Investigation of intranuclear inclusion bodies in the hepatocytes of the koala and potential association with herpesvirus infection

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Statement of Originality

I hereby certify that to the best of my knowledge, the content of this thesis is the product of my own work. All assistance received in preparing this thesis and sources have been acknowledged in the text. The content of this thesis has not been submitted in part or full for a degree at this or any other institution.

Krysten Lee

Artificial Intelligence Statement

Generative artificial intelligence was not used in the preparation of this thesis.

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Leung CW, **Lee K**, Mooney E, Wen Siow J, Donahoe SL. Mesenteric lymphatic malformation with chylothorax in a cat. JFMS Open Rep. 2025 Apr 19;11(1):20551169251327400. doi: 10.1177/20551169251327400.

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Thesis Abstract

Background: Koalas (*Phascolarctos cinereus*) are susceptible to two gammaherpesviruses, Phascolarctid gammaherpesvirus 1 (PhaHV-1) and Phascolarctid gammaherpesvirus 2 (PhaHV-2). More recently an alphaherpesvirus (PhaHV-3) has been discovered. Little is currently known about the clinical or pathological significance of these viruses in wild koala populations. Inclusion bodies are seen commonly in koala hepatocytes at postmortem examination, and proposed causes include herpesvirus infection, glycogen or protein accumulation, or cytoplasmic invaginations, however, to date no cause has been identified.

Aims: To characterise the epidemiology of koalas with inclusion bodies; identify associations with other pathological changes in the liver or disease states; and determine whether the presence of inclusion bodies is associated with infection by PhaHV-1 or PhaHV-2.

Methods: Retrospective review of postmortem records alongside prospective collection of samples from koalas undergoing postmortem examinations was performed. Epidemiologic data was gathered, and liver histopathology was analysed using a developed grading scheme. Formalin-fixed paraffin embedded (FFPE) liver and spleen samples (n = 14) and fresh liver and spleen samples (n = 34) were subjected to qPCR for PhaHV-1 and PhaHV-2.

Results: Queensland koalas in the study were more likely to have inclusions than those from NSW, but prevalence did not differ with age, sex, and captive/wild status. Koalas with chlamydiosis or neoplasia were more likely to have inclusions than koalas dying from trauma. Koalas with inclusions had increased levels of hepatocellular anisokaryosis. No association was detected between PhaHV-1 or PhaHV-2 presence and the presence of inclusions.

Conclusions: PhaHV-1 and PhaHV-2 are both considered unlikely to be direct causes of the inclusion bodies. The association with chronic disease and debilitation, along with greater frequency of anisokaryosis, suggests that increased hepatocellular turnover linked to increased demand for hepatic metabolism might play a role in the development of inclusions.

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ACT – Australian Capital Territory

Ct – Cycle threshold

DNA – Deoxyribonucleic acid

H&E – Haematoxylin & eosin

KoRV – Koala Retrovirus

NSW – New South Wales

PAS – Periodic-Acid Schiff

PCR – Polymerase Chain Reaction

qPCR – Quantitative Polymerase Chain Reaction

PhaHV-1 – Phascolarctid herpesvirus-1

PhaHV-2 – Phascolarctid herpesvirus-2

PhaHV-3 – Phascolarctid herpesvirus-3

Qld – Queensland

RNA – Ribonucleic acid

SA – South Australia

EM – Electron Microscopy

Vic - Victoria

Chapter 1

Literature Review

1.1 Introduction

The rapidly declining numbers of koalas along the eastern coast of Australia resulted in a change in conservation status from Vulnerable to Endangered in 2022 (Australian Government, 2023). Alongside anthropogenic causes for population decline, including loss of habitat and motor vehicle trauma (Gonzalez-Astudillo et al., 2017), koalas are susceptible to numerous infectious agents including *Chlamydia* sp., (Polkinghorne et al., 2013), koala retroviruses, (Denner and Young, 2013), *Cryptococcus* sp., (Krockenberger et al., 2003), trypanosomes (McInnes et al., 2011), and multiple recently discovered herpesviruses (Stalder et al., 2015). Chlamydiosis is well known to have significant impacts on both koala fertility and mortality, particularly in eastern koala populations (Gonzalez-Astudillo et al., 2017, Hulse et al., 2021). The clinical and ecological impacts of the other infectious agents, however, remain poorly defined, although potential roles in immunosuppression and co-infections leading to increased pathogenicity have been proposed, particularly for retroviruses and herpesviruses (Blyton et al., 2022, Stalder et al., 2015). To better inform conservation efforts, it is vitally important to further investigate the interactions between environment, host, and pathogens and how these contribute to koala morbidity and mortality.

Koalas rely heavily on the liver for detoxification of the plant secondary metabolites such as terpenes, cyanogenic glucosides, and tannins which are abundant in their highly specialised diet of eucalyptus leaves (Moore et al., 2005, Marschner et al., 2019). This is demonstrated by their greater cytochrome P450 activity relative to non-eucalypt feeders such as humans and rats (Jones et al., 2008). Therefore, any pathology found within the liver with the potential to affect liver function, and thus the appropriate detoxification and elimination of dietary toxins, may lead to adverse health outcomes. Koala livers are often examined histologically as part of thorough postmortem examinations, and common findings include accumulation of brown pigment and fibrosis often surrounding central veins, which are both currently regarded as incidental and likely age-related changes (Higgins et al., 2018), although there is no peer-reviewed literature to support this theory. In addition, hepatocytes are commonly found to contain intranuclear inclusion bodies (unpublished observations). These inclusion bodies are generally round, eosinophilic, and surrounded by a clear halo with marginated chromatin (Figure 1). This appearance on light microscopy strongly resembles the morphology of inclusion bodies associated with herpesvirus infection in other species (MacLachlan and Dubovi, 2011).

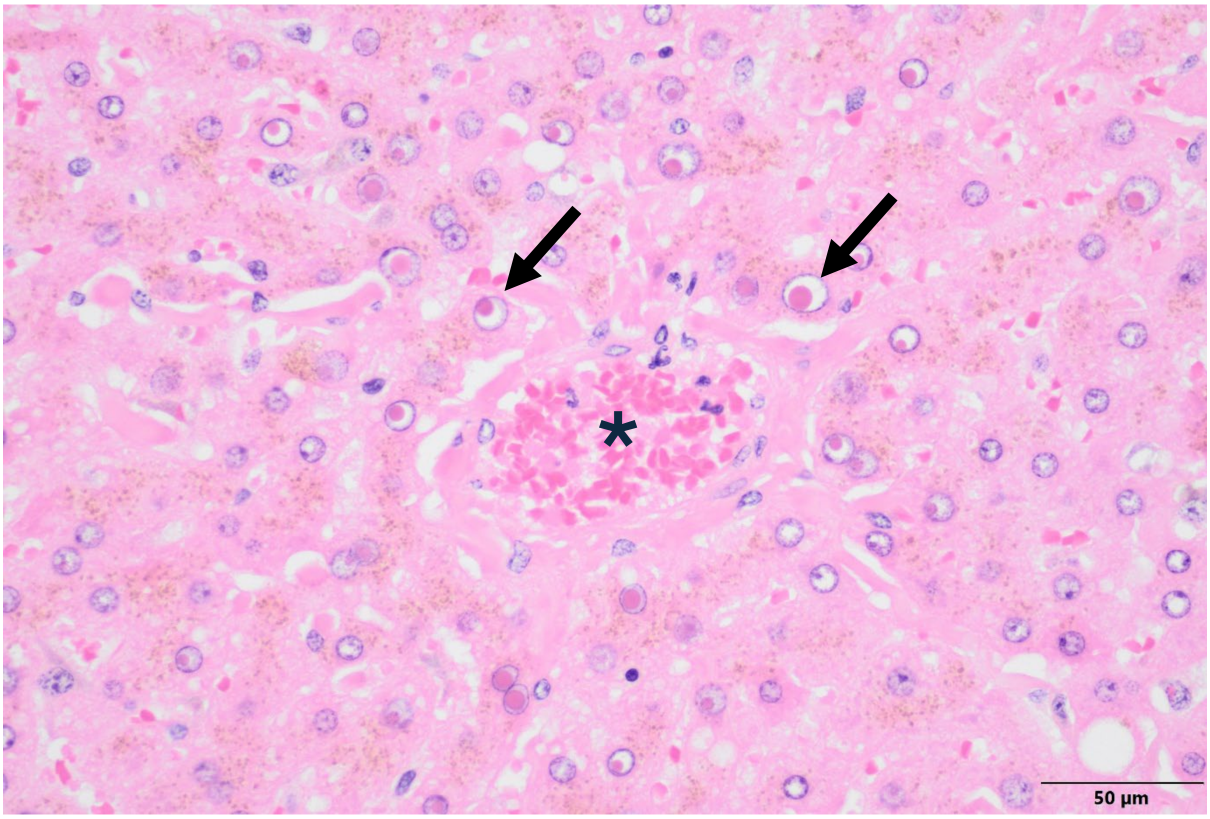

Figure 1: Intranuclear inclusion bodies (arrows) in the hepatocytes of a koala surrounding a central vein (asterisk). The inclusions are round, eosinophilic, and surrounded by a clear halo with margination of the chromatin. Haematoxylin & Eosin, 400x magnification.

Despite their frequent observation, there has been only a single published case report in the literature of these intranuclear inclusion bodies in the hepatocytes of a koala (Condron and Forsyth, 1986). This case report detailed a mature female koala who had been healthy the day prior to being found dead in a dam 50 kilometres northeast of Melbourne, Victoria. At necropsy, there was extensive oedema and emphysema of the lungs (consistent with drowning), and many hepatocyte nuclei were found to contain round intranuclear inclusion bodies. These inclusion bodies were Periodic-Acid Schiff (PAS) positive and unaffected by diastase treatment. Electron microscopy was performed on the formalin-fixed liver tissue, which demonstrated aggregates of discrete, 70-100nm diameter polygonal electron-dense particles, which had an irregular outline with fine granular substance dispersed between them. This was not considered characteristic of viral particles, given the irregular outline and varying size and shape, although formalin fixation is not ideal for ultrastructural assessment (Carson, 2018). Instead, they were considered more characteristic of glycogen particles, although this was inconsistent with their resistance to digestion by diastase and their morphologic appearance on light microscopy (Bennett et al., 2008). Therefore, at the time of publication, a precise aetiology for these inclusion bodies could not be identified, and they were determined most likely to be nuclear bodies – an umbrella term used for a variety of intranuclear structures which can be seen in normal tissues, a variety of tumours, viral infected tissues, and with immunological or hormonal stimulation (Condron and Forsyth, 1986).

Since this case report was published in 1986, these inclusion bodies have been identified commonly during postmortem examination of koala livers, and have been reported as an incidental finding, presuming them to be a marker of a metabolically active cell (Higgins et al., 2018). However, further investigations on a larger scale have not been performed to definitively conclude whether they are truly an incidental finding or whether they could be associated with infectious disease or other pathology, representing a significant gap in current knowledge.

Advancements in koala research and molecular techniques over the last 30 years have since identified two gammaherpesviruses that appear to be widespread amongst wild koala populations (Stalder et al., 2015, Vaz et al., 2011, Vaz et al., 2012), although the pathogenesis and clinical significance of these viruses are currently unknown. Considering this new information and the strong resemblance on light microscopy of these inclusions to herpesviral inclusions in other species, further investigating their aetiopathogenesis and potential link to herpesvirus infection has become warranted. Gammaherpesviruses in other species have been linked to immunomodulatory effects as well as lymphoproliferative disease including lymphoma (Ackermann, 2006), which is commonly diagnosed in koalas (Jondle and Tarakanova, 2020), and so understanding these comorbidities is of increasing importance.

The present study is the first to systematically investigate the aetiopathogenesis of these inclusion bodies and to explore a potential link to herpesvirus infection. By integrating histopathology with molecular techniques, this research revisits a phenomenon which has remained unexplained since the initial report (Condron and Forsyth, 1986). Regardless of whether the inclusions are linked to herpesvirus infection, investigating whether they have any impact on hepatocyte function or are truly an incidental finding will allow koala postmortem results to be interpreted more accurately than was previously possible. If the inclusions are associated with other pathologic changes, this may suggest an impact on the koala's health that requires further investigation. If there is a link to herpesviral infection, making this connection will allow enhanced diagnosis and monitoring of herpesviruses in both captive and wild animals by utilising a widely available sample type (formalin-fixed tissues), which can then better inform approaches to management and control of disease.

The following literature review begins with a discussion on intranuclear inclusion bodies and their various causes including both viral and non-viral aetiologies, their morphologic appearance, pathogenesis, and significance. Following this, the broader importance of koala conservation and reasons for decline in koala populations are addressed by way of context for the study. Finally, a discussion on infectious diseases affecting koalas and koala immunity provides context for the significance of herpesvirus infection in this species.

1.2 Intranuclear inclusion bodies

Intranuclear inclusion bodies can be caused by a variety of processes, which ultimately result in a change to the nuclear structure that is visible on light microscopy. The morphology of inclusion bodies on routine H&E staining is variable, from eosinophilic (containing more protein) to basophilic (containing more nucleic acids) (Chan, 2014). The most common causes of intranuclear inclusions include viral infection and non-viral changes to the nucleus such as cytoplasmic invagination (also referred to as pseudoinclusions), glycogen accumulation, or accumulation of various proteins, cytoplasmic materials, or secretory products (Ip et al., 2010). It is important to differentiate between these causes, as some have diagnostic significance (e.g., those associated with viral infections or certain types of tumours) whereas others are dismissed as incidental findings (Ip et al., 2010).

1.2.1 Viral intranuclear inclusions

Viruses are obligate intracellular parasites, and a frequent microscopic hallmark of viral-infected cells is the presence of inclusion bodies, which represent aggregates of viral and/or host proteins otherwise known as “viral replication factories” (Novoa et al., 2005). The location of the inclusion body can be either intranuclear or cytoplasmic, depending on the type of virus (DNA or RNA) and its replication strategy. The DNA viruses, including herpesviruses, adenoviruses, and parvoviruses commonly produce intranuclear inclusion bodies and these are further discussed below. Some DNA viruses such as poxviruses produce cytoplasmic inclusion bodies, while rabies and canine distemper virus are RNA viruses that produce cytoplasmic inclusions. Canine distemper virus is capable of producing both cytoplasmic and intranuclear inclusions (Miller and Zachary, 2017).

Herpesvirus inclusion bodies

Herpesviruses are large, enveloped, double-stranded DNA viruses with a genome ranging from 120-250kbp in size. Many herpesviruses produce characteristic intranuclear inclusion bodies, which are often globular and can occupy the majority of the nucleus (Netherton et al., 2007). Herpesvirus particles consist of four layers: a core containing the DNA, an icosahedral capsid, a tegument consisting of a poorly defined layer of protein, and a viral envelope which contains several glycoproteins. During viral replication, the genome synthesis and packaging as well as capsid assembly occur within the nucleus of the infected cell. The viral particles obtain tegument in either the nucleus or cytoplasm, or both, and then obtain an envelope in the cytoplasm (Netherton et al., 2007).

Herpesviruses enter the host cell *via* fusion of the nuclear envelope with the cell membrane, after which the capsid is released into the cytoplasm and migrates to nuclear pores (Boehmer and Nimonkar, 2003). Initial stages of productive herpesvirus infection within a cell are linked with nuclear structures called Nuclear Domain 10 (ND10) bodies in structures called nuclear aggresomes, which recruit proteins required for virus replication and assembly (Strnad et al., 2013). As early as 30 minutes post-infection, immediate early regulatory protein (ICP4) binds the viral DNA and forms discrete foci. A second type of inclusion with herpesvirus infection is known as an assemblon, which refers to the site where capsid proteins accumulate and assemble into nucleocapsids (Netherton et al., 2007). Therefore, herpesvirus inclusion bodies contain both host and viral-encoded proteins.

The microscopic appearance of herpesviral inclusion bodies is best described for human herpes simplex infection, in which they are divided into two types: Cowdry type A and Cowdry type B. Cowdry type A inclusions are believed to be the mature form and appear as an eosinophilic intranuclear inclusion body surrounded by a clear halo with marginated chromatin pushed to the nuclear membrane (Strnad et al., 2013), which is very similar in appearance to the inclusion bodies identified in koala hepatocytes. Cowdry type B inclusions are believed to represent an earlier form and consist of an enlarged nucleus with a ‘ground glass’ appearance and a fractured nuclear membrane (Ip et al., 2010).

Ultrastructurally, herpesvirus inclusion bodies have a variable appearance depending on the stage of infection, and may consist of condensed capsid proteins, electrolucent regions containing viral DNA, and mature virions (Strnad et al., 2013). Herpesvirus virions have icosahedral morphology, giving them a hexagonal outline, and are surrounded by limiting lipid bilayers. Herpesvirus virions are generally 100-120nm in diameter, with the complete structure including the envelope measuring approximately 150-180nm diameter (Farris et al., 2018). The identification of herpesvirus virions using electron microscopy can provide a useful diagnostic tool, even in cases where classic inclusion bodies are not visible on light microscopy, although in one study of confirmed human herpes simplex encephalitis cases virions were only detected in 7/9 specimens (White and Taxy, 1983). Herpesvirus virions were not detected in the single case of koala inclusion bodies subjected to electron microscopy (Condron and Forsyth, 1986), although it is possible that a larger sample size may have revealed them to be present. In addition, formalin fixation can reduce the quality of electron microscopy specimens (Carson, 2018).

Herpesviral DNA has been detected in many Australian wildlife species, including the koala, as well as macropods and other marsupials (Stalder et al., 2015). Herpesviral infections in koalas are discussed in further detail later in this chapter.

Adenovirus inclusion bodies

Adenoviruses are another family of DNA viruses known to form intranuclear inclusions. Adenoviruses are nonenveloped double-stranded DNA viruses, with virions measuring approximately 70-100nm diameter (Netherton et al., 2007). An example of an adenovirus causing intranuclear inclusions in hepatocytes is the fowl adenovirus causing inclusion body hepatitis (IBH) which occurs in birds, including pigeons (*Columba livia*) and chickens (*Gallus gallus*). These inclusions are large, eosinophilic to basophilic, and occasionally surrounded by a halo with peripheral aggregation of chromatin. On electron microscopy, viral particles arrange in paracrystalline arrays. These inclusions are usually associated with necrotising hepatitis (Goryo et al., 1988). Adenoviruses have not been detected in marsupial animals to date, although research is limited with no published investigations aiming to identify them in koalas or other marsupial species. Although an atadenovirus, within the *Adenoviridae* family, has been found in the faeces of a wild brush-tailed possum (*Trichosurus vulpecula*), the virus was not identified within the tissues of the animal and therefore there is no current evidence of active infection occurring (Thomson et al., 2002). Detection of viruses in gastrointestinal contents or faeces can represent ingestion rather than true infection characterised by replication in host tissues (Harrach et al., 2019), and given brush-tailed possums are classed as opportunistic omnivores (Cameron et al., 2013), ingestion of virus-containing animal tissues or vegetation contaminated with animal waste could provide an explanation for the presence of adenovirus in the faeces. A novel adenovirus was detected in a captive kowari (*Dasyuroides byrnei*, a dasyurid also known as the brush-tailed marsupial rat) in Hungary, which was associated with pneumonia and hepatic necrosis (Gál et al., 2017). This kowari was housed with a companion animal which was infected by the same virus but did not show clinical signs of disease. Altogether, considering the morphological and electron microscopy appearance (paracrystalline arrays) of adenovirus inclusions along with the lack of identified adenoviruses in koalas or closely related species, adenovirus infection was considered less likely to be a cause for the current koala inclusions, despite the similar size to the particles identified previously (Condron and Forsyth, 1986).

Parvovirus inclusion bodies

Parvoviruses are another common cause of intranuclear inclusion bodies in animals, with canine and feline parvoviruses usually forming inclusion bodies in the intestine, myocardium, and occasionally the liver. Parvovirus virions are very small, 18-25nm in diameter, and the inclusions are generally basophilic (as they contain more DNA than protein) and occupy the majority of the nucleus with or without an evident halo (Bestetti and Zwahlen, 1985). Parvovirus infections are usually associated with severe necrosis and inflammation of the infected tissues (Parrish, 1995). Sequences from the *Parvoviridae* family have been identified as endogenised within marsupial (including koala) genomes, meaning the virus has been incorporated in to the host germ cell genome and therefore become inheritable (Harding et al., 2021). This is considered to represent a possible ancient antiviral defence (Harding et al., 2021), but active infections have not been identified to date. Hence, parvovirus was also considered unlikely to present a possible cause for the current koala inclusions.

1.2.2 Non-viral intranuclear inclusions

Pseudoinclusions

Intranuclear pseudoinclusions, or nuclear cytoplasmic inclusions, are invaginations of the cell cytoplasm into the nucleus (Arora and Dey, 2012). These can be seen in various tumours, particularly well-recognised in people with papillary carcinoma of the thyroid, and present as a discrete variably shaped structure in the nucleus causing round or oval areas of pallor (Ip et al., 2010). The contents of the pseudoinclusion are cytoplasmic materials, and therefore on light microscopy they tend to appear as the same colour as the cytoplasm of the affected cell. On electron microscopy, cytoplasmic organelles such as rough endoplasmic reticulum, Golgi apparatus, or secretory granules can be identified within the pseudoinclusion, and it is surrounded by the invaginated nuclear membrane (Arora and Dey, 2012). In the present case of koala inclusions (Condron and Forsyth, 1986), pseudoinclusions were considered unlikely based on the deeply eosinophilic colour suggesting high protein content and the lack of surrounding nuclear membrane or cytoplasmic organelles on electron microscopy.

Glycogen

Glycogen inclusion bodies are common findings in the liver of some species, including humans (Strnad et al., 2013, Bennett et al., 2008). Glycogen should not stain with eosin, and therefore these inclusions tend to appear as a pale ‘clearing’ of the nucleus on routine H&E staining (Chan, 2014). Intranuclear inclusion bodies occur commonly in the hepatocytes of humans with non-alcoholic fatty liver disease and diabetes mellitus and are divided into two types. Type I inclusions contain degenerate cellular organelles and autophagy proteins, although are completely surrounded by a limiting membrane (rather than an invaginated nuclear membrane) which indicates that they are not simple cytoplasmic invaginations. Type 2 inclusions contain only glycogen deposits with no organelles or limiting membrane. Both types stain positively with PAS and are digested by treatment with diastase. In one study, the presence of both types of inclusions were associated with progression of liver disease in people (Schwertheim et al., 2020b).

Glycogen inclusion bodies have also been reported in western barred bandicoots (*Perameles bougainville*) (Bennett et al., 2008), in which abnormal hepatocyte nuclei with margination of chromatin and central pallor occurred commonly. These inclusions were PAS positive and completely digested by diastase treatment. On transmission electron microscopy, the nuclei did not contain viral particles, and characteristic glycogen beta-particles and alpha-rosettes were identified. Glycogen inclusion bodies have also been reported in canine parietal cells of the stomach (Silvestri et al., 2017), where they were similarly identified by PAS positivity and sensitivity to diastase along with electron microscopy. In both bandicoot hepatocytes and canine parietal cells, these glycogen inclusions were presumed to be an incidental finding.

Glycogen was considered as a possible cause for the koala intranuclear inclusions, due to the finely granular material present on electron microscopy, but was ultimately considered unlikely based on the lack of sensitivity to diastase treatment and the morphological appearance on light microscopy (densely eosinophilic rather than pale or clear) (Condron and Forsyth, 1986).

Intranuclear accumulation of cellular substances

Another non-infectious cause for intranuclear inclusion bodies is the accumulation of various cellular substances such as biotin, surfactant (specific to pneumocytes), immunoglobulins, or other host cell proteins within the nucleus (Ip et al., 2010). Biotin inclusions can be seen in human biopsies, particularly of the endometrium, manifesting as a lightly eosinophilic ground-glass material which replaces the entire nucleus. These are very similar in appearance to Cowdry type B inclusions seen with herpes simplex infection and can result in misdiagnosis of herpes infection in people. On electron microscopy, the nucleus contains characteristic microfilaments and the biotin composition can be confirmed by use of immunohistochemistry (Ip et al., 2010). Immunoglobulins can occasionally accumulate within the nuclei of plasma cells and less commonly B lymphocytes, with the resulting inclusions named Dutcher bodies. These are eosinophilic, PAS positive, and resistant to diastase (Ip et al., 2010).

Eosinophilic intranuclear inclusion bodies in renal tubular epithelial cells have been described in laboratory rats receiving various drugs including norepinephrine/serotonin reuptake inhibitors (Radi et al., 2013), as well as seemingly occurring spontaneously in non-treated rats (Shimoyama et al., 2015). In both instances, the accumulated protein was determined to be D-amino acid oxidase (DAO) via immunohistochemistry. DAO is a flavoenzyme that catalyses the oxidative deamination of D-amino acids, with a key role in catabolising D-serine, a neuromodulator, in the central nervous system (Pollegioni et al., 2018), although it is also present in high concentrations in the kidney and liver (Shimoyama et al., 2015). These inclusions are negative on PAS staining and have a finely granular homogenous appearance on electron microscopy with no limiting membrane. They are not considered pathological, as there are no associated degenerative or inflammatory renal changes and renal function is not affected.

A similar process of host protein accumulation within the nucleus could provide an alternative non-viral explanation for the koala inclusions, although identifying the specific protein involved could prove a challenge as many techniques used to label proteins, such as immunohistochemistry, require antibodies specific to the protein of interest (Magaki et al., 2019). Koala liver function is known to differ significantly from many eutherian mammals as well as other marsupials, likely due to the high levels of potentially toxic substances such as essential oil monoterpenes found in their specialised eucalyptus diet (Jones et al., 2008). The koala liver expresses higher levels of cytochrome P450 (CYP) enzymes when compared to rats and humans (Jones et al., 2008), and this enhanced metabolic capability leads to shorter half-lives for the metabolism of many exogenous substances such as pharmaceuticals. For example, the half-life of bromosulphthalein (a compound metabolised almost exclusively by the liver and used as a marker of liver function) in koalas was found to be significantly shorter than in either sheep or macropods (Pass and Brown, 1990). Similarly, rates of meloxicam clearance are much higher in marsupials than in dogs or rats, which is also presumed to be due to higher CYP activity (Kimble et al., 2014). Although proteins such as CYPs are expected to be found in higher quantities in koala livers, they are generally located in the cytoplasm embedded within the endoplasmic reticulum (Seliskar and Rozman, 2007). Therefore, if CYPs or other metabolic proteins are related to the inclusion bodies, a process of cytoplasmic invagination would need to be involved.

1.3 Koala key threatening processes

1.3.1 Population distribution and conservation status

As of March 2024, the National Koala Monitoring Program estimated the national population of koalas in Australia to be in the range of 224,000 – 524,000 individuals (National Koala Monitoring Program, 2024). This includes 95,000 – 238,000 in the listed (endangered) population (QLD, NSW, ACT), and 129,000 – 286,000 in the unlisted population (VIC, SA) (Figure 2). Koalas are highly specialised arboreal folivores, and their range is highly influenced by the availability of suitable vegetation for both food and shelter (Hailee et al., 2017). This restricts most populations to the coastal regions of Australia, and fertile riverine habitats west of the Great Dividing Range, which have also been the most highly impacted by European settlement, resulting in extensive anthropogenic loss of habitat (Griffith et al., 2013). This loss of habitat, along with habitat fragmentation, impacts of climate change such as changing weather patterns with increased severity and frequency of bushfire and flooding events, increased incidences of trauma including motor vehicle accidents and predation by domestic animals, and high levels of infectious disease, have all resulted in the decline of koala numbers over recent years (Gonzalez-Astudillo et al., 2019, Hailee et al., 2017). This has resulted in the listed population (QLD, NSW, and ACT) being upgraded from Vulnerable to Endangered in 2022 (Australian Government, 2023). The unlisted populations (VIC and SA) appear to have been less affected by the aforementioned factors, and as a result are not currently listed as Endangered. Maps of koala population distribution within Australia are available online via the National Koala Monitoring Program (National Koala Monitoring Program, 2024).

1.3.2 Reasons for rescue and mortality

Numerous retrospective studies conducted in multiple Australian states describe the causes for koala mortality and population decline and explore the reasons for differences between the listed and unlisted populations. In one study, mortality was proposed to be the major driver of population decline, as opposed to reduced reproduction, as birth rates in the eastern populations appeared to be consistent with those in the more abundant populations in the south (Rhodes et al., 2011). However, infertility and resultant reduced fecundity also contribute to population decline of wildlife species and, as a known consequence of chlamydial infection, also needs to be considered. This is discussed in further detail later in this chapter; the following discussion focusses on causes of mortality proposed to be driving population decline.

One of the largest retrospective studies investigating causes of mortality as well as hospital admission in 20,250 koalas was performed in Queensland, spanning seventeen years (1997-2013) (Gonzalez-Astudillo et al., 2017). In that study, chlamydiosis was the most common aetiology observed in submissions (52% of koalas), followed by trauma (15%) and wasting (14%). Wasting, or emaciation, can be due to debilitation caused by displacement, chronic disease, or age-related dental attrition, which results in decreased masticatory efficiency. Mastication is especially important in the koala to allow maximal microbial fermentation of eucalyptus leaves (Lanyon and Sanson, 2011). A combination of factors was reported in 39% of cases, with chlamydiosis frequently co-occurring with other causes of mortality. The aetiology considered to be the main overall cause of admission to koala hospitals and rehabilitation facilities was motor vehicle accidents, which may be overrepresented due to increased visibility to the public resulting in greater chances of rescue and admission. Motor vehicle trauma was also the most frequent reason for admission in a study investigating koala morbidity and mortality in the mid-north coast of NSW during 1975-2004 (Griffith et al., 2013). Interestingly, an increased probability of motor vehicle trauma was found after 1985, which correlates with increased urbanisation of this region of Australia. Chlamydiosis was the second most common cause of mortality and occurred more often in aged animals.

Dispersal patterns during seasonal breeding periods have been reported as causing a particularly elevated mortality rate in two to three-year-old males (Griffith et al., 2013, Rhodes et al., 2011), although this was not found as a contributing factor in the large-scale study in Queensland (Gonzalez-Astudillo et al., 2017). In contrast, another study from southeast Queensland spanning 2009-2014 (Burton and Tribe, 2016) investigated admission records from four major wildlife hospitals, and did find an increased rate of koalas brought into care during spring and early summer, coinciding with the breeding period. In this study, major threats were similarly found to be related to urbanisation (motor vehicle accidents, domestic animal attacks) and chlamydiosis, with most koalas admitted (66%) being euthanised or dying in care and only 27% being released back into the wild (the remaining 7% of koalas had unknown outcomes)

Similarly, another study investigating disease processes in koalas presented for necropsy in southeastern Queensland (Gonzalez-Astudillo et al., 2019) identified trauma as the most common diagnosis (42.7% of koalas), followed by infectious disease (31.8%), wasting of unknown cause (7.7%), and neoplasia (6.4%). Multiple infectious processes or co-occurring infectious processes with trauma or other diseases were also commonly identified, reinforcing the complex interactions between the various threats to koala health.

Relative to the endangered eastern populations, threats to the more abundant southern populations present both similarities and differences. In a South Australian study of 85 koalas (Speight et al., 2018), 57% died from trauma (motor vehicle accident, dog attack, or bushfire burns), 32% had oxalate nephrosis (in contrast to the relatively uncommon presentation of this condition in the eastern states), and 12% had chlamydiosis, a rate much lower than that seen in the eastern states. Similarly to the eastern states, 45% of koalas presented with two or more abnormalities.

As evidenced by these studies, koala populations are facing multiple threats leading to population decline, which vary based on geographical location but commonly demonstrate an interplay between various factors including both infectious disease and anthropogenic factors. To ensure the conservation of this species and address population decline, strategies to address each of these factors are required.

1.4 Koala infectious disease and immunity

As evidenced by the retrospective studies above, infectious diseases, particularly chlamydiosis, are a major threat to the endangered eastern koala populations. Therefore, it is vitally important to understand more about koala immunity and how co-infections and chronic stress contribute to morbidity and mortality. If a link between the presence of inclusion bodies and herpesvirus infection is present, the inclusion bodies may represent an important diagnostic feature that would improve our capability to diagnose infection utilising a widely available sample type (formalin-fixed postmortem tissues). This could then allow many retrospective cases of koalas with inclusion bodies to be examined for associations between herpesvirus infection and other co-infections, providing valuable insight into how these herpesviruses may affect immunity by comparing herpesvirus positive and herpesvirus negative koalas.

Koalas are also susceptible to infection with koala retroviruses (KoRV), members of the genus Gammaretrovirus, which are closely related to other retroviruses that are known to cause immunomodulatory effects (Joyce et al., 2022), including feline leukaemia virus (FeLV), porcine endogenous retrovirus (PERV) (Denner and Young, 2013), and gibbon ape leukaemia virus (GALV) (Alfano et al., 2016). In addition, an association between herpesvirus infection and chlamydiosis has been identified in multiple studies (Stalder et al., 2015, Vaz et al., 2019), with the presence of *Chlamydia pecorum* DNA strongly associated with the presence of herpesvirus DNA in Victorian koalas. Therefore, both herpesviruses and retroviruses may play important roles in koala immunity and susceptibility to infectious diseases, though causative relationships have not been established. This means that if the inclusion bodies are linked to viral infection, this could have important secondary consequences such as increased susceptibility to other infectious diseases, leading to poorer health outcomes. Furthermore, retroviruses are known to insert into the host genome where they modify gene expression and hence protein production (Denner and Young, 2013), which could be linked to an aggregation of host proteins as another possible cause of inclusion bodies. Common koala infectious agents are discussed in further detail in the following sections.

1.4.1 Chlamydiosis

As the major infectious disease threat to koalas, chlamydiosis has been well-studied. Two chlamydial species have been shown to infect koalas, *Chlamydia pneumoniae* and *Chlamydia pecorum*, of which the latter has been reported in nearly all mainland koala populations (Polkinghorne et al., 2013). *C. pneumoniae* is usually isolated from the conjunctiva, often at low levels, and does not appear to be associated with high-grade infections. It is extremely rarely detected in wild populations (Hulse et al., 2018). In contrast, *C. pecorum* is commonly associated with overt clinical ocular and urogenital disease (Gonzalez-Astudillo et al., 2017).

The disease complex chlamydiosis is associated with four clinical syndromes: rhinitis-pneumonia, ocular, reproductive, and urinary tract disease. The most prevalent manifestations are urinary tract and reproductive disease (Gonzalez-Astudillo et al., 2017). Urinary tract disease often presents with brown urine staining and wetness around the rump due to cystitis, leading to use of the colloquial terms “wet bottom” or “dirty tail” to describe the clinical presentation (Polkinghorne et al., 2013). In contrast, reproductive disease is rarely apparent on external examination. In the female koala, ovarian bursal cysts are commonly identified via ultrasound and are a common indicator of infertility and reproductive loss. Although not a direct cause of mortality, under some government guidelines the presence of bursal cysts is an indication for euthanasia on the basis of infertility (Gonzalez-Astudillo et al., 2017). This has resulted in poorer clinical outcomes in females compared to males, in which the reproductive lesions are not as evident (Polkinghorne et al., 2013). However, chlamydiosis also impacts male fertility with a measurable reduction in semen quality characterised by reduced DNA quality and morphologically abnormal spermatozoa (Hulse et al., 2021).

Ocular disease results from infection of the mucosae of the eye, leading to inflammation with purulent discharge, conjunctival hyperplasia, and in chronic stages, fibrosis. Blindness can be a sequela of severe disease, either due to the hyperplastic conjunctiva covering the eye, scarring and oedema of the cornea or, more rarely, ophthalmitis and ensuing globe rupture (Polkinghorne et al., 2013). This is thought likely to then lead to increased rates of both starvation and predation (Gonzalez-Astudillo et al., 2017), though evidence is not available to support this.

Transmission of chlamydiosis is widely considered to be primarily horizontal, through sexual transmission or vertical, from mother to joey, probably via the faecal-oral route during pap feeding to establish the joey's gut flora. Diagnosis of the syndrome is commonly based on the clinical manifestation, using scoring systems to subjectively grade the severity of lesions around the rump and eyes against images or criteria (Phillips et al., 2024). However, many infections occur without overt clinical signs, and therefore for accurate diagnosis PCR testing of urogenital and conjunctival swabs is recommended (Polkinghorne et al., 2013).

1.4.2 Koala retroviruses

Koala retroviruses are members of the genus Gammaretrovirus, some of which are associated with the onset of neoplasia and, through their immunomodulatory effects, infectious disease (Joyce et al., 2022). A key feature of retroviruses is their ability to exist as endogenous (integrated into the host genome of germ cells and able to be inherited vertically) and exogenous (spread horizontally and may be integrated into somatic but not germ cells) forms. Endogenous retroviruses are often not pathogenic, in contrast to exogenous forms which are more often associated with disease (Gifford and Tristem, 2003), although endogenous viruses can still exert beneficial or deleterious effects on gene expression through insertion into the genome.

Twelve subtypes of KoRV have been described to date: KoRV A to KorV I, and KoRV K to KoRV M (Blyton et al., 2022). Sequencing of the *env* gene (Chappell et al., 2017) has indicated that the long list of subtypes can be classified into three clades: KoRV A, KoRV B/J, and a diverse subtype KoRV D (which includes previously described subtypes KoRV C and E, and novel subtypes F, G, H, I, and K-M). KoRV A is widespread in northern koalas in the endogenous (integrated) form, with all koalas tested in Qld, NSW, and the ACT positive for this subtype (Blyton et al., 2022). In contrast, endogenous KoRV A is absent in southern (SA and Vic) populations, and instead KoRV A is only occasionally present and at low copy numbers per host cell, suggestive of exogenous infection (Blyton et al., 2022). The other subtypes have high levels of genetic diversity, more consistent with ongoing exogenous replication and horizontal transmission, although this may change as ongoing endogenisation events occur (Chappell et al., 2017).

Although multiple studies have shown a significant association between KoRV infection (of subtypes other than A) with neoplasia (Denner and Young, 2013, Fabijan et al., 2020, Zheng et al., 2020), the strongest evidence supporting oncogenesis comes from clustering of retroviral insertions near cancer-inducing oncogenes in association with neoplastic lesions (McEwen et al., 2021).

It has also been suggested that high retroviral loads may lead to a higher incidence of infectious diseases such as chlamydiosis. A 2020 study (Fabijan et al., 2020) of Queensland and South Australian koalas indicated urinary tract disease was more severe in the Queensland koalas, despite similar *C. pecorum* loads, although other host or *Chlamydia* differences cannot be ruled out as a factor. In the SA koalas, KoRV viral load correlated positively with chlamydiosis severity, although this was not reproducible in the Queensland population. In another study, greater KoRV viral load (of exogenous subtypes) increased the predicted probability of northern koalas having disease signs from 25% to over 80%, with the predicted probability of *C. pecorum* infection increasing from 40% to over 80% (Blyton et al., 2022). In one study, viral load correlated positively with splenic lymphoid area (measured mean area of lymphoid follicles and periarteriolar lymphoid sheaths), with the authors suggesting that splenic lymphoid tissue may be a key site for KoRV viral replication (Fabijan et al., 2020).

1.4.3 Koala herpesviruses

A defining attribute of herpesviruses is their ability to establish latent infections, persisting indefinitely within their specific vertebrate host (Boehmer and Nimonkar, 2003). This is due to the long-standing evolutionary relationship whereby the virus is well-adapted to survive within the host without causing severe illness or death, allowing prolonged survival and transmission (Grinde, 2013). Active replication tends to induce a host immune response, often resulting in the death of the infected cell. During latency, the viral genome becomes circularised and is packed in histones, the viral DNA is copied by host DNA polymerase when the cell is undergoing mitosis, and viral protein expression is dampened by the host's epigenetic mechanisms. This is important for the avoidance of immune detection (Cohen, 2020). In contrast, during active replication the genome becomes linearised and viral DNA polymerase replicates the DNA, constituting a cell takeover (Grinde, 2013). Herpesviruses are classified into alpha-, beta-, or gammaherpesviruses on the basis of genomic features and infection characteristics.

Alphaherpesviruses are characterised by active replication at epithelial and mucosal sites, and tend to establish latency within neurons (Kuny and Szpara, 2021). Examples of veterinary alphaherpesviruses include canine herpesvirus-1 (CHV-1), which causes reproductive disorders including abortion and mortality in neonatal puppies commonly associated with hepatic necrosis (Decaro et al., 2008), Marek's disease in poultry caused by gallid alphaherpesvirus-2 (GaHV-2) which causes lymphoproliferative disease (Zelník et al., 2013), and macropodid herpesvirus-1 (MaHV-1) which causes conjunctivitis, pneumonia, and hepatic and splenic necrosis in macropods (Finnie et al., 1976).

Phascolarctid herpesvirus-3 (PhaHV-3) is an alphaherpesvirus that was identified in 2022 (Bowater et al., 2022), in one captive koala from Queensland, which was admitted to hospital due to severe respiratory disease and anorexia. Postmortem examination of this animal identified chronic interstitial pneumonia along with multifocal necrotising adrenalitis and hepatitis. Intranuclear inclusion bodies were detected in cells of the bronchi, alveoli, hepatocytes, biliary epithelium, and adrenal glands in this case, often associated with foci of necrosis. Universal family-level herpesvirus PCR was performed on fresh lung tissue, and the product sequence had 97% similarity to macropodid alphaherpesvirus-1. *Cryptococcus gattii* was also detected in the lung by histopathology and confirmed by culture and PCR. To date, no further cases of PhaHV-3 have been published, and no specific diagnostic qPCR test is currently available for this virus. The intranuclear inclusions in the liver and spleen associated with regions of necrosis was consistent with the lytic reproduction strategy of alphaherpesviruses well-recognised in other species.

In contrast, gammaherpesviruses are lymphotropic and tend to establish latency in lymphocytes, with latency and reactivation an important survival strategy (MacLachlan and Dubovi, 2011, Ackermann, 2006). Active replication tends to occur within epithelial cells of the oropharynx and/or genital tract (Cohen, 2020). Veterinary examples of gammaherpesviruses include ovine herpesvirus-2, the causative agent of malignant catarrhal fever in cattle, which is associated with lymphoproliferative disease; equid herpesvirus-5, which is the cause of progressive multinodular pulmonary fibrosis in horses (MacLachlan and Dubovi, 2011); and macropodid herpesvirus-3, which does not appear to cause significant disease in eastern grey kangaroos (Smith et al., 2008). Some gammaherpesviruses are linked with oncogenic transformation, such as human herpesvirus-8 (Kaposi's sarcoma-associated herpesvirus), a gammaherpesvirus that contains viral genes that can override normal cell cycle arrest and result in Kaposi sarcoma, multicentric Castleman disease, or B-cell lymphoma. These conditions in people are frequently associated with retroviral coinfection (Radu and Pantanowitz, 2013). This is interesting given the frequent infection of koalas with koala retroviruses, which raises the question of the importance of co-infection of these two viruses and whether they play a role in the susceptibility of koalas to lymphoproliferative disease. Although many gammaherpesviruses are not associated with significant disease in immunocompetent hosts, immunocompromise can result in pathologic conditions associated with increased numbers of latently infected lymphocytes (Ackermann, 2006), thus reinforcing the importance of investigating co-infections.

There are two currently identified koala gammaherpesviruses – phascolarctid herpesvirus-1 (PhaHV-1) and phascolarctid herpesvirus-2 (PhaHV-2). PhaHV-1 was first identified in 2011 (Vaz et al., 2011) in postmortem samples from three wild koalas collected from three different sites in Victoria during 2010. PhaHV-1 was isolated from the liver and spleen of one koala, a nasal scraping from the second koala, and the spleen of the third koala. The sequence of the amplified DNA products from each koala were identical and matched to other viruses in the subfamily *Gammaherpesviridae*. There were no overt clinical signs in the affected koalas, although histopathology did reveal severe lymphoid depletion in the spleen and lymph nodes, consistent with prolonged stress which could be caused by environmental stressors and/or chronic infection. Discovery of PhaHV-2 occurred shortly afterwards in 2012 (Vaz et al., 2012), in one of the Victorian koalas already shown to be infected with PhaHV-1. Further analysis of the extracted DNA from the nasal swab of this koala identified a second distinct gammaherpesvirus. This was the first report of a coinfection with two different gammaherpesviruses in an Australian marsupial.

Since the discovery of PhaHV-1 and PhaHV-2, a small number of studies have aimed to investigate the prevalence of these viruses within various koala populations. In South Australia, active shedding (determined by oropharyngeal swabs) of either PhaHV-1 or PhaHV-2 was detected in 72.5% of wild-caught koalas in the Mount Lofty Ranges (Kasimov et al., 2020). In Victoria, herpesvirus DNA was detected from swabs of 33.3% of koalas tested in one study (Stalder et al., 2015), while in another the swab prevalence varied widely between populations, from 1 to 55% (Vaz et al., 2019). In Queensland and New South Wales, the only published prevalence data is for PhaHV-1, and there was a notable difference between samples collected from rescued koalas (26%) and field-collected samples from wild koalas (8%), which was suggested to reflect increased shedding in koalas admitted to hospitals, possibly due to stress (Wright et al., 2024). Investigation of the prevalence of latent gammaherpesvirus infections is limited by the need to test tissues rich in lymphocytes, such as the spleen, which is only available through postmortem examination, and therefore latent infection could be more widespread than has been reported in the current literature.

Multiple studies have found that adult and older animals are more likely to be positive for PhaHV-1 than juveniles (Vaz et al., 2019, Wright et al., 2024). Although previously a similar age relationship was not detected for PhaHV-2 (Vaz et al., 2019), with a difference in transmission between the two viruses suggested, a more recent study did find that older koalas were also more likely to test positive for PhaHV-2 (Church et al., 2025). This highlights that much is still unknown about the transmission, epidemiology, and clinical significance of these viruses in koala populations.

1.5 Hypotheses, aims, and objectives

To summarise, there are multiple phascolarctid herpesviruses that are commonly identified in koalas. Intranuclear inclusion bodies are frequently detected in koala hepatocytes and are currently reported as an incidental finding, but their aetiopathogenesis and significance remains unclear. The morphology of these inclusions (round, eosinophilic, and often surrounded by a clear halo with margination of chromatin) could be consistent with herpesvirus inclusions which are often observed in other species.

Ultimately, the work in this thesis aims to achieve the following three objectives:

1. To characterise the epidemiological features of koalas with inclusion bodies.
2. To determine whether the presence of inclusion bodies has significance for the animal, by identifying associations with other pathological changes in the liver or disease states.
3. To determine whether the presence of inclusion bodies is associated with herpesvirus (PhaHV-1 or PhaHV-2) infection.

The hypotheses are that the presence of inclusion bodies will be positively associated with diseased koalas and increased levels of pathology within the affected livers, and that koalas with inclusion bodies will be more likely to test positive for herpesvirus infection.

Chapter 2

Materials & Methods

2.1 Case recruitment

Koala Health Hub Formalin-Fixed-Paraffin-Embedded Tissue Archive

A retrospective review of postmortem records was conducted via the University of Sydney Veterinary Pathology Diagnostic Services (VPDS) laboratory records software (Integrated Laboratory Management Software, ILMS) and archived research data. Cases comprised captive koalas that had died, or koalas presented to wildlife care facilities and then subjected to necropsy following death or euthanasia for welfare reasons, independent of researcher influence.

Inclusion and exclusion criteria

All koala postmortem records from 2000 to 2023, inclusive, were examined for suitability for inclusion in the study. Inclusion criteria were that liver histopathology slides and/or tissue blocks were available for review, and tissue was of adequate diagnostic quality (excluding those with severe autolysis or freeze-thaw artefact). Retrospective cases were further selected for qPCR based on the availability of the liver FFPE tissue block and the presence of intranuclear inclusion bodies in hepatocytes on light microscopy.

Prospective sample collection

Similar cases were gathered prospectively throughout 2024 with the addition of fresh (frozen) liver and spleen specimens for PCR analysis.

Data collection

Information recorded for each case included the date of postmortem examination (month/year), location of origin of the koala (where available), submitting organisation, captive or wild status, age of koala (based on standardised tooth wear scores where available, or estimated age as provided by the submitter), sex, gross pathology, and histopathology results.

Data was categorised further based on the following criteria. Age from records, originally based on tooth wear, was categorised as juvenile (<1 year), adult (estimated 1-8 years) or aged (estimated 8+ years). Where detailed age data was not available, animals were categorised as juvenile if the terms ‘back young’, ‘young’, ‘juvenile’, or ‘joey’ were used; adult if ‘young adult’ or ‘adult’; and aged if advanced tooth wear was mentioned. The most significant gross and histological postmortem results for each case were categorised into one of seven categories: presumed or confirmed chlamydiosis, trauma, neoplasia, pneumonia, kidney disease, unknown (no significant lesions), or other (diagnoses with five or fewer koalas per category, including cryptococcosis, cardiac disease, lymphoid depletion, septicaemia, old age, and skin disease). Many koalas were diagnosed with more than one pathology, and in these cases the most significant or severe pathology was used, usually corresponding to the presumed cause of death. As trauma was assumed not to affect formation of intranuclear inclusions or the hepatic pathologies measured in this study, they were included as a “normal” cohort. Therefore, where trauma was the likely cause of death, but a significant additional, more chronic, pathology was present, the additional pathology was used, such that animals in the trauma category were free of any other significant structural disease on gross and histopathologic examination.

The presence or absence of inclusion bodies was reviewed for every case by scanning each liver section in entirety at 100x magnification to identify regions possibly containing inclusion bodies, then further examining ten high-powered (400x) fields (equivalent to a field of view of 2.37mm^2); five centred on a portal tract and five centred on a central vein.

2.2 Histopathological grading

To investigate association of inclusion bodies with liver pathology, a standardised grading scheme was developed and applied to each case (Table 1). The average number of inclusions was calculated for five high-powered (400x) fields centred on a portal tract, five centred on a central vein, and then overall across all ten of the high-powered fields. Brown pigment in the hepatocyte cytoplasm was graded on a scale of 0 - 3 by comparison with image standards of each grade, developed in this study (Appendix 1). Periportal fibrosis and necrosis/inflammation were graded based on an adapted Batts-Ludwig system (Batts and

Ludwig, 1995), which is used for chronic hepatitis in people. Examples of each grade, as used in the current study, are presented in Appendix 1. Binucleated hepatocytes were counted and recorded as a proportion of 100 hepatocytes, ensuring that both periportal and centrilobular hepatocytes were included in the count by counting in one centrilobular field and one periportal field for each case. Anisokaryosis was evaluated *via* nuclear diameter measurements, performed using cellSens Entry microscopy software (version 4.3.1, Olympus, Australia) and reported as a ratio of the largest nuclear diameter to the smallest. Measurements were performed in the field with the most variation in nuclear size of the total ten high-powered fields examined, at a magnification of 400x. The diameter of the largest nucleus in this field (in µm) was divided by the diameter of the smallest nucleus (in µm) to provide the ratio. The presence or absence of hepatocellular vacuolation of any type (without differentiating lipid, glycogen, or hydropic) was recorded as a binary variable, as was the presence of neoplasia of any type within the liver section. Limitations of the use of retrospective tissue collection for histopathology are addressed in the Discussion. The histopathological grading scheme is summarised below in Table 1.

Table 1: Histopathological grading scheme developed for use in the current study.

Factor	Grades	Description
Brown pigment	0	Eosinophilic cytoplasm, no identifiable pigment.
	1	Mild pigment is <50% of hepatocytes.
	2	Moderate pigment in 50-90% of hepatocytes.
	3	Strong pigment in >90% of hepatocytes.
Fibrosis stage	0	No periportal fibrosis.
	1	Mild periportal fibrosis.
	2	Moderate periportal fibrosis.
	3	Severe periportal fibrosis.
Necrosis/inflammation grade	0	No necrosis or inflammation.
	1	Minimal/patchy necrosis or inflammation.
	2	Mild necrosis or inflammation (some or all portal tracts, scattered necrosis).
	3	Moderate necrosis or inflammation (all portal tracts, necrotic clusters).
	4	Severe, prominent inflammation and/or diffuse hepatocellular damage.
Binucleates	Continuous	Counted as a proportion of 100 hepatocytes across one centrilobular field and one periportal field.
Hepatocellular vacuolation	No	No hepatocellular vacuolation.
	Yes	Presence of hepatocellular vacuolation of any type (lipid, glycogen, or hydropic).
Anisokaryosis	Continuous	Ratio of smallest to largest nuclear diameter.
Hepatic neoplasia	No	No neoplasia in sections available.
	Yes	Neoplasia of any type present in sections available.

2.3 Detection of herpesviral DNA

Both liver and spleen were tested for PhaHV-1 and PhaHV-2, where available. The desired sample size for detection of herpesvirus was calculated using an online calculator (Epitools, https://epitools.ausvet.com.au/), with a total population size of 42 (the number of available koala records with inclusion bodies), an assumed test sensitivity of 80%, desired sensitivity for detection of 95%, and an expected prevalence of 30% (using the estimated prevalence of PhaHV-1 in the general koala population (Stalder et al., 2015, Wright et al., 2024)). Given these assumptions, this resulted in a desired sample size of 11 to ensure detection of at least one positive case, and so 14 retrospective cases were tested. Following prospective sample collection, all available frozen tissues (including those with and without inclusion bodies on histopathology, total 34 koalas) were also subjected to qPCR for PhaHV-1 and PhaHV-2.

Prior to sectioning and between each sample, the FFPE tissue blocks, microtome, glass slides, and heating block were cleaned with 70% ethanol. Multiple 8μm thick sections were cut from FFPE tissue blocks containing liver and spleen and placed on a glass slide, which was then placed on a heating block to melt the paraffin wax allowing the tissue of interest to be collected into a sterile 1.5mL Eppendorf tube. DNA was then extracted using a commercial kit (Qiagen DNeasy Blood and Tissue Kit) according to the manufacturer's instructions, with the modification of omitting the xylene pre-treatment on the basis of a small validation study indicating negligible reduction in DNA yield (see Appendix 2). The same kit and protocol were used for the fresh liver and spleen samples. Extracted DNA was suspended in 100μL elution buffer and stored at -20°C until use (up to 6 months). Each extraction was carried out alongside a negative control of PBS only.

The extracted DNA was subjected to qPCR for PhaHV-1 and PhaHV-2, performed using the primers and conditions described in Wright et al. (2023) and Church et al. (2025) (summarised below). Briefly, 20μL reactions included forward and reverse primers at a concentration of 250nM (Table 2), 10μL 2x SYBR Green Supermix (Bio-Rad, Australia), 7.5μL PCR-grade water, and 2μL of the DNA template. The koala gene coding for beta-actin was used as a control for the presence of sample DNA of sufficient quality. Samples testing negative for beta-actin on the first run were re-tested at a 1:10 dilution with PCR-grade water to dilute any inhibitors and, if still negative, were excluded from analysis on the basis of insufficient DNA quality. Fresh liver and spleen samples were all run at a 1:10 dilution due to the large quantity of DNA present. Samples were run in duplicate, and each run included positive controls for beta-actin, PhaHV-1, and PhaHV-2 (synthetic controls constructed using pMG-Amp vector containing the target region), as well as a no template control (PCR-grade water). Initial denaturation was performed at 98°C for 3 minutes, followed by 40 cycles of denaturation (98°C, 10 sec), and annealing/extension (57°C, 20 sec), with a final melt curve of 57-95°C at 0.5°C increments of two seconds each.

Table 2: Details of primers used in herpesviral qPCR reactions. Beta-actin was included as a control housekeeping gene for the presence of adequate DNA in the sample.

Primer	Sequence 5'-3'	Length (bp)	Target fragment length (bp)	Reference
PhaHV-1_fwd	GGGAAGAACTATGTTGGAACGC	22	103	(Wright et al., 2023)
PhaHV-1_rev	TGAGTCCTTTTCGCTTGGGA	20		
PhaHV-2_fwd	GGTGACGTGCAATTCAGTGT	20	87	(Church et al., 2025)
PhaHV-2_rev	TTTCGAGCATCATGCGTCCT	20		
Beta-actin_fwd	CTCAGATTATGTTTGAGACCTTC	23	144	(Hulse et al., 2018)
Beta-actin_rev	CCTTCATAGATGGGCACA	18		

For quantification of herpesviral DNA, the delta Ct method was used to compare relative abundance of herpesviral DNA to the control reference gene beta-actin, as has been previously described (Livak and Schmittgen, 2001). This was done to normalise results, accounting for variations in sample DNA yield and quality. Limitations and assumptions of the delta Ct method are addressed in the Discussion.

2.4 Statistical analysis

Case data was recorded in a spreadsheet (Excel) and epidemiologic variables as well as PhaHV-1 and PhaHV-2 status were analysed using descriptive statistics and Chi-squared analyses (https://www.socscistatistics.com) with a significance level of 95%. All other statistical analyses were conducted in Jamovi (2.3.28), with a p value <0.05 considered significant. Fisher's exact test was used in place of Chi-squared to investigate differences between age categories due to the presence of categories with zero counts. The association between the outcome (presence of inclusions) and cause of death, as well as pathologic variables, was assessed using logistic regression with an underlying binomial distribution. For pathologic variables, univariable analyses were conducted and any variables with a *p* value <0.25 were considered for inclusion in the multivariable model. A stepwise backwards elimination was performed until all terms in the final model were significant. Post-hoc Tukey's tests were performed to determine pairwise differences.

Chapter 3
Results

3.1 Summary statistics

Records of koalas with sufficient data for inclusion in the study (n = 221) were identified from 2000 to 2024, inclusive of retrospective cases from 2000-2023 and prospectively collected cases in 2024 (supplementary data). Of these, 50 were from 2001 due to a research project being conducted in that year (Figure 2). Koalas were mainly submitted from NSW (160) and Qld (66), with the remainder from Victoria (3) and Hong Kong (1), and the submitter of one koala was unknown. Most of the koalas submitted were from one of four organisations across NSW and Qld: Koala Conservation Hospital, Port Macquarie NSW (PMKH; 81 koalas), Moggill Koala Rehabilitation Centre Qld (Moggill; 40 koalas), Port Stephens Koala Hospital NSW (PSKH; 27 koalas), and Australia Zoo Qld (AZ; 24 koalas) (Figure 3). Overall, 69/221 (31.2%) koalas had inclusions. Fifteen (6.8%) were juvenile, 144 (65.2%) were adult, 57 (25.8%) were aged, and 5 (2.3%) were of unknown age. One hundred and thirty-five (61.1%) were female and 86 (38.9%) were male. Twenty-seven (12.2%) were captive, 190 (86.0%) were wild, and 4 (1.8%) were of unknown captive/wild status. The most common major concurrent pathology diagnosed was presumed chlamydiosis, with 92/221 (41.6%) of koalas either diagnosed definitively or suspected of having the disease based on gross and/or histopathologic lesions. The next most common major pathologies were trauma (23/221, 10.4%), neoplasia (19/221, 8.6%), pneumonia (18/221, 8.1%), and other (38/221, 17.2%). Summary statistics are presented in Table 3, below.

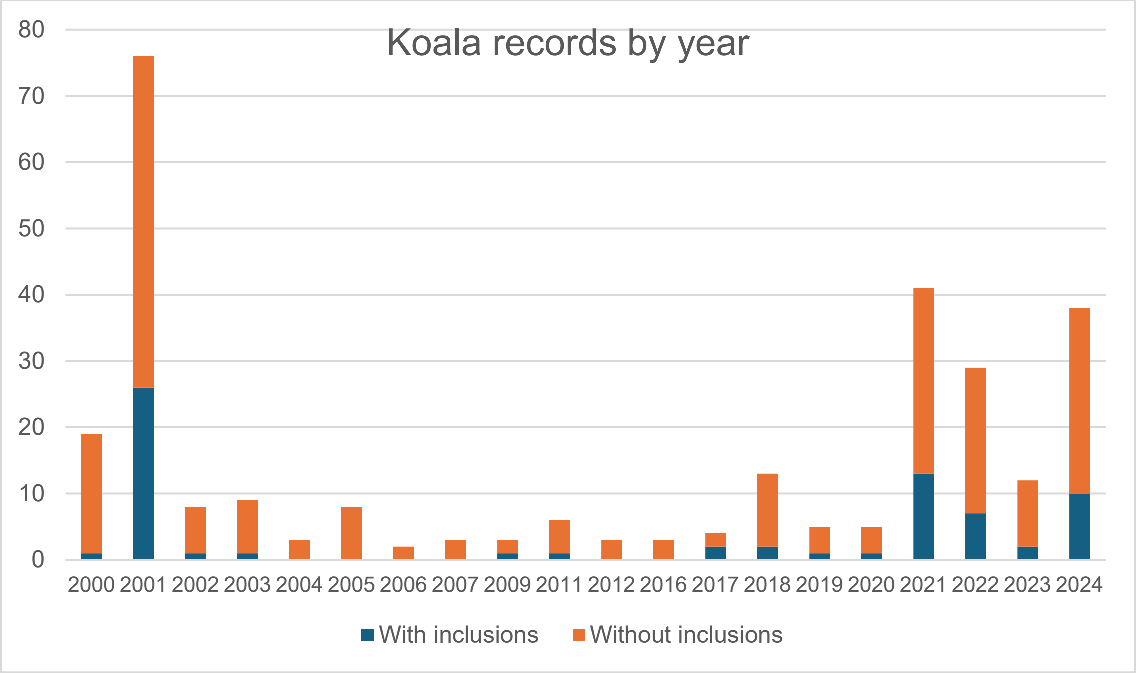

Figure 2: Koala records included in the study by year. Note the targeted sampling effort in 2001, when most of the Moggill (Qld) and Port Macquarie (NSW) data was collected.

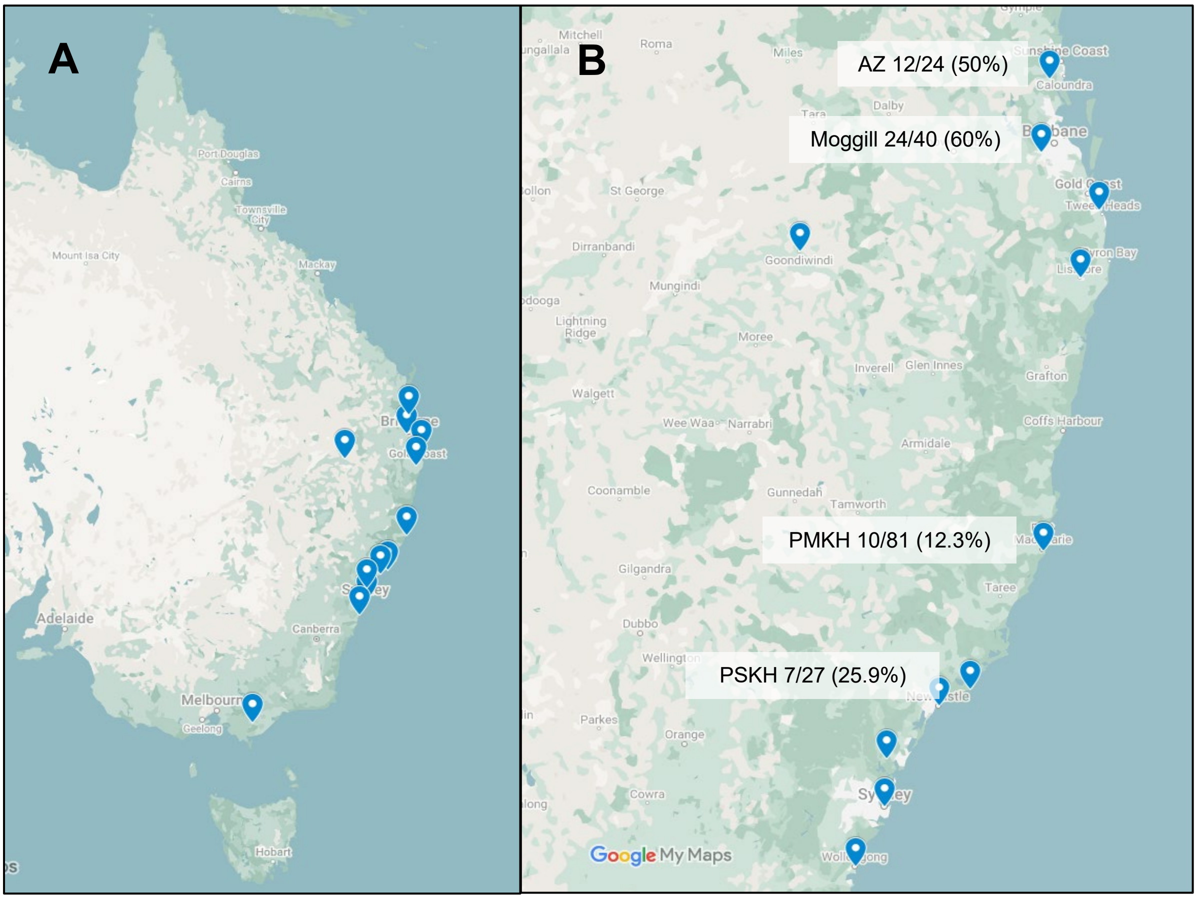

Figure 3: A – Location of submitters of koala postmortems across eastern Australia. B – Detailed map of submissions from northern NSW and southern Qld. The number of koalas with inclusions as a percentage of total koalas submitted from each organisation is indicated. AZ = Australia Zoo, Moggill = Moggill Koala Rehabilitation Centre, PMKH = Port Macquarie Koala Hospital, PSKH = Port Stephens Koala Hospital.

Table 3: Results of summary statistics. The total column includes the low numbers of koalas from Victoria, Hong Kong, and unknown locations.

Factor		NSW	Qld	Total
Age	Juvenile	12 (8.0%)	0 (0%)	15 (6.8%)
	Adult	85 (56.6%)	57 (86.4%)	144 (65.2%)
	Aged	48 (32.0%)	9 (13.6%)	57 (25.8%)
	Unknown	5 (3.3%)	0 (0%)	5 (2.3%)
Sex	Male	60 (40.0%)	24 (36.4%)	86 (38.9%)
	Female	90 (60.0%)	42 (63.6%)	135 (61.1%)
Captive/wild	Captive	24 (16.0%)	1 (1.5%)	27 (12.2%)
	Wild	122 (81.3%)	65 (98.5%)	190 (86.0%)
	Unknown	4 (2.7%)	0 (0%)	4 (1.8%)
Major pathology	Trauma	17 (11.3%)	5 (7.6%)	23 (10.4%)
	Presumed chlamydiosis	44 (29.3%)	47 (71.2%)	92 (41.6%)
	Neoplasia	14 (9.3%)	5 (7.6%)	19 (8.6%)
	Pneumonia	16 (10.7%)	0 (0%)	18 (8.1%)
	Kidney disease	14 (9.3%)	0 (0%)	15 (6.8%)
	Other	35 (23.3%)	3 (4.5%)	38 (17.2%)
	Unknown	10 (6.7%)	6 (9.1%)	16 (7.2%)
Totals		150	66	221

3.2 Epidemiology

Prevalence of inclusions did not differ between female (41/135; 30.4%) and male (28/86; 32.6%) koalas ($p = 0.732$). A significant difference between age categories was detected via Fisher's exact test ($p = 0.003$), with post-hoc analysis revealing a significant difference between juvenile (0/15, 0%) and adult (45/144, 31.3%) koalas ($p = 0.0065$). Prevalence of inclusions did not differ between adult and aged (24/57; 42.1%) koalas ($p = 0.187$) (Figure 4). Prevalence of inclusions did not differ between captive (5/27, 18.5%) and wild (64/190, 33.7%) koalas ($p = 0.113$). There was a significant difference in the prevalence of inclusions between the four major koala postmortem submitters on 4x2 Chi square analysis ($p < 0.00001$). Post-hoc analysis showed that there was no significant difference between the two Qld sites ($p = 0.435$) or the two NSW sites ($p = 0.093$), and therefore these categories were combined to state level for final analysis. Overall Qld koalas in the study were 4.25 times more likely (95% CI 2.29 – 7.91) than those from NSW to have inclusions ($p < 0.001$). Given the difference in age distribution between NSW and Qld (12 juveniles in NSW and none in Qld), juveniles were removed from the model and significance was maintained ($p < 0.001$). Koalas from Vic, Hong

Kong, and an unknown submitter were not included in statistical analysis due to low case numbers. A summary of epidemiological findings and their statistical significance is presented in Table 4.

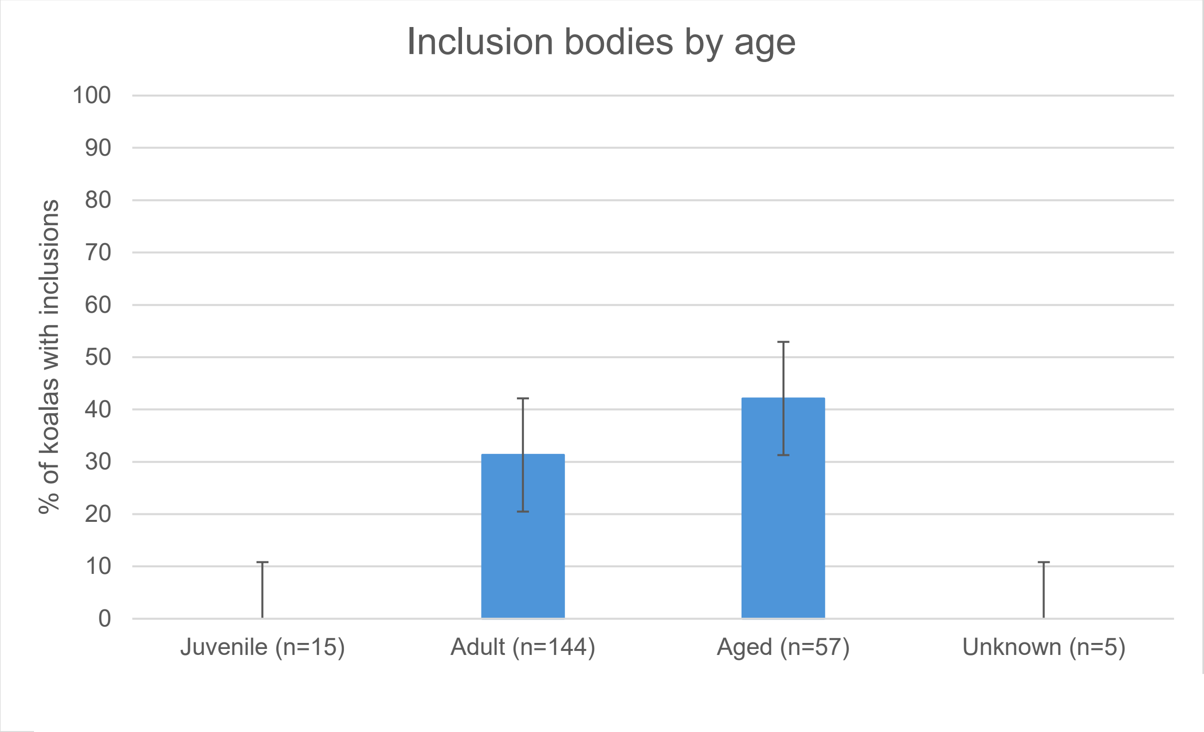

Figure 4: Percentage of koalas in each age category with inclusion bodies. Juveniles were less likely to have inclusion bodies than adult koalas (p = 0.0065). No statistically significant difference was found between adult and aged koalas (p = 0.187).

Table 4: Epidemiological variables and their association with the presence of hepatocellular intranuclear inclusions. p-values are given as the result of Chi-square analysis, excluding age category where Fisher's exact test was used due to the 0 counts, at 95% confidence. No difference was detected for sex or captive/wild status. Significant differences were present between age categories and location.

Demographic		**Prevalence of inclusions**
Sex (*p* = 0.732)	Female	41/135 (30.4%)
	Male	28/86 (32.6%)
Captive/wild (*p* = 0.113)	Captive	5/27 (18.5%)
	Wild	64/190 (33.7%)
Age (*p* = 0.003)	Juvenile	0/15 (0%)
	Adult	45/144 (31.3%)
	Aged	24/57 (42.1%)
	Unknown	0/5 (0%)
Location (*p* <0.001)	Qld	36/66 (55%)
	NSW	33/150 (22%)

Koalas with presumed chlamydiosis were 4.27 (95% CI 1.19 – 15.47) times more likely to have inclusions than koalas dying from trauma (*p* = 0.026), and koalas with neoplasia were 4.85 (95% CI 1.06 – 22.11) times more likely to have inclusions than those dying from trauma (*p* = 0.041) (Table 5).

Table 5: Association of the presence of inclusions with most significant pathology. Koalas with presumed chlamydiosis and neoplasia (bold) were both more likely to have inclusions compared to those animals dying from trauma.

Major pathology/cause of death	Total number of koalas	Number with inclusions (percentage)	Odds ratio (95% confidence interval)	*p*-value
Trauma[a]	23	3 (13.0%)	1.00	
Presumed chlamydiosis	**92**	**36 (39.1%)**	**4.27 (1.19 – 15.47)**	**0.026**
Neoplasia	**19**	**8 (42.1%)**	**4.85 (1.06 – 22.11)**	**0.041**
Pneumonia	18	4 (22.2%)	1.91 (0.368 – 9.87)	0.443
Kidney disease	15	2 (13.3%)	1.03 (0.15 – 7.00)	0.979
Other	38	12 (31.6%)	3.08 (0.76 – 12.39)	0.114
Unknown	16	4 (25.0%)	2.22 (0.43 – 11.68)	0.346

[a] *Reference level*

3.3 Histopathologic analysis

At the univariable analysis level, fibrosis stage (*p* = 0.661), binucleates (*p* = 0.365), and hepatocellular vacuolation (*p* = 0.744) were not associated with the presence of inclusions. All other variables (p < 0.25) were considered for inclusion in the multivariable model. Hepatic neoplasia (distinct from neoplasia as a cause of death) was removed from the model due to the low number of koalas in this category with inclusions (n = 1). After removal of each variable that was not significant in the multivariable analysis, only anisokaryosis remained significant (Table 6). For every unit increase in the anisokaryosis ratio, koalas were 29.79 (95% CI 7.96 – 111.44) times more likely to have inclusions (*p* <.001). The distribution of anisokaryosis data is presented in Figure 5.

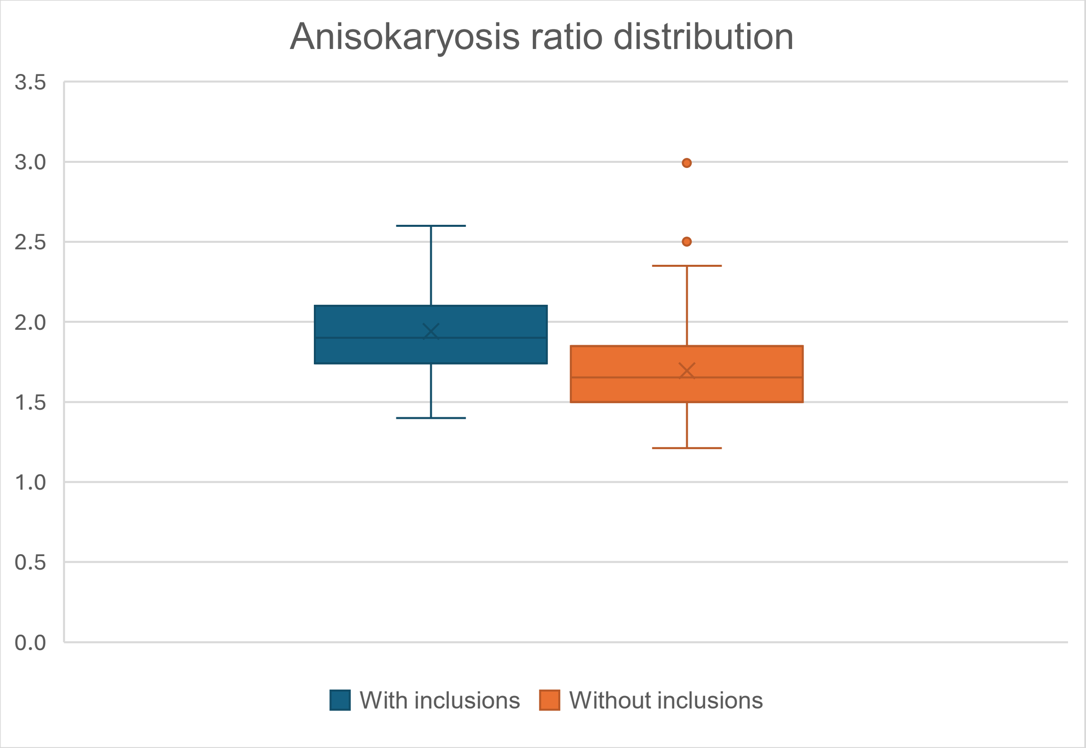

Figure 5: Distribution of anisokaryosis ratio results for koalas with and without inclusions. Note increased anisokaryosis ratio in koalas with inclusion bodies. Anisokaryosis calculated as a ratio of the largest to smallest hepatocellular nuclear diameter.

Table 6: Association of the presence of inclusions with pathological variables in multivariable analysis. Koalas with increasing anisokaryosis (bold) were more likely to have inclusions. p-values are reported as the result of univariable analysis (fibrosis stage, binucleates, hepatocellular vacuolation, anisokaryosis), or multivariable analysis (brown pigment, necroinflammation grade). Hepatic neoplasia was removed from the model due to the low number of koalas with inclusions (n = 1).

Factor	Levels	Total number of koalas	Number with inclusions (percentage)	Odds ratio (95% confidence interval)	*p*-value
Brown pigment	0[a]	21	1 (4.8%)		0.061
	1	64	21 (32.8%)		
	2	95	35 (36.8%)		
	3	41	12 (29.3%)		
Fibrosis stage	0[a]	107	31 (29.0%)		0.661
	1	80	26 (32.5%)		
	2	29	11 (37.9%)		
	3	2	1 (50%)		
Necrosis/inflammation grade	0[a]	143	51 (35.7%)		0.437
	1	39	10 (25.6%)		
	2	17	3 (17.6%)		
	3	18	5 (27.8%)		
	4	1	0 (0%)		
Binucleates	Continuous				0.365
Hepatocellular vacuolation	No[a]	193	61 (31.6%)		0.744
	Yes	28	8 (28.6%)		
Anisokaryosis	**Continuous**			**29.79 (7.96 – 111.44)**	**<0.001**
Hepatic neoplasia	No[a]	213	68 (31.9%)		
	Yes	8	1 (12.5%)		N/A

[a]*Reference level for odds ratios*

3.4 qPCR analysis on FFPE tissues

FFPE samples from 14 koalas with inclusion bodies were tested for beta-actin, PhaHV-1, and PhaHV-2, with 14 liver samples and 12 spleen samples available for analysis. Archived FFPE blocks were prepared between 2001 and 2022 (storage time 1-22 years). Three of fourteen liver samples and 3/12 spleen samples did not amplify beta-actin on initial testing and were excluded from analysis. Beta-actin Ct values of the remaining samples ranged from 27-38. Two of the nine spleen samples amplified a very low level of PhaHV-1 DNA (Ct 37-38), although in both samples this result was not repeatable between duplicates. Eleven liver samples and seven spleen samples were negative for PhaHV-1.

Samples that failed to amplify beta-actin in liver or spleen were not used subsequently for PhaHV-2 testing, leaving 11 koalas (11 liver samples and 8 spleen samples) that were tested for PhaHV-2. Four of eleven liver samples failed to amplify beta-actin after re-testing and were subsequently excluded. This discrepancy in beta-actin recovery was likely due to the low level of viable DNA present (consistent with high Ct values >35) which was close to the limit of detection of the test. 5/8 spleen samples amplified PhaHV-2 DNA at Ct values ranging from 37-39 and with melt curves in the expected region, but no positive results were repeatable between duplicates. Seven liver samples and three spleen samples were negative for PhaHV-2. Results of qPCR testing on FFPE tissues are presented in Appendix 3, although these results were not further used in analysis due to the low sensitivity of host DNA recovery and low reproducibility of results.

3.5 qPCR analysis on fresh tissues

Fresh liver and spleen samples were available from 11 koalas with inclusions and 23 koalas without inclusions. All samples amplified satisfactory beta-actin levels, indicative of adequate DNA presence and quality (Ct 16.68 – 31.79). For overall herpesviral detection, cases were considered positive if viral DNA was present in the liver, spleen, or at both sites. Most positive cases were positive at both sites (19/22, 86% of PhaHV-1 cases, 6/7, 86% of PhaHV-2 cases).

There was no significant difference in PhaHV-1 detection between koalas with inclusions (9/11, 81.8%) and without inclusions (13/23, 56.5%) (*p* = 0.149). Similarly, there was no significant difference in PhaHV-2 detection between koalas with inclusions (1/11, 9.1%) and without inclusions (6/23, 26.1%) (*p* = 0.252). Results of qPCR testing of fresh tissues are presented in table 7 below with detailed results available in Appendix 3.

Table 7: PhaHV-1 and PhaHV-2 qPCR results on fresh liver and spleen samples of koalas with and without inclusion bodies. Cases considered positive were positive in either liver, spleen, or both sites. No significant difference in herpesviral detection was present between koalas with and without inclusions.

		Number of cases positive (percentage)	***p*-value**
PhaHV-1	With inclusions	9/11 (81.8%)	0.149
	Without inclusions	13/23 (56.5%)	
PhaHV-2	With inclusions	1/11 (9.1%)	0.252
	Without inclusions	6/23 (26.1%)	

In PhaHV-1 and PhaHV-2 positive koalas, there was also no association between viral abundance in the liver, as measured by delta Ct, and the number of inclusion bodies present (Figure 6).

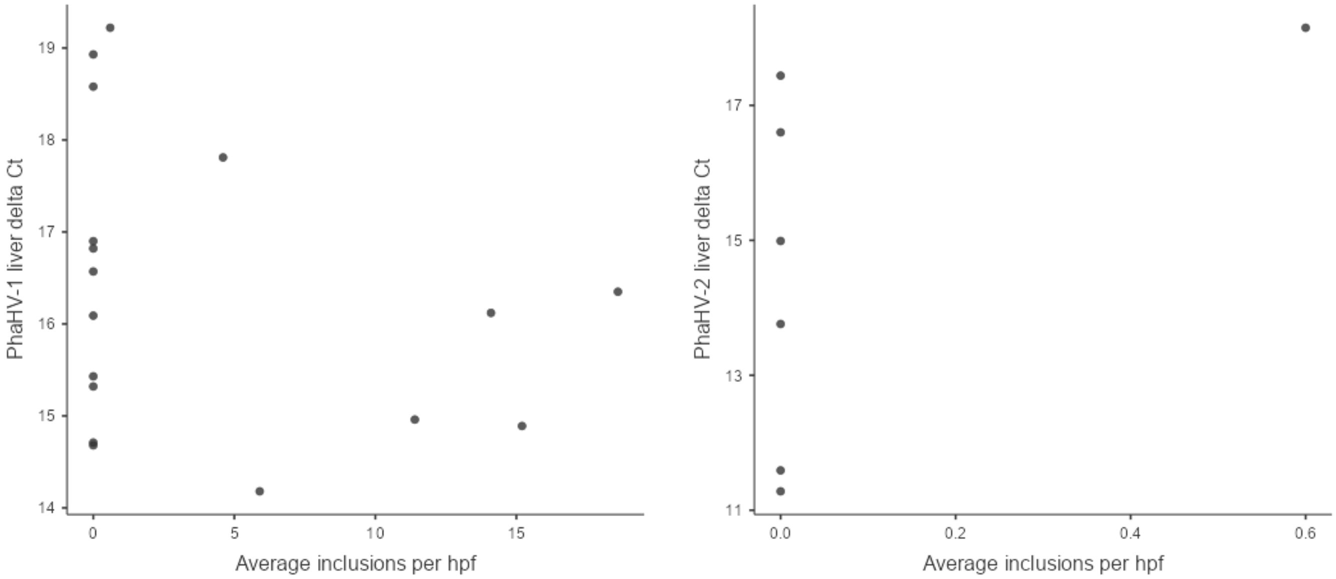

Figure 6: Scatterplots showing no association between PhaHV-1 (left) and PhaHV-2 (right) abundance (measured by delta Ct) in the liver and average number of inclusions per high-powered field (hpf).

Chapter 4
Discussion

This investigation began with the broad approach of characterising the epidemiologic features of koalas with hepatocellular inclusion bodies. The most significant findings were that the Queensland koalas sampled were more likely than those from NSW to have inclusions and that koalas with suspected or diagnosed chlamydiosis or neoplasia were both more likely to have inclusions than koalas dying with trauma alone. Although prevalence of inclusions did not differ between adult and aged koalas, none of the juveniles in the study had inclusions. Histopathological analysis revealed that koalas with inclusions were more likely to have higher anisokaryosis ratios. There was no association with other pathologies such as necrosis, inflammation, or fibrosis. Of the possible aetiologies for these inclusion bodies, gammaherpesvirus infection was considered a possibility, and two gammaherpesviruses (PhaHV-1 and PhaHV-2) were tested for by qPCR. qPCR testing of FFPE tissues was mostly unsuccessful due to poor host DNA recovery, but qPCR testing of fresh liver and spleen samples was successful and indicated no significant difference in PhaHV-1 or PhaHV-2 detection between koalas with and without inclusions, and we therefore consider these herpesviruses are unlikely to play a direct role in the pathogenesis of the inclusions. Other potential aetiologies remain as directions for future research.

Epidemiology

Investigation of epidemiological factors related to the presence of inclusion bodies in koala hepatocytes revealed that the koalas sampled from Queensland were much more likely to have inclusion bodies than those from New South Wales, with rates of up to 60% detected in the Moggill Koala Hospital cohort compared to as low as 12.3% in koalas from the Port Macquarie region. Too few koalas from Victoria or other locations were included in the study to further investigate this potential difference in distribution along the coast of Australia. The reason for this difference was unclear. Temporal clustering appears unlikely given the majority of Qld samples were collected with similar prevalence as far apart as 2001 (Moggill) and 2024 (Australia Zoo). The Qld koalas had higher rates of chlamydiosis (71.2% compared to 29.3% in the NSW koalas), and this could be contributing to observed higher rates of inclusion bodies in this population (see further discussion on chronic disease below). There were also no juveniles in the Qld sample cohort compared to 12 in NSW; however, significance was maintained (*p* <0.001) after removal of the juveniles from the model and therefore an age bias was not a significant factor in this difference.

The fact that none of the juveniles in the study had inclusions, with a significant difference detected between juveniles and adults (*p* = 0.0065), may suggest that there is a minimum period of time for inclusion bodies to develop, but the fact that no significant difference was detected between the adult and aged categories suggests that they do not continue to become more common after adulthood. However, the sample size of juveniles was small (n=15) and therefore it is possible that inclusions do occur in juveniles but at a lower rate than adults or aged koalas. This analysis was limited by the fact that detailed age data (i.e. tooth wear scores or estimated ages) were frequently unavailable in the retrospective records, and thus further accurate stratification was impossible resulting in most koalas falling into a broad ‘adult’ age category.

The significantly higher presence of inclusions in those animals dying of presumed chlamydiosis or neoplasia compared to those dying of trauma (*p* = 0.026 and *p* = 0.041, respectively) could be associated with the greater rates of chlamydiosis in the Qld population compared to the NSW koalas, although the rates of neoplasia were comparable in both populations (7.6% in Qld and 9.3% in NSW). Demographic biases were considered for their potential to play a role in this difference as koalas suffering from trauma have been found in some studies to be younger and more likely to be male, due to increased dispersal during breeding periods (Burton and Tribe, 2016), in comparison to koalas with chlamydiosis or neoplasia which are more likely to be older. However, in this dataset there was no significant difference in age category or sex between koalas dying from trauma and those with chlamydiosis or neoplasia, or in the presence of inclusions, and so the effect of potential demographic bias on the results is expected to not be significant. Trauma was selected as the closest available category to a control group as, given its peracute nature, trauma was considered unlikely to influence formation of the inclusions. Although koalas with significant underlying illness diagnosed on gross and/or histopathological examination were excluded from the trauma category, the possibility of non-structural (and thus undiagnosed) underlying illness contributing to a greater likelihood of suffering trauma was not considered which is another limitation of this analysis.

An association of inclusions with chlamydiosis and neoplasia, if truly present, may reflect general debilitation of the animal, associated with the presence of chronic disease. Other, less chronic, causes of death such as pneumonia, GI disease, cardiac disease, and septicaemia were not associated with inclusions. The link between chronic disease or debilitation and the presence of inclusions is not studied but hepatocellular regeneration can be stimulated by a variety of mitogenic signalling factors, including growth factors (particularly hepatocellular growth factor [HGF] and epidermal growth factor [EGF]), inflammatory cytokines such as interleukin-6 (IL-6) and TNFα, and hormones such as insulin (Michalopoulos and Bhushan, 2021). Increased TNFα expression has been reported in association with chlamydial infection in koalas (Mathew et al., 2013), which could provide a potential explanation for increased hepatocellular turnover with chlamydiosis, and similar inflammatory cytokine profiles may also be associated with neoplasia (Lan et al., 2021). Intranuclear inclusions have been detected in the hepatocytes of people with hepatocellular carcinoma and found to contain accumulations of ß-catenin (Schwertheim et al., 2020a). Activation of the ß-catenin-Wnt pathway is involved in both liver regeneration and development of neoplasia (Xu et al., 2022), and therefore activation of this pathway or others involved in regeneration may lead to accumulations of host proteins within the nucleus. Histopathology findings of the current study support association between hepatocellular turnover and presence of inclusion bodies.

Histopathology

A link between increased hepatocellular turnover and the presence of inclusion bodies is supported by the strong association between presence of inclusions and increasing anisokaryosis (*p* <0.001). Anisokaryosis can be a normal finding in the liver and varies between species, being more common in humans and rodents than in most domestic animals. In people and rodents anisokaryosis often reflects tetraploidy or other degrees of polyploidy (Cullen and Stalker, 2016), and thus can be associated with hepatocellular regeneration (Miyaoka and Miyajima, 2013). Anecdotally, an increased level of anisokaryosis is commonly identified in koala liver specimens, both with and without inclusion bodies, compared to other domestic animals. This may be due to the increased metabolic workload of the koala liver, associated with hepatic metabolism of dietary eucalypt toxins (Jones et al., 2008). Thus, an increase in anisokaryosis in koalas with inclusions may be associated with a debilitated state and/or an increased toxin load from the diet increasing hepatocellular regeneration and therefore polyploidy.

None of the other pathological variables investigated, including brown pigment, fibrosis, necroinflammation, binucleate count, hepatocellular vacuolation, or hepatic neoplasia, were associated with inclusions. This suggests that the presence of inclusions is not directly associated with hepatocellular injury. Alphaherpesviruses, such as PhaHV-3, are usually associated with lytic reproduction in epithelial cells, and thus hepatic necrosis and resultant inflammation would be expected, as reported in the single published case (Bowater et al., 2022). In contrast, gammaherpesviruses such as PhaHV-1 and PhaHV-2 are often not associated with significant necrosis or inflammation in infected tissues (Ackermann, 2006), and therefore despite the lack of significant pathology in affected livers, gammaherpesvirus infection was still considered a possible aetiology and qPCR for these viruses was pursued.

While it was not feasible for this investigation to validate the pathologic grading scheme for reproducibility and agreement between different pathologists (Avallone et al., 2021), we implemented measures to maximise its validity. For variables that required a count, such as binucleates, the counting method was standardised and representation maximised by counting in both portal and centrilobular regions. However, the number of binucleates could vary to an unknown extent in different sections and different lobes of the liver and this may explain why binucleates, which would be expected to be present in greater numbers with increased hepatocellular turnover, were not increased along with inclusions and anisokaryosis. This was a limitation of utilising retrospective tissues for examination, as collection and processing of tissues was not standardised, and therefore the size and number of sections collected and location within the liver (i.e. lobes) varied between cases.

Necrosis and inflammation were combined as ‘necrosis/inflammation’ based on the human grading scheme used for chronic hepatitis in people (Batts and Ludwig, 1995) and to simplify grading. However, this did result in the aggregation of different presentations such as acute random necrosis due to septicaemia, acute centrilobular necrosis due to hypoxia, and chronic portal hepatitis, all of which have very different pathogenic mechanisms. To further stratify these different presentations was beyond the scope of the current study, but it is possible that an association with one of these presentations may have been present (e.g. chronic but not acute inflammation) and remained undetected due to the combined category.

Formalin-fixed paraffin embedded qPCR

The finding that 3/14 (21%) FFPE liver samples and 3/12 (25%) spleen samples did not contain sufficient beta-actin DNA for detection predicts a very low sensitivity of herpesviral detection in this sample type, which was the case. Of the samples that did test positive, Ct values ranged from 37-39 and no positive results were replicated between both duplicates. The limit of detection for the PhaHV-1 qPCR assay has been previously determined as 12 copies per reaction, correlating to a Ct value of 37.5 (Wright et al., 2023). This is likely to provide an explanation for the lack of repeatability between duplicates within runs and between different runs, as the levels of DNA present for both beta-actin and herpesviruses were very close to the limit of detection of the assay. At such low levels, there may be several false negative results in other samples, including the liver samples, which does not allow any definite conclusions about the presence or absence of herpesviral DNA to be drawn from the FFPE samples and hence these results were not used further in analysis.

Formalin fixation can affect the sensitivity of PCR reactions in several ways. Formalin fixation creates DNA-protein crosslinks, which inhibit the PCR reaction and increase sensitivity of DNA to mechanical stress. This then increases levels of fragmentation and reduces the ability of enzymes to access the strands (Dietrich et al., 2013). Oxidization of formalin to formic acid, can also cause DNA strand breaks as well as depurination (the loss of purine bases adenine and guanine). The severity of DNA degradation is affected by many factors including the length of fixation, type of fixative used (including formalin concentration and pH), and temperature (Lenze et al., 2012). These variables result in a diverse range of sample quality amongst FFPE tissues. The length of formalin fixation time was unknown for these historical cases, but samples taken postmortem are often fixed for a longer period than biopsy submissions from live patients, given the lack of urgency for results. This may have caused more severe DNA degradation than is reported in many studies which frequently utilise FFPE biopsy specimens (Dupont et al., 2023, Kokkat et al., 2013).

DNA fragmentation can result in a lack of amplification of the target fragment, particularly for amplicons over 250-300bp in length (Dietrich et al., 2013), which are common in conventional PCR. This can be partially overcome by using qPCR assays, which typically amplify shorter targets, and the qPCR assays used in this experiment had short target fragments of 103bp for PhaHV-1, 87bp for PhaHV-2, and 144bp for beta-actin. However, fixation of samples at higher temperatures, in unbuffered formalin, or for a prolonged time can result in DNA fragments of 100bp or less (Lenze et al., 2012), and this was likely a major cause of the lack of beta-actin amplification in many samples. The longer length of the beta-actin target compared to the herpesviral targets means that this was able to act as an appropriate control gene to account for DNA fragmentation.

High levels of PCR inhibitors may also be present in FFPE tissues. The major inhibitors in FFPE tissues are the DNA cross-links caused by formalin-fixation, but generation of random short DNA debris by fragmentation can also directly inhibit DNA polymerase (Dietrich et al., 2013). Samples were diluted with PCR-grade water, which theoretically should dilute the concentration of inhibitors, but limited improvement in DNA amplification was achieved, supporting irreversible cross-links or fragmentation as the cause of low qPCR sensitivity. Samples that were tested were between 1-22 years old, and there was no association between the age of the sample and the likelihood of DNA recovery, again supportive of irreversible cross-links from prolonged formalin fixation as the major factor affecting test sensitivity. The use of fresh frozen tissues overcomes these problems with formalin fixation, optimising sensitivity for molecular diagnostics (Lüder Ripoli et al., 2016). Given the very low levels of herpesviral DNA detected in the few positive spleen samples, this was decided as the optimal next step for investigation.

Fresh tissues qPCR

Prospective recruitment of fresh liver and spleen samples provided a small sample size (11 koalas with inclusions and 23 without). The use of fresh tissues overcame the problem of sensitivity with FFPE tissues, with abundant beta-actin detected in both liver and spleen samples suggesting high sensitivity for detection of both viruses. PhaHV-1 was detected at a much higher rate (56.5 – 81.8%) than PhaHV-2 (9.1 – 26.1%), and both viruses were detected at a higher rate than previous studies have reported on urogenital swabs collected from NSW and Qld (Wright et al., 2024, Church et al., 2025). This is to be expected, given that testing tissue sites rich in lymphocytes detects viral latency, rather than active replication and shedding reflected by detection on swabs (Kasimov et al., 2020).

qPCR analysis of fresh tissues suggested that PhaHV-1 and PhaHV-2 are both unlikely to be direct causes of the inclusion bodies. No significant differences in PhaHV-1 or PhaHV-2 detection were found between koalas with and without inclusions ($p = 0.149$ and $p = 0.252$, respectively). Similarly, there was no association of relative viral abundance (measured by delta Ct) with the number of inclusions in each sample. The delta Ct method is commonly used for relative quantification of qPCR results (Livak and Schmittgen, 2001); however, this method does have limitations. Comparing viral abundance to beta-actin abundance, to approximate pathogen load per quantum of host cells, assumes that the sample comprises only pathogen and host cells, which may not be the case, particularly if the sample includes higher numbers of cells which are not targets for the virus, such as inflammatory cells or connective tissue. The delta Ct method also assumes that the target gene and the reference gene amplify with the same efficiency (typically 100%) and are equally affected by any inhibitors in the sample (Rao et al., 2013). Despite these limitations, delta Ct was considered a useful method for comparison in this experiment as liver is generally a homogenous tissue with limited variation in cell types present.

Alternative hypotheses and future directions

Given the lack of association between PhaHV-1 and PhaHV-2 and the presence of inclusion bodies, these two viruses appear unlikely to be direct causes of the inclusions. Testing for PhaHV-1 and PhaHV-2 was a very targeted and specific approach, based on the information that is currently known about koala herpesviruses including their prevalence and distribution in wild koala populations. These results do not exclude the possibility of a viral cause for the inclusions, with possible alternate hypotheses of as-yet undiscovered viruses such as other herpesviruses, adenoviruses, or parvoviruses. PhaHV-3 was not tested for in this study due to the lack of a validated test and no published information on its distribution in wild koalas. In addition, PhaHV-3 is an alphaherpesvirus and thus was considered less likely to play a role in the pathogenesis of these inclusions due to the absence of an association with necrosis or inflammation which are common features of alphaherpesvirus infections (MacLachlan and Dubovi, 2011). To fully exclude a viral cause, possible future investigations could include broader methods of viral detection such as electron microscopy, RNAseq, or PCR utilising degenerate family-level primers followed by sequencing.

Electron microscopy (EM) is a helpful method to detect viral particles and can often identify the genus of viruses involved in lesions. As well as detecting viral presence, EM is able to localise the viral particles to the lesion of interest and is thus often used when investigating inclusion bodies (Wild, 2008). EM was initially planned for this study but constraints in accessing appropriate sample types and EM facilities meant this was unable to be completed.

In the initial case report of these inclusions (Condron and Forsyth, 1986), EM was performed on the formalin-fixed liver tissue following washing in cacodylate buffer and post-fixation with 1% osmium tetroxide. Ideally tissues for electron microscopy are preserved in glutaraldehyde, rather than formalin (Carson, 2018), although post-fixation can still provide reasonable results on formalin-fixed tissues (Wild, 2008). EM can also be performed on FFPE tissues; however, this is the least ideal method for cell preservation. In these cases of less ideal preservation, viral particles are often the most well-preserved in comparison to cellular organelles, particularly those high in lipids such as the Golgi body (Wild, 2008), and therefore EM could be feasibly pursued on more cases of inclusions to further exclude a viral aetiology.

In most cases, ultrastructural features are sufficient to identify a virus to the family level (Popov et al., 2019). Electron microscopy of herpesviruses generally reveals arrays of virus capsids within the nucleus (corresponding to intranuclear inclusion bodies visible on light microscopy), enveloped capsids in the cytoplasm, and possibly viral particles along the nuclear or plasma membrane which may represent budding events (Shabman et al., 2016, White and Taxy, 1983). The family of viral particles found can be differentiated by size, with herpesviruses measuring 100-120nm diameter, or up to 150-180nm diameter including the envelope (Farris et al., 2018), and shape, with herpesvirus virions having a classic icosahedral appearance (Strnad et al., 2013). However, the morphological appearance does not vary significantly between alpha-, beta-, and gammaherpesviruses, and thus molecular techniques such as PCR and sequencing are required to then definitively identify the virus involved.

The use of degenerate (virus family-level, i.e., not specific to individual viral species) primers for PCR was also considered for this study, particularly to search for as yet unknown herpesviruses. Ultimately it was decided to pursue the more specific approach of qPCR for PhaHV-1 and PhaHV-2 due to multiple factors. Firstly, the prevalence data available for PhaHV-1 in NSW and Qld koalas (Wright et al., 2024) appeared to be similar to the prevalence of inclusion bodies. Secondly, the lack of associated necrosis and inflammation was consistent with a gammaherpesvirus infection. Finally, the short target fragment length of the qPCR assays was anticipated to assist in overcoming the issue of fragmentation due to formalin fixation of the FFPE tissues, although ultimately this was unsuccessful. Previous investigations have used degenerate family-level primers to identify herpesviruses in koala swabs and/or tissues (Vaz et al., 2011, Vaz et al., 2012, Stalder et al., 2015); however, these studies were performed on Victorian koala populations and did not include koalas from NSW or Qld. This method could also be used to detect PhaHV-3, which currently lacks a specific validated test. However, as mentioned previously, an alphaherpesvirus was considered unlikely to be associated with the inclusions due to the lack of hepatic necrosis or inflammation.

Following these broader investigations, if a virus was suspected as a cause of the inclusions, in-situ hybridisation (ISH) could be considered to further localise the virus to the hepatocytes with inclusions. ISH is a technique which requires the use of a specific DNA probe which would bind to the sequence of interest in the tissue, and therefore the specific virus with a known DNA sequence would need to be known in advance in order to design an appropriate probe (Levsky and Singer, 2003).

If viral causes were fully excluded, the remaining alternate hypothesis is that these inclusions are composed of an accumulation of host protein or cellular substance, which would be more difficult to investigate. An alternative option to cast a broader net for investigation of both viral and non-viral causes is the use of RNAseq (RNA sequencing), which uses deep-sequencing technology to measure transcriptomes (Wang et al., 2009), the complete set of transcripts in a cell. This includes mRNAs, non-coding RNAs, and small RNAs, and is able to quantify the expression level of each transcript under various conditions (Wang et al., 2009). Comparison of the transcriptome between koalas with and without inclusions might suggest changes in either viral or host gene expression which could provide further insight into the composition of the inclusions.

Although retroviruses do not directly cause inclusion bodies, a possible association between KoRV infection and the presence of inclusion bodies could be considered on the basis of altered gene expression secondary to viral genome insertions. Insertion site analysis can be performed to identify if viral DNA is integrated in the vicinity of important host genes (McEwen et al., 2021), and this could then be compared with gene expression levels and the histological presence of inclusion bodies.

Finally, genome-wide association studies (GWAS) can be performed to identify associations between genotypes and phenotypes by looking for differences in allele frequency between different individuals (Uffelmann et al., 2021), thus investigating if a genetic difference is involved in why some koalas develop inclusion bodies while others do not. These studies often require very large sample sizes to identify significant associations, and therefore require a significant financial and time investment (Uffelmann et al., 2021) which would be unlikely to be feasible without a clear association between the inclusions and disease or poor health outcomes in koalas.

Conclusion

This study represents the first systematic investigation into the epidemiologic and pathologic features of koala hepatocellular intranuclear inclusions, and the first attempt to evaluate their potential association with phascolarctid herpesvirus infection. Through the combination of histopathology and molecular analysis, this research provides novel insights into a long-recognised but previously unexplained pathological finding in this species.

Epidemiologic investigation revealed for the first time that Queensland koalas were more likely than NSW koalas to have inclusions. In the absence of a clear cause for the inclusions, the reason for the geographical difference in prevalence is unclear. Although there were no differences between age, sex, and captive/wild status, koalas with chronic disease processes, specifically chlamydiosis or neoplasia, were more likely to have inclusions than koalas dying from acute trauma. This suggests that the inclusions may develop secondary to chronic physiological stress, debilitation, or altered metabolic demand, rather than being a true incidental or age-related finding as previously thought. Pathologic investigation showed an association with increased anisokaryosis, supporting this hypothesis and implying heightened hepatocellular turnover, possibly driven by increased demand for detoxification (dietary toxin load) or systemic illness. Importantly, this study provides strong evidence against a direct causal relationship between inclusions and infection with PhaHV-1 or PhaHV-2. Despite their morphological resemblance to herpesviral inclusions described in other species, molecular testing revealed no significant association between the presence of inclusions and herpesviral detection. However, other viral infections cannot be definitively ruled out due to the specificity of the current approach.

The results of this study hold important relevance for koala diagnostics and conservation. From a diagnostic perspective, these results refine the interpretation of postmortem findings by demonstrating a lack of association with herpesviral infection, preventing overinterpretation or misdiagnosis. At the same time, recognising these inclusions as potential markers of systemic stress or altered hepatic metabolism could help pathologists to interpret liver pathology in the context of systemic disease. From a conservation perspective, the work contributes to understanding the complex interplay between chronic disease, physiological stress, and hepatic function in a species already under considerable environmental pressure. Improved diagnostic clarity based on these results will support more accurate health surveillance in both wild and captive koala populations.

Future research should build on these findings through several approaches. To more comprehensively explore potential infectious causes, broader and less target-specific methods of viral detection such as electron microscopy, degenerate family-level PCR and sequencing, or RNA-seq could be pursued. These techniques could detect as-yet-undiscovered viral agents or clarify subtle viral-host interactions not detectable by targeted qPCR. If viral involvement is definitively excluded in the pathogenesis of the inclusions, the focus should shift toward investigating host-derived mechanisms such as accumulation of proteins, nuclear transport disruption, or altered metabolic regulation. This could be achieved via proteomic characterisation of the inclusion contents, ultrastructural analysis, or genome-wide association studies to identify metabolic or genetic predispositions.

Ultimately, the findings of this study highlight the need to integrate ecological, pathological, and molecular data to explore the factors influencing koala health. By further defining the characteristics of the inclusions and the lack of evidence for a PhaHV-1 or PhaHV-2 cause, this research has provided new information on a long-observed pathologic phenomenon and establishes a basis for further investigation into their exact composition.

References

ACKERMANN, M. 2006. Pathogenesis of gammaherpesvirus infections. *Vet Microbiol,* 113**,** 211-22.

ALFANO, N., KOLOKOTRONIS, S. O., TSANGARAS, K., ROCA, A. L., XU, W., EIDEN, M. V. & GREENWOOD, A. D. 2016. Episodic Diversifying Selection Shaped the Genomes of Gibbon Ape Leukemia Virus and Related Gammaretroviruses. *J Virol,* 90**,** 1757-72.

ARORA, S. K. & DEY, P. 2012. Intranuclear peudoinclusions: morphology, pathogenesis, and significance. *Diagn Cytopathol,* 40**,** 741-4.

AUSTRALIAN GOVERNMENT. 2023. *Koala listing under national environmental law* [Online]. Available: https://www.dcceew.gov.au/environment/biodiversity/threatened/species/koalas/listing-under-national-environmental-law [Accessed 15/01/2025].

AVALLONE, G., RASOTTO, R., CHAMBERS, J. K., MILLER, A. D., BEHLING-KELLY, E., MONTI, P., BERLATO, D., VALENTI, P. & ROCCABIANCA, P. 2021. Review of Histological Grading Systems in Veterinary Medicine. *Vet Pathol,* 58**,** 809-828.

BATTS, K. P. & LUDWIG, J. 1995. Chronic hepatitis. An update on terminology and reporting. *Am J Surg Pathol,* 19**,** 1409-17.

BENNETT, M. D., WOOLFORD, L., NICHOLLS, P. K., WARREN, K. S. & O'HARA, A. J. 2008. Hepatic intranuclear glycogen inclusions in western barred bandicoots (Perameles bougainville). *J Vet Diagn Invest,* 20**,** 376-9.

BESTETTI, G. & ZWAHLEN, R. 1985. Generalized parvovirus infection with inclusion-body myocarditis in two kittens. *Journal of Comparative Pathology,* 95**,** 393-397.

BLYTON, M. D. J., PYNE, M., YOUNG, P. & CHAPPELL, K. 2022. Koala retrovirus load and non-A subtypes are associated with secondary disease among wild northern koalas. *PLoS Pathog,* 18**,** e1010513.

BOEHMER, P. E. & NIMONKAR, A. V. 2003. Herpes virus replication. *IUBMB Life,* 55**,** 13-22.

BOWATER, R. O., HORWOOD, P. F., PICARD, J., HUISMAN, I., HAYES, L., MACKIE, T. & TAYLOR, J. D. 2022. A novel alphaherpesvirus and concurrent respiratory cryptococcosis in a captive koala (Phascolarctos cinereus). *Aust Vet J,* 100**,** 329-335.

BURTON, E. & TRIBE, A. 2016. The Rescue and Rehabilitation of Koalas (Phascolarctos cinereus) in Southeast Queensland. *Animals (Basel),* 6.

CAMERON, K., BIZO, L. & STARKEY, N. 2013. Food Preferences of the Brushtail Possum (Trichosurus vulpecula). *International journal of comparative psychology / ISCP; sponsored by the International Society for Comparative Psychology and the University of Calabria,* 26**,** 324-336.

CARSON, F. L. 2018. Formaldehyde as a Fixative for Light and Electron Microscopy. *Microscopy Today,* 8**,** 30-31.

CHAN, J. K. C. 2014. The Wonderful Colors of the Hematoxylin–Eosin Stain in Diagnostic Surgical Pathology. *International Journal of Surgical Pathology,* 22**,** 12-32.

CHAPPELL, K. J., BREALEY, J. C., AMARILLA, A. A., WATTERSON, D., HULSE, L., PALMIERI, C., JOHNSTON, S. D., HOLMES, E. C., MEERS, J. & YOUNG, P. R. 2017. Phylogenetic Diversity of Koala Retrovirus within a Wild Koala Population. *J Virol,* 91.

CHURCH, C., CASTERIANO, A., MUIR, Y. S., KROCKENBERGER, M., 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,* 15**,** 6136.

COHEN, J. I. 2020. Herpesvirus latency. *J Clin Invest,* 130**,** 3361-3369.

CONDRON, R. J. & FORSYTH, W. M. 1986. Hepatic inclusion bodies in the liver of a koala (Phascolarctos cinereus). *Aust Vet J,* 63**,** 387-8.

CULLEN, J. M. & STALKER, M. J. 2016. Chapter 2 - Liver and Biliary System. *In:* MAXIE, M. G. (ed.) *Jubb, Kennedy & Palmer's Pathology of Domestic Animals: Volume 2 (Sixth Edition).* W.B. Saunders.

DECARO, N., MARTELLA, V. & BUONAVOGLIA, C. 2008. Canine adenoviruses and herpesvirus. *Vet Clin North Am Small Anim Pract,* 38**,** 799-814, viii.

DENNER, J. & YOUNG, P. R. 2013. Koala retroviruses: characterization and impact on the life of koalas. *Retrovirology,* 10**,** 108.

DIETRICH, D., UHL, B., SAILER, V., HOLMES, E. E., JUNG, M., MELLER, S. & KRISTIANSEN, G. 2013. Improved PCR Performance Using Template DNA from Formalin-Fixed and Paraffin-Embedded Tissues by Overcoming PCR Inhibition. *PLOS ONE,* 8**,** e77771.

DUPONT, M. E., CHRISTIANSEN, S. N., JACOBSEN, S. B., KAMPMANN, M.-L., OLSEN, K. B., TFELT-HANSEN, J., BANNER, J., MORLING, N. & ANDERSEN, J. D. 2023. DNA quality evaluation of formalin-fixed paraffin-embedded heart tissue for DNA methylation array analysis. *Scientific Reports,* 13**,** 2004.

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. *Journal of Comparative Pathology,* 176**,** 50-66.

FARRIS, A. B., SELIG, M. K. & NIELSEN, G. P. 2018. 5 - Ultrastructural Diagnosis of Infection. *In:* KRADIN, R. L. (ed.) *Diagnostic Pathology of Infectious Disease (Second Edition).* Elsevier.

FINNIE, E. P., LITTLEJOHNS, I. R. & ACLAND, H. M. 1976. Mortalities in Parma Wallabies (Macropus Parma) Associated with Probable Herpesvirus. *Australian Veterinary Journal,* 52**,** 294-294.

GÁL, J., MÁNDOKI, M., SÓS, E., KERTÉSZ, P., KOROKNAI, V., BÁNYAI, K. & FARKAS, S. L. 2017. Novel adenovirus detected in kowari (Dasyuroides byrnei) with pneumonia. *Acta Microbiol Immunol Hung,* 64**,** 81-90.

GIFFORD, R. & TRISTEM, M. 2003. The Evolution, Distribution and Diversity of Endogenous Retroviruses. *Virus Genes,* 26**,** 291-315.

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. *Sci Rep,* 7**,** 42587.

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. *Sci Rep,* 9**,** 17494.

GORYO, M., UEDA, Y., UMEMURA, T., HARUNA, A. & ITAKURA, C. 1988. Inclusion body hepatitis due to adenovirus in pigeons. *Avian Pathol,* 17**,** 391-401.

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. *J Wildl Dis,* 49**,** 18-28.

GRINDE, B. 2013. Herpesviruses: latency and reactivation – viral strategies and host response. *Journal of Oral Microbiology,* 5**,** 22766.

HAILEE, J., MURRAY, M. & CROWTHER, M. 2017. A review of climatic change as a determinant of the viability of koala populations. *Wildlife Research,* 44**,** 458-470.

HARDING, E. F., RUSSO, A. G., YAN, G. J. H., WATERS, P. D. & WHITE, P. A. 2021. Ancient viral integrations in marsupials: a potential antiviral defence. *Virus Evol,* 7**,** veab076.

HARRACH, B., TARJÁN, Z. & BENKŐ, M. 2019. Adenoviruses across the animal kingdom: a walk in the zoo. *FEBS Letters,* 593.

HIGGINS, D., ROSE, K. & SPRATT, D. 2018. Chapter 18 - Monotremes and Marsupials. *In:* TERIO, K. A., MCALOOSE, D. & LEGER, J. S. (eds.) *Pathology of Wildlife and Zoo Animals.* Academic Press.

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.

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**,** 523-529.

IP, Y. T., DIAS FILHO, M. A. & CHAN, J. K. 2010. Nuclear inclusions and pseudoinclusions: friends or foes of the surgical pathologist? *Int J Surg Pathol,* 18**,** 465-81.

JONDLE, C. N. & TARAKANOVA, V. L. 2020. Innate immunity and alpha/gammaherpesviruses: first impressions last a lifetime. *Curr Opin Virol,* 44**,** 81-89.

JONES, B. R., EL-MERHIBI, A., NGO, S. N., STUPANS, I. & MCKINNON, R. A. 2008. Hepatic cytochrome P450 enzymes belonging to the CYP2C subfamily from an Australian marsupial, the koala (Phascolarctos cinereus). *Comp Biochem Physiol C Toxicol Pharmacol,* 148**,** 230-7.

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**,** 15787.

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.

KIMBLE, B., LI, K. M., VALTCHEV, P., HIGGINS, D. P., KROCKENBERGER, M. B. & GOVENDIR, M. 2014. In vitro hepatic microsomal metabolism of meloxicam in koalas (Phascolarctos cinereus), brushtail possums (Trichosurus vulpecula), ringtail possums (Pseudocheirus peregrinus), rats (Rattus norvegicus) and dogs (Canis lupus familiaris). *Comp Biochem Physiol C Toxicol Pharmacol,* 161**,** 7-14.

KOKKAT, T. J., PATEL, M. S., MCGARVEY, D., LIVOLSI, V. A. & BALOCH, Z. W. 2013. Archived formalin-fixed paraffin-embedded (FFPE) blocks: A valuable underexploited resource for extraction of DNA, RNA, and protein. *Biopreserv Biobank,* 11**,** 101-6.

KROCKENBERGER, M. B., CANFIELD, P. J. & MALIK, R. 2003. Cryptococcus neoformans var. gattii in the koala (Phascolarctos cinereus): a review of 43 cases of cryptococcosis. *Med Mycol,* 41**,** 225-34.

KUNY, C. V. & SZPARA, M. L. 2021. Alphaherpesvirus Genomics: Past, Present and Future. *Curr Issues Mol Biol,* 42**,** 41-80.

LAN, T., CHEN, L. & WEI, X. 2021. Inflammatory Cytokines in Cancer: Comprehensive Understanding and Clinical Progress in Gene Therapy. *Cells,* 10.

LANYON, J. & SANSON, G. 2011. Koala (Phascolarctos cinereus) dentition and nutrition. II. Implications of tooth wear in nutrition. *Journal of Zoology,* 209**,** 169-181.

LENZE, D., MÜLLER, H.-H. & HUMMEL, M. 2012. Considerations for the use of formalin-fixed and paraffin-embedded tissue specimens for clonality analysis. *Journal of Hematopathology,* 5**,** 27-34.

LEVSKY, J. M. & SINGER, R. H. 2003. Fluorescence in situ hybridization: past, present and future. *J Cell Sci,* 116**,** 2833-8.

LIVAK, K. J. & SCHMITTGEN, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods,* 25**,** 402-8.

LÜDER RIPOLI, F., MOHR, A., CONRADINE HAMMER, S., WILLENBROCK, S., HEWICKER-TRAUTWEIN, M., HENNECKE, S., MURUA ESCOBAR, H. & NOLTE, I. 2016. A Comparison of Fresh Frozen vs. Formalin-Fixed, Paraffin-Embedded Specimens of Canine Mammary Tumors via Branched-DNA Assay. *Int J Mol Sci,* 17.

MACLACHLAN, N. J. & DUBOVI, E. J. 2011. Chapter 9 - Herpesvirales. *In:* MACLACHLAN, N. J. & DUBOVI, E. J. (eds.) *Fenner's Veterinary Virology (Fourth Edition).* San Diego: Academic Press.

MAGAKI, S., HOJAT, S. A., WEI, B., SO, A. & YONG, W. H. 2019. An Introduction to the Performance of Immunohistochemistry. *Methods Mol Biol,* 1897**,** 289-298.

MARSCHNER, C., KROCKENBERGER, M. B., HIGGINS, D. P., MITCHELL, C. & MOORE, B. D. 2019. Ingestion and Absorption of Eucalypt Monoterpenes in the Specialist Feeder, the Koala (Phascolarctos cinereus). *J Chem Ecol,* 45**,** 798-807.

MATHEW, M., BEAGLEY, K. W., TIMMS, P. & POLKINGHORNE, A. 2013. Preliminary characterisation of tumor necrosis factor alpha and interleukin-10 responses to Chlamydia pecorum infection in the koala (Phascolarctos cinereus). *PLoS One,* 8**,** e59958.

MCEWEN, G. K., ALQUEZAR-PLANAS, D. E., DAYARAM, A., GILLETT, A., TARLINTON, R., MONGAN, N., CHAPPELL, K. J., HENNING, J., TAN, M., TIMMS, P., YOUNG, P. R., ROCA, A. L. & GREENWOOD, A. D. 2021. Retroviral integrations contribute to elevated host cancer rates during germline invasion. *Nat Commun,* 12**,** 1316.

MCINNES, L. M., GILLETT, A., HANGER, J., REID, S. A. & RYAN, U. M. 2011. The potential impact of native Australian trypanosome infections on the health of koalas (Phascolarctos cinereus). *Parasitology,* 138**,** 873-83.

MICHALOPOULOS, G. K. & BHUSHAN, B. 2021. Liver regeneration: biological and pathological mechanisms and implications. *Nat Rev Gastroenterol Hepatol,* 18**,** 40-55.

MILLER, M. A. & ZACHARY, J. F. 2017. Chapter 1 - Mechanisms and Morphology of Cellular Injury, Adaptation, and Death11For a glossary of abbreviations and terms used in this chapter see E-Glossary 1-1. *In:* ZACHARY, J. F. (ed.) *Pathologic Basis of Veterinary Disease (Sixth Edition).* Mosby.

MIYAOKA, Y. & MIYAJIMA, A. 2013. To divide or not to divide: revisiting liver regeneration. *Cell Div,* 8**,** 8.

MOORE, B. D., FOLEY, W. J., WALLIS, I. R., COWLING, A. & HANDASYDE, K. A. 2005. Eucalyptus foliar chemistry explains selective feeding by koalas. *Biol Lett,* 1**,** 64-7.

NATIONAL KOALA MONITORING PROGRAM. 2024. *NKMP Updates* [Online]. Available: https://www.nkmp.org.au/populations.php [Accessed 15/01/2025].

NETHERTON, C., MOFFAT, K., BROOKS, E. & WILEMAN, T. 2007. A guide to viral inclusions, membrane rearrangements, factories, and viroplasm produced during virus replication. *Adv Virus Res,* 70**,** 101-82.

NOVOA, R. R., CALDERITA, G., ARRANZ, R., FONTANA, J., GRANZOW, H. & RISCO, C. 2005. Virus factories: associations of cell organelles for viral replication and morphogenesis. *Biol Cell,* 97**,** 147-72.

PARRISH, C. R. 1995. Pathogenesis of feline panleukopenia virus and canine parvovirus. *Baillieres Clin Haematol,* 8**,** 57-71.

PASS, M. A. & BROWN, A. S. 1990. Liver function in normal koalas and macropods. *Aust Vet J,* 67**,** 151-2.

PHILLIPS, S., MADDEN, D., GILLETT, A., QUIGLEY, B. L., JELOCNIK, M., BOMMANA, S., O'MEALLY, D., TIMMS, P. & POLKINGHORNE, A. 2024. Koala ocular disease grades are defined by chlamydial load changes and increases in Th2 immune responses. *Front Cell Infect Microbiol,* 14**,** 1447119.

POLKINGHORNE, A., HANGER, J. & TIMMS, P. 2013. Recent advances in understanding the biology, epidemiology and control of chlamydial infections in koalas. *Veterinary Microbiology,* 165**,** 214-223.

POLLEGIONI, L., SACCHI, S. & MURTAS, G. 2018. Human D-Amino Acid Oxidase: Structure, Function, and Regulation. *Front Mol Biosci,* 5**,** 107.

POPOV, V. L., TESH, R. B., WEAVER, S. C. & VASILAKIS, N. 2019. Electron Microscopy in Discovery of Novel and Emerging Viruses from the Collection of the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA). *Viruses,* 11.

RADI, Z. A., STEWART, Z. S., GRZEMSKI, F. A. & BOBROWSKI, W. F. 2013. Renal pathophysiologic role of cortical tubular inclusion bodies. *Toxicol Pathol,* 41**,** 32-7.

RADU, O. & PANTANOWITZ, L. 2013. Kaposi sarcoma. *Arch Pathol Lab Med,* 137**,** 289-94.

RAO, X., HUANG, X., ZHOU, Z. & LIN, X. 2013. An improvement of the 2ˆ(-delta delta CT) method for quantitative real-time polymerase chain reaction data analysis. *Biostat Bioinforma Biomath,* 3**,** 71-85.

RHODES, J. R., NG, C. F., DEIDRÉ, L., HARRIET, J. P., CLIVE, A. M. & POSSINGHAM, H. P. 2011. Using integrated population modelling to quantify the implications of multiple threatening processes for a rapidly declining population. *Biological Conservation,* 144**,** 1081-1088.

SCHWERTHEIM, S., JASTROW, H., KÄLSCH, J., HEROLD, T., THEURER, S., TING, S., SCHMID, K. & BABA, H. 2020a. β-catenin in intranuclear inclusions of hepatocellular carcinoma. *Hepatoma Research,* 6.

SCHWERTHEIM, S., KÄLSCH, J., JASTROW, H., SCHAEFER, C. M., THEURER, S., TING, S., CANBAY, A., WEDEMEYER, H., SCHMID, K. W. & BABA, H. A. 2020b. Characterization of two types of intranuclear hepatocellular inclusions in NAFLD. *Scientific Reports,* 10**,** 16533.

SELISKAR, M. & ROZMAN, D. 2007. Mammalian cytochromes P450--importance of tissue specificity. *Biochim Biophys Acta,* 1770**,** 458-66.

SHABMAN, R. S., SHRIVASTAVA, S., TSIBANE, T., ATTIE, O., JAYAPRAKASH, A., MIRE, C. E., DILLEY, K. E., PURI, V., STOCKWELL, T. B., GEISBERT, T. W., SACHIDANANDAM, R. & BASLER, C. F. 2016. Isolation and Characterization of a Novel Gammaherpesvirus from a Microbat Cell Line. *mSphere,* 1.

SHIMOYAMA, N., NAKATSUJI, S., ANDOH, R., YAMAGUCHI, Y., TAMURA, K. & HOSHIYA, T. 2015. Spontaneously Occurring Formation of Intranuclear and Cytoplasmic Inclusions in Renal Proximal Epithelium Due to Accumulation of d-Amino Acid Oxidase in Wistar Hannover Rats. *Toxicologic Pathology,* 43**,** 675-680.

SILVESTRI, S., LEPRI, E., DALL'AGLIO, C., MARCHESI, M. C. & VITELLOZZI, G. 2017. Nuclear Glycogen Inclusions in Canine Parietal Cells. *Vet Pathol,* 54**,** 520-526.

SMITH, J. A., WELLEHAN, J. F., JR., POGRANICHNIY, R. M., CHILDRESS, A. L., LANDOLFI, J. A. & TERIO, K. A. 2008. Identification and isolation of a novel herpesvirus in a captive mob of eastern grey kangaroos (Macropus giganteus). *Vet Microbiol,* 129**,** 236-45.

SPEIGHT, K. N., HICKS, P., GRAHAM, C., BOARDMAN, W., BREED, W. G., MANTHORPE, E., FUNNELL, O. & WOOLFORD, L. 2018. Necropsy findings of koalas from the Mount Lofty Ranges population in South Australia. *Aust Vet J,* 96**,** 188-192.

STALDER, K., VAZ, P. K., GILKERSON, J. R., BAKER, R., WHITELEY, P., FICORILLI, N., TATARCZUCH, L., PORTAS, T., SKOGVOLD, K., ANDERSON, G. A. & DEVLIN, J. M. 2015. Prevalence and Clinical Significance of Herpesvirus Infection in Populations of Australian Marsupials. *PLoS One,* 10**,** e0133807.

STRNAD, P., NURALDEEN, R., GULDIKEN, N., HARTMANN, D., MAHAJAN, V., DENK, H. & HAYBAECK, J. 2013. Broad spectrum of hepatocyte inclusions in humans, animals, and experimental models. *Compr Physiol,* 3**,** 1393-436.

THOMSON, D., MEERS, J. & HARRACH, B. 2002. Molecular confirmation of an adenovirus in brushtail possums (Trichosurus vulpecula). *Virus Res,* 83**,** 189-95.

UFFELMANN, E., HUANG, Q. Q., MUNUNG, N. S., DE VRIES, J., OKADA, Y., MARTIN, A. R., MARTIN, H. C., LAPPALAINEN, T. & POSTHUMA, D. 2021. Genome-wide association studies. *Nature Reviews Methods Primers,* 1**,** 59.

VAZ, P., 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). *J Wildl Dis,* 48**,** 226-9.

VAZ, P., 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). *J Wildl Dis,* 47**,** 787-91.

VAZ, P. K., LEGIONE, A. R., HARTLEY, C. A. & DEVLIN, J. M. 2019. 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.

WANG, Z., GERSTEIN, M. & SNYDER, M. 2009. RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet,* 10**,** 57-63.

WHITE, C. L. & TAXY, J. B. 1983. Early morphologic diagnosis of herpes simplex virus encephalitis: Advantages of electron microscopy and immunoperoxidase staining. *Human Pathology,* 14**,** 135-139.

WILD, P. 2008. Chapter 25 Electron Microscopy of Viruses and Virus-Cell Interactions. *Methods in Cell Biology.* Academic Press.

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**,** 1223.

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**,** e0286407.

XU, C., XU, Z., ZHANG, Y., EVERT, M., CALVISI, D. F. & CHEN, X. 2022. β-Catenin signaling in hepatocellular carcinoma. *J Clin Invest,* 132.

ZELNÍK, V., LAPUNÍKOVÁ, B. & KÚDELOVÁ, M. 2013. Marek´s disease: rapid progress in research with unclear biological implementations. *Acta Virol,* 57**,** 265-70.

ZHENG, H., PAN, Y., TANG, S., PYE, G. W., STADLER, C. K., VOGELNEST, L., HERRIN, K. V., RIDEOUT, B. A. & SWITZER, W. M. 2020. Koala retrovirus diversity, transmissibility, and disease associations. *Retrovirology,* 17**,** 34.

Appendix 1 – Photomicrographs of koala livers representing each grade for brown pigment, fibrosis, and necrosis/inflammation

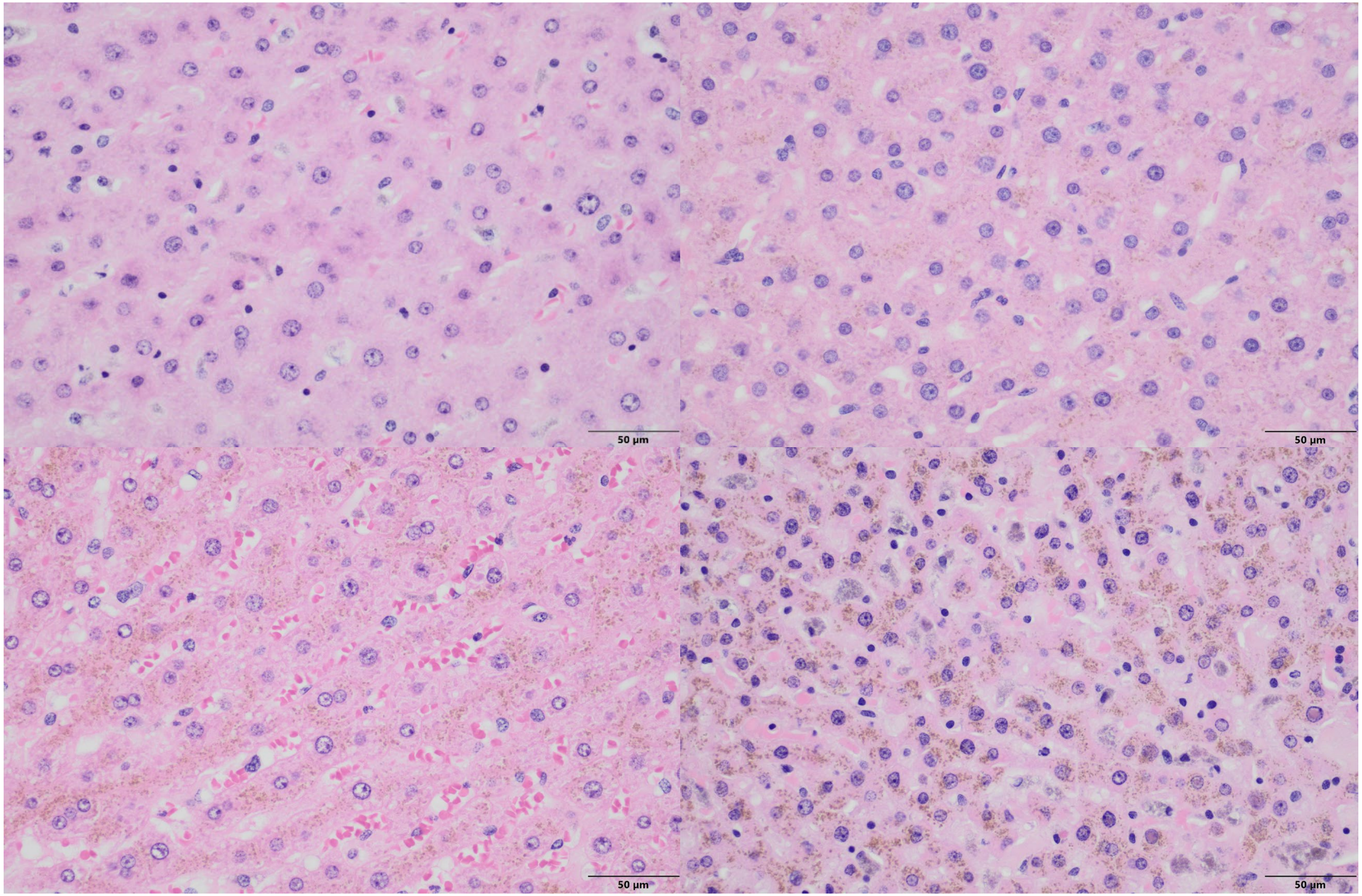

Figure 1: Photomicrographs of representative koala livers for each brown pigment grade. Top left: Grade 0. Eosinophilic cytoplasm, no identifiable pigment. Top right: Grade 1. Mild pigment in less than 50% hepatocytes. Bottom left: Grade 2. Moderate pigment in 50-90% of hepatocytes. Bottom right: Grade 3. Strong pigmentation in >90% hepatocytes All images Haematoxylin & Eosin, 400x magnification.

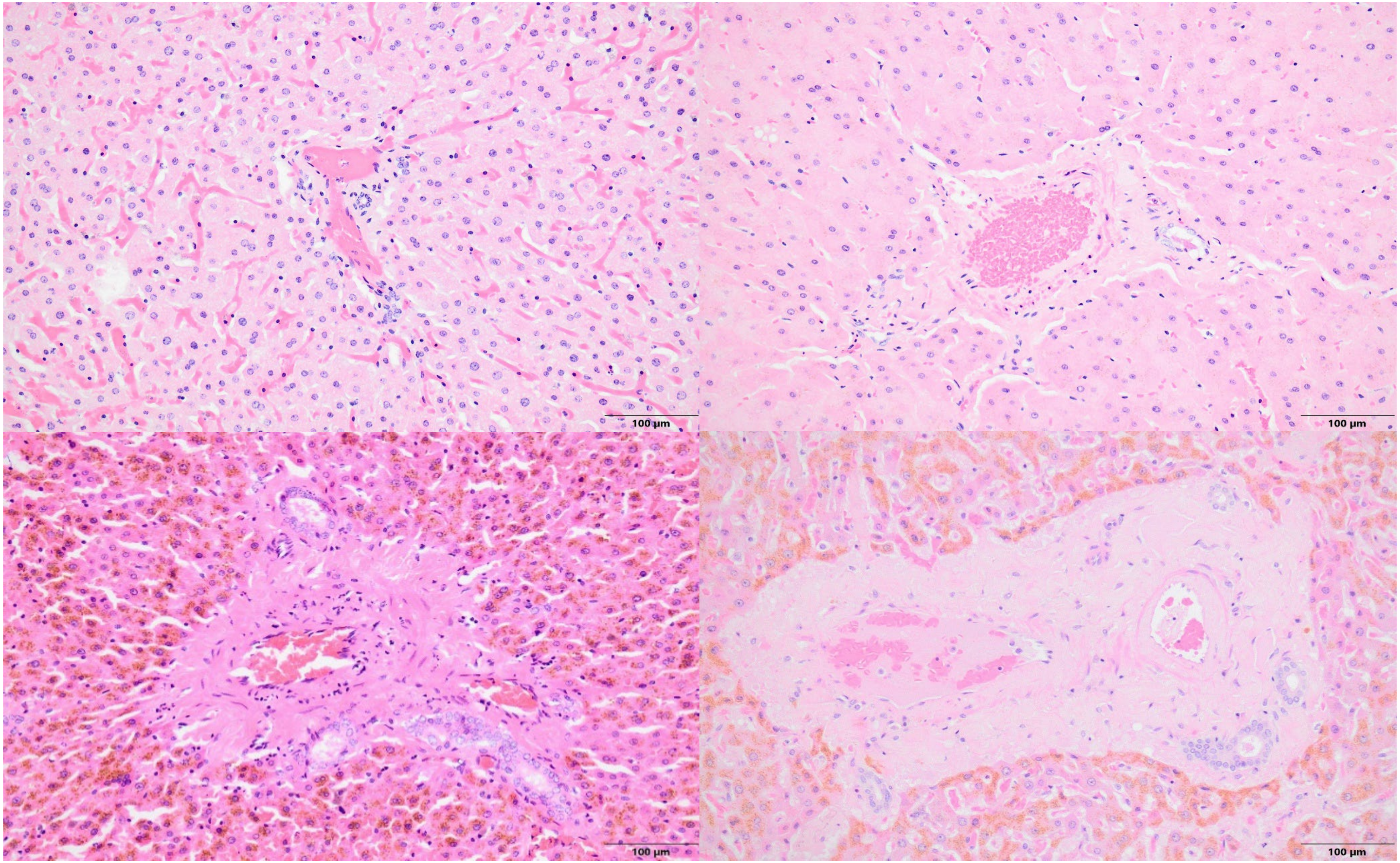

Figure 2: Photomicrographs of representative koala livers for each periportal fibrosis grade. Top left: Grade 0. No fibrosis. Top right: Grade 1. Mild portal fibrosis. Bottom left: Grade 2. Moderate portal fibrosis. Bottom right: Grade 3. Severe portal fibrosis. All images Haemtoxylin & Eosin, 200x magnification.

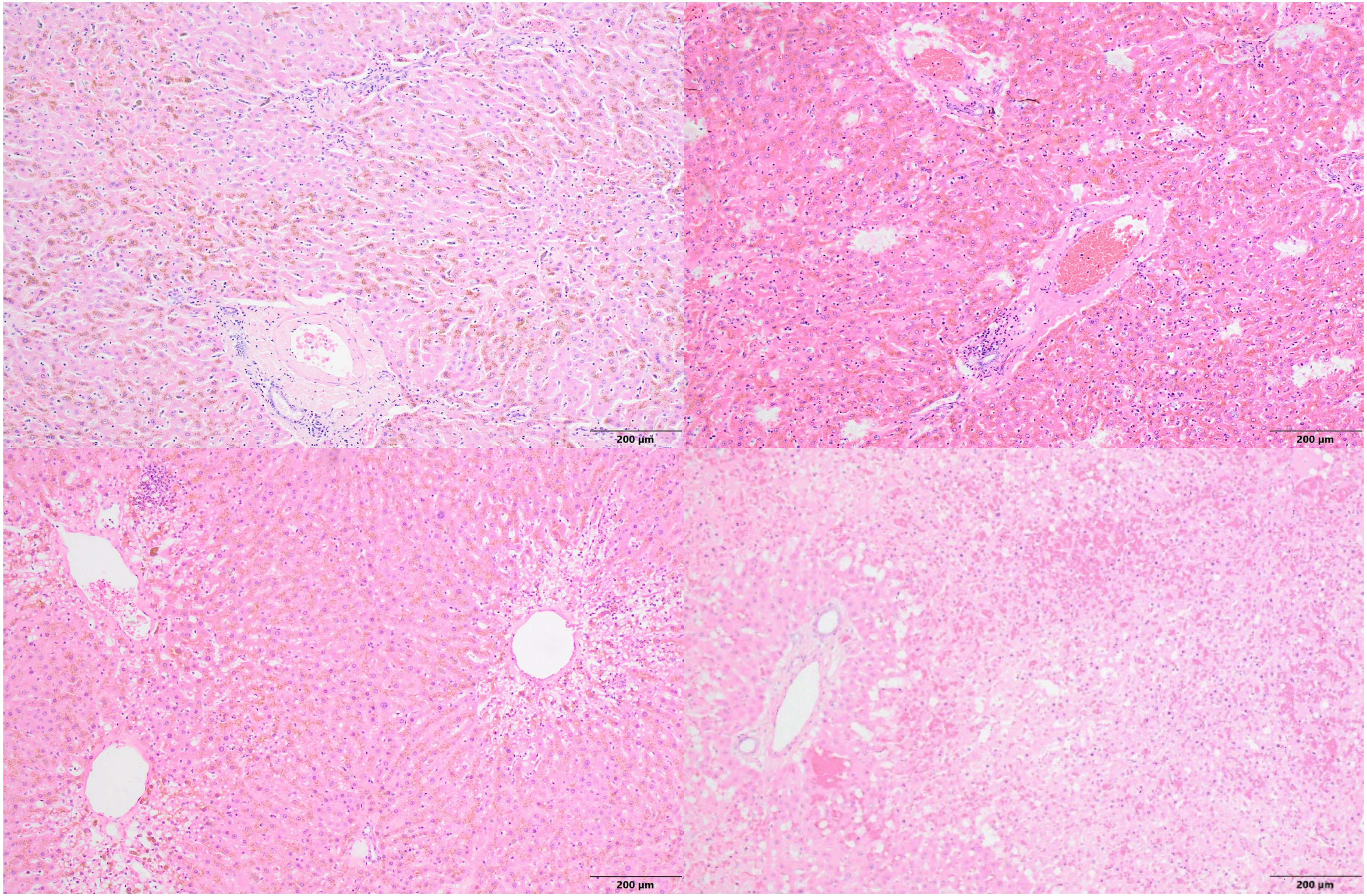

Figure 3: Photomicrographs of representative koala livers for each necrosis/inflammation grade. Not pictured: Grade 0, none. Top left: Grade 1. Minimal/patchy. Top right: Grade 2. Mild (some or all portal tracts, scattered necrosis). Bottom left: Grade 3. Moderate (all portal tracts, necrotic clusters). Bottom right: Grade 4. Severe, prominent inflammation and diffuse hepatocellular damage. All images Haematoxylin & Eosin, 100x magnification.

Appendix 2 – FFPE extraction validation trial

DNA extraction for qPCR tests was performed using the Qiagen DNeasy Blood & Tissue Kit. The manufacturer's instructions recommend a pre-treatment protocol for paraffin-embedded tissue, utilising xylene to remove residual paraffin. Due to chemical handling concerns with the use of xylene and lack of suitable facilities for this work, an initial trial was performed comparing the extraction of two different samples with the xylene pre-treatment compared to omitting the xylene deparaffinisation step and comparing beta-actin qPCR results (Table 1).

Table 1: Beta-actin qPCR results comparing extraction with xylene and without xylene.

Sample		Ct beta-actin	Ct difference
22-0726	With xylene	36.52	1.67
	Without xylene	38.19	
22-0870	With xylene	33.82	1.44
	Without xylene	35.26	

Given the relatively minor differences in Ct values, it was decided to continue the rest of the trial without the use of the xylene pre-treatment to minimise safety risks. Following the completion of FFPE qPCR, many samples had insufficient DNA quality with no beta-actin detection, and it was considered whether the omission of the xylene pre-treatment may have played a role in this lack of sensitivity. However, following the completion of qPCR testing on fresh liver and spleen samples, it was found that the delta Ct between PhaHV-1/PhaHV-2 and beta-actin was often between 10-19, requiring a minimum beta-actin Ct of approximately 27 to detect herpesviruses at the limit of detection of the test (Ct ~37). Therefore, it was considered unlikely that the FFPE tissues would have represented a useful sample type for detection even with the additional use of xylene. The limitations of FFPE tissues for qPCR analysis are further considered in the Discussion of this thesis.

Appendix 3 – PhaHV-1 and PhaHV-2 qPCR results on FFPE and fresh tissues

Table 1: PhaHV-1 and PhaHV-2 qPCR results on historical FFPE tissues of koalas with inclusion bodies.

		PhaHV-1					PhaHV-2		
Animal ID	**Tissue**	**Ct actin**	**Ct PhaHV-1**	**1:10 dilution Ct actin**	**1:10 dilution Ct PhaHV-1**	**Result**	**Ct actin**	**Ct PhaHV-2**	**Result**
1057-01	Liver	N/A	N/A	38.58, N/A	N/A	Invalid	NP	NP	NP
	Spleen	N/A	N/A	N/A, 38.59	N/A	Invalid	NP	NP	NP
1124-01	Liver	34.50	N/A	37.37	N/A	Negative	34.09	39.14, N/A	Negative*
	Spleen	N/A, 38.17	N/A	NP	NP	Invalid	NP	NP	NP
1249-02	Liver	N/A	N/A	N/A	N/A	Invalid	NP	NP	NP
	Spleen	36.3	N/A	NP	NP	Negative	NP	NP	NP
678-09	Liver	38	N/A	N/A, 37.21	N/A	Negative	N/A	NA, 37.79	Invalid
	Spleen	35.86, N/A	N/A	N/A	N/A	Invalid	37.85	N/A, 37.14	Low positive
17-1655	Liver	29.66	N/A	NP	NP	Negative	29.67	N/A	Negative
	Spleen	31.3	N/A	NP	NP	Negative	30.39	N/A	Negative
18-1329	Liver	NP	NP	35.71, N/A	N/A	Negative	38.52	N/A	Negative
	Spleen	NP	NP	NP	NP	NP	NP	NP	NP
19-0766	Liver	N/A	N/A	N/A	N/A	Invalid	NP	NP	NP
	Spleen	29.68	N/A	NP	NP	Negative	NP	NP	NP
21-0948	Liver	NP	NP	34.92	N/A	Negative	38.75, N/A	N/A	Invalid
	Spleen	NP	NP	NP	NP	NP	NP	NP	NP
22-0198	Liver	N/A	N/A	31.13	N/A	Negative	29.67	N/A	Negative
	Spleen	29.8	N/A, 38.18	NP	NP	Low positive	29.8	N/A, 38.18	Low positive
22-0254	Liver	NP	NP	37.68	N/A	Negative	N/A	N/A	Invalid
	Spleen	37.29	N/A	37.84, N/A	N/A	Negative	38.32	39.17, N/A	Low positive
22-0558	Liver	36.11	N/A	NP	NP	Negative	36.11	N/A	Negative
	Spleen	30.4	N/A	NP	NP	Negative	30	N/A, 39.28	Low positive
22-0726	Liver	N/A	N/A	29.19	N/A	Negative	30.85	N/A	Negative
	Spleen	28.4	N/A, 37.34	28	N/A, 38.17	Low positive	27.92	39.77, N/A	Low positive
22-0870	Liver	N/A	N/A	32.73	N/A	Negative	N/A	N/A	Invalid
	Spleen	28.8	N/A	NP	NP	Negative	28.63	N/A	Negative
22-1071	Liver	31.5	N/A	NP	NP	Negative	31.5	N/A	Negative
	Spleen	27.5	N/A	NP	NP	Negative	27.32	N/A	Negative

N/A = no DNA amplification. NP = not performed. Ct values are provided as the mean of both duplicates. Where only one duplicate resulted in a measurable Ct and the other did not, both the Ct value and N/A are recorded.
* This sample was regarded as negative despite low-range DNA amplification due to the melt curve location inconsistent with the positive control.

Table 2: PhaHV-1 and PhaHV-2 qPCR results on fresh liver and spleen samples of koalas with and without inclusion bodies.

Animal ID	Tissue	Avg inclusions per hpf	Ct actin	Ct PhaHV-1	delta Ct	Result	Ct PhaHV-2	delta Ct	Result
24-0437	Liver	4.6	19.12	36.93	17.81	Positive	N/A	N/A	Negative
	Spleen		20.76	33.47	12.71	Positive	N/A	N/A	Negative
24-0568	Liver	11.4	18.32	33.28	14.96	Positive	N/A	N/A	Negative
	Spleen		19.11	33.10	13.99	Positive	N/A	N/A	Negative
24-0617	Liver	15.5	18.94	36.00	17.06	Positive	N/A	N/A	Negative
	Spleen		20.41	32.77	12.36	Positive	N/A	N/A	Negative
24-0762 G	Liver	7.6	17.97	N/A	N/A	Negative	N/A	N/A	Negative
	Spleen		19.71	N/A	N/A	Negative	N/A	N/A	Negative
24-0762 H	Liver	18.6	19.97	36.3	16.35	Positive	N/A	N/A	Negative
	Spleen		19.99	31.8	11.82	Positive	N/A	N/A	Negative
24-0762 I	Liver	14.1	18.30	34.4	16.12	Positive	N/A	N/A	Negative
	Spleen		22.77	36.2	13.47	Positive	N/A	N/A	Negative
24-0707	Liver	5.9	19.35	33.53	14.18	Positive	N/A	N/A	Negative
	Spleen		20.72	33.72	13.00	Positive	N/A	N/A	Negative
24-0748	Liver	15.2	17.29	32.18	14.89	Positive	N/A	N/A	Negative
	Spleen		20.51	31.49	10.98	Positive	N/A	N/A	Negative
23-0394	Liver	0.6	18.55	37.77	19.22	Low positive	36.70	18.15	Positive
	Spleen		17.03	37.44	20.41	Low positive	N/A	N/A	Negative
24-0908 B	Liver	18.1	17.85	N/A	N/A	Negative	N/A	N/A	Negative
	Spleen		17.96	N/A	N/A	Negative	N/A	N/A	Negative
24-0762 A	Liver	0	18.88	N/A	N/A	Negative	33.86	14.99	Positive
	Spleen		19.15	N/A	N/A	Negative	37.01	17.87	Low positive
24-0762 B	Liver	0	19.11	N/A	N/A	Negative	N/A	N/A	Negative
	Spleen		23.13	N/A	N/A	Negative	N/A	N/A	Negative
24-0762 C	Liver	0	20.12	36.94	16.82	Positive	N/A	N/A	Negative
	Spleen		20.24	35.77	15.53	Positive	N/A	N/A	Negative
24-0762 D	Liver	0	17.82	36.40	18.58	Positive	N/A	N/A	Negative
	Spleen		18.09	31.94	13.86	Positive	N/A	N/A	Negative
24-0762 E	Liver	0	19.15	N/A	N/A	Negative	N/A	N/A	Negative
	Spleen		22.92	N/A	N/A	Negative	N/A	N/A	Negative
24-0762 F	Liver	0	19.80	N/A	N/A	Negative	N/A	N/A	Negative
	Spleen		20.18	N/A	N/A	Negative	N/A	N/A	Negative
24-0762 J	Liver	0	21.48	N/A	N/A	Negative	N/A	N/A	Negative
	Spleen		16.68	32.29	15.61	Positive	N/A	N/A	Negative
24-0708	Liver	0	18.53	37.11	18.58	Positive	N/A	N/A	Negative
	Spleen		17.14	32.09	14.95	Positive	N/A	N/A	Negative
23-0395	Liver	0	21.15	36.58	15.43	Positive	32.43	11.28	Positive
	Spleen		19.63	33.44	13.81	Positive	29.15	9.52	Positive
24-0573	Liver	0	19.22	35.97	16.75	Positive	N/A	N/A	Negative
	Spleen		18.69	32.47	13.78	Positive	N/A	N/A	Negative
24-0703	Liver	0	20.97	33.60	12.63	Positive	N/A	N/A	Negative
	Spleen		21.75	34.86	13.11	Positive	N/A	N/A	Negative
24-0699	Liver	0	20.45	N/A	N/A	Negative	N/A	N/A	Negative
	Spleen		18.53	N/A	N/A	Negative	N/A	N/A	Negative

Animal ID	Tissue	Avg inclusions per hpf	Ct actin	Ct PhaHV-1	delta Ct	Result	Ct PhaHV-2	delta Ct	Result
24-0908 A	Liver	0	19.34	34.65	15.32	Positive	N/A	N/A	Negative
	Spleen		31.79	N/A	N/A	Negative	N/A	N/A	Negative
24-0908 C	Liver	0	18.82	N/A	N/A	Negative	N/A	N/A	Negative
	Spleen		24.25	N/A	N/A	Negative	N/A	N/A	Negative
24-0908 D	Liver	0	18.75	N/A	N/A	Negative	N/A	N/A	Negative
	Spleen		18.02	N/A	N/A	Negative	N/A	N/A	Negative
24-0908 E	Liver	0	19.87	36.43	16.57	Positive	31.45	11.59	Positive
	Spleen		20.38	33.14	12.77	Positive	31.9	11.53	Positive
24-0908 F	Liver	0	19.95	36.85	16.90	Positive	N/A	N/A	Negative
	Spleen		19.31	35.11	15.80	Positive	N/A	N/A	Negative
24-0908 G	Liver	0	20.51	36.60	16.09	Positive	37.10	16.60	Low positive
	Spleen		21.38	33.49	12.12	Positive	35.69	14.32	Positive
24-0908 H	Liver	0	19.18	N/A	N/A	Negative	N/A	N/A	Negative
	Spleen		17.65	N/A	N/A	Negative	N/A	N/A	Negative
24-0908 I	Liver	0	19.58	34.25	14.68	Positive	33.33	13.76	Positive
	Spleen		19.28	29.47	10.19	Positive	35.60	16.32	Positive
24-0908 J	Liver	0	22.01	N/A	N/A	Negative	N/A	N/A	Negative
	Spleen		19.24	N/A	N/A	Negative	N/A	N/A	Negative
24-0908 K	Liver	0	18.05	36.97	18.93	Positive	35.48	17.44	Positive
	Spleen		18.60	36.22	17.63	Positive	34.01	15.42	Positive
24-0727	Liver	0	19.69	34.39	14.71	Positive	N/A	N/A	Negative
	Spleen		NP	NP	NP	NP	NP	NP	NP

hpf = high-powered (400x) field. N/A = no DNA amplification. NP = not performed. Ct values are provided as the mean of both duplicates.