2 qPCR

qPCR Assay	Gene	Amplicon size (bp)	End	Sequence	Reference
Chlamydia multiplex Probe qPCR	*Chlamydia* (23S rRNA)	*137*	Forward	5'-GCTCACCAATCGAGAATC-3'	
		137	Reverse	5'-CCAACACTCCTTTCGGTA-3'	
		137	Probe	ROX-CTGAATACTACGCTCTCCTACCGC-BHQ2	
	C. pecorum (*ompB* gene)	*141*	Forward	5'–CCAAGCATAATCGTAACAA-3'	
		141	Reverse	5'-CGAAGCAAGATTCTTGTC-3'	(Hulse *et al.*, 2018)
		141	Probe	FAM-ACTTGTTGGCAATTCTTCTCTTCACA-BHQ1	
	Koala *B-actin* mRNA	*145*	Forward	5'-CTCAGATTATGTTTGAGACCTTC-3'	
		145	Reverse	5'-CCTTCATAGATGGGCACA-3'	
		145	Probe	HEX-ACCATCACCAGAGTCCATCACAAT-BHQ1	
Phascolarctid herpesvirus 1 & 2 SYBR qPCR	PhaHV-1 *dpol*	*22*	Forward	5'-GGGAAGAACTATGTTGGAACGC-3'	(Wright *et al.*, 2023)
		20	Reverse	5'-TGAGTCCTTTTCGCTTGGGA-3'	
	PhaHV-2 *dpol*	*20*	Forward	5'-GGTGACGTGCAATTCAGTGT-3'	(Church *et al.*, 2025)
		20	Reverse	5'-TTTCGAGCATCATGCGTCCT-3'	
	Koala *B-actin* mRNA	*145*	Forward	5'-CTCAGATTATGTTTGAGACCTTC-3'	(Hulse *et al.*, 2018)
		145	Reverse	5'-CCTTCATAGATGGGCACA-3'	

Chapter 5 Supplementary material references:

Church, C., Casteriano, A., Muir, Y. S. S., Krockenberger, M. B., Vaz, P. K., Higgins, D. P., & Wright, B. R. (2025). New insights into the range and transmission dynamics of a koala gammaherpesvirus, phascolarctid gammaherpesvirus 2. Sci Rep, In press.

Currumbin Wildlife Sanctuary. (2022). Currumbin Wildlife Hospital Leading the Way in Urgent Koala Research. https://currumbinsanctuary.com.au/wildlife-hospital/koala-chlamydia-vaccine-research-trial

Ellis, W. A. H., & Carrick, F. N. (1992). Total body water and the estimation of fat in the koala (Phascolarctos cinereus). Australian Veterinary Journal, 69(9), 229-231. https://doi.org/10.1111/j.1751-0813.1992.tb09933.x

Gordon, G. (1991). Estimation of the age of the Koala, Phascolarctos cinereus (Marsupialia: Phascolarctidae), from tooth wear and growth. Australian Mammalogy, 14(1), 5-12.

Hulse, L. S., Hickey, D., Mitchell, J. M., Beagley, K. W., Ellis, W., & Johnston, S. D. (2018). Development and application of two multiplex real-time PCR assays for detection and speciation of bacterial pathogens in the koala. J Vet Diagn Invest, 30(4), 523-529. https://doi.org/10.1177/1040638718770490

Phillips, S., Hanger, J., Grosmaire, J., Mehdi, A., Jelocnik, M., Wong, J., & Timms, P. (2024a). Immunisation of koalas against Chlamydia pecorum results in significant protection against chlamydial disease and mortality. npj Vaccines, 9(1), 139. https://doi.org/10.1038/s41541-024-00938-5

Wright, B. R., Jelocnik, M., Casteriano, A., Muir, Y. S. S., Legione, A. R., Vaz, P. K., Devlin, J. M., & Higgins, D. P. (2023). Development of diagnostic and point of care assays for a gammaherpesvirus infecting koalas. PLoS One, 18(6), e0286407. https://doi.org/10.1371/journal.pone.0286407

Chapter 6 Supplementary Materials:

Table 7.30: Summary table of key associations from the thesis according to indicator with reference to the respective chapter.

Indicator group	Indicator	Association	Chapter
Infectious agent	Mucosal *C. pecorum* shedding	Detection associated with clinical chlamydiosis	2
		Detection associated with reproductive disease	2
		Lack of detection associated with greater odds of survival in wild koalas from a site with high morbidity	5
		Detection associated with greater odds of mortality in koalas with clinical chlamydiosis and/or detectable mucosal *C. pecorum* shedding	5
	Mucosal PhaHV-1 shedding	Detection associated with the presentation of clinical chlamydiosis	2
		Detection associated with the presentation of reproductive disease	2
		Detection associated with the presentation of untreatable chlamydiosis (euthanised)	2
		Detection associated with greater odds of survival in koalas with clinical chlamydiosis and/or detectable mucosal *C. pecorum* shedding	5
	Circulating *T. irwini*	Detection inversely associated with the presentation of clinical chlamydiosis	2
	Circulating KoRV B *env*	Detection associated with the detection of mucosal *C. pecorum* shedding	2
		Detection associated with greater odds of survival in koalas with clinical chlamydiosis and/or detectable mucosal C. pecorum shedding	5
	Viral KoRV *pol*	Increased transcription associated with the detection of mucosal *C. pecorum* shedding	2
		Increased transcription associated with increased odds of euthanasia at triage	3
	Proviral KoRV *pol*	Increased proviral loads associated with the presentation of reproductive disease	2
Host gene	CD3G, CD8B, CD4, CD79B, TLR7, MHCII DAB, MHCII DBB, DICER1, SAMHD1, SLC29A1, and inversely IL18 and a Cathelicidin-like gene (LOC110217150)	Increased dimension score in clusters of koalas with greater frequency of survival at triage	3
	CD3G, CD8B, CD4, CD79B, TLR7 and IL8	Increased dimension score associated with greater odds of survival in koalas with clinical chlamydiosis and/or detectable mucosal *C. pecorum* shedding	5
		Increased dimension score associated with greater odds of survival in koalas treated for chlamydiosis	5
	IL10R, IFNGR1, IL4R, IL17RA, CARD9, TLR2, TLR4, and KoRV A	Increased dimension score associated with greater odds of mortality in koalas with clinical chlamydiosis and/or detectable mucosal *C. pecorum* shedding	5
	FKBP5, IL1R2, IL3RA, RENBP and a Cathepsin-like gene (LOC110197639)	Increased dimension score associated with greater odds of mortality in wild koalas from a site with high morbidity	5
		Increased dimension score associated with greater odds of survival in koalas with clinical chlamydiosis and/or detectable mucosal *C. pecorum* shedding	5
	SAMHD1 and KoRV A	Increased dimension score associated with greater odds of mortality in wild koalas from a site with low morbidity	5
	KoRV pol, KoRV D and a Cathelicidin-like gene (LOC110217150)	Increased dimension score associated with greater odds of mortality in wild koalas from a site with low morbidity	5
	CLEC4E, MHC II DAB and MHC II DBB	Increased dimension score associated with greater odds of mortality in wild koalas treated for chlamydiosis	5
	TRIM24	Increased transcription at triage associated with survival at triage and post-treatment	3
	IL18	Transcription at triage inversely correlated to genes within the adaptive lymphocyte dimension	3
		Increased transcription at triage associated with greater odds of euthanasia at triage	3
		Increased transcription post-treatment associated with greater odds of survival at post-treatment	3

Indicator group	Indicator	Association	Chapter
	IL18	Increased expression at triage in koalas with clinical chlamydiosis	4
	FKBP5	Increased expression at triage in koalas that were euthanised	4
	IL1R2	Increased expression at triage in koalas with clinical chlamydiosis	4
		Increased expression at triage in koalas that were euthanised	4
	MARCO	Increased expression at triage in koalas with clinical chlamydiosis	4
		Increased expression at triage in koalas that were euthanised	4
	RARRES1	Increased expression at triage in koalas with clinical chlamydiosis	4
		Increased expression at triage in koalas that were euthanised	4
	MYO1B	Increased expression at triage in koalas with clinical chlamydiosis	4
		Increased expression at triage in koalas that were euthanised	4
Other health determinants	Clinical chlamydiosis	Associated with greater odds of mortality in koalas with detectable circulating *C. pecorum*	5
	Sub-clinical chlamydiosis	Associated with greater odds of survival in koalas with detectable mucosal *C. pecorum shedding*	5
	Age	Increased age associated with greater odds of mortality in koalas with clinical chlamydiosis and/or detectable mucosal *C. pecorum* shedding	5
	Body condition score ≤ 2	Associated with greater odds of mortality in koalas from a site of high morbidity	5
		Associated with greater odds of mortality in koalas with clinical chlamydiosis and/or detectable mucosal *C. pecorum* shedding	5
	Future chlamydiosis treatment	Associated with greater odds of survival in koalas from a site with high morbidity	5
	Current chlamydiosis treatment	Associated with greater odds of survival in koalas with clinical chlamydiosis and/or detectable mucosal *C. pecorum* shedding	5
	Chlamydiosis treatment at any point	Associated with reduced odds of survival in koalas with detectable circulating *C. pecorum*	5
	Originating from a site with high morbidity	Associated with greater odds of mortality in koalas with detectable circulating *C. pecorum*	5
	Previous or future ovariohysterectomy	Associated with reduced odds of survival in koalas with detectable circulating *C. pecorum*	5