Prevalence and pathological findings associated with *Chlamydia pecorum* and koala retrovirus infections in South Australian koala populations

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Abstract

Two infectious pathogens contributing to northern Australian (Queensland and New South Wales) population declines are *Chlamydia pecorum* and koala retrovirus (KoRV). *C. pecorum* infection causes conjunctivitis and blindness, urinary tract infections or reproductive tract disease causing infertility. In northern populations, KoRV-A is 100% prevalent and an active endogenous virus. High KoRV viral load and exogenous KoRV-B have been associated with lymphosarcoma and chlamydial disease. In South Australia (SA), less is known about *C. pecorum* and KoRV prevalence and disease, though early evidence has suggested that chlamydial disease is less severe and KoRV prevalence is low. SA koalas may therefore provide an opportunity to further investigate the association of KoRV with disease.

The prevalence of *C. pecorum* and KoRV was determined in wild-caught SA koalas. The Kangaroo Island (KI) population was shown to be *C. pecorum*-free by qPCR (n=170) and in historical clinical data (n=13,000). In the Mount Lofty Ranges (MLR), 46.7% (35/75) of koalas were *C. pecorum* positive, and whilst only 4.0% (3/75) were observed with disease, *C. pecorum* infection was significantly associated with female reproductive inactivity. The prevalence of KoRV in KI and MLR was 42.4% (72/170) and 65.3% (49/75), respectively and only KoRV-A and not KoRV-B was detected by PCR. The median (range) KoRV proviral load in KI and MLR was low, at 113 (2- 12,641) and 35 (1- 574) copies/10^3 *β*-actin copies, respectively. There was no association between *C. pecorum* infection or disease and KoRV provirus, however koalas with concurrent infections were over three times more likely to develop chlamydial disease. Subclinical *C. pecorum* and KoRV infections were shown to have no effect on haematology values, allowing for the development of the first southern koala haematology reference intervals (n=138).

An extensive comparative pathological investigation was conducted on KoRV-positive koalas from the MLR (n=92) and Queensland (n=67). Lymphosarcoma was observed in 4.3% (4/92) of MLR and 7.5% (5/67) of Queensland koalas, with the same morphology and high KoRV proviral and viral loads, that may suggest common oncogenic pathways. Ocular chlamydial disease was severe in both populations, but urinary tract disease was less severe in MLR. There was no association between chlamydial disease severity or load and KoRV proviral load, but in MLR there was a positive correlation between chlamydial disease severity and KoRV viral load. In both populations, KoRV proviral and viral loads were positively correlated with lymphocyte and metarubricyte counts and negatively correlated with erythrocyte and neutrophil counts which may suggest that KoRV can infect bone marrow and disrupting cellular differentiation. KoRV viral load was positively correlated with splenic lymphoid area suggesting that spleen may also be a site for KoRV replication.

The prevalence of *C. pecorum* and KoRV in mainland SA was higher than expected and severe chlamydial disease and lymphoid neoplasia do occur. No clear association between *C. pecorum* disease and KoRV were identified, however KoRV infection appears to be highly complex and differs between northern and southern populations. Southern populations with a reduced prevalence of disease may therefore ensure the longevity of the species.

Thesis declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Signed

Jessica Fabijan

Date: 4th November 2019

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Preamble

This thesis consists of five research articles which resulted from two collaborative research ventures. The first part of my PhD was funded by my supervisor Dr Natasha Speight in collaboration with other researchers from the University of Adelaide and the University of the Sunshine Coast, to conduct the first health survey of wild-caught South Australian koalas (MLR and KI). The second collaborative 'koala retrovirus pathogenesis project' was led by Dr Greg Simmons from the University of Queensland and involved researchers from the University of Adelaide and University of Nottingham, UK, and aimed to compare koala retrovirus infection between necropsied koalas from South Australia and Queensland. Through these collaborations I have been involved in a number of publications involving koala health, as indicated by the list of publications produced during my candidature which are not incorporated into this thesis.

This thesis presents a series of research articles investigating the role of *Chlamydia pecorum* and koala retrovirus infections in South Australian koala populations. Chapter 1 is a literature review which describes the background and objectives of this thesis. The first two articles (Chapters 2 and 3) are from my own work investigating the prevalence of *C. pecorum* and the koala retrovirus in wild-caught South Australian koala populations. These wild-caught koalas were then used to develop new haematological reference intervals for southern koalas (Chapter 4). Chapter 5 includes two research articles, the first (Chapter 5.1) was the first report of reproductive chlamydial disease, lymphosarcoma and koala retrovirus infection in a South Australian koala, and Chapter 5.2 describes disease in KoRV positive necropsied South Australian koalas, and how these findings compare to koalas from Queensland, as part of the collaborative koala retrovirus pathogenesis project.

List of Publications

Research articles by the author incorporated into the thesis

1. **Fabijan J**, Caraguel C, Jelocnik M, Polkinghorne A, Boardman WSJ, Nishimoto E, Johnsson G, Molsher R, Woolford L, Timms P, Simmons G, Hemmatzadeh F, Trott DJ, Speight, KN. 2019. *Chlamydia pecorum* prevalence in South Australian koala (*Phascolarctos cinereus*) populations: Identification and modelling of a population free from infection. *Sci Rep.* 9(6261) https://doi.org/10.1038/s41598-019-42702-z
2. **Fabijan J**, Miller D, Olagoke O, Woolford L, Boardman WSJ, Timms P, Polkinghorne A, Simmons G, Hemmatzadeh F, Trott DJ, Speight KN. 2019. Prevalence and clinical significance of koala retrovirus in two South Australian koala (*Phascolarctos cinereus*) populations. *J Med Microbiol.* https://doi.org/10.1099/jmm.0.001009
3. **Fabijan J**, Speight KN, Boardman WSJ, Hemmatzadeh F, Trott DJ, Woolford L. 2020. Hematological reference intervals in clinically healthy, wild koalas (*Phascolarctos cinereus*). *Aust Vet J*. https://doi.org/10.1111/avj.12923
4. **Fabijan J**, Woolford L, Lathe S, Simmons G, Hemmatzadeh F, Trott DJ, Speight N. 2017. Lymphoma, koala retrovirus infection and reproductive chlamydiosis in a koala (*Phascolarctos cinereus*) *J Comp Pathol.* 157(2). 188-192. https://doi.org/10.1016/j.jcpa.2017.07.011
5. **Fabijan J**, Sarker N, Speight KN, Owen H, Meers J, Simmons G, Seddon JM, Emes RD, Tarlinton R, Hemmatzadeh F, Woolford L, Trott DJ. 2020. Pathological findings in koala retrovirus positive northern and southern koalas (*Phascolarctos cinereus*): a comparative study. *J Comp Pathol*. Accepted 6th February 2020.

Additional research articles as a co-author but not incorporated into the thesis

1. Butcher R, Pettett LM, **Fabijan J**, Ebrahimie E, Mohammadi-Dehcheshmeh M, Speight KN, Boardman WSJ, Bird PS, Trott DJ. 2020. Periodontal disease in free-ranging koalas (*Phascolarctos cinereus*) from the Mount Lofty Ranges, South Australia and its association with koala retrovirus infection. *Aust Vet J*. https://doi.org/10.1111/avj.12919
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4. Pettett LM, Wilson GJ, Nicolson V, Boardman WSJ, Speight KN, **Fabijan J**, Trott DJ, Bird PS. 2019. The malocclusions of the koala (*Phascolarctos cinereus*). *Aust Vet J*. 97(11). 473-481. https://doi.org/10.1111/avj.12863
5. Sarker N, **Fabijan J**, Emes RD, Hemmatzadeh F, Meers J, Moreton J, Owen H, Seddon JM, Simmons G, Speight N, Trott DJ, Woolford L, Tarlinton R. 2018. Identification of stable reference genes for quantitative PCR in koalas. *Sci Rep*. 8(1). 3364. https://doi.org/10.1038/s41598-018-21723-0
6. Sarker N, **Fabijan J**, Owen H, Seddon JM, Simmons G, Speight KN, Kaler J, Woolford L, Emes RD, Hemmatzadeh F, Trott DJ, Meers J, Tarlinton R. 2020. Koala retrovirus viral load and disease burden in distinct northern and southern koala populations. *Sci Rep*. 10. 263. https://doi.org/10.1038/s41598-019-56546-0

7. Sarker N, **Fabijan J**, Seddon JM, Tarlinton R, Owen H, Simmons G, Thia J, Speight KN, Kaler J, Emes RD, Woolford L, Trott DJ, Hemmatzadeh F, Meers J. Genetic diversity of KoRV env gene subtypes: Insights into Queensland and South Australian koala populations. *J Gen Virol*. https://doi.org/10.1099/jgv.0.001304
8. Taggart P, Fancourt BA, **Fabijan J**, Peacock D, Speight KN, Caraguel C, McAllister M. Comparison of *Toxoplasma gondii* seroprevalence in koalas (*Phascolarctos cinereus*) on a large Australian island and the adjacent mainland. *J Parasitol*. 105(4). 638-641. https://doi.org/10.1645/19-40
9. Tarlinton RE, Sarker N, **Fabijan J**, Dottorini T, Woolford L, Meers J, Simmons G, Owen H, Seddon J, Hemmatzedah F, Speight N, Trott DJ, Emes RD. 2017. Differential and defective expression of Koala Retrovirus reveal complexity of host and virus evolution. *bioRxiv*. https://doi.org/10.1101/211466

Conference presentations relevant to the thesis

1. **Fabijan J**, Sarker S, Simmons G, Waugh C, Polkinghorne A, Timms P, Boardman WSJ, Hemmatzadeh F, Speight N, Trott DJ. Occurrence of the Koala retrovirus (KoRV) and *Chlamydia pecorum* in the Mount Lofty Ranges population. Wildlife Disease Association, Twin Waters, Queensland, Australia. 26-30 July 2015. Poster presentation.
2. **Fabijan J**, Sarker S, Simmons G, Waugh C, Polkinghorne A, Timms P, Boardman WSJ, Hemmatzadeh F, Speight N, Trott DJ. Occurrence of the Koala retrovirus (KoRV) and *Chlamydia pecorum* in the Mount Lofty Ranges population. Natural Resource Management Science Conference, Adelaide, Australia, 13 April 2016. Poster presentation.
3. **Fabijan J**, Speight N, Woolford L, Boardman WSJ, Simmons G, Hemmatzadeh

F, Trott DJ. Prevalence and pathological features of the Koala retrovirus and Chlamydia pecorum in the South Australian Mount Lofty Ranges and Kangaroo Island koala populations. The 2nd National Koala Conference, Port Macquarie, New South Wales, Australia, 2-4 June 2017. Oral presentation.

4. **Fabijan J**, Sarker S, Simmons G, Hemmatzadeh F, Woolford L, Boardman WSJ, Owen H, Tarlinton R, Polkinghorne A, Timms P, Seddon J, Meers J, Trott DJ, Kaler J, Emes RD, Speight N. Koala retrovirus and *Chlamydia pecorum* infection: a South Australian perspective. Wildlife Disease Association Australasian section, Falls Creek, Victoria, Australia, 24-29 2017. Oral presentation.
5. Sarker S, Owen H, Simmons G, Seddon J, Tarlinton R, **Fabijan J**, Speight N, Trott DJ, Emes RD, Kaler J, Meers J. Comparative analysis of Koala retrovirus (KoRV) pathogenesis between Queensland and South Australian koalas. Australian Society of Microbiology, Canberra, Australia, 12-15 July 2015. Poster presentation.
6. Sarker S, **Fabijan J**, Emes RD, Simmons G, Meers J, Seddon J, Owen H, Speight N, Trott, DJ, Kaler J, Tarlinton R. Application of transcriptome analysis to identify Koala retrovirus (KoRV) variant diversity within Queensland and South Australian koalas. Australian Society of Microbiology, Hobart, Australia, 2-5 July 2017. Poster presentation.
7. Butcher RG, Pettett LM, Ebrahimie E, Speight KN, Boardman WJS, **Fabijan J**, Bird PS, Trott DJ. Is there an association between Koala Retrovirus (KoRV) infection and periodontal disease (PD) in free-ranging South Australian koalas (Phascolarctos cinereus) from the Mount Lofty Ranges? Wildlife Disease Association Australasian section, Falls Creek, Victoria, Australia, 24-29 2017. Oral presentation.

8. Sarker S, **Fabijan J**, Owen H, Meers J, Seddon J, Speight N, Trott DJ, Hemmatzadeh F, Tarlinton R, Kaler J, Emes RD, Simmons G. Comparison of Koala retrovirus (KoRV) proviral DNA and viral RNA levels between Queensland and South Australian koalas. Australasian Virology Society Meeting AVS9, Adelaide, Australia, 4-7 December 2017. Poster presentation.
9. Meers J, Sarker N, Boardman W, Emes R, **Fabijan J**, Hemmatzadeh F, Jozani R, Kaler J, Owen H, Seddon J, Simmons G, Speight N, Tarlinton R, Trott D, Woolford L. Koala retrovirus infection in Queensland and South Australian koala populations. Australian Society of Microbiology, Brisbane, Australia, 1-4 July 2018. Oral presentation.

Chapter 1

Literature review

1.1. Introduction

The koala (*Phascolarctos cinereus*) is a folivorous, arboreal marsupial and the only remaining member of the family, *Phascolarctidae* (Lee and Martin 1996). Historically, koala populations were distributed across the eastern and south-eastern coast of Australia, however, their distribution was significantly reduced post-European settlement primarily due to hunting for the fur trade, with localised extinctions in the southern part of their range (Phillips 1990). In an effort to conserve the species, a series of translocations were performed that introduced koalas to a number of previously unoccupied regions, including Kangaroo Island and the Mount Lofty Ranges, South Australia.

Today, koala populations in northern Australia (Queensland and New South Wales) are in significant decline and are classified as vulnerable, while southern Australian (Victoria and South Australia) koalas are overabundant (DSEWPC 2012). One leading cause for the northern population declines is mortality from disease (Rhodes *et al.* 2011). *Chlamydia pecorum* is a key infectious pathogen of northern koalas with a high prevalence in wild populations and overt chlamydial disease is commonly observed (Polkinghorne *et al.* 2013). Overt *C. pecorum* disease can develop as conjunctivitis causing blindness (Wan *et al.* 2011), pneumonia (Mackie *et al.* 2016), urinary tract infections observed as cystitis and nephritis (Wan *et al.* 2011) and reproductive tract infections causing infertility in male (Johnston *et al.* 2015) and female koalas (McColl *et al.* 1984; Obendorf and Handasyde 1990).

Koala retrovirus (KoRV), a gammaretrovirus, is another pathogen of concern for northern koala populations. KoRV is 100% prevalent in northern koala populations (Simmons *et al.* 2012) and has been associated with the development of lymphoid neoplasia (Tarlinton *et al.* 2005; Xu *et al.* 2013) which is the most common neoplasia reported in the koala (Spencer and Canfield 1996). Also, like other retroviruses KoRV may modulate the immune system, predisposing the koalas to disease from secondary

pathogens (Tarlinton *et al.* 2005). KoRV modulation of the immune system, or immunosuppression, may therefore explain the high prevalence of severe *C. pecorum* disease in northern koalas, however investigating this association in northern koalas is difficult due to the ubiquitous nature of overt chlamydial disease and KoRV infection in these populations.

In southern koala populations the prevalence and severity of *C. pecorum* and KoRV appears to be lower. In South Australia, the Kangaroo Island population is believed to be *C. pecorum*-free based on ten koalas tested by PCR two decades ago (Polkinghorne *et al.* 2013), while the prevalence in necropsied Mount Lofty Ranges koalas was recently found to be 88% (n=65) with a low prevalence (12/65) of mild ocular and urinary tract disease observed (Speight *et al.* 2016). KoRV was found to be 14.8% prevalent (n=162) on Kangaroo Island in 2012 (Simmons *et al.* 2012) and the Mount Lofty Ranges has not been investigated. Therefore, the South Australian koala populations may provide a unique opportunity to compare *C. pecorum* infection and disease between KoRV infected and non-infected koalas.

1.2. Establishment of koalas in South Australia

At the turn of the 20th century, the koala fur trade was at its economic peak. Populations in northern Australia were significantly reduced in size, while localised extinctions occurred in southern Australia, including the extant koala populations in south-eastern South Australia (Phillips 1990). It became apparent conservation efforts would need to be implemented to conserve the species before the koala became extinct Australia-wide. In an attempt to ensure the koala's survival, a number of translocations occurred which introduced koalas to new, previously unoccupied island and mainland regions (Martin and Handasyde 1990; Phillips 1990). Koalas were introduced to Magnetic Island in Queensland, French Island, Philip Island and Cape

Otway in Victoria, and Kangaroo Island, the Mount Lofty Ranges, Eyre Peninsula and the Riverland in South Australia.

South Australia was populated by koalas primarily from Victoria, with some reports of northern koalas also being released in the state. Koalas were first translocated from mainland Victoria to French Island and Philip Island in the 1880s (Robinson *et al.* 1989). These populations flourished and between 1923 and 1925 eighteen koalas were translocated and introduced to Kangaroo Island. Initially these koalas were in captivity, however, they escaped and by 1948 they were observed in abundance across Kangaroo Island (Robinson 1978). This population also exceeded capacity, resulting in considerable defoliation of the manna gum forests (Robinson 1978). To reduce the size of the Kangaroo Island population, koalas were translocated to a number of locations on mainland South Australia, including the Eyre Peninsula, the Riverland, lower south-eastern South Australia and the Mount Lofty Ranges (Phillips 1990). The Mount Lofty Ranges koala population was initially founded by six koalas from Kangaroo Island. Within the Mount Lofty Ranges, there are also reports of an illegal captive population of koalas that originated from New South Wales, that may have escaped into the wild (Robinson 1978). Additional koalas bred in Adelaide were also released in the 1940s into the Mount Lofty Ranges and Kangaroo Island populations. These koalas were descendants from Queensland and Victorian koalas (Lindsay 1950).

The translocations of koalas throughout southern Australia was thought to have caused a series of genetic bottlenecks which resulted in reduced genetic diversity of southern koalas, however, recent work suggests these bottlenecks may not have been as severe as initially thought. A late 1990s study showed allelic diversity to be significantly reduced in the southern populations compared with koalas from northern populations (Houlden *et al.* 1996). However, two recent studies using advanced

genomics compared koalas across Australia and both showed that southern koalas still have considerable genetic diversity (Neaves *et al.* 2016; Kjeldsen *et al.* 2018). Neaves *et al.* (2016) showed that southern and northern koalas had comparable mtDNA diversity and Kjeldsen *et al.* (2018) also showed that southern koalas had comparable genetic diversity to northern populations using SNP markers. Whilst northern and southern koalas are not genetically separate subspecies, it appears that they fall into three (Kjeldsen *et al.* 2018) to four (Neaves *et al.* 2016) genetic lineages; two northern lineages in Queensland, one lineage in northern New South Wales, and the fourth southern linage which encompasses southern New South Wales, Victoria and South Australia.

1.3. Current koala population stability

Today, koala numbers are still low and declining in northern Australia and were classified as 'vulnerable' by the Australian Government in 2012 (DSEWPC 2012). These populations are facing a number of threatening processes which are causing koala mortality. Deforestation and habitat fragmentation due to urban expansion is a key driver of population decline (Dique *et al.* 2003) and urbanisation is also increasing mortality by more vehicle collisions and dog attacks as koalas travel between fragmented habitat (Griffith *et al.* 2013; Rhodes *et al.* 2014; Gonzalez-Astudillo *et al.* 2017). Fire (Lunney *et al.* 2007) and climate change (Seabrook *et al.* 2011) are also becoming of increasing concern. However, disease could be considered the most important factor contributing to population decline, particularly *C. pecorum* (Rhodes *et al.* 2011) and possibly KoRV (Tarlinton *et al.* 2005; Simmons *et al.* 2012).

Southern populations are not considered to be vulnerable as the introduced populations have all thrived and are now considered overabundant (DSEWPC 2012). Some populations in Victoria and South Australia have become so overabundant that evidence of defoliation is apparent (Bryan 1996; Masters *et al.* 2004). Population

control measures have been implemented in some Victorian and South Australian populations to reduce fertility, including hormone implants (Hynes *et al.* 2010) and surgical sterilisation (Duka and Masters 2005; Molsher 2017) to reduce the rate of population growth. In South Australia, while the Kangaroo Island (Masters *et al.* 2004) and Mount Lofty Ranges populations (Bryan 1996; Sequeira *et al.* 2014) are considered to be the two largest populations, few studies have reported on their health and disease status. Little is known about pathogens affecting koalas in the Kangaroo Island population, with few reports of the presence of the KoRV but not associated with disease (Tarlinton *et al.* 2006; Simmons *et al.* 2012). In the Mount Lofty Ranges, oxalate nephrosis has recently been identified as a prevalent kidney disease (Speight *et al.* 2012), and chlamydiosis has been reported as a possible emerging disease (Funnell *et al.* 2013; Speight *et al.* 2016).

1.4. *Chlamydia*

Chlamydiaceae are a family of obligate, intracellular bacteria with a bi-phasic life cycle. Chlamydial infection occurs by acquiring an infectious, metabolically inactive elementary body which is taken up by the host cell, usually an epithelial cell or macrophage, into a cell-derived vacuole. Differentiation of the elementary bodies into a metabolically active reticulate body occurs and begins to replicate by binary fission. Upon replication of eight to twelve rounds, the reticulate bodies return to the inert elementary bodies which are released from the host cell into the extracellular space to further infect and replicate within the host (Madigan *et al.* 2009). There are currently eleven known species of *Chlamydia*; *C. abortus, C. avium, C. caviae, C. felis, C. gallinacea, C. muridarum, C. pecorum, C. pneumoniae, C. psittaci, C. suis*, and *C. trachomatis* (Polkinghorne *et al.* 2013; Sachse *et al.* 2014; Sachse *et al.* 2015).

Early investigations into koala ocular and urinary disease implicated *Chlamydia psittaci* as a causative agent based on morphology and microbiological

techniques (Cockram and Jackson 1974; McColl *et al.* 1984; Brown *et al.* 1987). Two subtypes were later recognised (I and II) (Girjes *et al.* 1993) and with advancements in DNA analysis, two new species of *Chlamydia* were identified, *C. pneumoniae* (Grayston *et al.* 1989) and *C. pecorum* (Fukushi and Hirai 1992), which were found to be *C. psittaci* type I (*C. pneumoniae*) and type II (*C. pecorum*) (Glassick *et al.* 1996).

Since the identification of the two chlamydial species that infect koalas, *C. pneumoniae* and *C. pecorum*, studies of associated disease have shown *C. pecorum* to have an increased pathogenicity. In a study which graded the severity of chlamydial disease as 1 (no disease) to 4 (severe disease), *C. pecorum* infection developed into grade 4 disease, whereas *C. pneumoniae* infection only developed into grade 2 disease (Jackson *et al.* 1999). This was supported by another study which found a higher prevalence of disease in koalas with *C. pecorum* infection when compared to *C. pneumoniae* (Devereaux *et al.* 2003). Based on this evidence, *C. pecorum* has since been regarded as the virulent species causing the severe disease observed in the koala and considered of greatest concern for koala health (Polkinghorne *et al.* 2013).

1.4.1. Overt disease and subclinical infection

C. pecorum and *C. pneumoniae* infections can cause disease in four anatomical regions of the koala; ocular, urinary, reproductive and respiratory tract sites (Cockram and Jackson 1974; McColl *et al.* 1984; Brown *et al.* 1987; Girjes *et al.* 1988). Chlamydial infection and disease may develop independently in one anatomical region or concurrently with other regions. Ocular and respiratory diseases were commonly reported concurrently in necropsied koalas from New South Wales (Canfield 1987), but the most frequent concurrent infections are urogenital disease (urinary and reproductive tracts) as reported in koalas from Queensland (Gonzalez-Astudillo *et al.* 2017), New South Wales (Canfield 1989; Griffith and Higgins 2012; Griffith *et al.* 2013) and Victoria (Obendorf 1983; Patterson *et al.* 2015). The close proximity of these

organs may account for the high frequency of concurrent infections at these anatomical sites. Disease can be difficult to treat, often involving extended hospitalisation periods for antibiotic therapy and occasionally surgical intervention (Robbins *et al.* 2018). Euthanasia is common in severe cases based on poor prognosis and animal welfare concerns.

Ocular disease can develop either unilaterally or bilaterally and primarily infects the conjunctiva. Inflammation and hyperaemia of the conjunctiva are common, and a mucopurulent discharge may occur in chronic infections (Cockram and Jackson 1974). Histological examination of infected conjunctiva shows epithelial thickening and villous hyperplasia and hypertrophy, which increases with the severity of the inflammation (Hemsley and Canfield 1997). The cornea may or may not be involved, showing oedema, ulceration and pannus (Brown *et al.* 1987; Wan *et al.* 2011), however corneal damage usually occurs indirectly from the physical changes of the conjunctiva or by the koala scratching and rubbing the eye (Obendorf 1983; Canfield 1990; Wan *et al.* 2011). Ocular disease can lead to blindness and mortality in the koala.

Urinary tract infections are a common condition for the koala. Commonly referred to as “wet-bottom” or “dirty tail” disease, cystitis and urethritis result in urinary incontinence, soiling and ulceration of the external perineal skin (Canfield 1989; Wan *et al.* 2011). Cystitis does not always result in overt clinical signs and can develop severely with no outward sign of infection (Canfield 1989; Wan *et al.* 2011). Inflammation caused predominantly by lymphocyte and plasma cell infiltration within the mucosa, submucosa and muscularis commonly lead to fibrosis and loss of normal architecture (Hemsley and Canfield 1997). Infection can ascend from the bladder to the kidneys, resulting in ureteritis, pyelonephritis, glomerulonephritis and fibrosis (Higgins *et al.* 2005b), however, kidney disease can also present without concurrent

cystitis (Canfield 1989). Severe cases of urinary tract infections may lead to renal failure and death of the koala.

Reproductive tract infections can develop inapparently, where clinically overt changes develop internally without outward signs of infection and ultimately lead to infertility. The pathologies observed in infected female koalas include paraovarian and bursal cysts, pyometra, metritis and endometritis (McColl *et al.* 1984; Higgins *et al.* 2005b; Wan *et al.* 2011). Histological examination of infected tissues often identifies marked inflammation and infiltration with neutrophils, lymphocytes, macrophages and plasma cells, with occasional purulent exudate within the vaginal, uterine and salpingeal lumen (Obendorf 1981; Canfield *et al.* 1983). Infected females may exhibit a purulent discharge from the cloaca (Canfield *et al.* 1991), which may be considered the only externally overt clinical sign of reproductive tract infection in females. Disease manifests in male koalas as orchitis, epididymitis, prostatitis and prostatic and/or penile urethritis (Johnston *et al.* 2015; Palmieri *et al.* 2018) and has recently been shown to cause inflammation within the bulbourethral glands (Palmieri *et al.* 2018). Orchitis observed during histological examination of the testis displayed loss of normal epithelial structure of the seminiferous tubules with or without luminal exudate, fibrosis of interstitial tissue and occasionally granulomatous reactions with aggregates of macrophages and lymphocytes. Epididymitis is characterised by fibrosis and loss of normal tubule structure, or tubule architecture may remain with interstitial fibrosis and granulomatous aggregation (Johnston *et al.* 2015). Infertility arises in both females and males due to the changes in reproductive tract architecture which prevents the organs from functioning. In females, clinical changes around the ovaries may physically prevent ovulation (Higgins *et al.* 2005a), and in males, fibrosis within the testis prevents spermatogenesis (Johnston *et al.* 2015). While reproductive tract disease is not as overt as ocular or urinary tract infections, the implications of infertility have significant consequences for koala health and population stability.

While chlamydial ocular, urinary and reproductive tract diseases are well documented, less is known about chlamydial respiratory tract disease. *Chlamydia psittaci* was first detected in the respiratory tract of diseased koalas with rhinitis and pneumonia (Brown and Grice 1986; Wardrop *et al.* 1999) and *C. pecorum* has also been shown to infect and cause disease of the respiratory tract (Mackie *et al.* 2016). Respiratory disease due to *C. pneumoniae* was commonly reported in captive populations but appeared to be less prevalent in wild koala populations (Wardrop *et al.* 1999; Blanshard and Bodley 2008). Early reports of mass mortality events due to respiratory disease (Rahman 1957; Backhouse and Bolliger 1961) have now become infrequent and reports of respiratory disease in necropsied koalas are low, at 12% in Queensland (Gonzalez-Astudillo *et al.* 2017), 6.3% in New South Wales (Canfield 1987), 0% in Victoria (Obendorf 1983) and 4% in South Australian, Mount Lofty Ranges koalas (Speight *et al.* 2018). Clinically, koalas with respiratory disease present with rhinitis and pneumonia, with purulent nasal discharge (Wardrop *et al.* 1999).

Chlamydial infection does not always result in the development of clinical disease and subclinical carriage of *Chlamydia* may occur (Ladds 2009). There are likely many factors which contribute to the expression of clinical disease, which would involve chlamydial and host immune factors. Persistent subclinical infections can occur in the koala with both *C. pecorum* and *C. pneumoniae* infections (Hogan *et al.* 2004). Persistence may occur spontaneously, or as a result of reduced nutritional availability to the bacterium, which may prevent chlamydial replication from occurring (Hammerschlag 2002; Dewannieux and Heidmann 2013). Persistence may also be attributed to the host's immune system, where the host is unable to clear the infection (Hammerschlag 2002). Elimination of chlamydial bodies requires a strong Th1 immune response due to the intracellular nature of *C. pecorum* (Perry *et al.* 1997). A number of recent studies have shown that koalas possess and express key cytokines for Th1 (Mathew *et al.* 2013b; Maher *et al.* 2014), Th2 (Mathew *et al.* 2013a; Maher *et al.* 2014)

and TH17 (Mathew *et al.* 2014; Maher and Higgins 2016) pathways, with early evidence highlighting upregulation of interferon gamma (INFϒ) (Mathew *et al.* 2013b), tumour necrosis factor alpha (TNFα), interleukin 10 (IL-10) (Mathew *et al.* 2013a) and IL-17a (Mathew *et al.* 2014) in koalas with overt disease compared to no disease. There are many other chlamydial and host factors which may contribute to subclinical infection which require further investigation.

1.4.2. Transmission of *C. pecorum*

Chlamydiales are known to transmit between hosts via direct contact or by aerosol (Everett *et al.* 1999). Transmission of *C. pecorum* between koalas is thought to primarily occur through sexual transmission, as koalas are more likely to become infected as they age and become sexually active (Jackson *et al.* 1999). Furthermore, *C. pecorum* is commonly found on swabs of the cloaca of females and penile urethra of males (Polkinghorne *et al.* 2013; Patterson *et al.* 2015) which supports the likelihood of sexual transmission. Sexually immature young can also become infected (Russell *et al.* 2018), which is likely from direct contact from an infected mother (Jackson *et al.* 1999; Legione *et al.* 2016b) or through the ingestion of pap (Blanshard and Bodley 2008). Koalas with subclinical chlamydial infection had higher chlamydial loads than koalas with overt disease (Nyari *et al.* 2019), which suggests that koalas with subclinical infection may be key in chlamydial transmission between koalas.

1.4.3. Prevalence of *Chlamydia* in Australia

Chlamydia spp. have been found in nearly all koala populations throughout Australia, including wild and captive populations. Koala populations in Queensland and New South Wales have shown a higher prevalence of *C. pecorum*, 52% and 49%, respectively, with a high prevalence of overt disease also reported in these populations (Polkinghorne *et al.* 2013). The prevalence of *C. pecorum* in wild Victorian koalas was found to be lower, with 15% infected and a low prevalence of mild urinary tract

infections observed (Patterson *et al.* 2015; Legione *et al.* 2016b). Magnetic Island, Queensland (Hirst *et al.* 1992) and French Island, Victoria (Patterson *et al.* 2015) were thought to be *Chlamydia*-free, however *C. pecorum* has recently been detected in two koalas from French Island with urinary incontinence (Legione *et al.* 2016a) and no recent studies have been conducted on Magnetic Island. The discovery of *C. pecorum* on French Island may be isolated cases of trans-species transmission, where koalas may have been infected by sheep infected with *C. pecorum* (Jelocnik *et al.* 2015) or the early detection of the emergence of *C. pecorum* in the French Island population. Continued monitoring and surveillance of *Chlamydia*-free populations is warranted to ensure early detection of the emergence of *C. pecorum* in these populations.

1.4.4. *Chlamydia* in South Australia

There have been fewer investigations into prevalence and pathology of chlamydial infections in South Australian koala populations. In the Kangaroo Island population, the first evidence for the presence of *Chlamydia* was based on radiographic opacities in the abdomen of a few female koalas which were thought to have been paraovarian cysts (Brown *et al.* 1984). Early serology provided conflicting evidence. One study did not detect anti-*Chlamydia* antibodies (n=63) (Robinson *et al.* 1989), but another study at the same time did detect anti-*Chlamydia* antibodies (Brown *et al.* 1987). Another serological study conducted in the late 1990s determined the seroprevalence of *Chlamydia* to be 18% (n=201) however no clinical signs were observed (Whisson and Carlyon 2010). Also, in the late 1990s, ten koalas were *C. pecorum* negative by PCR (Polkinghorne *et al.* 2013). With the lack of clinical signs observed, the populations high fecundity (Masters *et al.* 2004) and that these koalas originated from the previously *Chlamydia*-free French Island population (Robinson *et al.* 1989; Legione *et al.* 2016a), a general consensus was reached that the Kangaroo Island population was *Chlamydia*-free.

In the Mount Lofty Ranges there have been more recent studies into the presence of *Chlamydia* within the population. Initially, the study by Brown *et al.* (1984) also performed radiographs of female koalas from the Mount Lofty Ranges and found opacities which could have been paraovarian cysts. Serology was also performed and detected anti-*Chlamydia* antibodies in an unspecified number of koalas from the population, whether these were wild or captive koalas is unknown (Brown *et al.* 1987). The presence of *C. pecorum* was confirmed in the Mount Lofty Ranges in small numbers of healthy, free ranging koalas by PCR (6/6) (Houlden and St John 2000). Another survey at the same time based in captive koalas found 88% and 53% were *C. pecorum* and *C. pneumoniae* positive, respectively (n=17), however no clinical disease was reported in these koalas (Polkinghorne *et al.* 2013). Between 1997 and 2012, there were no studies undertaken to detect *C. pecorum*, and anecdotally there were no reports of chlamydial disease observed by local veterinarians (N. Speight pers coms). The first cases of overt ocular disease were observed in three koalas from the Mount Lofty Ranges in 2012. These koalas presented to local veterinarians with bilateral conjunctivitis and were diagnosed with *C. pecorum* by PCR (Funnell *et al.* 2013). As overt disease had not previously been reported, these first cases raised the questions as to whether *C. pecorum* disease was becoming more prevalent, or if a low level of disease had been present since the introduction of the Queensland and New South Wales koalas into the population (Lindsay 1950; Robinson 1978).

A subsequent study in euthanised, necropsied koalas reported 88% of koalas to be infected (57/65) with 21% (n=12) having overt ocular or urinary tract disease which was mild in nature (Speight *et al.* 2016). Another study of necropsied koalas reported 12% (10/85) had mild ocular and urogenital chlamydial disease (Speight *et al.* 2018). These studies showed clinical disease in the Mount Lofty Ranges was possibly emerging, as cases were becoming more prevalent in rescued koalas. The implications of increasing disease prevalence in this population are unknown. As these

reports have been within rescued, euthanased koalas, the prevalence of *C. pecorum* disease may be over-represented. No studies have been conducted in wild-caught koalas, therefore future studies should determine the prevalence of *C. pecorum* and clinical disease in the wild Mount Lofty Ranges koala population.

1.4.5. Comparisons of northern and southern chlamydial infections

The reasons behind the highly prevalent, severe chlamydial disease in northern koalas compared with the low prevalence of mild disease in southern koalas is unknown. Chlamydial infections in other species are predominantly subclinical, where clinical disease development is often attributed to the opportunistic nature of *Chlamydiaceae* (Ladds 2009). Opportunistic pathogens do not normally cause disease within the host, and disease usually only develops when there is a reduction in the efficiency of the host's immune system (Ladds 2009). This suggests there may be factors in northern koala populations which predispose the koalas to commonly develop severe clinical disease.

Factors which may influence disease development include variation in *C. pecorum* isolates, koala genetics, stress, the presence of concurrent infections with other pathogens and immunosuppression. *C. pecorum* has been found to possess a plasmid, *pCpec*, which may carry a virulence factor. The plasmid was found to be considerably more prevalent in *C. pecorum* isolates from Queensland, New South Wales and Victoria, at 72.7%, 84.2% and 78.5%, respectively, than in isolates from the Mount Lofty Ranges, where the prevalence of *pCpec* was 11% (Jelocnik *et al.* 2015) and was weakly associated with the development of overt chlamydial disease (Phillips *et al.* 2018). Disease may also vary in its development and progression due to genetic differences between koala populations (Neaves *et al.* 2016; Kjeldsen *et al.* 2018), where genetic differences in the immune system may interact in different ways to control *C. pecorum* infection. Studies have shown upregulation of Th1 and Th17

pathways in relation to chlamydial disease in Queensland koalas (Mathew *et al.* 2013a; Mathew *et al.* 2014) but no investigation has occurred in southern koala populations. Another immune modulator is chronic stress (Brown *et al.* 1984; Lanyon and Sanson 1986), which could occur from habitat fragmentation and urbanisation, and contribute to the development of clinical chlamydial disease (Canfield *et al.* 1989; Jackson *et al.* 1999). However, Griffith *et al.* (2013) did not identify an increase in overt clinical disease in New South Wales koalas, over a 30-year period in a continually urbanised area.

Concurrent infections may also initiate chlamydial disease development. The Koala retrovirus (KoRV), also a prevalent pathogen in northern koalas (Simmons *et al.* 2012), may predispose the koalas to the development of clinical chlamydial disease through immune modulation (Tarlinton *et al.* 2005). Retroviruses, such as human immunodeficiency virus (HIV), are well known for causing immune suppression in their respective hosts (Denner 1998), where secondary infections are often observed (Oostendorp *et al.* 1993). It is therefore possible that northern koalas are more susceptible to chlamydial disease due to concurrent KoRV infection. As the prevalence of KoRV in southern koalas is lower (Simmons *et al.* 2012), these populations may provide an opportunity to investigate the interactions between KoRV and *C. pecorum* in koalas.

1.5. Koala retrovirus (KoRV)

Koala retrovirus (KoRV), a gammaretrovirus (Hanger *et al.* 2000), is an enveloped virus containing two single stranded, positive sense RNA genomes. Upon infection, retroviruses fuse with the host cell membrane of mononuclear cells (leukocytes) (Murphy *et al.* 2018) and the capsid is released into the host cell. This fusion stimulates viral reverse transcriptase to transcribe the RNA genome into a complementary double stranded DNA provirus which can be inserted into the host

chromosomal genome by the viral protein, integrase. Once inserted into the host genome, the provirus can be transcribed and translated using host cellular mechanisms to produce viral proteins and copies of the viral genome, that come together to form new virus particles upon exocytosis from the host cell (Boeke and Stoye 1997). KoRV possess three key genes; *gag*, *pro*/*pol* and *env*; the *gag* gene codes for structural proteins, including capsid, nucleocapsid and matrix proteins; the *pro* gene codes for protease proteins which cleave the cellular lipid bilayer around the capsid and beneath the envelope upon cell entry; the *pol* gene codes for integrase and reverse transcriptase which are vital for proviral insertion into the host cellular DNA, and the *env* gene, which codes for surface and transmembrane proteins. Additionally, KoRV contains transcriptional promotor regions termed long terminal repeats (LTR) that can act on both viral and host cellular genes (Boeke and Stoye 1997).

1.5.1. Transmission of retroviruses and KoRV

Endogenous retroviruses are a key feature of all mammalian species and are indirectly pathogenic (Maksakova *et al.* 2008). Endogenous retroviruses were once exogenous retroviruses that transmitted horizontally between hosts (Hoover *et al.* 1976). These exogenous retroviruses infected a germline cell which consequently produced offspring and resulted in the retrovirus being present in every somatic and germline cell, and the formation of a heritable endogenous retrovirus (Denner and Young 2013). Ancient endogenous retroviruses which integrated into the host genome centuries ago have since become inactivated due to mutation (Lander *et al.* 2001) or silencing through methylation (Rowe *et al.* 2010; Turelli *et al.* 2014). Some endogenous retroviruses which intergrated more recently may become reactivated and pathogenic by concurrent infections with another exogenous retrovirus, such as human endogenous retrovirus K (HERV-K) which can be reactivated by human immunodeficiency virus (HIV) (Young *et al.* 2018) and feline endogenous retroviral

elements which recombine with exogenous feline leukaemia virus A (FeLV-A) and produce pathogenic FeLV-B (Hartmann 2011).

KoRV is a unique retrovirus, which is endogenous in northern koala populations and apparently exogenous in southern koala populations (Tarlinton *et al.* 2006; Simmons *et al.* 2012). KoRV was shown to be endogenous in northern koalas by southern blotting methods, where KoRV was present in all northern koala tissues, whilst koalas from the southern Kangaroo Island population were KoRV-free (Tarlinton *et al.* 2006). The proviral copy number, or proviral load, was also consistently high in northern koalas with approximately 165 copies per cell, while in Victorian koalas the proviral load was as little as 1 copy per million cells, which suggests an exogenous, infectious virus is present in southern populations (Simmons *et al.* 2012). The mechanisms by which KoRV is transmitted exogenously are unknown, however direct contact is likely, through bodily fluids, such as saliva which is how FeLV infects cats (Hartmann 2012) or by transmission vectors, such as Arthropoda (Manet *et al.* 1989) that were shown to transmit KoRV from an infected blood sample to a non-infected blood sample (Simmons 2011).

The introduction of KoRV into the koala population likely occurred thousands of years ago, with molecular clock analysis and proviral insertion sites providing evidence for endogenisation events. However, this analysis has proved difficult for KoRV due to the two transmission methods. When analysed as an endogenous retrovirus, KoRV would have entered the koala genome millions of years ago, whereas an exogenous KoRV would have entered only decades ago (Bromham 2002). One study estimated the maximum time for endogenisation initiation to be between 22,200-49,900 years ago (Ishida *et al.* 2015b). KoRV provirus has been detected in DNA isolated from koala skins that were harvested at the turn of the 20th Century and sourced from museums. KoRV was present in all northern koala skins, and two

southern koala skins were KoRV-free (Avila-Arcos *et al.* 2013). An ancient endogenous virus would also have consistent proviral insertion sites across individual koalas. A few studies have now investigated the proviral insertion site within live, modern day koalas from Queensland, New South Wales and Victoria and in koala museum skins. These studies have not identified any consistent proviral insertion sites between koalas across Australia (Tsangaras *et al.* 2014; Ishida *et al.* 2015b; Cui *et al.* 2016; Hobbs *et al.* 2017; Johnson *et al.* 2018). The evidence from these studies suggest KoRV is continuing active endogenisation in northern koalas, as proviral insertion sites are yet to stabilise within the population, and KoRV is in the early stages of endogenisation in some Victorian koalas

1.5.2. Origin of KoRV in Australia

The evidence for KoRV transmitting as both an endogenous and exogenous virus suggests that KoRV is undergoing active endogenisation of the koala genome and the recent evolution of KoRV. It is likely that KoRV was produced as a result of recent trans-species transmission. The most closely related retrovirus to KoRV is the exogenous gibbon ape leukaemia virus (GaLV), first identified in captive gibbons in south-east Asia (Kawakami *et al.* 1972; Delassus *et al.* 1989). Due to the vast geographical distance between GaLV and KoRV, and the genetic diversity between placental and marsupial mammals, it is unlikely this retrovirus transmitted directly between gibbons and koalas, and therefore an unknown intermediate vertebrate host must exist to have facilitated this trans-species transmission.

Rodents and bats are possible intermediate hosts as their range spans across all continents, therefore providing the opportunity to transfer gammaretroviruses from south-east Asia to northern Australia. An endogenous retrovirus in the Asian mouse (*Mus caroli*) was found to have a greater sequence identity to GaLV than *Mus musculus* retroviruses (Lieber *et al.* 1975). The *Melomys burtoni* retrovirus (MbRV) was

recently discovered in the Australian native rodent species *Melomys burtoni,* distributed through Queensland, and has high levels of sequence identity to GaLV and KoRV, but more identity to GaLV (Simmons *et al.* 2014a). These rodent retroviruses are possible candidates for transmission between rodents and gibbons to koalas (Simmons *et al.* 2014b). Bats also harbor a number of endogenous and exogenous retroviruses (Hayward *et al.* 2013). Recently a gammaretrovirus was found in the Australian black flying fox (*Pteropus alecto*) (McMichael *et al.* 2019), where both viral species had close sequence identity with the mammalian retroviral clade of retroviruses, including KoRV and GaLV. Further investigations of this clade of retroviruses in trans-continental species are required to understand this intriguing trans-species transmission, and investigations into KoRV in southern koala populations may shed light on the mechanisms of exogenous transmission, and the process of endogenisation.

The high prevalence of endogenous KoRV in northern koalas may be due to an extended period of contact between koalas and KoRV within these populations, if KoRV was first introduced into northern Australian koalas, as hypothesised from the similarities of MbRV and GaLV with KoRV. However, there may be other vectors or genetic factors present that differ between the northern and southern populations that may have facilitated or inhibited the spread of exogenous KoRV. Arthropoda are known transmission vectors of other retroviral infections, such as bovine leukaemia virus (BLV) (Manet *et al.* 1989), and may be a transmission vector for KoRV in northern populations, as mosquitoes, tabanid flies and paralysis ticks have been shown to transfer KoRV from one infected blood sample to another (Simmons 2011). Or koalas from the southern genetic lineage (Neaves *et al.* 2016) may have a variation in genetic immunity that has slowed the endogenisation and/or exogenous spread of KoRV. Understanding the susceptibility of southern koalas to exogenously acquired KoRV infection may highlight these differences between northern and southern koalas.

1.5.3. Prevalence of KoRV

It is possible KoRV was first introduced into the northern koala populations from Asia and has since migrated into southern koala populations, which correlates with the distribution pattern of KoRV throughout Australia. KoRV is 100% prevalent in northern Australian populations and endogenous, while the prevalence in southern koala populations is lower, thought to be exogenous, and appears to decrease from an east to west direction (Simmons *et al.* 2012). Initially the prevalence of KoRV was thought to be as high as 81.8% in mainland Victorian koalas (Simmons *et al.* 2012), however a recent study of KoRV in Victorian koalas found the prevalence to be 24.7% (160/648) but varied between 17-40% across the state (Legione *et al.* 2017). The only koala population to have been investigated in South Australia is Kangaroo Island, which was initially thought to be KoRV-free in 2006 (n=26) (Tarlinton *et al.* 2006) until the detection of KoRV in 2012 at 14.8% (24/162) (Simmons *et al.* 2012). This discovery may be due to an increased sample size as KoRV is present in the ancestral French Island population. The prevalence of KoRV in Mount Lofty Ranges koalas has not been investigated.

1.5.4. KoRV variants

Investigations of the KoRV genome have discovered the existence of multiple KoRV variants, which differ in the sequence of the *env* gene and have resulted in translational changes to the transmembrane proteins. There are currently 10 known KoRV variants, including KoRV-A, the first endogenous KoRV sequence identified (Tarlinton *et al.* 2006; Xu *et al.* 2013), and six other variants that are thought to be exogenously acquired, including; KoRV-B (Xu *et al.* 2013), KoRV-C, KoRV-D (Miyazawa *et al.* 2011), KoRV-E, KoRV-F (Xu *et al.* 2015) and KoRV-J (Shojima *et al.* 2013b) discovered in captive northern koalas. There are a number of similarities between KoRV-B and KoRV-J which suggests these viruses are of the same

phylogenetic clade (Young 2014). As retroviruses have no proof-reading mechanism of reverse transcriptase, a high error rate during viral transcription and proviral synthesis is common. These errors facilitate the evolution of retroviruses and allow the retrovirus to maintain infectivity (Rosenberg and Jolicoeur 1997) but can also result in mutations which facilitate the loss of virulence factors that may promote endogenisation within the host genome (Oliveira *et al.* 2007). The development of these variants is therefore an evolutionary process of KoRV and highlights that KoRV is still an active virus in northern koalas.

The few studies to investigate the prevalence of KoRV variants in southern koala populations have shown that some koalas may not be infected with KoRV-A. In Victorian koalas positive for the KoRV *pol* gene, KoRV-A was detected in 88.1% (141/160) of koalas and KoRV-B was not detected by conventional PCR (Legione *et al.* 2017), and for the 19 koalas that were negative for KoRV-A and KoRV-B, another variant may have been present as no simple diagnostic tests are currently available. In South Australia, one Kangaroo Island koala was investigated in 2007 where a sequence similar to KoRV-C was identified (Young 2014). Due to the translocations of koalas in the early 20th century, the prevalence of KoRV and KoRV variants that entered South Australia from Victoria may be highly variable. Perhaps some translocated koalas from French Island may have been free from KoRV-A infection, but infected with another variant, such as KoRV-C which may be more pathogenic in southern koalas compared to northern koalas. There is a strong need for investigation of KoRV variants in southern koalas, which may shed light on KoRV pathogenicity across Australia.

1.6. KoRV associated diseases

While many koalas with KoRV infection are clinically healthy, some koalas develop diseases that have been associated with retroviral infection in other species.

Lymphoid neoplasia is commonly observed in the koala (Canfield *et al.* 1990; Connolly *et al.* 1998) and has been reported in association with retroviral infection in other species including cows (Gillet *et al.* 2007), cats (Hartmann 2012) and gibbons (Kawakami *et al.* 1972). Additionally, retroviruses can cause immune suppression in their host which leads to diseases from secondary infections. *Chlamydia* has been associated with retroviral induced immune suppression in cats (O'Dair *et al.* 1994) and humans (Monno *et al.* 1997). Evidence is starting to accumulate to support an association between chlamydial disease and KoRV infection in the koala (Tarlinton *et al.* 2005; Waugh *et al.* 2017; Quigley *et al.* 2018a) which may account for the high prevalence of chlamydial disease in northern koala populations.

1.6.1. Disease-free KoRV infected koalas

Whilst KoRV is 100% prevalent in northern koala populations, many koalas are clinically healthy. In a study of captive northern koalas in Japan, six clinically healthy KoRV provirus-positive koalas were negative for KoRV viraemic RNA, which suggests these koalas were not actively transcribing the virus at the time of sampling. Another koala in this study initially was actively viraemic and had an elevated leukocyte count but was no longer viraemic with a normal leukocyte count when re-examined 5 months later, which may suggest a recent infection which has been subdued by the immune system (Kayesh *et al.* 2019). In a recent study of KoRV positive, wild Queensland koalas sampled between 2013 and 2017, 68.8% (192/279) of koalas were clinically healthy at veterinary examination, and disease observed included five koalas with lymphoid neoplasia, 75 koalas had overt chlamydial disease and seven koalas had disease attributed to immune suppression (Quigley *et al.* 2018b). The reason why some koalas develop disease and not others is likely to be a complex relationship that involves a number of viral and host immunity factors.

The immune response to retrovirus infection may be important in the health status of infected koalas. A study of cats inoculated with FeLV-A found that a number of cats that appeared clinically healthy had no detectable FeLV viraemia (Hartmann 2012). In studies of experimentally infected cats, it has been shown that the cat's first immune response to FeLV is critical in the development of lymphoid neoplasia, and as a result there are three categories of immune response in cats to FeLV. "Abortive" cats develop a strong neutralising antibody and cytotoxic T cell response within the first few weeks after infection which effectively eliminates FeLV viraemia, however cats can remain provirus positive (Flynn *et al.* 2002). In cats with a "regressive" infection, FeLV infects progenitor cells within the bone marrow early in infection which slowly disseminates through mononuclear leukocytes (Hartmann 2012), and while these cats can also eliminate FeLV viraemia, they have a latent infection that can be reactivated (Rojko *et al.* 1982). In "progressively" infected cats, the bone marrow is also infiltrated by FeLV soon after the infection (Hartmann 2012) and they do not produce an effective immune response; these cats exhibited a delay in cytotoxic T cell response of up to seven weeks post-infection, continuously shed FeLV (Flynn *et al.* 2002) and develop neoplasia within a few years of infection (Hartmann 2012). Regressive cats tended to have lower proviral loads while progressive cats had high proviral loads (Hartmann 2012). These three infection scenarios may highlight how KoRV causes disease in the koala, where koalas that are KoRV provirus-positive and RNA viraemia negative may have a regressive infection and koalas with a progressive infection develop lymphoid neoplasia. Longitudinal studies monitoring natural KoRV infection in individual koalas would provide an opportunity to investigate the immune response of koalas with recent exogenous infections, or the regression of a latent infection and disease development.

1.6.2. Lymphoid neoplasia

Lymphoid neoplasia, lymphosarcoma and leukaemia, are the most common forms of neoplasia in both captive and wild koala populations (Canfield 1990; Pye *et al.* 2014). The first reports of lymphoid neoplasia in koalas were in 1961 (Backhouse and Bolliger 1961; Heuschele and Hayes 1961), with further cases of lymphosarcoma reported in post-mortem studies (Arundel *et al.* 1977; McKenzie 1981; Canfield *et al.* 1988; Spencer and Canfield 1996). Up to 40% of deaths of captive northern koalas were attributed to lymphosarcoma (Gillett 2014). The prevalence in wild northern koala populations was previously reported to be between 3-6% (Backhouse and Bolliger 1961; McKenzie 1981; Spencer and Canfield 1996), however a recent study reported a lower prevalence of 1.0% (3/290) in wild Queensland koalas (Quigley *et al.* 2018b).

Lymphoid neoplasia can develop in numerous anatomical regions, in both lymphoid and non-lymphoid tissues. Koalas may develop primary lymphoid leukaemia, characterised by the lack of development of tumours, or lymphosarcoma with secondary leukaemia as a result of neoplastic bone marrow infiltration (Heuschele and Hayes 1961; Spencer and Canfield 1995; Canfield and Hemsley 1996; Connolly *et al.* 1998; Kido *et al.* 2012). It is not uncommon for wild koalas to present in a late stage of disease, in which the origin of leukaemia as primary or secondary cannot be deduced (Spencer and Canfield 1996).

Neoplastic lymphoid cells are usually small to medium in size (4-8 µm), with round to ovoid shaped nuclei, minimal cytoplasm, with variable nucleoli visibility and chromatin patterns (Backhouse and Bolliger 1961; Heuschele and Hayes 1961; Canfield and Hemsley 1996; Spencer and Canfield 1996; Connolly *et al.* 1998; Kido *et al.* 2012). Neoplastic lymphocytes may be observed on blood smear examination and are also observed infiltrating the affected organs (Backhouse and Bolliger 1961; Connolly *et al.* 1998). Bone marrow is often infiltrated to the degree that all normal

structure is lost, with few erythroid and myeloid regions remaining in the bone marrow (Canfield *et al.* 1987; Spencer and Canfield 1996), resulting in anaemia and blood cell morphology changes.

Lymphosarcoma predominantly affects peripheral lymph nodes and is characterised by neoplastic infiltration resulting in the loss of normal lymph node follicular structure (Heuschele and Hayes 1961; Canfield *et al.* 1987; Canfield and Hemsley 1996; Spencer and Canfield 1996). Lymph node tumours are usually enlarged, tan coloured, flattened and ovoid in shape (Heuschele and Hayes 1961; Canfield and Hemsley 1996). Lymphosarcoma may be isolated to the lymph nodes however multi-organ involvement is commonly observed (Backhouse and Bolliger 1961; Heuschele and Hayes 1961; Canfield *et al.* 1987; Canfield 1990; Spencer and Canfield 1996) whereby neoplastic cells diffusely infiltrate or develop defined foci within an organ (Canfield *et al.* 1987; Spencer and Canfield 1996). Anatomically, lymphosarcoma is generally classified as *multicentric*, involving multiple organs and tissues, or as *alimentary* which is predominantly isolated to alimentary lymphoid tissue but may metastasize into other organs (Spencer and Canfield 1996; Connolly *et al.* 1998). Affected organs may be observed with tan or white coloured nodules, with the splenic parenchyma and hepatic portal triads of the liver being common sites, and thymic involvement also reported (Backhouse and Bolliger 1961; Heuschele and Hayes 1961; Canfield *et al.* 1990; Canfield and Hemsley 1996; Spencer and Canfield 1996; Connolly *et al.* 1998). Other non-lymphoid tissues involved are usually diffusely infiltrated, and include adrenal glands, central nervous system, gastrointestinal tract, lungs, pancreas, skeletal muscle and urogenital tract (Heuschele and Hayes 1961; Canfield *et al.* 1987; Spencer and Canfield 1996; Connolly *et al.* 1998; Kido *et al.* 2012).

The immunophenotype of lymphoid neoplasia in koalas from Queensland and New South Wales was determined in a study by Connolly *et al.* (1998). T and B cell

lymphosarcomas were observed but T cell tumours more prevalent, observed at 51% and 24%, respectively. T-cell leukaemia was also more commonly observed than B cell leukemia. The study reported no correlation between the location of the tumour and immunophenotype, nor with age or sex of the koalas (Connolly *et al.* 1998).

Leukaemic changes can be observed through haematological analysis of koalas with lymphosarcoma. A study of lymphoid neoplasia observed haematological values of 22 koalas (Spencer and Canfield 1996). All koalas (n=17) with multicentric lymphosarcoma and bone marrow involvement and koalas with primary leukaemia presented with haematological changes, whilst koalas without bone marrow involvement had no haematological changes (n=5). Four of these five koalas had alimentary lymphosarcoma and the fifth koala had a splenic mass. Haematological changes observed in koalas with bone marrow involvement were variable with non-specific changes due to the anatomical location of lymphosarcoma (Spencer and Canfield 1996). Koalas may present with leucopaenia as a result of neutropaenia and lymphopaenia, while other koalas may present with leukocytosis due to lymphocytosis from circulating neoplastic lymphoid cells. Koalas may also present with regenerative and non-regenerative anaemia, thrombocytopaenia and hypoalbuminaemia (Heuschele and Hayes 1961; Canfield *et al.* 1987; Canfield *et al.* 1990; Spencer and Canfield 1996).

1.6.2.1. Association between KoRV and lymphoid neoplasia

With the increasing number of koalas reported with lymphoid neoplasia, it was hypothesised there may be viral involvement (Arundel *et al.* 1977). The first evidence was discovered in 1988 where type C oncovirus particles were discovered in the bone marrow of a captive female koala with leukaemia (Canfield *et al.* 1988). With the development of PCR, partial sequences of a retrovirus were discovered in the 1990s (O'Brien *et al.* 1997), which lead to the classification of KoRV in 2000 (Hanger *et al.*

2000). Further studies investigated the association between KoRV infection and lymphoid neoplasia in northern koalas, where koalas with lymphoid neoplasia had significantly higher KoRV viraemic loads than clinically healthy KoRV infected koalas (Tarlinton *et al.* 2005). KoRV-B and KoRV-J were detected in captive northern koalas with leukaemia (Shojima *et al.* 2013a), whilst another study found that 50% (3/6) of koalas with KoRV-B infection developed leukaemia (Xu *et al.* 2013). More recently in wild Queensland koalas, lymphoid neoplasia was significantly associated with KoRV-B infection, however interestingly, one koala with lymphosarcoma was KoRV-B negative (Quigley *et al.* 2018b).

All reports of lymphoid neoplasia have been in northern koalas and have presumably been KoRV positive, based on the 100% prevalence reported in these populations (Simmons *et al.* 2012). There have also been a number of reports of other neoplastic diseases in northern koalas which may also be attributed to KoRV infection. Other neoplastic diseases observed in both captive and wild koalas include osteochondroma, fibrosarcoma, mesothelioma, haemangiosarcoma and renal, gastrointestinal and mammary adenocarcinomas (Gillett 2014; Hanger and Loader 2014; Mulot 2014; Pye *et al.* 2014). There have been no reports of lymphoid neoplasia or other neoplastic diseases in southern koalas, which warrants investigation into the prevalence and types of neoplasia in southern KoRV-infected and KoRV-free koalas.

1.6.2.2. Tumourigenesis

There is currently no direct causal link between KoRV infection and tumourigenesis, however there is evidence to support this theory (Tarlinton *et al.* 2005; Xu *et al.* 2013). The processes of KoRV induced tumourigenesis are likely to be complex and a combination of both viral and host factors. Viral factors that may alter the pathogenicity of KoRV could include KoRV variant genome sequence variations and proviral insertion sites into the host genome. KoRV-A is considered to be non-

pathogenic, as endogenous infections eventually become defective viruses due to mutation (Gifford and Tristem 2003; Rowe *et al.* 2010; Turelli *et al.* 2014), while exogenous KoRV-B has been associated with lymphoid neoplasia in the koala (Xu *et al.* 2013). KoRV-B and KoRV-J have duplications in the LTR promotor region that are not present in KoRV-A, and these duplications may have a greater potential for tumourigenesis through upregulation of adjacent genes (Xu *et al.* 2013). Insertion site has particularly malignant consequences when proviral insertions occur adjacent to oncogenes, most commonly the host *myc* gene, resulting in over expression of the oncogene which in-turn leads to unregulated proliferation of the infected cell (Hartmann 2011). As the target site or sites of KoRV proviral insertion have shown considerable diversity (Tsangaras *et al.* 2014; Ishida *et al.* 2015b; Hobbs *et al.* 2017; Johnson *et al.* 2018), and a number of insertions were discovered in introns that would have little impact on gene expression (Johnson *et al.* 2018), proviral insertion site may not be a key role in KoRV induced tumourigenesis. Investigation into KoRV variants, the insertion site and orientation will aid in the understanding of viral factors associated with tumourigenesis in the koala.

Alternatively, lymphoid neoplasia may arise at the end stage of disease. Longitudinal studies have monitored the progression of disease in cats inoculated with feline immunodeficiency virus (FIV) (Eckstrand *et al.* 2016; Eckstrand *et al.* 2017a; Eckstrand *et al.* 2017b; Murphy *et al.* 2018). In a recent report, an 8-year-old cat developed lymphosarcoma after an eight-year period of asymptomatic infection. The cat's first clinical signs observed were weight loss, fever, progressive leucopaenia and non-regenerative anaemia with lymphosarcoma diagnosed at necropsy examination. This study was able to compare the FIV genome sequence in the tumours to the initial inoculum given to the cat. There was a single SNP mutation in the LTR promotor region which was thought to have had no effect on viral oncogenic potential. The proviral and viral loads were also compared post-mortem to biopsies of spleen, small intestines

(Eckstrand *et al.* 2017b) and lymph node (Eckstrand *et al.* 2016) collected ante-mortem 6 years post-infection, where the proviral load was higher in all tissues at necropsy (Murphy *et al.* 2018). While the cause for progression from asymptomatic to diseased in this cat was undetermined, this longitudinal study has shown that lymphosarcoma can develop in apparently healthy cats with a corresponding increase in proviral and viral loads.

1.6.3. Diseases associated with poor immune system function in koalas

KoRV infection may also predispose koalas to diseases from secondary pathogens by suppressing the koala's immune system. Immunosuppression is a common clinical sign of retroviral infection in other species and is associated with increasing viral load (Denner 1998; Torres *et al.* 2008), and therefore this may also occur in KoRV infected koalas.

Evidence for retroviruses to modulate the immune system can be found within the retroviral genome. In the genome of many mammalian retroviruses, including KoRV, there is a CETTG *env* protein motif (Oliveira *et al.* 2007; Xu *et al.* 2013), also known as the immunosuppressive domain (ISD) within the outer membrane protein, p15E (Cianciolo *et al.* 1985). The ISD genome sequence is identical between KoRV, GaLV, FeLV, porcine endogenous retrovirus (PERV) and murine leukaemia virus (MLV), with homologous regions in HIV and human T lymphotrophic virus (Cianciolo *et al.* 1985; Denner 1998; Fiebig *et al.* 2006; Ishida *et al.* 2015a) and has been shown to alter cytokine and cellular activity in cats (Wellman *et al.* 1984), humans (Denner 1998) and mice (Cianciolo *et al.* 1985) with retroviral infections. As the KoRV genome also includes the CETTG motif (Hanger *et al.* 2000), it is possible KoRV may also modulate the immune system by similar pathways in koalas.

Retroviruses, as intracellular pathogens, require a strong Th1 immune response to clear infections. $CD4^+$ T lymphocytes respond in one of two ways to MHC

II complexes; they will either activate as Th1 cells which promote phagocyte or cytotoxic T-cell activity or activate as Th2 cells which respond by stimulating B-cells to generate pathogen-specific immunoglobulins. $CD8^+$ T lymphocytes respond to MHC I complexes and differentiate into cytotoxic T cells which use cytolytic granules to rupture host cells with intracellular pathogens (Doan *et al.* 2008). It has been hypothesised that retroviral infections may promote a shift from Th1 to Th2 pathways (Clerici and Shearer 1993), which would reduce the activity of cytotoxic T-cells and prolong retroviral infection. This shift to a Th2 dominant response is shown in the downregulation of $CD4^+$ cells in humans infected with HIV (Mellors *et al.* 1997) and cats with FeLV (Hartmann 2012). The downregulation of $CD4^+$ cells then alters the CD4:CD8 ratio in both species (Mellors *et al.* 1997; Hartmann 2012), reducing the capacity of $CD4^+$ cells to promote cytotoxic T-cell activity.

Retroviruses are also known to modulate cytokine expression. Key cytokines of the Th1 pathway include interleukin 2 (IL-2), interferon gamma (INFϒ) and tumour necrosis factor alpha (TNFα) and in the Th2 pathway key cytokines include IL-4, IL-6 and IL-10: IL-2 which stimulates T-cell proliferation, IL-4 promotes differentiation of B-cells, IL-6 promotes inflammation, IL-10 stimulates the differentiation of Th2 cells, INFϒ enhances MHC I and II expression and acts as an antiviral agent and TNFα acts as a mediator for inflammation (Doan *et al.* 2008). *In vitro* studies of human lymphocytes infected with HIV promoted the downregulation of the Th1 pathway by reducing IL-2 and TNFα expression and upregulated the Th2 pathway by increased expression of IL-4, IL-10 and INFϒ (Denner 1998). However, in FeLV infected cats the Th1 to Th2 shift was less clear (Linenberger and Deng 1999; Graham *et al.* 2003). The Th1 pathway was altered by TNFα upregulation and inconsistent regulation of INFϒ, and in the Th2 pathway, IL-4 was downregulated (Hartmann 2012). In cats with naturally occurring FIV, no clear change in pathways was observed (Leal *et al.* 2015).

More recently, it has been proposed that retroviral infections may promote immune dysregulation rather than immune modulation (Tompkins and Tompkins 2008).

Limited studies of the koala's immune response to KoRV infection have been undertaken, with the first studies investigating whether endogenous KoRV infected koalas could produce anti-KoRV antibodies. One study on captive koalas found no antibody response to KoRV-A antigens (Fiebig *et al.* 2015) while in wild Queensland koalas anti-KoRV IgG antibodies were detected in vaccinated koalas (Waugh *et al.* 2016b). The only study to characterise cytokine and cell expression *in vitro* was performed on koala leukocytes collected from captive northern koalas in New South Wales (Maher and Higgins 2016). In this study, mRNA expression of cytokines involved in the Th1 (INFϒ, TNFα), Th2 (IL-4, IL-6, IL-10) and Th17 (IL-17a) pathways and $CD4^+$ and $CD8^+$ gene expression were compared between KoRV-B positive and negative koalas. There was no clear shift observed from a Th1 to Th2 pathway, the Th17 pathway was upregulated, and there was no change in CD4:CD8 ratio (Maher and Higgins 2016). These results suggested immune dysregulation rather than modulation, however further studies are needed to describe the processes of KoRV induced immune modulation in the koala.

Disease surveys in captive and wild koalas have highlighted a number of infections which may develop in immunocompromised koalas. In a study of captive northern koalas in a zoo in the United States, KoRV was suspected to have an underlying involvement in the euthanasia of 12.4% (21/169) of koalas that developed opportunistic infections, while KoRV associated disease (neoplasia) was the reason for euthanasia in 28.4% (48/169) of koalas (Pye *et al.* 2014). In mortality surveys of wild koalas, there have been a number of koalas with no definitive diagnosis for the cause of death (Obendorf 1983; Canfield 1990). In Victoria, these koalas were described as having a "koala stress syndrome" and commonly present with a poor

body condition, lethargy and depression, and decline during veterinary care with further weight loss and no response to antibiotics. These koalas were observed with leucopaenia and thrombocytopaenia which could be consistent with lymphoid leukaemia, however their KoRV status is unknown (Obendorf 1983). Another mortality survey of wild northern koalas from New South Wales reported similar findings in some koalas, with anaemia and hypoalbuminaemia but no post-mortem abnormalities (Canfield 1990). More recently, a study of wild Queensland koalas determined that seven koalas had KoRV-associated disease with chronic ill-thrift, poor body condition score, dermatitis, stomatitis, severe periodontal disease and gastrointestinal disease observed at clinical examination (Quigley *et al.* 2018b). It is apparent there are a number of potentially opportunistic or secondary pathogens in the koala, for which disease development may be predisposed to by KoRV infection. As these studies have all occurred in endogenous KoRV positive koalas, or koalas with unknown KoRV infection status, it is important for future studies to further investigate the association between KoRV and opportunistic/secondary pathogens in southern koalas, and to describe the immune response of koalas with exogenous KoRV infection.

1.6.4. Association between *C. pecorum* disease and KoRV infection

Chlamydia spp. commonly do not cause disease in their host (Madigan *et al.* 2009), however, as described previously, northern koalas are more likely to develop severe overt chlamydial disease than to present with subclinical carriage (Polkinghorne *et al.* 2013) where this high prevalence of disease may be due to concurrent KoRV infection. In a study of wild Queensland koalas, koalas with overt chlamydial disease were observed with higher KoRV viral loads ($6.9x10^8$ copies/mL plasma) than clinically healthy koalas ($7.7x10^7$ copies/mL plasma) (Tarlinton *et al.* 2005). Based on this finding it was hypothesised that KoRV may have immunosuppressive effects that may result in chlamydial disease development.

As *C. pecorum* is an intracellular pathogen (Madigan *et al.* 2009), a dominant Th1 pathway and strong $CD8^+$ cell response is required to eliminate infected host cells with intracellular *Chlamydia* (Perry *et al.* 1997; Doan *et al.* 2008). Chlamydial elimination from a host has been associated with a strong Th1 pathway response, while the Th2 pathway has been associated with chlamydial persistence and the development of disease (Perry *et al.* 1997). If KoRV promotes a shift from Th1 to a Th2 pathway as other retroviruses do (Clerici and Shearer 1993), this may promote *C. pecorum* persistence in the koala. Or, if KoRV dysregulates the immune system as investigated by Maher and Higgins (2016) this may also promote *C. pecorum* persistence.

A number of studies have investigated the immune response of northern koalas to *C. pecorum* infection (Higgins *et al.* 2005a; Morris *et al.* 2015; Mangar *et al.* 2016), and particularly in response to chlamydial vaccination (Carey *et al.* 2010; Kollipara *et al.* 2012; Mathew *et al.* 2013a; Mathew *et al.* 2013b; Mathew *et al.* 2014; Nyari *et al.* 2019). These studies have shown that KoRV positive koalas can mount an immune response to *C. pecorum* infection (Higgins *et al.* 2005a; Carey *et al.* 2010; Kollipara *et al.* 2012; Mathew *et al.* 2013a; Mathew *et al.* 2013b; Mathew *et al.* 2014; Morris *et al.* 2015; Mangar *et al.* 2016), however, when koalas with chlamydial disease were vaccinated, their immune response to *C. pecorum* was boosted, with increased titres of anti-chlamydial antibodies detected in plasma (Kollipara *et al.* 2012). Clinical outcomes may also be improved by vaccination, with ocular disease severity in Queensland koalas reduced after *C. pecorum* vaccination (Waugh *et al.* 2016a). This evidence suggests that while KoRV positive koalas can mount an immune response to *C. pecorum* infection, it may not be sufficient to eliminate intracellular chlamydial bodies, as severe disease has been associated with high chlamydial loads (Wan *et al.* 2011).

Studies have begun to investigate whether an association between *C. pecorum* and KoRV infections exists in the koala. In wild Queensland koalas, KoRV-B infection has been positively associated with *C. pecorum* disease (Waugh *et al.* 2017). Concurrent lymphoid neoplasia (presumably due to KoRV) and overt chlamydial disease have also been reported in wild, necropsied koalas from New South Wales populations (Spencer and Canfield 1996). A study in Victorian koalas found no association between *C. pecorum* infection or disease and KoRV-A but did report an association between KoRV-A and wet-bottom disease which was present in *C. pecorum* negative koalas (Legione *et al.* 2017). The processes of KoRV immune modulation and the development of overt chlamydial disease are likely to be complex and need further investigation to identify risk factors and key processes leading to disease development. As the prevalence of both *C. pecorum* and KoRV are lower in southern koala populations, these populations may provide a unique opportunity to investigate if an association between these two infectious pathogens exists.

1.7. Conclusion

C. pecorum and KoRV are two key infectious pathogens causing mortality of koalas. The prevalence of *C. pecorum* and KoRV in northern Australian koala populations is high; koalas with *C. pecorum* infection commonly develop overt disease and lymphoid neoplasia is associated with KoRV infection. KoRV, like other retroviruses, may modulate the immune system predisposing the koala to disease caused by secondary pathogens, such as *Chlamydia*. Studies in northern koalas have provided evidence for KoRV induced immune modulation, and for an association between *C. pecorum* disease and KoRV infection. However, as KoRV-A is 100% prevalent in these northern populations, drawing definitive conclusions is difficult.

In southern Australian koalas the prevalence of *C. pecorum* is lower with reduced chlamydial disease severity reported (Patterson *et al.* 2015; Legione *et al.*

2016b; Speight *et al.* 2016). The prevalence of KoRV is also low in southern koalas, where KoRV-free koalas exist within the populations (Simmons *et al.* 2012; Legione *et al.* 2017). There has only been one study to investigate KoRV associated diseases in Victoria (Legione *et al.* 2017), and no reports of lymphoid neoplasia in southern koalas. It is evident that there is a significant lack of understanding of the prevalence of KoRV infection and associated diseases in southern koala populations. While it is important to know the prevalence of both pathogens in southern koalas for conservation management efforts, these populations also provide a unique opportunity to investigate *C. pecorum* disease development in exogenously infected KoRV koalas and will shed further light on KoRV pathogenesis.

1.8. Aims and objectives of the present study

The aims of this thesis were; (i) to determine the prevalence and describe disease associated with *C. pecorum* and KoRV in wild-caught South Australian koalas, (ii) to develop southern Australia koala haematological reference intervals, and (iii) to describe and compare disease in KoRV-infected, necropsied koalas from the Mount Lofty Ranges and Queensland populations forming part of the koala retrovirus pathogenesis project conducted in collaboration with The University of Queensland to further understand the pathogenicity of KoRV.

Chapter 2 focuses on the prevalence and disease of *C. pecorum* infection in wild-caught koalas from the Kangaroo Island and Mount Lofty Ranges populations and Chapter 3 focuses on the prevalence of KoRV in these same koalas and determines if KoRV is a risk factor for the development of *C. pecorum* disease. Southern koala haematological reference intervals are reported in Chapter 4. Chapter 5 focuses on the findings from necropsied koalas from the Mount Lofty Ranges. Chapter 5.1 presents the first report of lymphosarcoma in a female koala and Chapter 5.2 presents the extensive comparative pathological investigations of KoRV-positive koalas from

the Mount Lofty Ranges and Queensland populations. The findings of these studies will provide valuable information on the prevalence of infection and disease in South Australian koala populations that are required for informed population management decisions. These studies will also further contribute the understanding of *C. pecorum* disease development and the pathogenicity of KoRV.

1.9. References

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Chapter 2

Chlamydia pecorum prevalence in South Australian koala (*Phascolarctos cinereus*) populations: Identification and modelling of a population free from infection

Statement of Authorship

Title of Paper	*Chlamydia pecorum* prevalence in South Australian koala (*Phascolarctos cinereus*) populations: Identification and modelling of a population free from infection
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Principal Author

Name of Principal Author (Candidate)	Jessica Fabijan
Contribution to the Paper	Contributed to the study design and development, organised, facilitated and coordinated the capture and sampling of koalas in the Mount Lofty Ranges (MLR). Coordinated and collected swab samples from koalas from Kangaroo Island (KI) koala populations. Coordinated the collection and transport of samples from the MLR and KI field sample sites to the Roseworthy campus for storage. Processed swab samples for DNA extraction, ran PCR for quality control and organised transport of samples for C. pecorum detection at the University of Sunshine Coast. Interpreted and performed statistical analysis on MLR and KI data. Reviewed and processed historical data collected from KI koalas over a 22 year period. Contributed to the development of the model to demonstrate freedom of *C. pecorum* in KI koalas. Wrote manuscript.
Overall percentage (%)	85%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 9/4/19

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Developed the demonstration of freedom from *C. pecorum* infection model for the KI koala population Assisted in editing the manuscript		
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SCIENTIFIC REPORTS

OPEN

Chlamydia pecorum prevalence in South Australian koala (*Phascolarctos cinereus*) populations: Identification and modelling of a population free from infection

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Chlamydia pecorum **is an established and prevalent infection that produces severe clinical disease in many koala populations, contributing to dramatic population declines. In wild South Australian koala populations, *C. pecorum* occurrence and distribution is unknown. Here, *C. pecorum*-specific real-time quantitative PCR (qPCR) was applied to ocular and urogenital swabs from targeted surveys of wild koalas from the mainland Mount Lofty Ranges (MLR) (n = 75) and Kangaroo Island (KI) (n = 170) populations. Historical data from 13,081 KI koalas (1997–2018) provided additional evidence for assessing the absence of *C. pecorum* infection. In the MLR population, 46.7% (CI: 35.1–58.6%) of koalas were *C. pecorum* positive by qPCR but only 4% had grade 3 clinical disease. MLR koala fertility was significantly reduced by *C. pecorum* infection; all reproductively active females (n = 16) were *C. pecorum* negative, whereas 85.2% of inactive females (n = 23) were positive ($P < 0.001$). KI koalas were *C. pecorum* negative and the population was demonstrated to be free of *C. pecorum* infection with 95% confidence. *C. pecorum* is a real threat for the sustainability of the koala and KI is possibly the last isolated, large *C. pecorum*-free population remaining in Australia. These koalas could provide a safeguard against this serious disease threat to an iconic Australian species.**

Chlamydia pecorum is recognised as the most significant pathogen causing mortality in koalas and a key contributor to the dramatic population declines in northern Australia (Queensland and New South Wales)[1,2]. *Chlamydia* are obligate intracellular bacteria, of which two species, *C. pecorum* and *C. pneumoniae*, can infect and cause disease in wild and captive koalas[3]. *C. pecorum* is the most pathogenic species, causing conjunctivitis and blindness[4,5], pneumonia[6], urinary tract infections (cystitis and nephritis) associated with urinary incontinence[7,8]; and reproductive tract infections resulting in infertility in both females and males due to, paraovarian cysts, endometritis and vaginitis[9], and orchitis, epididymitis and prostatitis, respectively[10]. *C. pneumoniae* infection can cause pneumonia and respiratory tract infections, however pneumonia is more commonly reported in captive koala populations, and the prevalence appears lower in wild koalas[7,11]. In northern koalas, *C. pecorum* is a prevalent pathogen (up to 90%) and severe overt chlamydial disease is commonly observed[1,3].

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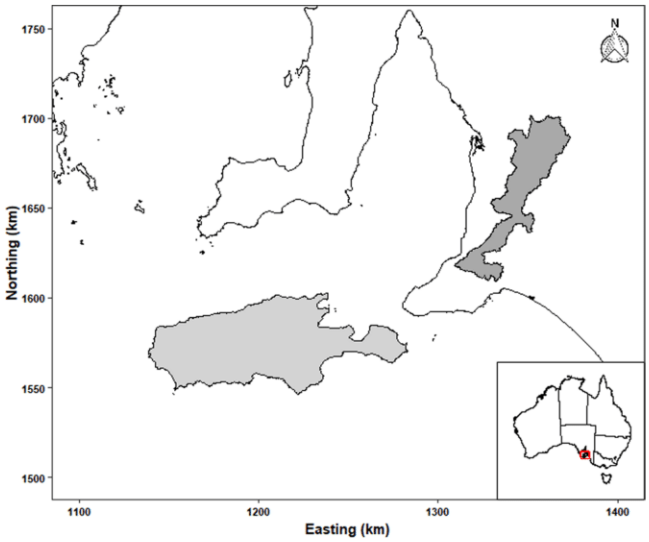

Figure 1. Geographical range of the Mount Lofty Ranges (dark grey) and the Kangaroo Island (light grey) koala populations, South Australia, Australia.

In South Australia, the two largest koala populations[12,13] are found on the mainland in the Mount Lofty Ranges (MLR) and on Kangaroo Island (KI) (Fig. 1). Although these populations are presumed to be healthy based on the overabundance of koalas within these populations[12,13], there have been limited reports of the occurrence of infectious diseases.

In MLR, two small scale surveys to investigate *Chlamydia* prevalence were performed two decades ago using conventional PCR; the first reported all six tested koalas to be positive for *Chlamydia*, of unknown species[14], and in the second 88% (15/17) of animals were *C. pecorum* positive with a low incidence of clinical disease reported[3]. The first clinical cases of *C. pecorum* disease were only recently reported in three MLR free-ranging koalas that all presented with severe conjunctivitis[15]. Subsequently, a post mortem survey of rescued koalas found a high prevalence of *C. pecorum* detection (88%, 57/65) in the MLR. However 28% (n = 16) of positive koalas had no disease associated with infection, 21% (n = 12) showed mild clinical signs of conjunctivitis and/or urinary tract infections and the remaining 51% (n = 29) had inapparent, predominantly microscopic, *C. pecorum* infection only[16].

On KI, previous investigations of *Chlamydia* used outdated techniques with low diagnostic sensitivity and specificity in comparison to PCR, which is regarded as the gold standard[3]. Radiography showed potential paraovarian cysts in eleven female koalas in 1984[17]. Serological studies reported conflicting results, with one study finding no anti-*Chlamydia* antibodies in 63 koalas in 1989[18] and the second a seroprevalence of 18% in 1997 (n = 201)[19]. The first survey to use direct detection of *C. pecorum* DNA was conducted in 1999 and surveyed ten koalas that were all found to be *C. pecorum* PCR negative[3]. Given that koalas were introduced into KI from French Island, Victoria in the 1920's[20], which was considered *Chlamydia*-free[21], KI has also been regarded as *Chlamydia*-free. However, *C. pecorum* has recently been detected in two French Island koalas[22] and other reports of introduction of koalas from Queensland to KI in the 1940s[23] raise doubts on the true status of *C. pecorum* in the KI koala population.

At a time when other mainland koala populations are seriously jeopardised by *C. pecorum* infection, disease and infertility, MLR koalas appear to have lower levels of overt disease, whilst KI may be the last large *C. pecorum*-free population in Australia. Hence this study aimed to determine the prevalence of *C. pecorum* in wild ranging koalas from the MLR and KI populations in South Australia and to describe any clinical disease associated with infection.

Results

Mount Lofty Ranges targeted survey. In the MLR, 30 male and 45 female koalas were captured and sampled. *C. pecorum* was detected by qPCR in 46.7% (35/75, Binomial Exact 95% CI: 35.1–58.6%) of koalas. *C. pecorum* was more likely to be detected with higher loads at the urogenital site (median, (range)) (34/35; 170 (10–30,600) copies/μL) compared to the ocular site (3/35; 30 (17–2,020) copies/μL). There was no significant difference in prevalence between sexes (females: 55.5%, 25/45; males: 36.7% 11/30; P = 0.156) or between sex and the site of infection (P = 0.339). Despite this, the three koalas that were qPCR positive at the ocular site were all female (3/45). At the urogenital site, females had a higher chlamydial load with median 409 copies/μL (range: 28–30,600 copies/μL) compared to males, 77 copies/μL (range: 10–645 copies/μL). Only 4% (3/75) of koalas presented with overt *C. pecorum* clinical disease and all were classified as severe (grade 3). These koalas were all female; one case of mucopurulent pyometra (TWC II), one case of cystitis with urine soiling and scalding of the rump of the koala (TWC VI) and one case of unilateral severe, conjunctivitis (TWC V) (Fig. 2a). Koalas which did not fit the definition of a clinical case included; grade 1 urogenital signs observed as fur discolouration with no scalding of the perineum in koalas qPCR positive (n = 10) and negative (n = 5) for *C. pecorum* infection, and grade 1 ocular signs observed in 22 *C. pecorum* PCR negative koalas (Fig. 2b).

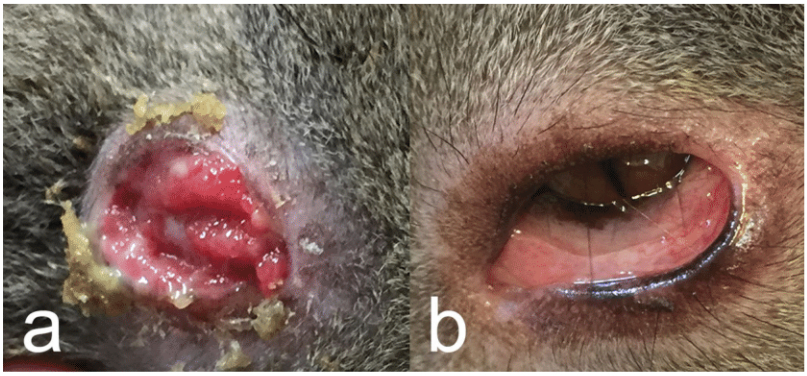

Figure 2. Conjunctival changes in koalas from the Mount Lofty Ranges, South Australia. (**a**) Female koala with unilateral grade 3 severe conjunctivitis, positive for *Chlamydia pecorum*. (**b**) Female koala with grade 1 reddened conjunctiva, negative for *C. pecorum* infection.

Kangaroo Island targeted survey. From the targeted survey, 170 female koalas were sampled over a 3-year period (2014–17) and all found to be *C. pecorum* negative by qPCR. The DEW koala program recorded observations for 13,373 individual koalas, surgically sterilised over a 22-year period (1997–2018). None of the clinical records fitted the definition of a clinical chlamydial disease case. Records for two koalas from the targeted survey and 292 koalas from the DEW koala program (all negatives) were not included in the demonstration of freedom analysis as information for age or sex was missing. Data for 10,160 females and 2,921 males were incorporated from the DEW koala program. Details about demographic strata (sex, age class), number of koalas sterilised each year and each surveillance component were reported into the model accessible elsewhere (https://figshare.com/s/590fe1b98c52a4778f83).

Fecundity of female koalas. Reproductive activity was recorded in female koalas on KI by pregnancy (observed during laparoscopic sterilisation) and the presence of pouch and back young and in MLR by the presence of pouch young, as back young had matured by this time and were not observed. On KI 79.2% (118/149) of sexually mature females were reproductively active, with 41 back young, 66 pouch young and 28 pregnant. Six koalas had both a back and pouch young, and eleven had a back young and were pregnant. The youngest pregnant female was aged 2–3 years (TWC II) and weighed 3.78 kg.

In the MLR, reproduction was significantly reduced due to *C. pecorum* infection, where only 37.2% (16/43) of female koalas had pouch young. No reproductively active females were infected with *C. pecorum*; while females without pouch young were significantly more likely to be infected with *C. pecorum* ($P < 0.001$). In addition, reproductively active females were five times more likely to be infected (RR = 5.0). Of the inactive females, 85.2% (23/27) were infected. Reproductively active females from both populations were more likely to be in excellent body condition ($P = 0.040$). There was no association between reproductive activity and age in either population (Fig. 3).

Grade 1 clinical disease observed in KI. In both the targeted survey and the DEW koala program, no KI koalas fitted the definition of a clinical case (>grade 2), however, some koalas were graded 1 for the ocular and urogenital sites. In the targeted survey, 12 (7%) koalas were observed with very mild ocular changes (qPCR negative for *C. pecorum*) which were graded with an ocular clinical score of 1. Within the DEW koala program clinical records, 1.08% of koalas (n = 141) were found to have clinical records graded at 1. Ocular changes were recorded in 0.5% of koalas (n = 67), with records such as conjunctivitis (n = 11), corneal scar, ulcer or opacity (n = 35) and periocular inflammation or oedema (n = 21). Urinary tract changes were recorded in 0.03% of female koalas (n = 4), with cystitis (n = 1), haematuria (n = 2) and kidney disease (n = 1) reported. Testicular aplasia was recorded in 0.2% of males (n = 27) and one male with testicular infection. Reproductive changes were recorded in 0.33% of female koalas (n = 43), where records were brief and lacked significant detail. 'Cystic ovary' was the most common record (n = 30), and of these koalas 13 were reproductively active; 'enlarged uterus' was reported in one female with pregnancy status not recorded; and 'reproductive adhesions or inflammation' was also recorded in females with unrecorded pregnancy status (n = 12).

Demonstration of freedom simulation model. The evidence of absence of *C. pecorum* on KI was collected through the 22 year DEW program and targeted surveys including qPCR over a 3-year period. This data supported that the KI koala population is free from *C. pecorum* infection in 2018, with an estimated probability of freedom with at least 95% confidence if the prevalence of *C. pecorum* on KI was at least 2% and if the yearly probability of introduction of the bacterium in the population is at most 7%. It was not possible to reach a minimum of 95% probability of freedom if the design prevalence was 1% or less. If the design prevalence is >2%, the probability of freedom was at least 95% regardless of the probability of introduction (Table 1).

The evolution over time of the sensitivity of the surveillance (SSe_i) and the probability of freedom (95% CI), with a 2% design prevalence and a risk of introduction at 7% is represented in Fig. 4. The *SSe* displayed is the average of 10,000 iterations in the model. The *SSe* was highly depended on the surveillance effort (i.e. the number of koalas screen and accuracy of detection). Over the first two years (1997–1998), the *SSe* was above 80%, but

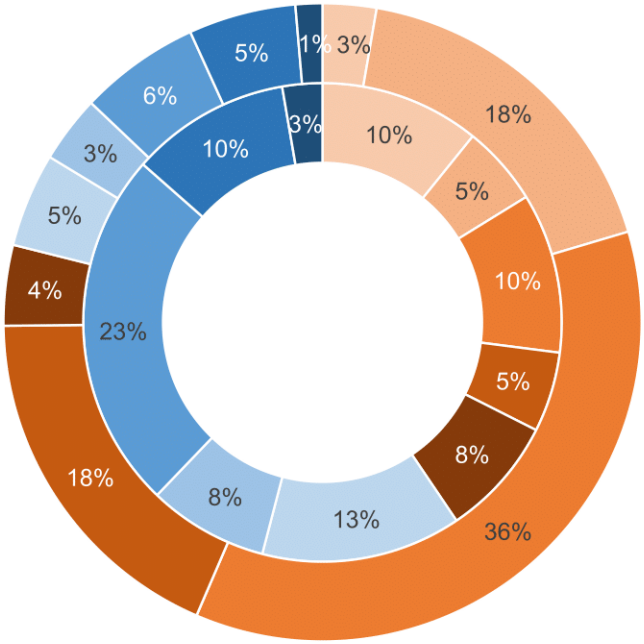

Figure 3. Reproductively active (orange) and inactive (blue) female koalas across age classes (TWC II (lightest) to VI (darkest)) from the Mount Lofty Ranges (inner circle) and Kangaroo Island (outer circle) populations.

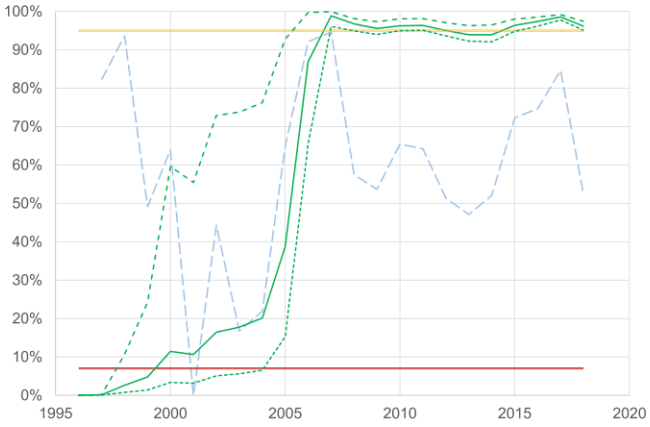

Figure 4. Demonstration of freedom from *Chlamydia pecorum* infection in the KI koala population. Mean estimates for surveillance sensitivity (*SSe*) and probability of freedom, $P(free)_i$ from *C. pecorum* between 1997 and 2018. The mean *P*(*free*) (solid, dark green line) with 95% CI as lower (shorted, dashed, dark green line) and upper (long, dashed, dark green line) confidence limits and *SSe* (long, dashed, light blue line) with a probability of introduction, *P*(*intro*) of 7% (solid double red line) and a design prevalence (*P**) of 2%, concluded in 2018 above the 95% limit (solid double yellow line).

Design Prevalence	Yearly probability of introduction			
	1%	7%	10%	20%
1%	96.5% (89.4–99.1%)	84.8% (66.5-93.5%)	76.6% (50.6–90.4%)	38.9% (9.4–78.2%)
2%	***99.5% (99.3–99.6%)***	**96.1% (95.1–97.4%)**	94.3% (92.8–96.1%)	87.7% (84.3–91.6%)
5%	***99.9% (99.8–99.9%)***	**99.0% (98.5–99.6%)**	**98.5% (97.8–99.4%)**	**96.7% (95.3–98.6%)**
10%	***99.9% (99.9–100%)***	***99.8% (99.7–99.9%)***	***99.8% (99.6–99.9%)***	***99.5% (99.1–99.9%)***
15%	***99.9% (99.9–100%)***	***99.9% (99.9–100%)***	***99.9% (99.9–100%)***	***99.9% (99.8–100%)***

Table 1. Sensitivity analysis of the probability of freedom (P_{free}) (mean, lower and upper limits) after 22-years of surveillance for given design prevalence (*P**) and probability of introduction (P_{intro}) values. Lower P_{free} limit above a 95% confidence estimate (bold), and above 99% confidence estimate (bold, italicised).

dropped to below 70% from 1999 to 2004 due to a smaller number of records for each year (maximum 564) and no koalas captured in 2001, reflecting the *SSe* to 0%. From 2005–2018, the *SSe* remained above 50%. The highest *SSe* was above 90% in 2006 and 2007 with the largest number of koalas observed in the DEW program, with 1,522 and 1,705 observed, respectively. The targeted survey from 2015 to 2017 increased the *SSe* to above 80% from below 70% in 2014 with a similar number of koalas captured.

The lower limit of the $P(free)_i$ was below 90% up until 2007. In 2007, with the large number of koalas captured, minimum $P(free)_i$ increased to 96.0% and approximated 95% with the last $P(free)_{2018}$ of 95.1% (Fig. 4).

Discussion

Chlamydia pecorum is a pathogen of critical, national importance to the conservation of the koala species. Koala populations in northern Australia (Queensland and New South Wales) are experiencing significant declines that have been attributed to a number of factors, including deforestation, urbanisation, trauma (vehicular and dog attacks)[1,24] and *C. pecorum*[25]. In the present study, we have shown that *C. pecorum* is well established in the wild Mount Lofty Ranges (MLR) population, with 46.7% of the surveyed koalas positive by PCR, but clinical disease was only observed in 4% of koalas. In KI koalas, the population was demonstrated to be free of *C. pecorum* infection with 95% confidence.

In wild MLR koalas, the prevalence of *C. pecorum* infection was found to be high, but was half of the prevalence reported in the previous study of necropsied koalas from the same area[16]. Overt *C. pecorum* disease was only observed in three koalas, which is substantially less than that seen in northern populations, where 20.8% (5/24) of free-ranging koalas from Queensland populations presented with overt *C. pecorum* disease[26]. Urogenital infections (2/3) were more common than ocular infections (1/3), which is also the trend reported in Victorian koalas[27]. Although *C. pecorum* infection was reported in the MLR population two decades ago[3,14], clinical disease associated with infection is only recently being reported more frequently with increasing disease severity[15,16]. Prior to 2012, overt clinical disease was not observed by local veterinarians treating rescued koalas from the MLR population (N. Speight pers. comm.). The increasing prevalence may be due to increased awareness of chlamydial disease but may also suggest a possible change in pathogenicity of *C. pecorum* or increased host susceptibility. Possible causes include the introduction of a new pathogenic variant of *C. pecorum*[28]; an upregulation in the transmission of a virulence plasmid, *pCpec*[29]; or the recent introduction of the Koala retrovirus[30], which may predispose koalas to develop clinical disease through immunosuppression[31,32]. Evidence has shown immunologically naïve koalas exposed to *C. pecorum* are at risk of high morbidity and mortality[33–35]. The implications for *C. pecorum* infection in the MLR population is unknown, but chlamydial infection was found in this study to significantly reduce female koala fecundity. Hence monitoring of the MLR population should continue to detect any future changes in chlamydial prevalence and pathogenicity that could cause the population to decline similarly to that observed in northern koala populations.

In KI, the model has demonstrated that the DEW koala program and targeted survey had sufficient surveillance sensitivity to detect an established *C. pecorum* infection in the KI population if the prevalence was greater than 2%. Hence it remains possible that *C. pecorum* may be present in the population at a very low prevalence, for instance if it had been recently introduced. The risk of introduction of *C. pecorum* into KI is unknown. Possible routes of introduction include from infected koalas, domestic livestock or other native species. *C. pecorum* is thought to transmit between koalas by direct contact[3,35], which is likely to include sexual transmission[26] and mother to offspring[27,36]. As the introduction of infected koalas to KI is unlikely to occur due to strict DEW regulations on koala care and movement, koala faeces infected with *C. pecorum*[37] may pose a threat, based on what is known in other species, as the faecal-oral route is thought to be the most likely route of transmission between domestic cattle (*Bovis taurus*) infected with *C. pecorum*[38].

This highlights the potential introduction of *C. pecorum* into KI through livestock. While the koala has a distinct clade of *C. pecorum* isolates which infect and cause disease in the koala, there are some genetically similar strains of *C. pecorum* shared between domestic cattle, sheep (*Ovis aries*) and the koala[39,40]. The recently discovered *C. pecorum* isolates in French Island koalas were found to be genetically related to livestock *C. pecorum* genotypes[22], suggesting potential transmission between species. Sheep farming is a common activity on KI, with up to 680,000 sheep[41], and while the presence of *C. pecorum* in KI sheep remains unknown, *C. pecorum* has been found in sheep in south-eastern South Australia[42]. Investigation into the presence of *C. pecorum* in livestock on KI is underway.

Transmission may occur either directly by exposure of koalas to infected livestock faeces when they travel on the ground between trees, or via an intermediate species such as has been hypothesised with possums[43]. It has been found that the mountain brushtail possum (*Trichosurus caninus*) in Victoria can be infected with *C. pecorum*[44] and due to its arboreal nature, it may introduce contaminated livestock faeces into the eucalypt trees on which koalas feed. While this species of possum is not present on KI, the common brushtail possum (*Trichosurus vulpecula*) is highly abundant and has also been shown to share some intestinal parasites with sheep on KI[45]. To ensure trans-species transmission does not occur with the koala on KI, *C. pecorum* in domestic livestock and native species on KI should be investigated through targeted surveillance, in conjunction with increased biosecurity measures implemented to ensure that *C. pecorum* is not brought onto the island.

The DEW sterilisation program on KI provides a sensitive surveillance tool for both ocular and urogenital disease. Review of the clinical data from the KI DEW koala program found 141 koalas (1.08%) with possible signs of chlamydial disease that were assigned a grade 1 chlamydial score. This was due to minimal details of clinical signs recorded, that no diagnostic PCR performed to confirm *C. pecorum* infection and that although these changes were consistent with chlamydial infection, they were not pathognomonic for *C. pecorum*, and could be explained by other causes. Ocular and urinary tract disease has been reported in southern Australian koalas without *C. pecorum* infection[16,21,27], and with recent analysis of the ocular and urogenital microbiomes in the koala, there are possibly other pathogens which cause similar clinical signs[46,47]. Kidney disease may be due to oxalate

nephrosis, which is highly prevalent in the MLR population[48,49], while testicular aplasia has been reported in association with reduced genetic diversity in KI koalas[50]. A common report was "ovary cyst" with no additional description of the cysts, such as size or quantity. As some koalas were also reproductively active, it cannot be determined if these described cysts were pathological[9], or a result of normal reproduction, such as large follicles[51].

The impacts of *C. pecorum* on the eastern Australian koala populations are devastating with considerable morbidity and mortality as a result of infection and declines in population numbers due to infertility. We have estimated freedom from infection on KI over a 22-year period with >95% confidence. Hence this large, isolated *C. pecorum*-free population of koalas holds significant importance as insurance for the future of the species. Every effort should be made to ensure the population remains *C. pecorum*-free so that these koalas could be used, in conjunction with the newly developed *C. pecorum* vaccine[52], to re-populate declining populations, and may ultimately ensure the survival of the koala for generations.

Materials and Methods

Chlamydia pecorum targeted surveys. In the MLR, 75 wild koalas were captured in April 2016 using ropes and poles as described previously[19], and relocated to a nearby sampling site. On KI, 170 wild koalas were sampled between November 2014 and February 2017 in conjunction with the South Australian Department for Environment and Water (DEW) Koala Sterilisation Program. The Koala Sterilisation Program was implemented in 1996 to monitor the overabundant KI population and uses surgical sterilisation as a means for population management[53]. These surveys were approved by The University of Adelaide Animal Ethics committee (S-2013-198, S-2015-138) with State Government DEW Scientific Research permits (Y26054-6, U26431-1) and completed in accordance with the University of Adelaide and State Government guidelines and regulations.

For clinical examination, MLR koalas were anaesthetised with either alfaxalone (Alfaxan, Jurox, United Kingdom) (3.5 mg/kg) IM or alfaxalone (2 mg/kg) with medetomidine (Domintor, Vetquinol, United Kingdom) (40 µg/kg) IM, and on KI, isoflurane (2–5%) and oxygen (2%/min) was used. Each koala was aged by the degree of wear of the upper premolar (Tooth wear class (TWC) I, 1–2 years; II, 2–3 years; III, 4 years; IV, 5–6 years; V, 10–12 years; VI, 12+ years[54]). For KI females, laparoscopic sterilisation was performed allowing visualisation of the reproductive tract for pregnancy or any pathological changes. Reproductive activity was recorded in MLR females by the presence of pouch young and on KI by pregnancy, pouch and/or back young. Koalas were classified as sexually immature if their bodyweight <3.90 kg[19]. Each koala was graded for ocular and urogenital clinical signs consistent with *C. pecorum* using a 4 scale system (grade 0, no disease; 1, mild disease; 2, moderate disease; 3, severe disease)[55]. Two dry aluminium shaft swabs (Copan Italia, Brescia, Italy) of the conjunctiva and cloaca were collected[7] and stored at −80 °C until *C. pecorum* detection.

Historical clinical examination on KI. The DEW koala program collected clinical examination data during routine surgical sterilisations of koalas conducted every year from approximately November to March for 22 years (1997–2018). The clinical data included sex (male or female), age class (as described above) and signs of disease. Individual clinical records were reviewed retrospectively and ocular and urogenital findings consistent with *C. pecorum* infection were graded as described above[55]. A koala was classified as a positive clinical case if the clinical record described grade 2 disease or above for either the ocular or urogenital sites. This case definition favours the specificity of the classification to minimise possible false positive cases from mild, non-pathognomonic clinical signs[46,48,50,51].

***Chlamydia pecorum* molecular detection.** Ocular and urogenital swabs from individual koalas were screened for *C. pecorum* detection. DNA was extracted using Qiagen DNA Mini kit (Qiagen, Hilden, Germany) and extracted DNA was pooled and amplified using a *C. pecorum*-specific qPCR targeting a 209 bp fragment of C. pec HP gene[56]. Positive pooled swabs were re-tested separately. Briefly, the qPCRs were performed in a final volume of 20 µl, including 10 µl iTaq master mix (Bio-Rad, California, USA), 1 µl of 10 µM each of forward and reverse primer (Sigma-Aldrich, Australia), 3 µl dH_2O and 5 µl template DNA. Cycling conditions consisted of 15 min at 95 °C, followed by 35 cycles of 15 sec at 94 °C, 15 sec at 57 °C and 30 sec extension at 72 °C. Samples were tested in duplicates, and negative control (dH_2O) and positive control (*C. pecorum* Marsbar DNA) were included in each assay. In each assay, infectious load was quantified by plotting the crossing points against a standard curve produced using a serial dilution from 10^6 to 10^0 copies/µl of the known standard, *C. pecorum* target amplicon. C. pec HP gene amplicon was characterised with a high-resolution melt (HRM) of 77.5 ± 0.5 °C. DNA quality was assessed by detecting a 122 bp fragment of the koala β–actin gene. The reaction was performed in 25 µl containing 5 µl of 5X Taq polymerase buffer (Bioline, Australia), 2 mM of magnesium chloride, 0.1 mM of dNTP mix, 1 mM of each of the published β-actin primers, 5′-AGATCATTGCCCCACCT-3′ (sense) and 5′-TGGAAGGCCCAGATTC-3′ (anti-sense)[57], 0.25 µL of MyTaq DNA polymerase (Bioline, Australia), and 10 µL of DNA template. The PCR conditions were performed as recommended by the Bioline PCR kit, with a 58 °C primer annealing temperature and final extension at 72 °C for 10 minutes.

Univariate analysis. Statistical analysis was performed using SPSS v.24 to determine significance based on sex, age and *C. pecorum* status ($\alpha = 0.05$). For continuous *Chlamydia* load variables, a Shapiro-Wilk test was performed to determine Gaussian distributions. For variables with normal distribution, an F-test was performed to determine equal variance prior to a two-way independent t-test. For non-parametric variables a Kruskal-Wallis H analysis was performed with post-hoc Mann-Whitney U test. Chi-squared analysis was performed to determine relationships and odds ratio between *C. pecorum* infection, sex, age and reproduction status.

Demonstration of freedom from *Chlamydia pecorum* on KI. The probability that the KI koala population is *C. pecorum*-free was estimated by collating qPCR results from the targeted survey and DEW koala

program historical clinical data in a scenario tree modelling approach[58]. The probability of freedom of a given year of surveillance i ($P(free)_i$) was calculated using the Bayesian approach from the probability of freedom from the prior year $i-1$ ($P(free)_{i-1}$) and the surveillance system sensitivity (SSe_i) and specificity (SSp_i), reflecting the strength of the evidence collected during the year i[58]:

$$P(free)_i = \frac{Prob(free)_{i-1} \times SSp_i}{Prob(free)_{i-1} \times SSp_n + (1 - P(free)_{i-1}) \times (1 - SSe_i)} \quad (1)$$

$P(free)_i$ was then adjusted for the probability of introduction ($P(intro)_i$) during the same year i[58]:

$$adjusted\ P(free)_i = 1 - ((1 - P(free)_i) + P(intro)_i - (1 - P(free)_i) \times P(intro)_i \quad (2)$$

Surveillance System Specificity (*SSp*). The diagnostic specificity of both diagnostic methods, qPCR and clinical examination, were perfect (100%) at the individual animal level. Therefore, the *SSp* relying on these two methods at the population level was deduced as also 100%. This deduction was further supported by the fact that none of the surveyed KI koalas were classified as positive (i.e. no potential false-positive).

Surveillance System Sensitivity (*SSe*). The *SSe* was calculated based on 12 month periods to match the yearly cycle of the surveillance. In a given year i, the SSe_i was calculated from the sensitivity of the *C. pecorum* targeted survey (CSe^{Survey}_i) and DEW koala program (CSe^{DEW}_i) surveillance components as follow[58]:

$$SSe_i = 1 - ((1 - CSe_i^{Survey}) \times (1 - CSe_i^{DEW})) \quad (3)$$

System Component Sensitivity (CSe). In a given year, CSe_i was calculated from the component unit sensitivity ($CSeU_i$, probability of detecting a single infected koala if sampled at a given period) of each surveillance component as follow[58]:

$$CSe_i = 1 - (1 - CSeU_i)^n \quad (4)$$

where n is the total number of koalas sampled during a given activity and given year.

Component Unit Sensitivity (CSeU). For each year i, the $CSeU_i$ was calculated from scenario trees representing all possible scenarios, including risk categories (sex and age class) and detection outcomes (positive or negative)[59]. Two separate trees were built for each surveillance component, the targeted survey and DEW koala program (Fig. 5). The probability of each scenario tree branch was calculated by multiplying the representation of its risk category class in the total sample, the adjusted risk (AR) of the risk category class, the design prevalence (assumed minimum prevalence of *C. pecorum* when the infection is established in a koala population) (P^*) and the probability of detection according to the diagnostic method accuracy (i.e. diagnostic sensitivity and specificity, *DSe* and *DSp* respectively).

Adjusted Risk (AR). For each risk category classes (sex and age classes), an $AR_{ageXsex}$ was calculated by multiplying the AR for sex and the AR for age class. The AR for sex were calculated using the relative risk of *C. pecorum* infection (RR) of males compared to females and the assumed population representation (PrP) of each sex as follow[58]:

$$AR_{Female} = \frac{1}{(RR_{Male} \times PrP_{Male}) \times PrP_{Female}} \quad (5)$$

$$AR_{Male} = AR_{Female} \times RR_{Male} \quad (6)$$

The AR for the six age classes were calculated similarly using the relative risk (RR) of each age class compare to the first age class (TWC I) and the assumed population representation (PrP) of each class for a given sex[58]:

$$AR_{TWC1/sex} = \frac{1}{\prod_2^6 (RR_{TWCJ/sex} \times PrP_{TWCJ/sex}) \times PrP_{TWC1/sex}} \quad (7)$$

$$AR_{TWCJ/sex} = AR_{TWC1/sex} \times RR_{TWCJ/sex} \quad (8)$$

where J is the age class ranging from TWC II to VI.

Diagnostic sensitivity and specificity (*DSe* and *DSp*). *DSe* and *DSp* of the qPCR (DSe_{PCR} and DSp_{PCR}) were estimated in-house by comparing the proportions of positive and negative results when screening known positive and negative standards respectively. The *DSe* and *DSp* of the clinical examination (DSe_{Clin} and DSp_{Clin}) were deduced by crossing the results from the clinical examination with the qPCR from koalas surveyed in the MLR (population infected with *C. pecorum*). DSe_{Clin} and DSp_{Clin} were calculated using the following formulae[60]:

$$DSe_{Clin} = \frac{n_1 DSp_{PCR} - c}{nDSp_{PCR} - m_0} \quad (9)$$

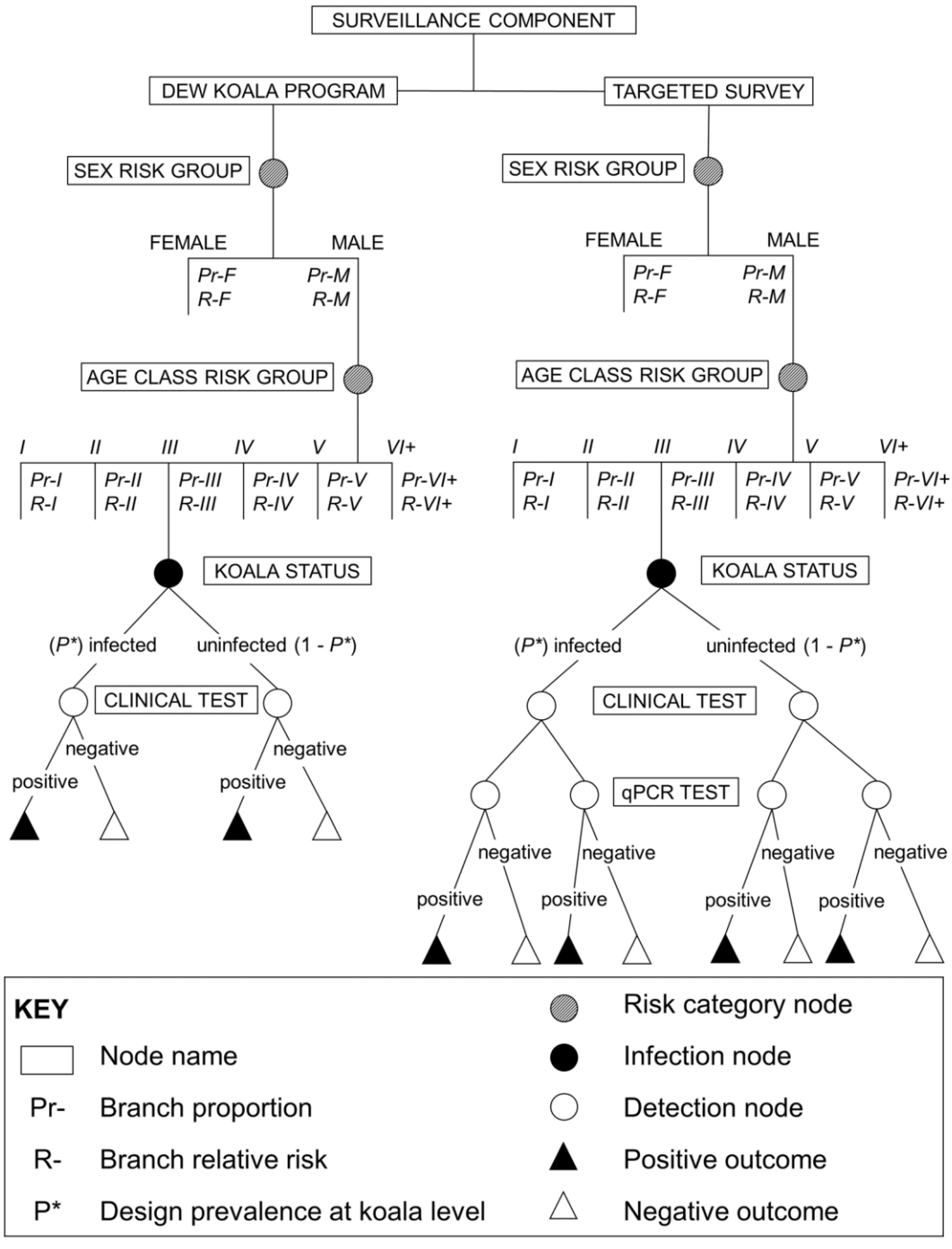

Figure 5. Structure of scenario trees used to estimate the Component Unit Sensitivity to detect *Chlamydia pecorum* infection in individual koalas during the DEW Koala Program or *C. pecorum* Targeted Survey, respectively.

$$DSp_{Clin} = \frac{n_0 DSe_{PCR} - b}{nDSe_{PCR} - m_1} \quad (10)$$

where n is the total number of koalas tested in the MLR; n_1 is the count of individuals with clinical signs present (positive clinical cases); n_0 is the count with no clinical signs (negative clinical cases); b is the count of PCR positive koalas without clinical signs; c is the count of PCR negative koalas with clinical signs; m_1 is the total count of PCR positive and m_0 is the total count of PCR negative koalas.

Model stochasticity. To account for the uncertainty about model parameters, stochasticity was built into the model by allocating a distribution to the parameters using the PopTools Excel add-in v3.2 (PopTools

Model parameters	Estimate	95%CI lower limit	95%CI upper limit	Distribution	Source
Population proportion					
PrP_{Female}	50.7%	54.4%	47.1%	Beta (365.9257, 355.3342)	55
$PrP_{TWC\ I/Female}$	38.8%	33.9%	43.9%	Beta (138.1517, 217.4571)	55
$PrP_{TWC\ II/Female}$	15.6%	12.1%	19.6%	Beta (52.5587, 280.6404)	55
$PrP_{TWC\ III/Female}$	22.2%	18.1%	26.7%	Beta (76.5331, 266.265)	55
$PrP_{TWC\ IV/Female}$	17.9%	14.2%	22.2%	Beta (61.1473, 276.0853)	55
$PrP_{TWC\ V/Female}$	4.0%	2.2%	6.4%	Beta (15.8344, 360.9804)	55
$PrP_{TWC\ VI/Female}$	1.6%	0.6%	3.4%	Beta (6.9566, 371.3016)	55
PrP_{Male}	49.3%	45.6%	52.9%	Beta (354.923, 365.5023)	55
$PrP_{TWC\ I/Male}$	44.3%	39.1%	49.5%	Beta (156.1654, 196.1467)	55
$PrP_{TWC\ II/Male}$	13.9%	10.5%	17.8%	Beta (44.9898, 274.427)	55
$PrP_{TWC\ III/Male}$	20.4%	16.4%	24.9%	Beta (67.8923, 262.326)	55
$PrP_{TWC\ IV/Male}$	14.7%	11.2%	18.7%	Beta (47.83, 273.3078)	55
$PrP_{TWC\ V/Male}$	3.0%	1.5%	5.3%	Beta (11.8921, 354.4976)	55
$PrP_{TWC\ VI/Male}$	3.8%	2.1%	6.3%	Beta (14.8459, 351.1047)	55
Relative risk					
RR_{Female}[a]	1.00	—	—	—	27
RR_{Male}	1.52	1.08	2.15	Pert (1.52, 1.08, 2.15)	27
$RR_{TWC\ I}$[b]	1.00	—	—	—	27
$RR_{TWC\ II}$	1.00	—	—	—	27
$RR_{TWC\ III}$	1.29	0.74	2.25	Pert (1.29, 0.74, 2.25)	27
$RR_{TWC\ IV}$	1.29	0.74	2.25	Pert (1.29, 0.74, 2.25)	27
$RR_{TWC\ V}$	2.77	1.44	5.37	Pert (2.77, 1.44, 5.37)	27
$RR_{TWC\ VI}$	2.77	1.44	5.37	Pert (2.77, 1.44, 5.37)	27
Test accuracy					
DSe_{PCR}	100.0%	83.9%	100.0%	Beta (21.00000977, 1)	Jelocnik, unpublished
DSp_{PCR}	100.0%	76.8%	100.0%	Beta (14.000000485, 1)	Jelocnik, unpublished

Table 2. Model parameters respective reported uncertainty (estimate sand 95% CI), allocated distribution for the stochastic modelling and source of information. PrP, expected proportion of sex and age within sex, of the population. TWC, tooth wear class. RR, relative risk. DSe_{PCR}, probability of qPCR to test positive if the koala is truly infected. DSp_{PCR}, probability of qPCR to test negative if the koala is truly non-infected. [a]Sex risk of *C. pecorum* in a koala relative to the risk of *C. pecorum* in females. [b]Age risk of *C. pecorum* in a koala relative to the risk of a koala with *C. pecorum* in TWC I.

2011). Distribution used in the model and the source of the information used to parametrise the distribution are reported in Table 2. Probability parameters were allocated Beta distribution parametrised with BetaBuster v1.0 software (Su 2012 https://www2.vetmed.ucdavis.edu/cadms/local_resources/docs/betabuster012006.zip) according to their exact Binomial 95%CI. Other model parameters were allocated a Pert distribution according to their reported 95% CI. The simulation was run for 10,000 iterations and the model outputs' distributions were reported with the mean, 2.5th and 97.5th percentiles. A population of koalas was deemed free from the infection if the 2.5th percentile (lower limit) the estimated probability of freedom was >95%.

Sensitivity Analysis. No robust estimates of the design prevalence (P^*) and the probability of introduction ($P(intro)$) could be sourced. A sensitivity analysis was conducted by varying both parameters and assessing the impact of the final probability of freedom estimate and 95% CI. As the risk of introduction may vary with time, we assumed in the model that the probability of introduction was at its maximum possible value for the entire surveillance period.

The model to assess freedom from *C. pecorum* was implemented in MS Excel (2013) and a copy is accessible online (https://figshare.com/s/590fe1b98c52a4778f83).

Data Availability

The dataset generated and analysed during this study is available in the Figshare repository [https://figshare.com/s/590fe1b98c52a4778f83]. Further data generated during this study is available from the corresponding author on reasonable request.

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Author Contributions

J.F. contributed to the study design, development and implemented the field work, processed samples for PCR, interpreted the targeted survey and KI historical data, assisted with the development of the demonstration of freedom model and wrote the manuscript. C.C. developed the demonstration of freedom model. M.J. and A.P. performed qPCR. W.S.J.B. contributed to study design and performed clinical examinations on MLR koalas. E.N. and G.J. performed current and historical clinical examinations on KI koalas. R.M. assisted in koala capture and provided the KI historical data. L.W. assisted in classifying clinical chlamydial disease. P.T., G.S., F.H. and D.J.T. contributed to the study design. N.S. was awarded the project funds, contributed to the study design and development of sampling, performed clinical examinations on MLR and KI koalas, assisted in data interpretation and analysis. All authors reviewed the manuscript.

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Chapter 3

Prevalence and clinical significance of koala retrovirus in two South Australian koala (*Phascolarctos cinereus*) populations

Statement of Authorship

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Overall percentage (%)	90%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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MICROBIOLOGY SOCIETY

Prevalence and clinical significance of koala retrovirus in two South Australian koala (*Phascolarctos cinereus*) populations

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Abstract

Purpose. Koala retrovirus (KoRV-A) is 100 % prevalent in northern Australian (Queensland and New South Wales) koala populations, where KoRV-B has been associated with *Chlamydia pecorum* disease and the development of lymphosarcoma. In southern populations (Victoria and South Australia), KoRV-A is less prevalent and KoRV-B has not been detected in Victoria, while the current prevalence in South Australian populations is unknown but is thought to be low. This study aimed to determine (i) the prevalence of KoRV in the two largest South Australian koala populations [Kangaroo Island (KI) and Mount Lofty Ranges (MLR)], (ii) KoRV subtype and (iii) if an association between KoRV and *C. pecorum* exists.

Methodology. Wild koalas were sampled in KI (*n* =170) between 2014 and 2017 and in MLR (*n* =75) in 2016. Clinical examinations were performed, with blood collected for KoRV detection and typing by PCR.

Results. KoRV prevalence was 42.4 % [72/170, 95 % confidence interval (CI): 34.9–49.8 %] in KI and 65.3 % (49/75, 95 % CI: 54.6–76.1 %) in MLR. Only KoRV-A, and not KoRV-B, was detected in both populations. In MLR, there was no statistical association between KoRV and *C. pecorum* infection (*P* =0.740), or KoRV and *C. pecorum* disease status (*P*=0.274), although KoRV-infected koalas were more likely to present with overt *C. pecorum* disease than subclinical infection (odds ratio: 3.15, 95% CI: 0.91–5.39).

Conclusion. KoRV-A is a prevalent pathogen in wild South Australian koala populations. Future studies should continue to investigate KoRV and *C. pecorum* associations, as the relationship is likely to be complex and to differ between the northern and southern populations.

INTRODUCTION

Koala retrovirus (KoRV), a gammaretrovirus, was discovered to infect koalas in 2000 [1] and was found to be most closely related to gibbon ape leukaemia virus (GaLV) from South-East Asia [1] and *Melomys burtoni* virus (MbRV) found in Queensland [2]. Due to these similarities with GALV and MbRV, it is thought that KoRV must have entered Australia from the north and spread in a southern direction [3]. KoRV is highly prevalent in northern (Queensland and New South Wales) koala populations, with up to 100 % of koalas infected [3]. In these populations, KoRV has been shown to be endogenous, with vertical transmission of the virus to offspring with ubiquitous high KoRV proviral copy numbers in all tissues [3, 4]. Recent studies have found a number of KoRV variants, denoted A to I, which differ in the envelope (*env*) gene and long terminal repeat (LTR) enhancer regions [5, 6]. KoRV-A is endogenous in northern koala populations, while the other variants, B to I, are less prevalent, with lower viral loads [5]. To date, all northern koalas have been found to be infected with KoRV-A, with multiple other concurrent variant infections [5, 6].

Evidence is building around the pathogenicity of KoRV, whereby in northern koalas, high KoRV viraemia has been associated with the development of lymphoid neoplasia [7, 8], the most common type of cancer reported in koalas [9–12]. KoRV-B is thought to be the most pathogenic

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Keywords: *Chlamydia pecorum*; koala; koala retrovirus; Phascolarctidae; South Australia.

Abbreviations: BCS, body condition score; CI, confidence interval; CP, conservation park; KI, Kangaroo Island; KoRV, koala retrovirus; MLR, Mount Lofty Ranges; NP, national park; TWC, tooth wear class.

variant, and has been associated with lymphoid neoplasia in captive northern koalas [8]. Further, in northern koalas, KoRV infection is thought to be immunosuppressive, leading to disease from otherwise opportunistic pathogens, such as *Chlamydia pecorum*, which causes clinical chlamydiosis [7], and which has also recently been associated with KoRV-B [13].

A high prevalence of severe *C. pecorum* disease has been reported in northern populations, with a prevalence of up to 90 % in some populations [14–16]. Chlamydial diseases recorded include: conjunctivitis, which leads to blindness [14]; respiratory tract infections [17]; urinary tract infections [18, 19]; and reproductive tract infections, which cause infertility in male and female koalas [20–22]. In Queensland, a study of admission trends found that koalas with *C. pecorum* disease were more likely to be euthanized than released [16], which is likely due to the difficulties encountered in treating intracellular *C. pecorum* infection with antibiotics [23].

In comparison to northern populations, the prevalence of both KoRV and *C. pecorum* in southern Australian (Victoria and South Australia) koala populations is lower, with a reduced prevalence of disease. The prevalence of KoRV in Victorian koalas was 25 % (*n*=648), and only KoRV-A and not KoRV-B was detected [24]. The prevalence of *C. pecorum* in the Victorian koala population was 15 % (*n*=820) [25] with low prevalence of mild clinical disease reported [25, 26]. No association was found between KoRV and *C. pecorum* infection in Victorian koalas [24].

In South Australia, the prevalence of KoRV has previously only been investigated on Kangaroo Island (KI) in 2012, and was found to be 14.8 % (*n*=162) [3]. In the Mount Lofty Ranges (MLR), KoRV has been reported in conjunction with lymphosarcoma and overt *C. pecorum* disease in a female koala [27], but the prevalence and disease manifestations of KoRV, such as association with *C. pecorum*, are otherwise unknown in this population. The prevalence of *C. pecorum* in South Australian koalas was recently reported, with 46.7 % of wild koalas from the MLR (*n*=75) being infected, and overt chlamydial disease only being observed in three female koalas (4 %), while the KI population was found to be *C. pecorum*-free [28].

The high KoRV prevalence and severity of *C. pecorum* clinical disease in northern koala populations is in stark contrast to what has been observed in southern populations, but this is based primarily on Victorian studies. Hence the aim of this study was (i) to determine the current prevalence of KoRV in wild koalas from the two main South Australian populations, the KI and MLR populations; (ii) to determine whether KoRV-A or B was present; and (iii) to describe any associations between KoRV and *C. pecorum* infection and disease.

METHODS

Sample collection

Wild koalas (*n*=170) from KI (35.7752° S, 137.2142° E) were sampled whilst under anaesthesia for the State Government, Department for Environment and Water (DEW) Koala Sterilisation Program between 2014 and 2017. Koalas were sampled from five koala management zones designated by the DEW Koala Sterilisation Program as reported by Molsher [29]. The five zones were North Coast, Cygnet River and Birchwood Lagoon, Eleanor River and Timber Creek, South West and Rocky River.

In April 2016, 75 wild koalas from the MLR (34.9736° S, 138.7086° E; 30 males and 45 females) were sampled across 4 national (NP) and conservation (CP) parks: Belair NP, Cleland CP, Horsnell Gully CP and Morialta Falls CP. Koalas were captured using a modified version of the noose and flag method [30] and transported to a base station to be examined under anaesthesia. All KI and MLR sampling was approved by the University of Adelaide Animal Ethics Committee (S-2013–198 and S-2015–138) in conjunction with the DEW Scientific Research approval (Y26054-6 and U26431-1).

Clinical examination for routine health checks recorded observations of illness or disease and included sex, age estimated by dentition [wear on the upper premolar: tooth wear class (TWC) I, 1–2 years; TWC II, 2–3 years; TWC III, 4 years; TWC IV, 5–6 years; TWC V, 10–12 years; TWC VI, 12+ years; [31]) and body condition score (BCS; muscle mass over the scapula graded as poor, good or excellent [18]). Up to 5 ml of blood was collected from the cephalic or femoral vein into an EDTA BD Vacutainer blood collection tube (Becton, Dickinson and Company, New York, NJ, USA) and used for KoRV provirus detection and proviral load quantification. Using a dry aluminium shaft swab (Copan, Melbourne, Australia) the conjunctiva and cloaca were sampled for *C. pecorum* testing as previously described [18]. Clinical signs consistent with *C. pecorum* disease were graded as no disease (0), mild disease (1), moderate disease (2) and severe disease (3) for the ocular and urogenital sites as described by [32]. *C. pecorum* detection was subsequently performed with extracted DNA screened with a *C. pecorum* species-specific qPCR assay [33], as described elsewhere [28]. For biosecurity measures, and to reduce the risk of cross-contamination, each veterinary work station and koala carrier was cleaned with a veterinary grade disinfectant (F10SC, Johannesburg, South Africa) between each koala.

Koala retrovirus detection nested PCR

Koala retrovirus detection was performed using conventional nested PCR on DNA extracted from whole blood (QIAamp DNA Mini kit, Qiagen, Hilden, Germany). The two-step reaction was performed in a Bio-Rad T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA). The first reaction was performed in 50 µl containing 10 µl of 5× *Taq* polymerase buffer (Bioline, Sydney, Australia), 2 mM of magnesium chloride, 0.1 mM of dNTP mix, 1 mM of each of the previously published KoRV polymerase (*pol*) gene long primers, 5′- CCTTGGACCAAGAGACTTTTGA-3′ (sense) and 5′- TCAAATCTTGGACTGGCCGA-3′ (antisense) [4], 0.5 µl of MyTaq DNA polymerase (Bioline, Sydney, Australia) and 150 ng of DNA template. The second, nested

reaction was performed in 25 µl containing 5 µl of 5× *Taq* polymerase buffer, 1 mM of the previously published KoRV *pol* gene internal primers 5′-TCATGGCTCCAACTCTTTCC-3′ (sense) and 5′-TACCAGAATCCCCAAATCCA-3′ (anti-sense) [3], and 0.25 µl of MyTaq DNA polymerase with 8 µl of the initial reaction for DNA template (24 ng DNA). A positive control (140348) and dH_20 for the negative control were included in each reaction. PCR conditions were initiated by denaturation at 95 °C for 3 min followed by 35 cycles of denaturation at 95 °C for 15 s; annealing at 61 °C for 15 s; and DNA elongation at 72 °C for 10 s with a final extension at 72 °C for 10 min. Storage of the PCR products occurred at 4 °C until gel electrophoresis for separation and visualization of amplicons using a UV transilluminator.

Koala retrovirus proviral load q-PCR

To quantify the number of KoRV proviral inserts in koala DNA from white blood cells, two qPCRs were performed on DNA extracted from whole blood, one for the quantification of the KoRV pol gene and the second for amplification of the koala *β*-actin gene to aid in proviral normalization. KoRV proviral load was reported as KoRV proviral copies/103 copies of *β*-actin. The reaction was performed in 5 µl triplicates dispensed in an Eppendorf epMotion 5075 LH model (Eppendorf, Hamburg, Germany) in a 7900HT Sequence Detection System (Applied Biosystems, Singapore). Each reaction contained 2.5 µl of Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 1 µl of each primer and 1.5 µl of DNA template diluted 1 : 10 or 1 : 100. The primers used to detect KoRV *pol* provirus were 5′-TTGGAGGA GGAATACCGATTACAC-3′ (sense) and 5′-CCACTCCCAT-ACCTGCCTT-3′ (anti-sense) [7]. The koala *β*-actin primers were 5′-GAGACCTTCAACACCCCAGC-3′ (sense) and 5′GTGGGTCACACCATCACCAG-3′ (anti-sense) [34]. Reactions were performed with known concentrations of 10^2, 10^4, 10^6 and 10^8 of the target sequences for KoRV and *β*-actin, with purified PCR product from a known KoRV-positive koala (140348). The results are reported as KoRV copies per 10^3 copies of *β*–actin.

Koala retrovirus genotyping

Koala retrovirus-positive samples were genotyped as KoRV-A or KoRV-B from genomic DNA from whole blood using conventional PCR. Genotype-specific primers were used to target 321 bp (KoRV-A) and 271 bp (KoRV-B) fragments of the KoRV genomic *env* gene [13].

Necropsy examinations

Up to 2 years after sampling in the MLR, seven koalas from this cohort were rescued by members of the public and taken to local veterinarians for clinical examinations, where they were subsequently euthanized on humane grounds. A comprehensive necropsy examination was performed within 24 h of euthanasia at the University of Adelaide, Roseworthy campus to assess for signs of infection or disease. Tissue samples were collected and fixed into 10 % neutral buffered formalin before processing for histopathological examination of haematoxylin and eosin-stained sections.

Statistics

Due to the binary nature of KoRV (present or absent) and *C. pecorum* disease status a generalized linear model with the logit link function was used to examine the effect of sex, age group (young – TWC I and II; adult – TWC III and IV; senior – TWC V and VI), BCS (poor, good, excellent) and location (KI or MLR). All two-way interactions were included in the model. Type III sums of squares and a 5 % level of significance were used to assess whether any of the terms were significant. All statistical analyses were performed using SPSS version 24. For KoRV proviral load, a Shapiro–Wilk test was performed to determine Gaussian distributions, where any distributions that deviated from normal were log-transformed. A general linear model with sex, age group, BCS and location included as fixed effects and all significant (P<0.05) two-way interaction included in the model.

RESULTS

Kangaroo Island

The prevalence of KoRV provirus in the KI population was 42.4 % [72/170, 95 % confidence interval (CI): 34.9–49.8 %] (Fig. 1). Conventional PCR detected KoRV provirus in 36.5 % (62/170, CI: 29.2–43.7 %) of koalas, while an additional 10 koalas were found to be KoRV provirus-positive by qPCR. The median proviral copy number was 113.3 KoRV copies/10^3 *β*-actin copies (minimum–maximum: 1.4–12 641.2 KoRV copies/10^3 *β*-actin copies). Koalas that were positive for KoRV provirus by conventional PCR (n=62) were screened for the KoRV-A and KoRV-B proviral *env* gene. KoRV-A was detected in 93.7 % (59/62) of KoRV-positive koalas, while all koalas were KoRV-B-negative. Risk analysis for KoRV infection in the KI population found no association between KoRV infection and age group or BCS (Table 1).

Nineteen mother and back young pairs were sampled. Six mother–young pairs were KoRV provirus-positive and eight pairs were KoRV provirus-negative. The remaining five pairs did not match; in two pairs the mother was KoRV-positive and in three pairs the offspring was KoRV-positive. All KoRV-positive koalas were infected with KoRV-A.

The highest prevalence of KoRV was in the North Coast management zone (87.5%), followed by the Cygnet River and Birchwood Lagoon management zone (53.1%) (Table 1), with koalas significantly more likely to be KoRV provirus-positive if from the North Coast zone by pairwise comparison (P=0.005). There was no difference in proviral load between the management zones (P=0.853), with median proviral loads of 107.1 KoRV copies/10^3 *β*-actin copies in the North Coast zone; 175.1 KoRV copies/10^3 *β*-actin copies in the Cygnet River and Birchwood Lagoon zone; 101.4 KoRV copies/10^3 *β*-actin copies in the Eleanor River and Timber Creek zone; 127.6 KoRV copies/10^3 *β*-actin copies in the South Coast

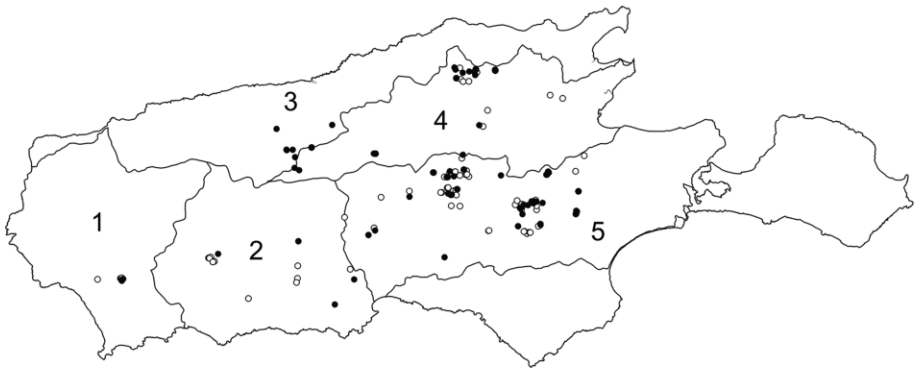

Fig. 1. The location of KoRV-positive (black circles) and -negative (white circles) koalas from the KI koala population, in relation to the Department for Environment and Water koala management zones (1, Rocky River; 2, South West; 3, North Coast; 4, Cygnet River and Birchmore Lagoon; 5, Eleanor River and Timber Creek). Due to scale, overlap has been allowed with KoRV-positive/-negative as the topmost layer. Map not to scale.

zone; and 143.0 KoRV copies/10^3 β-actin copies in the Rocky River zone.

The KI koalas (100 % female) had a mean TWC (±standard error) of 3.66±0.098 and the majority of koalas were in excellent health, with 84.1 % recording an excellent BCS (143/170). Only three koalas (1.7%) presented with clinical abnormalities, including dental disease (n=1) and old trauma (n=2). No clinical or subclinical infections of *C. pecorum* were found, and these results are presented elsewhere [28].

Mount Lofty Ranges

The prevalence of KoRV provirus in the MLR was 65.3 % (49/75, CI: 54.6–76.1 %). The conventional PCR detected KoRV in 52.0 % (39/75, CI: 40.7–63.3 %) of koalas, while the qPCR detected KoRV provirus in an additional 10 koalas.

Table 1. Univariate logistic regression analysis for the association of location, age and body condition with KoRV proviral infection in koalas from the KI population

Variable	KoRV prevalence (%)	Odds ratio (95 % CI)	*P*-value
Management unit			**0.005**
North Coast	7/8 (87.5)	21.00 (18.36–23.64)	
Cygnet River and Birchwood lagoon	17/32 (53.1)	3.40 (1.66–5.14)	
Eleanor River and Timber Creek	38/101 (37.6)	1.81 (0.16–3.46)	
South West	7/20 (35.0)	1.62 (0.00–3.46)	
Rocky River	2/8 (25.0)	1.00	
Age group			0.431
Young	17/32 (53.1)	1.00	
Adult	34/93 (36.6)	1.97 (1.15–2.78)	
Senior	20/43 (46.5)	1.30 (0.39–2.22)	
Not recorded	1/2 (50.0)		
Body condition score			0.810
Excellent	62/143 (43.4)	1.00	
Good	8/21 (38.1)	1.24 (0.30–2.18)	
Poor	2/6 (33.3)	1.53 (0.00–3.26)	

Bold *P*-value, $P<0.05$.

Table 2. Univariate logistic regression analysis for the association of location, age and body condition with KoRV proviral infection in koalas from the MLR population

Variable	KoRV occurrence (%)	Odds ratio (95 % CI)	*P*-value
Park			**0.017**
Belair NP	13/29 (44.8)	1.00	
Cleland CP	11/12 (91.7)	13.54 (11.36–15.71)	
Horsnell Gully CP	17/24 (70.8)	2.99 (1.84–4.13)	
Morialta CP	8/10 (80.0)	4.92 (3.21–6.64)	
Sex			0.517
Male	19/30 (63.3)	1.00	
Female	30/45 (66.7)	1.16 (0.19–2.13)	
Age group			**0.030**
Young	9/19 (47.4)	1.00	
Adult	18/32 (56.3)	1.43 (0.29–2.57)	
Senior	17/19 (89.5)	9.44 (7.72–11.16)	
Not recorded	5/5 (100)		
Body condition score			0.980
Excellent	22/36 (61.1)	1.00	
Good	14/22 (63.6)	1.11 (0.02–2.21)	
Poor	13/17 (76.5)	2.07 (0.76–3.37)	

Bold *P*-value, $P<0.05$.

The median proviral copy number was 35.12 copies/10^3 β-actin copies (minimum–maximum: 0.9–574.0 KoRV copies/10^3 β-actin copies). Of the 39 KoRV-positive koalas, 79.5 % (n=31) were positive for KoRV-A, and no koalas were positive for KoRV-B.

Within the parks, the prevalence of KoRV provirus was significantly lower in Belair NP, where 44.8 % of koalas were KoRV provirus-positive (P=0.017) (Table 2). The median KoRV proviral load within the parks was: 39.8 KoRV copies/10^3 β-actin copies in Belair NP, 35.1 KoRV copies/10^3 β-actin copies in Cleland CP, 168.1 KoRV copies/10^3 β-actin copies in Horsnell Gully CP and 28.9 KoRV copies/10^3 β-actin copies in Morialta CP. There was no statistical difference in KoRV proviral load between the national parks (P=0.680).

Our assessment of the risk factors for KoRV proviral infection in the MLR found an association with age. Koalas were significantly more likely to be KoRV-positive with increasing age group (P=0.030), where the odds of a senior koala (TWC V and VI) being KoRV-positive were over nine times greater than those for a young koala (TWC I and II). There was no association between KoRV proviral infection and sex or BCS (Table 2). There was no association between log-transformed KoRV proviral load and sex (P=0.957), age group (P=0.631) or BCS (P=0.924).

In the MLR, the mean TWC (±standard error) observed was 3.56±0.18. Most of the koalas presented in good health, with 61.1 % of koalas showing an excellent BCS (n=36) and 74.7 % of koalas (n=56) showing no abnormalities on clinical examination. Clinical abnormalities were recorded in 25.3 % (n=19) of koalas, and included presumptive sarcoptic mange and cardiac arrhythmia (n=1), crepitus in one or both stifles (n=7), reduced gut fill (n=1), testicular asymmetry (n=3), previous trauma (n=4) and overt chlamydiosis (n=3). Full results of *C. pecorum* PCR testing was provided elsewhere [28]. Within the MLR region, all koalas from Morialta CP were clinically healthy, while in Belair NP, Cleland CP and Horsnell Gully CP, 72.4 % (21/29), 66.7 % (8/12) and 70.8 % (17/24) of koalas were healthy, respectively.

The three koalas with overt *C. pecorum* disease were transported to local veterinary clinics for treatment, but all were subsequently euthanized. Of these, one koala was available for necropsy after euthanasia (M-48). In addition, up to 2 years after the initial capture, six koalas were rescued by carers after being reported by the public as unwell, and were subsequently euthanized by local Adelaide veterinarians for humane reasons. Overt chlamydial disease was the reason for euthanasia in six koalas, while the seventh koala (M-74) presented with lethargy and a poor BCS, and was

Table 3. Summary of necropsy and histopathological findings in koalas from the MLR that were euthanized up to 2 years following field sampling in April 2016

ID	Sex	TWC	BCS	KoRV*	*C. pecorum**	Necropsy date	Park	Diagnosis
M-05	M	4	3	+	+	19/03/2018	Morialta CP	Pyelonephritis
M-12	F	4	3	+	+	1/05/2018	Cleland CP	Cystitis, endometritis, paraovarian cysts
M-16	M	6	2	+	–	17/05/2016	Cleland CP	Unilateral conjunctivitis
M-17	M	5	2	+	+	7/06/2017	Cleland CP	Epididymitis
M-48	F	2	3	–	+	16/05/2016	Belair NP	Endometritis
M-64	M	2	4	+	–	24/04/2018	Horsnell Gully CP	Cystitis
M-74	M	3	4	+	+	31/10/2017	Horsnell Gully CP	Lymphosarcoma

*+, positive result; –, negative result.
BCS, body condition score; CP, conservation park; NP, national park; TWC, tooth wear class.

subsequently diagnosed with lymphosarcoma at necropsy. The necropsy findings for these seven koalas are reported in Table 3.

From the 75 koalas captured in April 2016, a total of 21 (28.0 %) had since presented to Adelaide veterinary clinics with clinical abnormalities, and 42.9 % (9/21) of these koalas had subsequently died or been euthanized, with *C. pecorum* disease the reason for euthanasia in 88.9 % of the koalas (8/9). Of the 21 koalas with clinical abnormalities, 13 were KoRV-positive and 8 KoRV-negative, and among the 54 clinically healthy koalas, 36 were KoRV-positive and 18 were KoRV-negative. There was no association between KoRV infection and clinical abnormality by pairwise comparison (P=0.697).

Associations between KoRV and *C. pecorum* infection and disease in MLR

To determine whether an association exists between KoRV infection and *C. pecorum* infection or chlamydial disease in MLR koalas, all koalas identified with clinical chlamydial disease since 2016 were incorporated into the analysis. Of 49 KoRV-positive koalas, 44.9 % were *C. pecorum*-negative (n=22), 40.8 % had subclinical chlamydial infection (n=20) and 14.3 % had overt disease (n=7). Of the 26 KoRV-negative koalas, 61.5 % were *C. pecorum*-negative (n=16), 34.6 % had subclinical chlamydial infection (n=9) and 3.8 % had overt disease (n=1). There was no statistical association between KoRV and *C. pecorum* infection (P=0.740, odds ratio: 1.67, 95 % CI: 0.70–2.64) or KoRV and *C. pecorum* disease status (P=0.274), although the odds of a KoRV-infected koala presenting with overt *C. pecorum* disease were over three times those for presenting with subclinical infection (odds ratio: 3.15, 95 % CI: 0.91–5.39). There were no associations between log-transformed KoRV proviral load with *C. pecorum* infection (P=0.535) or *C. pecorum* disease status (P=0.443). There was also no association between *C. pecorum* urogenital chlamydial load and KoRV provirus infection (P=0.674).

DISCUSSION

The current prevalence of KoRV in both the KI and MLR koala populations of South Australia was found to be considerably higher than expected. On KI, KoRV was found to affect 42.4 % of koalas, which is almost three times the previously reported prevalence (14.8%) [3]. The prevalence of KoRV in the MLR (65.3%) was previously unknown but had been thought to be low due to what had been previously reported on KI. Only KoRV-A, not KoRV-B, was identified in both populations, even in MLR koalas with *C. pecorum* infection. Overt *C. pecorum* disease was the most commonly observed clinical abnormality in MLR koalas, and KoRV-infected koalas had an increased risk of developing overt *C. pecorum* disease. KI koalas have recently been found to be *C. pecorum*-free [28] and were in good health.

The presence of only KoRV-A in South Australian koalas is a similar finding to that of a recent Victorian study, which also looked at the interaction of KoRV and *C. pecorum* and found an association between KoRV and clinical wet-bottom disease in the absence of KoRV-B [24]. In contrast, in northern koalas, which are all infected with endogenous KoRV-A [5, 35], recent studies have shown that the exogenous KoRV-B is the pathogenic variant associated with chlamydial disease and neoplasia [8, 13]. This suggests that the pathogenicity of KoRV-A may change with the mode of transmission, where an endogenous infection may not be pathogenic (as observed in northern koalas), but an exogenously acquired infection may cause immunosuppression and associated diseases. Hence in southern koalas, KoRV-A may in fact be the exogenous variant and play a role in the increased risk of developing overt *C. pecorum* disease. Also, a single MLR koala in the current study developed lymphosarcoma, which is the second MLR koala reported to have neoplastic disease and KoRV-A in this population [27].

These differences in KoRV pathogenicity between northern and southern koalas may be partly explained by the common origin of Victorian and South Australian koalas. The koala

populations in South Australia were established with koalas that predominantly originated from mainland Victoria. Koalas were first introduced onto KI and later translocated to populate the MLR [36]. However, additional koalas from northern populations were introduced into South Australian populations, including the descendants of Queensland and Victorian koalas bred in captivity in Adelaide that were later introduced into both regions [37], and reports suggest that koalas of New South Wales origin may also have been introduced into the MLR [36]. While these additional introductions into the MLR have diluted the gene pool, with a greater diversity in haplotypes compared to the KI population, South Australian koalas are considered to be from the same gene pool as Victorian koalas, in a southern koala clade [38]. This may imply that genetic differences between northern and southern koalas may play a role in the variability in susceptibility to KoRV.

The mechanisms of KoRV transmission had not been studied in South Australian koala populations, but Victorian koalas were thought to be exogenously infected due to considerably lower copy numbers of KoRV provirus when compared to northern koalas [3]. In this study, the KoRV proviral load in MLR koalas (median 35.12 copies/10^3 β-actin copies, min–max: 0.9–574.0 KoRV copies/10^3 β-actin copies) was similar to that in Victorian koalas, where the median proviral load was 10 copies/10^3 β-actin copies (min–max: 0.1 to 398 copies/10^3 β-actin copies) [24]. This supports the idea that KoRV may be transmitted exogenously within the MLR population. Furthermore, age was found to be a significant risk factor for infection in MLR koalas, as koalas are more likely to become exogenously infected with KoRV as they age, due to having more contact with other koalas.

The transmission of KoRV-A in the KI population is less clear. It was previously thought that KoRV transmits exogenously in KI koalas due to the low prevalence and low proviral load [3]. However, in this study the prevalence of KoRV had significantly increased since the last survey [3], and some koalas were found to have high KoRV-A proviral loads, providing evidence to support both exogenous and endogenous transmission. Some KI koalas had high proviral loads at similar levels to northern koalas with endogenous KoRV-A [3], which may suggest endogenous infection in these KI koalas, while some KI koalas had low proviral loads, suggesting recent infection with exogenous KoRV-A. The increased KoRV prevalence may be linked to the significant population growth of KI koalas since the last survey. A census report by the South Australian State Government estimated the population size to be 14 270±759 (standard error) in 2010, and 48 506±5975 koalas in 2015 [29]. This rapid population growth could have facilitated the spread of KoRV both exogenously and endogenously. The spread of exogenous KoRV, which could have been introduced with the original koalas from French Island, Victoria that were brought to populate KI [24], may have been facilitated by the increased rate of contact between koalas during the breeding season.

This high reproduction rate in KI koalas may also account for the increased prevalence of an endogenous KoRV, with more koalas being born with the endogenous virus. Endogenous KoRV may have been introduced with the last koalas translocated onto KI, which originated from Queensland [37]. These were released into Cygnet River region and in this study, a higher prevalence of KoRV was found in the Cygnet River and North Coast zones compared to the western end of the island. While there were no significant differences in proviral load and management zones, the median proviral load was highest in the Cygnet River zone (175.1 KoRV copies/10^3 β-actin copies).

The KoRV status of five mother–joey pairs from KI did not match. This mismatch may suggest that transmission of KoRV may be more complex than direct contact. Two KoRV-positive mothers may have been exogenously infected and KoRV was not transmitted to their joeys. KoRV-positive joeys with KoRV-negative mothers may have acquired KoRV exogenously from another koala. KoRV-positive joeys may also have been infected endogenously through Mendelian inheritance from an infected sperm. Similarly, if KoRV is spread endogenously, KoRV-negative joeys may have developed from KoRV-negative oocytes. Future studies should investigate the nature of KoRV transmission in South Australian koalas through sequencing and transcriptomics studies to further understand whether KoRV infection is endogenous or exogenous.

Research on KoRV variants has shown high complexity, and it is possible that exogenous KoRV-A infection, or another exogenous KoRV variant, may have greater implications for southern koala health. In this study, 11 koalas, 3 from KI and 8 from MLR, were PCR-positive for the proviral KoRV *pol* gene, but not for the proviral *env* KoRV-A or B genes. This is in contrast to northern koalas, which are all infected with KoRV-A [5], but similar to Victorian koalas, where 2.93 % (19/648) of koalas were also observed to be both KoRV-A and B-negative [24]. This suggests that in these southern koalas another KoRV variant, different from A or B, was present but not detected. These abnormalities cannot be explained by the recently described recKoRV, as the *pol* gene is not present in recKoRV [39], but perhaps some unknown overlap between the two retroviruses is occurring. Unfortunately, PCR tests are not currently available for the other variants, which have to be detected by sequencing. Future work should employ sequencing of full-length KoRV and recKoRV in KI and MLR koalas, which would shed light on the KoRV variants present in South Australian koalas.

This study has identified KoRV to be a prevalent pathogen in wild South Australian koala populations, although the role of KoRV infection in clinical diseases, particularly *C. pecorum* and neoplasia, in southern koalas appears to be complex. Unlike the northern koala population, in which KoRV-B has been associated with clinical chlamydial disease [13] and lymphosarcoma [8], KoRV-B was not detected in either South Australian population, only KoRV-A. Future studies should continue to investigate the possible association between other KoRV variants and *C. pecorum* infections, as the relationships are likely to differ between the northern

and southern populations. These findings could have important outcomes for koala conservation, given that southern koalas have lower prevalence of KoRV and are less affected by chlamydial disease.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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Chapter 4

Haematological reference intervals of wild southern Australian koalas (*Phascolarctos cinereus*)

Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Jessica Fabijan
Contribution to the Paper	Contributed to the study design and development, organised, facilitated and coordinated the capture and sampling of koalas in the Mount Lofty Ranges (MLR) Coordinated and collected samples from koalas from Kangaroo Island (KI) koala populations Coordinated the collection and transport of blood samples from the MLR and KI field sample sites to the Roseworthy campus for processing and storage Performed blood smear reviews Developed koala hematological reference intervals (RI) Interpreted and performed statistical analysis Wrote manuscript
Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Contributed to the study design of koala hematological RIs Assisted in blood smear reviews Assisted with hematological RI development and statistical analysis Assisted in editing the manuscript		
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ORIGINAL ARTICLE

Haematological reference intervals of wild southern Australian koalas (*Phascolarctos cinereus*)

J Fabijan,* N Speight, WSJ Boardman, F Hemmatzadeh, DJ Trott and L Woolford

Objective Current haematology reference intervals (RIs) for koalas were developed in northern Australian koalas, using low numbers and/or individuals of unknown *Chlamydia pecorum* and koala retrovirus (KoRV) status. This study developed haematological RIs for wild, clinically healthy southern Australian koalas of known *C. pecorum* and KoRV infection status and investigated the effects of population, age and sex.

Methods Haematological RIs were determined for 138 clinically healthy South Australian koalas (Mount Lofty Ranges [MLR], n = 68; Kangaroo Island, n = 70) examined in April 2016 and February 2017, respectively. *C. pecorum* and KoRV status were determined by PCR.

Results RIs for southern koala haematological parameters were established for all koalas based on the finding that there were limited differences in haematological values in koalas with subclinical *C. pecorum* or KoRV infections ($P > 0.05$), except KoRV-infected koalas had a lower haematocrit than noninfected koalas. MLR koalas had significantly lower erythrocyte mass and leucocyte counts than Kangaroo Island koalas. Young koalas had significantly lower haemoglobin, haematocrit and higher mean cellular haemoglobin concentration and lymphocyte counts than adult koalas. MLR male koalas had elevated erythrocyte, leucocyte and neutrophil counts compared with MLR females.

Conclusion The haematological RIs developed in this study are based on a large number of clinically healthy koalas, where subclinical *C. pecorum* and KoRV infections had no effect on haematological values and will be a valuable tool during clinical examination and disease investigation by veterinarians and researchers Australia-wide.

Keywords chlamydia; gammaretrovirus; health assessment; marsupial; wildlife

Abbreviations HCT, haematocrit; HGB, haemoglobin; KI, Kangaroo Island; KoRV, koala retrovirus; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; MCV, mean cell volume; MLR, Mount Lofty Ranges; N:L, neutrophil: lymphocyte; nRBC, nucleated red blood cell; PLT, platelet; RBC, red blood cell/erythrocyte; RI, reference interval; TWC, tooth wear class; WBC, white blood cell/leucocyte

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The koala, a folivorous, arboreal marsupial, is one of Australia's most valued species; however, there is growing concern for their future.[1] Prior to European settlement, koalas occupied habitat along the eastern and south-eastern coast of Australia, but by the turn of the 20th century their distribution was dramatically reduced, with localised extinctions occurring in the southern parts of Australia.[2] Today, koala populations in northern Australia (New South Wales and Queensland) are continuing to decline and are considered a vulnerable species by the Australian Government.[3] Factors contributing to this decline include habitat fragmentation, trauma from vehicle collision and dog attacks and fire and climate change.[1] Diseases are also key contributors to decreasing population numbers, particularly *Chlamydia pecorum* which causes ocular and urogenital infections,[4] and koala retrovirus (KoRV) which may cause the development of lymphoid neoplasia and immunosuppression.[5] Southern (Victoria and South Australia) koala population numbers are considered much more stable,[3] but similarly to northern koalas, trauma and diseases[6,7] result in the rescue of a large number of wild koalas by members of the public each year, for treatment at local wildlife and veterinary hospitals.[6]

Haematology is routinely performed during clinical examination of koalas.[8] In 1960, the first investigation of koala haematology compared values between 47 healthy and diseased individuals in northern koala populations.[9] Two subsequent studies developed haematological reference intervals (RIs) for northern koalas, which are still used by commercial veterinary laboratories Australia wide.[10,11] Dickens[11] developed RIs in 1976 using more than 200 wild and captive koalas from Queensland, New South Wales and Victoria by manual methods; however, the health status of these koalas was not reported, and haematological variables have been shown to differ between captive and wild animals in some species, such as fish[12] and river turtles.[13] The most widely used RIs, of Canfield et al,[10] were developed in 1989 from 45 wild koalas in apparent good health from New South Wales. Since the publication of these studies over three decades ago, new guidelines exist for the development of haematological RIs in animals.[14] Furthermore, no reference intervals have yet been developed for southern koala populations, which share a single southern lineage, and differ to northern koalas.[15]

In southern koala populations, the prevalence of infectious diseases is lower in both South Australia[6,16] and Victoria[17] than that reported in the north. In northern Australian populations, *C. pecorum* affects up to 90% of koalas, and causes blindness, infertility and death.[4] Also, KoRV is highly prevalent in northern populations, where 100% of koalas are infected with endogenous KoRV-A,[5] and the exogenous variant, KoRV-B, which was 24% prevalent in a wild Queensland

population,[18] has been associated with the development of lymphoid neoplasia[19] and overt chlamydial disease.[18]

In contrast, the Mount Lofty Ranges (MLR) and Kangaroo Island (KI) koala populations, the two largest South Australian populations,[20,21] were recently shown to have a lower prevalence of *C. pecorum*[22] and KoRV.[23] In the MLR, 46.7% (35/75) of koalas were *C. pecorum* positive and 65.3% (49/75) were KoRV-A positive, while the KI population was *C. pecorum*-free and 42.4% (72/170) of koalas were KoRV-A positive. The median (range) KoRV proviral loads of koalas from the MLR and KI populations were 35 (0.9–574) and 113 (1.4–12,641) KoRV provirus copies/10^3 β-actin copies.[23] Furthermore, clinical disease associated with these pathogens was low; only three MLR koalas (4.0%) presented with overt chlamydial disease[22] and KoRV-B was not detected in either population,[23] which along with low proviral loads.[23] Similar findings have also been reported in Victorian koalas; the prevalence of both *C. pecorum* (15%)[24] and KoRV (24.7%) was low, with low proviral loads (median, range: 10, 0.1–398 KoRV provirus copies/10^3 β-actin copies) and KoRV-B not detected.[17] The low prevalence of *C. pecorum*, low KoRV proviral loads and KoRV-A-based infection may contribute to the lower prevalence of disease in southern koalas.

This large cohort of clinically healthy southern koalas, a population with a significantly lower prevalence of infectious diseases (*C. pecorum*, KoRV) than northern populations, therefore provided the opportunity to review koala haematological RIs, using current guidelines[14] and investigated variations attributable to subclinical *C. pecorum* and KoRV-A infection, population, age and sex.

Materials and methods

Sample collection

Blood samples for haematological analysis were collected from wild-caught koalas from the MLR (n = 74) population in April 2016 and KI (n = 71) population in February 2017 as part of a study to assess wild koala health. Koalas were captured by a modified version of the noose and flag method.[25] In the MLR population, koalas were captured, sampled and released on the same day. The KI koalas were all female as they were sampled in conjunction with the state government Department for Environment and Water Koala Sterilisation Program, where koalas were captured and housed overnight prior to surgical sterilisation. Sampling of wild koalas was approved by the University of Adelaide Animal Ethics committee, S-2013-198 and S-2015-138 with DEW Scientific Research approval, Y26054-6 and U26431-1.

For clinical examination, MLR koalas were anaesthetised using injectable agents (alfaxalone and medetomidine) and KI koalas were anaesthetised by mask inhalation using isoflurane and oxygen. At examination, sex was recorded, age was estimated by dentition (tooth wear class [TWC]; TWC I, 1–2 years; TWC II, 2–3 years; TWC III, 4 years; TWC IV, 4–9 years, TWC V, 10–12 years, TWC VI, 12+ years)[26] and body condition score was estimated by the degree of musculature over the scapular spine (poor, fair, excellent).[8] Blood was collected from the cephalic or femoral vein into EDTA (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and chilled prior to transport. Blood smears were made at sample collection with fresh blood. Dry swabs were collected from the conjunctiva and cloaca for *Chlamydia pecorum* detection and whole blood was used for KoRV detection.

C. pecorum and KoRV qPCR detection

The methods of detection and prevalence for *C. pecorum* and KoRV in the MLR and KI koalas used in this study have previously been reported.[22,23] Briefly, *C. pecorum* was detected by qPCR with primers that targeted a 209 bp fragment of the CpeC HP gene[22] and KoRV was detected by qPCR targeting a 111 bp fragment of the *pol* gene and standardised against the koala β-actin gene.[5,27]

Haematology

Blood samples were chilled and transported back to the Veterinary Diagnostics Laboratory, Roseworthy Campus. The University of Adelaide for analysis. Blood samples collected in MLR and KI from Monday to Thursday were processed the following day (24 h, n = 73), and samples collected on Friday were processed the following Monday (72 h, n = 65). Haematological analysis was performed using a Cell-Dyn 3700 haematology analyser (Abbott Diagnostics Division, Ramsey, MN, USA). All analyses were performed by trained medical scientists at the Veterinary Diagnostics Laboratory. Internal quality controls are performed daily at 0900 hours using three levels (low, normal and high). The Veterinary Diagnostics Laboratory participates in two external quality control evaluation programs: the Randox International Quality Assessment Scheme on a monthly basis and Veterinary Laboratory Association Quality Assurance Program (Atlantic Veterinary College, University of Prince Edward Island) on a quarterly basis.

The analyser measured erythrocyte (red blood cell/erythrocyte [RBC]) count, haemoglobin (HGB), haematocrit (HCT), mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), automated leucocyte (WBC) count and automated platelet (PLT) count. Blood smears were stained with Wright-Giemsa stain (Kinetik, Narangba, Queensland, Australia) and differential cell counts performed which included nucleated RBC count (nRBC, cells/100 WBC) at 400× magnification, manual PLT estimate and cell morphology examination were performed at 1000× magnification on a BX43 microscope (Olympus, Notting Hill, Vic, Aust). Automated PLT counts were excluded from analyses if significant PLT clumping was observed on blood film review. Absolute nRBC count was calculated from manual nRBC count and automated WBC, and WBC count was corrected for n > 5nRBC/100 WBC. Neutrophil: lymphocyte (N:L) ratio was calculated for each koala to establish a RI for this parameter, which has in previous studies been used as an indicator for stress or disease.[7,11]

As a quality control measure, haematological values were assessed based on sampling to analysis time interval. Haematology values were compared using a general linear model with type I sums of squares ($\alpha = 0.05$), and included population, the interaction between population and sex, age group and days and all parameters were found to be comparable for the samples analysed 24 h after collection with those analysed 72 h after collection ($P > 0.1$), except for some erythrocyte parameters. Samples analysed 24 h (n = 73) after collection had significantly higher RBC count (mean ± SD: 3.68 ± 0.32 × 10^{12} cells/L) than samples analysed after 72 h (n = 65) (mean ± SD:

$3.56 \pm 0.33 \times 10^{12}$ cells /L) (P = 0.028). Samples at 24 h also had significantly lower MCV (mean ± SD: 102.4 ± 4.46 fL) and MCH (mean ± SD: 31.9 ± 1.24 g/L) counts than samples analysed after 72 h (mean ± SD: MCV, 104.4 ± 2.62 fL; MCH; 32.5 ± 1.30 g/L), P = 0.001 and 0.037, respectively. Despite these parameters showing statistical significance, the differences were of little effect and not considered to be of clinical significance.

Outlier removal and criteria for inclusion in RI

Koalas were included in RI development if there was an absence of injury or disease and if they presented with a fair or excellent body condition score. For each haematologic parameter, histograms of the reference values were examined for initial assessment of distribution and identification of potential outliers. Outliers identified through visual inspection of the histogram were verified to original laboratory reports to identify and correct any transcription errors. Outliers were also detected by Reference Value Advisor© (RefVal), version 2.1 (National Veterinary School of Toulouse, Toulouse, Haute-Garonne, France)[28] using Dixon's and Tukey's range test. Aberrant observations were removed, however there was a general tendency to retain outliers rather than remove.[14]

RI development

RIs were developed from the entire koala cohort (including both populations, all ages, and both sexes) following the 2011 guidelines by the American Society for Veterinary Clinical Pathology[14] and using RefVal.[28] For n ≥ 120 samples, nonparametric methods were used to generate upper and lower reference limits and respective 90% confidence intervals.[14] For PLT count, where 40 ≤ n ≤ 120, the RI was calculated by Robust methods and confidence intervals determined by Bootstrap method.[14] Partitioning was performed if there was a clear clinical or physiological reason to partition, for sample sizes with n > 40, and when <0.9% or >4.1% of the subgroup population was outside of the population RI.[29]

Statistical analysis

For all haematological parameters, a Shapiro–Wilk test along with histograms and standardised residual plots were reviewed to determine normal distribution, and non-Gaussian parameters were log-transformed and distribution re-assessed. Native Gaussian distributions were identified for RBC, HGB, HCT, MCV, MCH, MCHC, absolute nRBC, WBC, neutrophil, lymphocyte, monocyte and manual PLT. Parameters which did not achieve normal distribution with log transformation were band, eosinophil and basophil counts. Univariate general linear models were used with a type I sums of squares ($\alpha = 0.05$) to determine if pre-analytical koala factors had an effect on haematological values. Two models were performed to compare the effects of *C. pecorum* and KoRV on haematological values independently. In each model, for each of the haematological parameters pre-analytical factors were ordered; (1) population (MLR and KI), (2) sex nested within population (as an interaction as all KI koalas were female), (3) age group (young – TWC I and II; adult – TWC III and IV; senior – TWC V and VI) and (4) infectious agent detection, *C. pecorum* or KoRV (present or absent). All koalas (n = 138) were included in both models. For nonparametric variables, a Mann-Whitney or Kruskal-Wallis test was utilised. All statistical analyses were performed in SPSS version 24 (SPSS Inc., Chicago, IL, USA).

Results

A total of 145 blood samples were collected from wild-caught South Australian koalas from the MLR (n = 74) and KI (n = 71) populations. In the MLR, five koalas were excluded from this study; three koalas due to overt *C. pecorum* disease, one due to sample haemolysis and another as an outlier, and from KI only one sample was excluded, due to haemolysis. The reference population therefore included 138 samples from clinically healthy koalas, 68 from the MLR and 70 from the KI populations. Of the entire cohort, the majority of koalas were adults and most were female (as all KI koalas were female). The prevalence of *C. pecorum* within the final cohort was low as the KI population was shown to be *C. pecorum*-free,[22] while the prevalence of KoRV was moderately high[23] (Table 1).

Statistical analysis showed that there were no changes in haematological values due to subclinical *C. pecorum* infection (P > 0.05) (Table S1, Supporting information). The only haematological value which differed in koalas with subclinical KoRV infection was HCT (P = 0.042), which was lower in KoRV positive koalas (mean ± SD: 0.37 ± 0.03 L/L) compared to KoRV negative koalas (mean ± SD: 0.39 ± 0.04 L/L) (Figure 1). However, other markers of RBC mass (RBC count, HGB and MCV) were not affected by KoRV infection (Table S1).

Haematology RIs were developed for the entire koala cohort (Table 2). Histograms showing the spread of raw values for each haematological parameter are presented in Data S1. Nucleated RBCs were commonly observed. Circulating nRBCs were observed in 95.7% (132/138) of koalas, ranging between 1 and 49 nRBC/100 WBC (median, 7.0 nRBCs/100 WBCs; mean, 9.3 nRBCs/100 WBCs), where absolute nRBC is presented (Table 2). Neutrophils and lymphocytes were the most common circulating leucocytes, and the proportion of neutrophils (median, range: 53, 8%–82%) was more often greater than the proportion of lymphocytes (median, range: 37, 8%–

Table 1. Demographics of clinically healthy, wild caught South Australian koalas included in the development of koala haematology reference intervals

Demographic	Cohort n (%)	MLR n (%)	KI n (%)
Sex	138	68	70
Female	110 (79.7)	40 (58.8)	70 (100)
Male	28 (20.3)	28 (41.2)	0 (0)
Age group	132	63	69
Young	27 (20.5)	17 (27.0)	10 (14.5)
Adult	71 (53.8)	30 (47.6)	41 (59.4)
Senior	34 (25.7)	16 (25.4)	18 (26.1)
Chlamydia pecorum	138	68	70
Positive	28 (20.3)	28 (41.2)	0 (0)
Negative	110 (79.7)	40 (58.8)	70 (100)
Koala retrovirus	138	68	70
Positive	71 (51.4)	44 (62.9)	27 (38.6)
Negative	67 (48.6)	24 (37.1)	43 (61.4)

KI, kangaroo Island; MLR, Mount Lofty Ranges.

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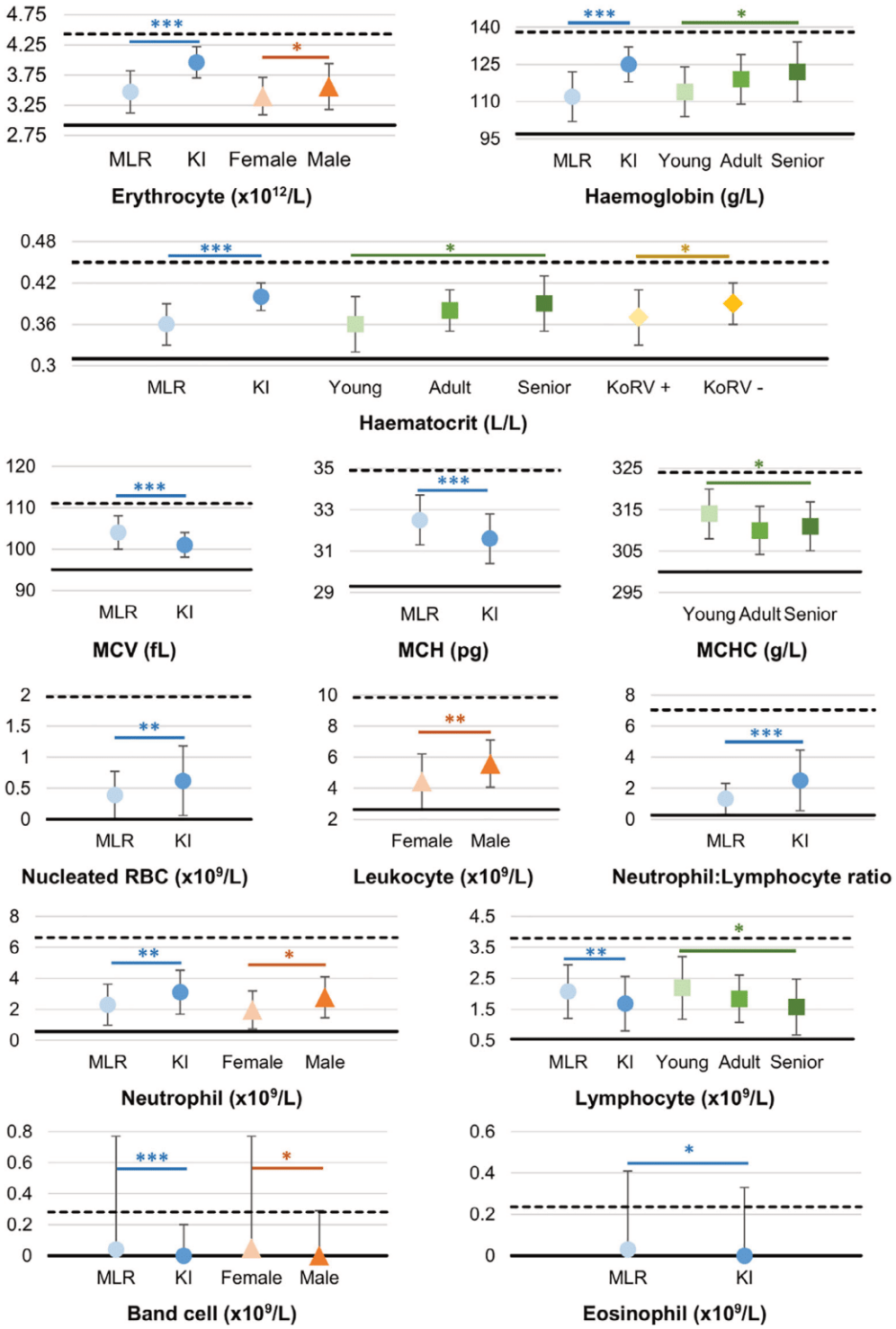

Figure 1. Comparison of statistically significant (P < 0.05, *: P < 0.01,**: P < 0.001,***) differences between haematological values (mean ± SD) and age (green square; light green-young, medium green-adult, dark green-senior), koala retrovirus (KoRV) infection status (yellow diamond; light yellow-KoRV negative, dark yellow-KoRV positive), population (blue circle; light blue-Mount Lofty Ranges [MLR], dark blue-Kangaroo Island [KI]) and sex (red triangle; light red-female, dark red-male) compared to reference intervals developed in the current study (upper interval-broken line; lower interval-solid line). Band and eosinophil counts are presented as median, minimum and maximum values.

86%) as presented by the N:L ratio (median, range: 1.46, 0.09–10.24). Mild RBC anisocytosis and polychromasia was a common finding with the presence of nRBCs (132/138). The presence of occasional RBC with spherocyte-like morphology was seen in 15 koalas; however, no evidence to support haemolysis or immune-mediated destruction was seen. Howell-Jolly bodies were observed

Table 2. Haematological reference intervals generated for 138 South Australian koalas using a Cell-Dyn 3700 automated haematology analyser

Parameter	Units	n	RI	90% LCI	90% UCI	Mean (SD)	Median
RBC	10^{12}/L	138	2.92–4.43	2.72–3.12	4.29–4.58	3.72 (0.4)	3.76
HGB	g/L	138	97–138	95.2–101	135–145	119 (11)	120
HCT	L/L	138	0.31–0.45	0.3–0.32	0.44–0.47	0.38 (0.04)	0.38
MCV	fL	138	95–111	93.8–96.8	110–114	103 (3.8)	103
MCH	Pg	138	29.3–34.9	29.1–29.9	34–35.6	32 (1.3)	32.0
MCHC	g/L	138	300–324	293–302	320–332	311 (5.9)	311.0
nRBC	10^9/L	138	0–1.97	0–0.04	1.63–2.62	0.5 (0.49)	0.36
WBC	10^9/L	138	2.62–9.84	1.92–2.82	8.54–10.3	5.05 (1.76)	4.81
Neutrophil	10^9/L	138	0.57–6.62	0.40–0.91	5.2–8.13	2.71 (1.42)	2.48
Lymphocyte	10^9/L	138	0.54–3.79	0.33–0.70	3.37–5.87	1.87 (0.89)	1.77
N:L ratio		138	0.26–7.04	0.09–0.46	4.73–10.25	1.91 (1.66)	1.46
Band	10^9/L	138	0–0.281	0–0	0.195–0.771	0.045 (0.09)	0
Monocyte	10^9/L	138	0.06–1.08	0–0.09	0.74–1.52	0.38 (0.24)	0.33
Eosinophil	10^9/L	138	0–0.236	0–0	0.174–0.410	0.039 (0.066)	0
Basophil	10^9/L	138	0–0.068	0–0	0.056–0.148	0.006 (0.021)	0
Automated PLT[a]	10^9/L	69	73–225	63–85	211–239	151 (38)	148
Manual PLT[a]	10^9/L	70	153–508	128–182	477–538	329 (88)	338

[a]RI generated using parametric, Robust method. RI were generated using nonparametric methods for n > 120. HCT, haematocrit; HGB, haemoglobin; LCI, lower confidence interval; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; MCV, mean cell volume; N:L, neutrophil:lymphocyte; nRBC, nucleated red blood cell; PLT, platelet; RI, reference interval; UCI, upper confidence interval; WBC, white blood cell/leucocyte.

occasionally to frequently. WBC morphology was consistent with previously described morphology for koalas[30] (Figure 2).

Based on koalas in this study originating from two South Australian populations, the MLR and KI, haematological values were compared for population differences. Strong statistical differences were identified for a number of parameters (Table S1). MLR koalas had lower means than KI koalas for RBC, HGB, HCT, absolute nRBC, neutrophil counts and N:L ratio, and higher means for MCV, MCH, band, lymphocyte, and eosinophil counts (Figure 1).

There were also a number of differences in haematological values between the three age groups (Table S1). In young koalas, MCHC and lymphocyte count were significantly higher compared to adult and senior koalas. Additionally, HGB and HCT were significantly lower in young koalas compared to adult and senior koalas (Figure 1). There were no other differences between haematological values between the three age groups.

As all KI koalas were female, sex differences were compared between MLR male and female koalas only. Male koalas had significantly higher RBC, WBC and neutrophil counts than female koalas (Figure 1). There were no other differences between male and female MLR koalas (Table S1).

Discussion

Presented here are haematological RIs for the koala, utilising 138 clinically healthy southern koalas as the reference population. Subclinical *C. pecorum* and KoRV infections were found to have little to no effect on haematological parameters. While HCT was marginally decreased in KoRV positive koalas, this finding was not reflected in other markers of RBC mass. However, there were a number of significant differences identified between the two koala populations, as well as age and sex. Of these, the majority showed only marginal differences in haematological parameters; however, age (HGB, HCT, MCHC and lymphocyte count)- and sex (RBC, WBC and neutrophil count)-related differences were quite marked and may have a biological basis.

The RIs developed in this study were comparable to those previously published in northern koalas by Canfield et al[10] (Figure 3). RIs for indicators of RBC mass showed general agreement between the studies although were higher and narrower in this study. The upper RI for WBC count was lower in this study compared with Canfield et al;[10] however, the most notable differences were observed for lymphocyte, monocyte and eosinophil count RIs, where lymphocyte and eosinophil RIs were narrowed and monocyte count expanded in the current study. Some differences between the two studies are likely due to the increased sample size of koalas used in this study (n = 138) compared to the previous study (n = 45).[10] Differences in the lymphocyte RIs may be due to more exhaustive health assessments of koalas in this study where koalas were tested for both *C. pecorum* and KoRV infections. The health status of some koalas in the previous study was unknown and underlying disease due to *C. pecorum* of KoRV which was not tested for may have been missed.[10] The eosinophils RI in this study was significantly narrowed

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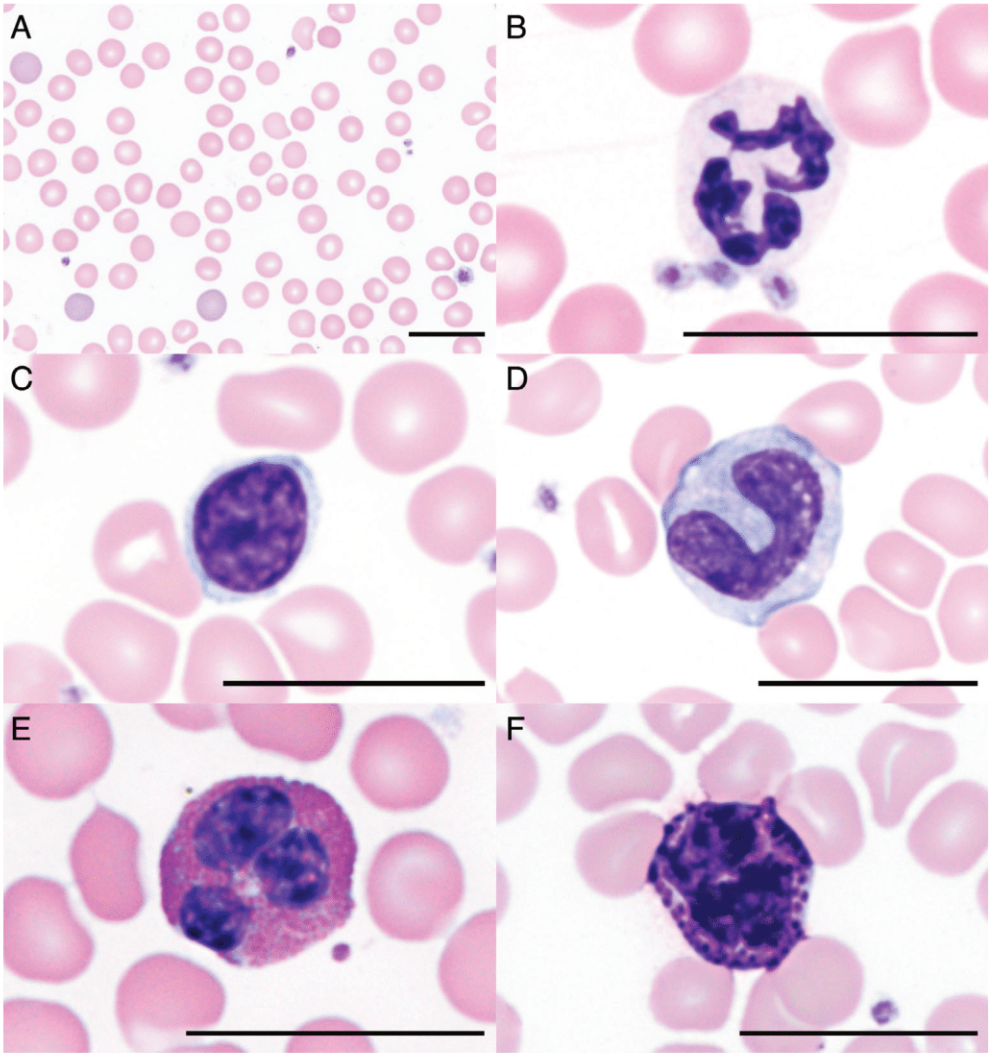

Figure 2. Typical koala peripheral blood morphology. (A) Erythrocyte morphology showing mild anisocytosis and mild polychromasia, (Wright-Giemsa stain), bar 20 µm. (B) Neutrophil with multi-lobed nucleus consisting of coarsely clumped chromatin and pale cytoplasm, (Wright-Giemsa stain), bar 20 µm. (C) Medium sized lymphocyte with central, round nucleus with coarsely clumped dense chromatin and scant basophilic cytoplasm, (Wright-Giemsa stain), bar 20 µm. (D) Monocyte with indented, bean-shaped nucleus and basophilic cytoplasm with multiple small vacuoles, (Wright-Giemsa stain), bar 20 µm. (E) Eosinophil with 3-lobed nucleus consisting of coarsely clumped chromatin and round eosinophilic granules present within the cytoplasm, (Wright-Giemsa stain), bar 20 µm. (F) 100× objective, basophil with large, round basophilic granules present within the cytoplasm which obscures the lobulated nucleus, (Wright-Giemsa stain), bar 20 µm.

and lowered. Eosinophils may increase in response to parasitic infections/ migration, and there are a number of parasitic infections reported in koalas from New South Wales, particularly the intestinal tapeworm *Bertiella obesa*,[31] where the study by Canfield et al[10] was conducted. As this has not been reported in southern koalas, this may account for the narrowed eosinophil RI in this study. Reasons behind the increased monocyte RI are unknown but may be due to immune differences between northern and southern koalas, or simply due to the larger sample of koalas used in this study.

Subclinical or carrier *C. pecorum* and KoRV infections are common in clinically healthy koalas and were found in this study to have no effect on haematological parameters. Only HCT was lower in KoRV positive koalas (0.37 ± 0.03 L/L) than KoRV negative koalas (0.39 ± 0.04 L/L), but as HCT is calculated from RBC and MCV, and there were no differences between KoRV infected and noninfected koalas for RBC or MCV counts, the lower HCT is unlikely to have any clinical significance. While northern koalas are observed with subclinical *C. pecorum* infections,[4] subclinical infections are much more common in southern koalas.[16,22] Furthermore, while the prevalence of KoRV is 100% in northern populations[5] and also high in these southern populations,[23] the majority of southern population koalas are clinically healthy. In northern koalas, only 4.7% (6/127) of koalas were observed with lymphoid neoplasia[31] and only 1.3% (1/75) of wild MLR koalas were observed with lymphoid neoplasia.[23] For koalas with overt chlamydial disease as assessed with RIs developed by Canfield et al,[10] koalas with cystitis commonly presented with anaemia and neutrophilia and a single koala with conjunctivitis also presented with anaemia.[32] Anaemia has also been reported in Victorian koalas affected with wet-bottom disease.[7] Koalas with lymphoid neoplasia have also been reported with anaemia and may present with leucopoenia or leucocytosis.[32] Therefore, in koalas presented for veterinary assessment with haematological values outside of the RIs developed by this study, the changes would likely reflect disease processes requiring further investigation.

Early studies reported a clear relationship with koala health and the N:L ratio; however, this relationship may not be as clear as initially thought. Dickens[11] reported that the proportion of lymphocytes was always higher than the proportion of neutrophils in healthy koalas, and Obendorf[7] showed a clear linear relationship with increasing proportion of neutrophils and disease. However, Canfield et al[10]

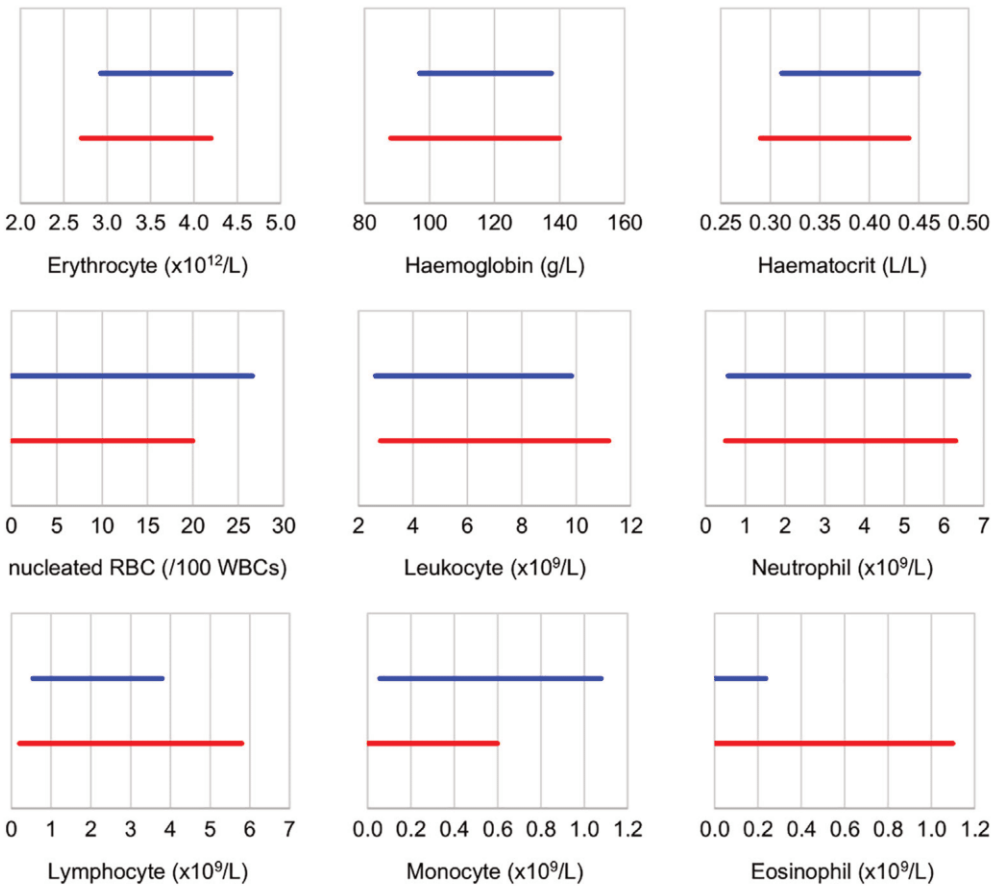

Figure 3. Comparison between haematological reference intervals for southern koalas in the current study (blue line) and haematological reference intervals for northern koalas (red line) published in 1989 by Canfield et al.[10] Absolute nucleated RBC count was not calculated in the previous study.

found neutrophil and lymphocyte proportions to vary considerably in healthy animals and in the current study, koalas commonly had a higher proportion of neutrophils than lymphocytes in circulation. Additionally, no relationship was found between N:L ratio and subclinical/ carrier *C. pecorum* or KoRV infection. Although the findings of the current study suggest that the N:L ratio may not be a reliable indicator of health status, future studies of diseased koalas may shed further light on the usefulness of this haematological parameter.

Haematological values showed a number of differences between the MLR and KI populations. MLR koalas had lower RBC mass parameters and total leucocyte and neutrophil parameters than KI koalas. Despite these differences, partitioning was not performed as the criteria (<0.9% or >4.1% of the subgroup population is required to be outside of the population RI)[29] were not met for these parameters. These differences are likely attributed to the differences in sampling methods between the populations. In the MLR, koalas were captured, sampled and released on the same day, where the maximum amount of time between capture and sampling was 4 h. In contrast, the KI koalas were captured and housed overnight prior to surgical sterilisation; therefore, there was a minimum of 15 h prior to clinical examination and sample collection. In previous studies, samples collected from koalas 6 h post-capture had significantly lower RBC, HGB and packed cell volume (PCV) counts and higher MCV than koalas sampled at 24 h postcapture.[33] These changes were also observed between the MLR koalas sampled within 6 h compared to the KI koalas sampled after 24 h and may reflect the time between capture and sampling, and possible effects of physiological stress or excitement such as adrenalin mediated splenic contraction and changes in haemodynamics. Hajduk et al[33] observed a stress leukogram in koalas sampled at 6 h compared to 24 h postcapture, where WBC, neutrophil and monocyte counts were elevated and lymphocyte count lowered at 6 h. However, these leucocyte differences were not consistently observed between the MLR and KI populations. While the population differences in erythrocyte parameters are consistent with previous studies of capture sampling intervals, the leucocyte differences are not and may be due to other factors.

The method of anaesthesia was different in each population, where MLR koalas were anaesthetised with injectable agents while KI koalas were anaesthetised by isoflurane gas. Both injectable agents in cats[34] and isoflurane in sheep[35] and flying foxes[36] have been shown to reduce erythrocyte and leucocyte values compared to when animals were unanaesthetised. However, to our knowledge, there has been no direct comparison of injectable and inhalational agents on haematological values. If injectable agents have a greater tendency to reduce haematological values than isoflurane, for example, through blood pressure-related changes in haemodynamics or splenic function, this may explain the findings in this study.

Due to constraints of field research in this study, haematological samples could not be analysed for 24 h when collected from Monday

to Thursday, or for 72 h if collected on a Friday. Samples analysed after 72 h showed marginal RBC lysis, with lower RBC counts, and elevated MCV and MCH as evidence of RBC swelling which has been shown to occur in stored human blood in EDTA.[37] There were no differences in leucocyte values between the samples analysed after 24 and 72 h, which was similarly found in a study that compared the effect of canine blood storage at 2–4°C over a 60 h period on haematological values.[38] Haematological analysis of wild species sampled in the field will often be delayed due to constraints of working in remote locations. These findings show that when koala blood samples are refrigerated for 72 h prior to analysis differences may be observed and should be taken into consideration when interpreting results.

Differences in haematological values were observed between koala age groups. Young koalas had elevated MCV and lymphocyte counts, and lower HGB and HCT compared to adult and senior koalas. These findings are consistent with other studies in northern koalas, where elevated lymphocyte and lowered HGB and PCV have been reported in younger koalas.,[11,39] and similar age-related changes are also reported for domestic and other wildlife species.[40] For this instance, partitioning was not performed as the criteria to partition[29] was not met for these parameters.

Comparison between male and female koalas was performed within the MLR population only, as no male KI koalas were sampled. MLR male koalas had significantly higher RBC, WBC and neutrophil counts than female koalas. No differences were reported between male and female koalas by Canfield et al;[10] however, Dickens[11] partitioned RIs by sex. Males had significantly higher RBC, HGB and PCV than females ($P < 0.01$), and no differences in leucocyte values.[11] Partitioning was not performed in this study for sex differences as the criteria for partitioning[29] were not met for these parameters. Male koalas may be more susceptible to capture stress, where elevated WBC and neutrophil counts may be indicative of an acute physiological or stress leukogram.[33] Koalas have been shown to have elevated WBC and neutrophil counts when excited after capture.[10] The stress of capture may also have caused adrenergic splenic contraction in male koalas, which would have elevated their RBC count. Relatively increased RBC mass in males is also a known androgenic effect in other species.[40] Future field studies should consider capture stress of male koalas when interpreting haematology values.

The haematological RIs developed in this study will be a valuable tool for clinical examination and disease investigation in koalas for veterinarians, and researchers will assist veterinary clinical pathologists in haematology interpretation and provide a comparison for studies published on northern koalas.

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Conflict of interest and sources of funding

The authors declare no conflicts of interest or sources of funding for the work presented here.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site: http://onlinelibrary.wiley.com/doi/10.1111/avj.12923/suppinfo.

Data S1. Xxxx.

Table S1. Variation observed in koala haematological values between *Chlamydia pecorum* and koala retrovirus infections, populations, age groups and sex.

(Accepted for publication 6 January 2020)

Table S1: Variation observed in koala haematological values between *Chlamydia pecorum* and koala retrovirus infections, populations, age groups and sex

		C. pecorum		**KoRV**		**Population**	
		Negative	**Positive**	**Negative**	**Positive**	**MLR**	**KI**
n		110	28	67	71	60	70
Parameter	**Units**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
RBC	10^{12}/L	3.46 ± 0.33	3.48 ± 0.37	3.82 ± 0.37	3.62 ± 0.4	3.47 ± 0.35	3.96 ± 0.26
HGB	g/L	112 ± 9	113 ± 12	121 ± 10	117 ± 11	112 ± 10	125 ± 7
HCT	L/L	0.36 ± 0.03	0.36 ± 0.04	0.39 ± 0.03	0.37 ± 0.04	0.36 ± 0.03	0.40 ± 0.02
MCV	fL	104 ± 4	105 ± 4	102 ± 4	103 ± 4	104 ± 4	101 ± 3
MCH	pg	32.4 ± 1.4	32.6 ± 1.0	31.8 ± 1.3	32.2 ± 1.3	32.5 ± 1.2	31.6 ± 1.2
MCHC	g/L	311 ± 6	311 ± 6	311 ± 6	312 ± 6	311 ± 6	311 ± 6
nRBC	10^9/L	0.27 ± 0.30	0.55 ± 0.42	0.55 ± 0.51	0.46 ± 0.46	0.39 ± 0.38	0.62 ± 0.56
WBC	10^9/L	5.13 ± 1.73	4.56 ± 1.80	5.18 ± 1.90	4.93 ± 1.62	4.90 ± 1.77	5.20 ± 1.75
Neutrophil	10^9/L	2.46 ± 1.32	2.07 ± 1.30	3.00 ± 1.54	2.43 ± 1.23	2.30 ± 1.32	3.10 ± 1.41
Lymphocyte	10^9/L	2.13 ± 0.92	1.97 ± 0.78	1.75 ± 0.82	1.98 ± 0.94	2.07 ± 0.86	1.68 ± 0.88
N:L ratio		1.36 ± 0.93	1.25 ± 1.13	2.15 ± 1.58	1.69 ± 1.72	1.31 ± 1.01	2.5 ± 1.95
Monocyte	10^9/L	0.40 ± 0.27	0.43 ± 0.30	0.35 ± 0.22	0.42 ± 0.26	0.41 ± 0.28	0.36 ± 0.19
PLT	10^9/L	335 ± 115	296 ± 86	336 ± 81	323 ± 94	315 ± 102	353 ± 53
		Median (range)	**Median (range)**	**Median (range)**	**Median (range)**	**Median (range)**	**Median (range)**
Band	10^9/L	0.04 (0-0)	0.04 (0-0)	0 (0-0)	0 (0-0)	0.04 (0-0.77)	0 (0-0.2)
Eosinophil	10^9/L	0 (0-0)	0.03 (0-0)	0 (0-0)	0 (0-0)	0.03 (0-0.41)	0 (0-0.33)
Basophil	10^9/L	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0.07)	0 (0-0.15)

		Age group			Sex[a]	
		Young	**Adult**	**Senior**	**Female**	**Male**
n		27	71	34	40	28
Parameter	**Units**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
RBC	10^{12}/L	3.53 ± 0.39	3.76 ± 0.34	3.8 ± 0.46	3.4 ± 0.31	3.56 ± 0.38
HGB	g/L	114 ± 10	119 ± 10	122 ± 12	111 ± 9	114 ± 11
HCT	L/L	0.36 ± 0.04	0.38 ± 0.03	0.39 ± 0.04	0.36 ± 0.03	0.37 ± 0.04
MCV	fL	103 ± 3	102 ± 4	103 ± 4	105 ± 4	104 ± 4
MCH	pg	32.3 ± 1.2	31.8 ± 1.2	32.1 ± 1.4	32.7 ± 1.1	32.3 ± 1.3
MCHC	g/L	314 ± 6	310 ± 5.8	311 ± 5.9	311 ± 6	311 ± 6
nRBC	10^9/L	0.32 ± 0.30	0.54 ± 0.48	0.61 ± 0.60	0.43 ± 0.44	0.32 ± 0.27
WBC	10^9/L	5.26 ± 1.76	5.07 ± 1.57	4.70 ± 1.78	4.42 ± 1.79	5.58 ± 1.52
Neutrophil	10^9/L	2.60 ± 1.57	2.78 ± 1.36	2.64 ± 1.30	1.96 ± 1.22	2.78 ± 1.32
Lymphocyte	10^9/L	2.19 ± 1.01	1.84 ± 0.76	1.57 ± 0.90	1.94 ± 0.78	2.24 ± 0.95
N:L ratio		1.38 ± 0.89	1.95 ± 1.71	2.36 ± 1.97	1.17 ± 0.90	1.51 ± 1.14
Monocyte	10^9/L	0.38 ± 0.23	0.37 ± 0.22	0.37 ± 0.21	0.37 ± 0.29	0.46 ± 0.26
PLT	10^9/L	308 ± 125	330 ± 83	343 ± 76	297 ± 87	344 ± 121
		Median (range)	**Median (range)**	**Median (range)**	**Median (range)**	**Median (range)**
Band	10^9/L	0 (0-0.18)	0 (0-0.29)	0 (0-0.77)	0.05 (0-0.77)	0 (0-0.29)
Eosinophil	10^9/L	0 (0-0.23)	0 (0-0.33)	0.01 (0-0.41)	0.03 (0-0.23)	0.02 (0-0.41)
Basophil	10^9/L	0 (0-0.15)	0 (0-0.06)	0 (0-0.07)	0 (0-0.07)	0 (0-0)

HCT, haematocrit; HGB, haemoglobin; KI, Kangaroo Island; KoRV, Koala retrovirus; MCV, mean cell volume; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; MLR, Mount Lofty Ranges; nRBC, nucleated red blood cell; N:L, neutrophil:lymphocyte; PLT, platelet; RBC, erythrocyte; WBC, leukocyte

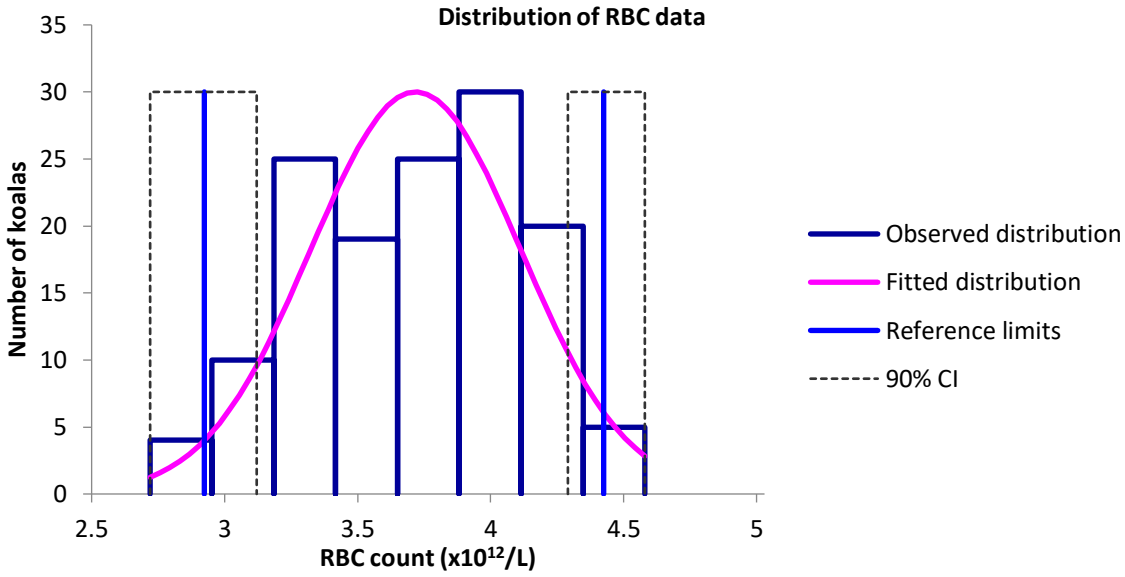
[a]Mean and median values are of koalas from the MLR population only as all KI koalas were female

Bold P-values indicate P<0.05

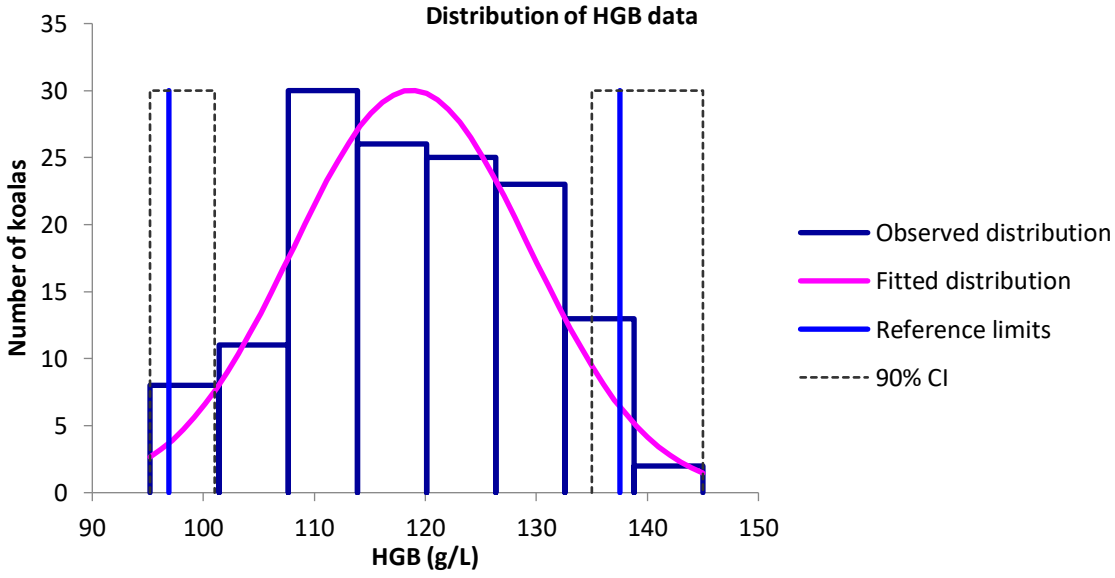
Supplementary information

Below are histograms for each haematological parameter that show the distribution of the raw data in reference to the haematological reference intervals and 90% confidence interval for the upper and lower reference intervals. Histograms were generated using RefVal[27].

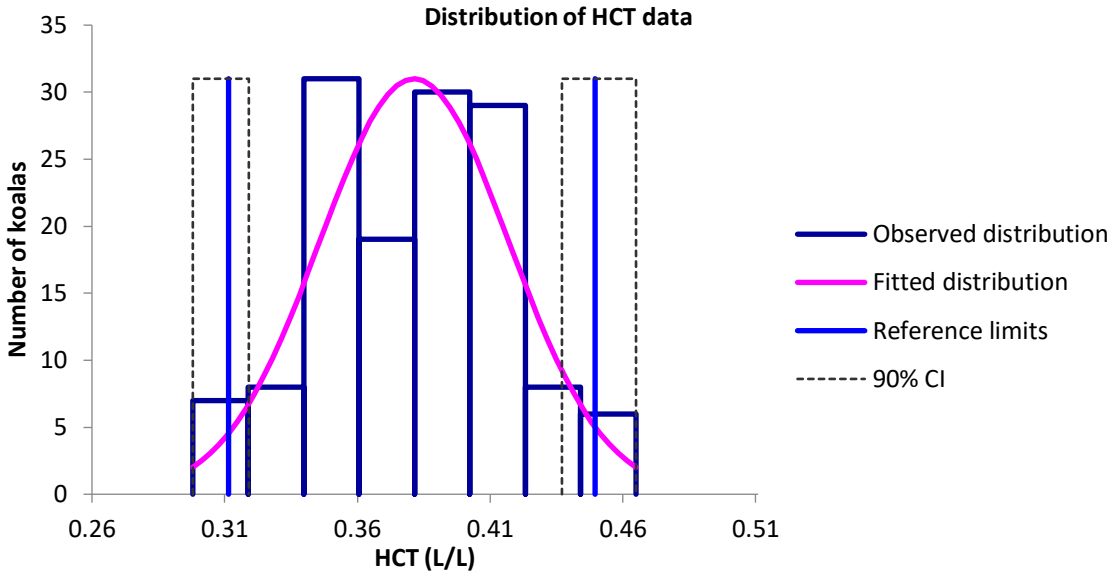
Erythrocyte (RBC)



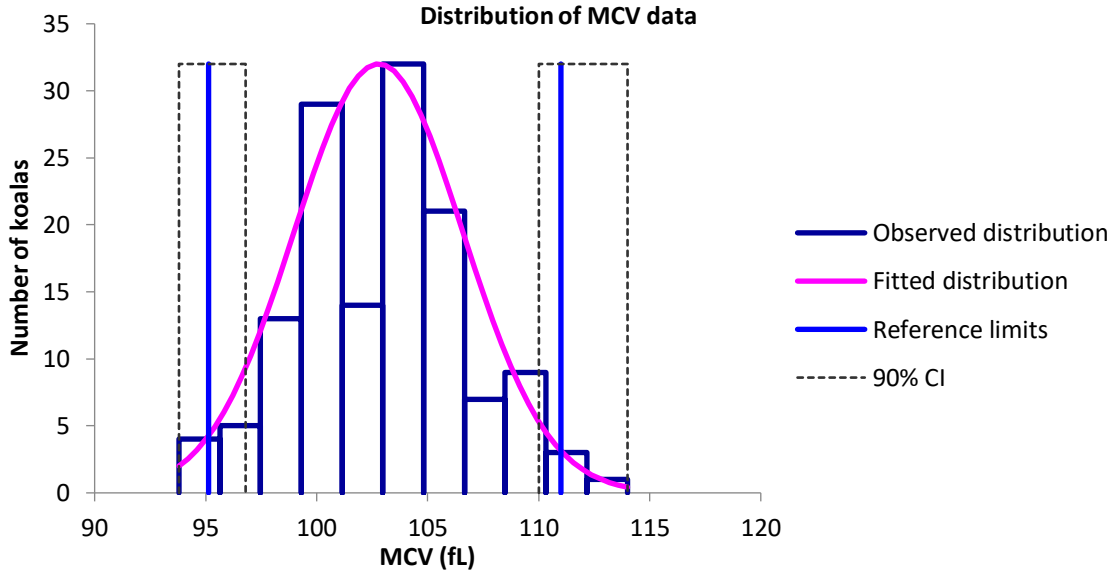
Haemoglobin (HGB)



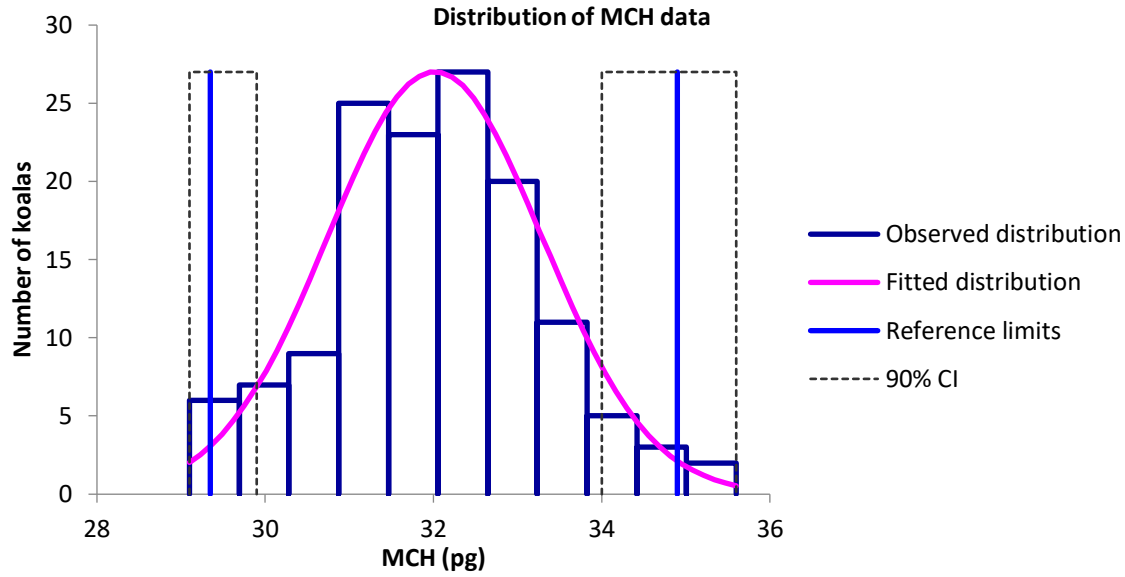
Haematocrit (HCT)



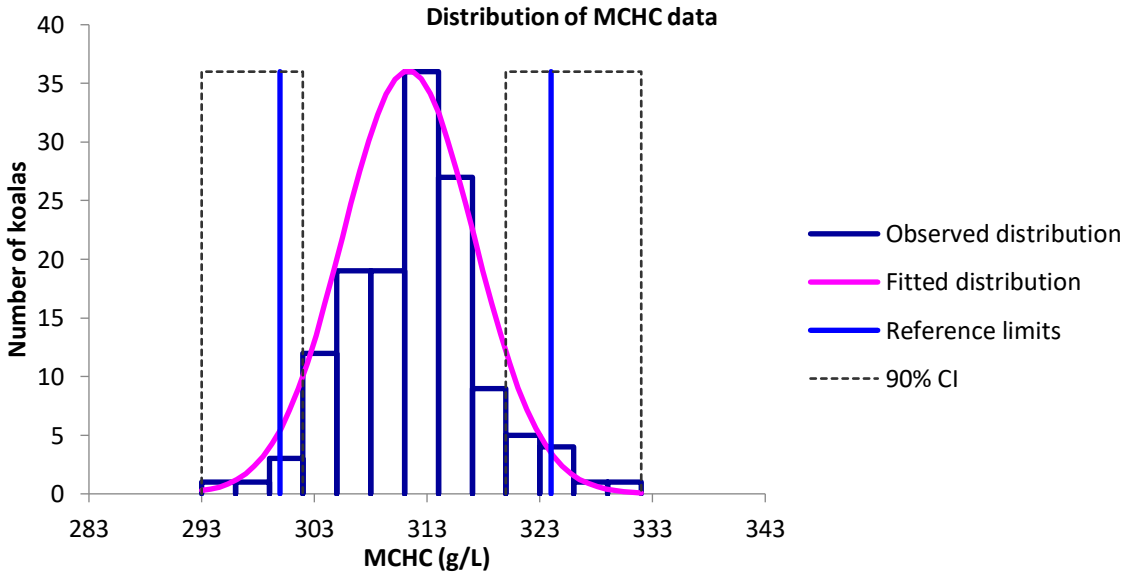
Mean Cellular Volume (MCV)



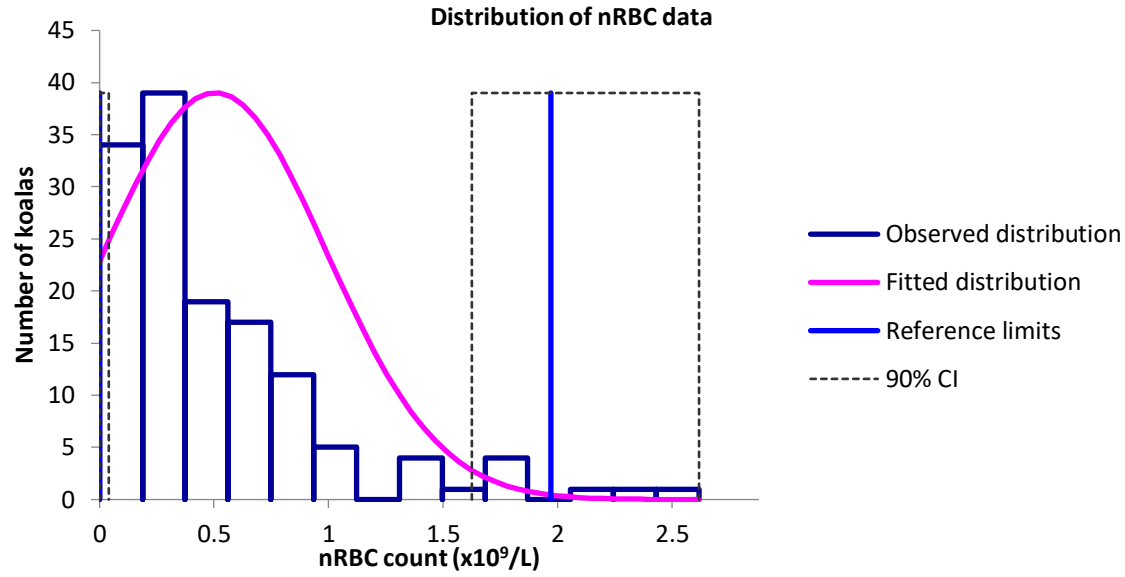
Mean Cellular Haemoglobin (MCH)



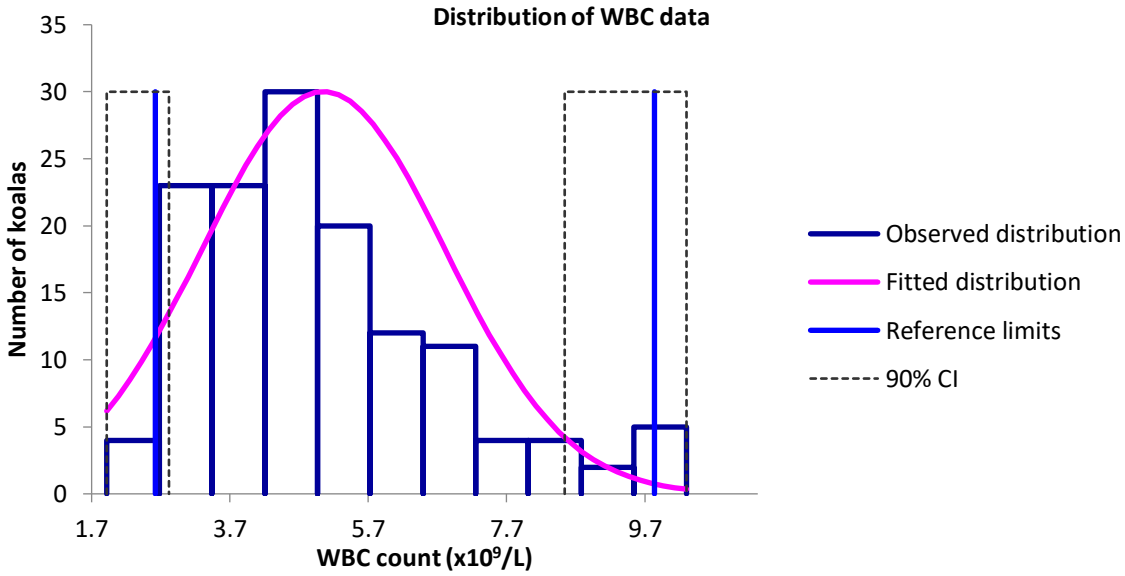
Mean Cellular Haemoglobin Concentration (MCHC)



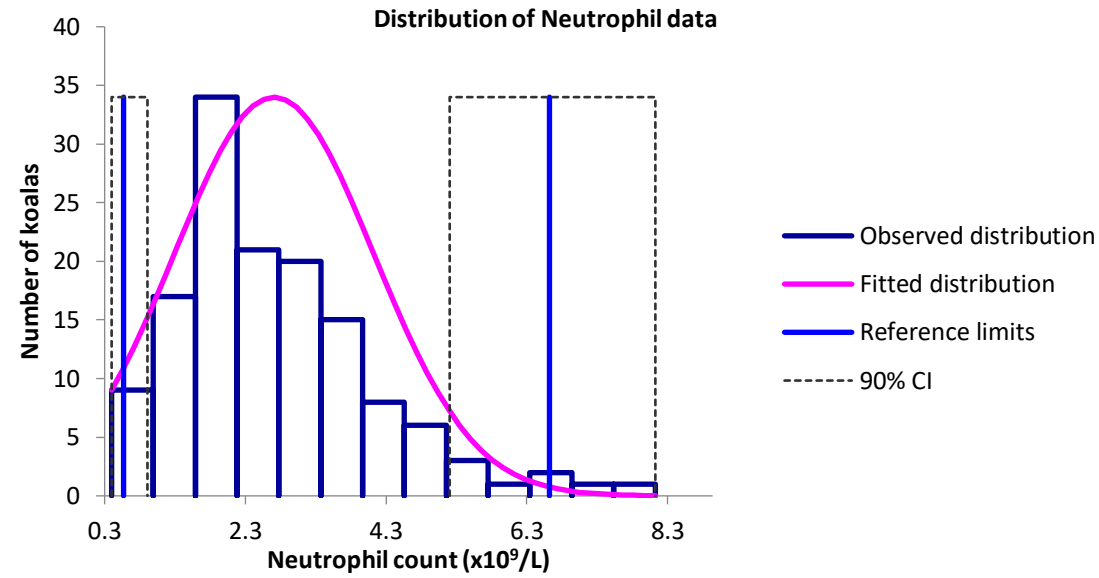
Nucleated erythrocyte (nRBC)



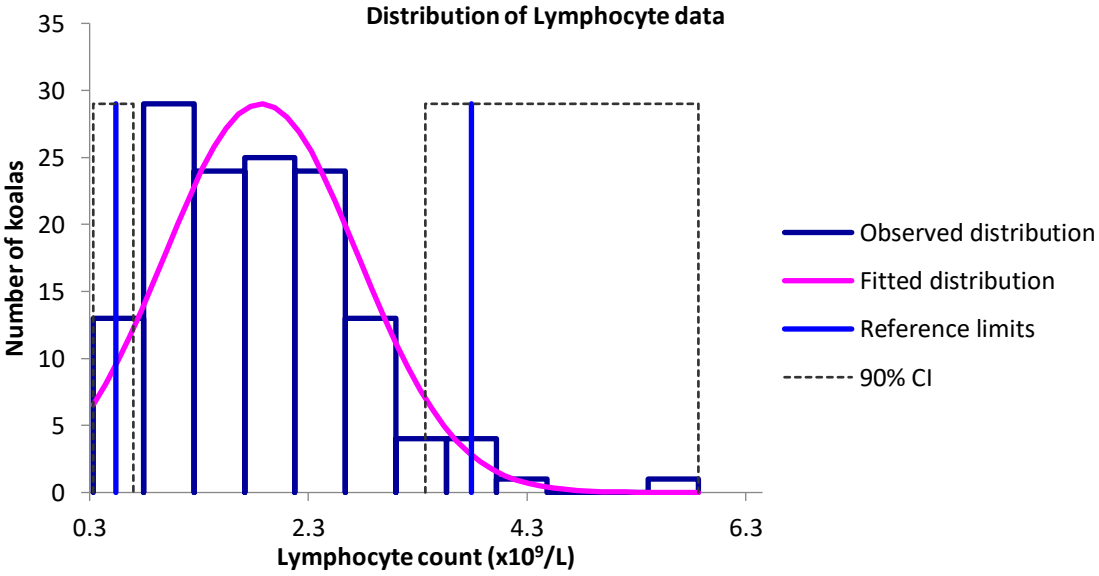
Leukocyte (WBC)



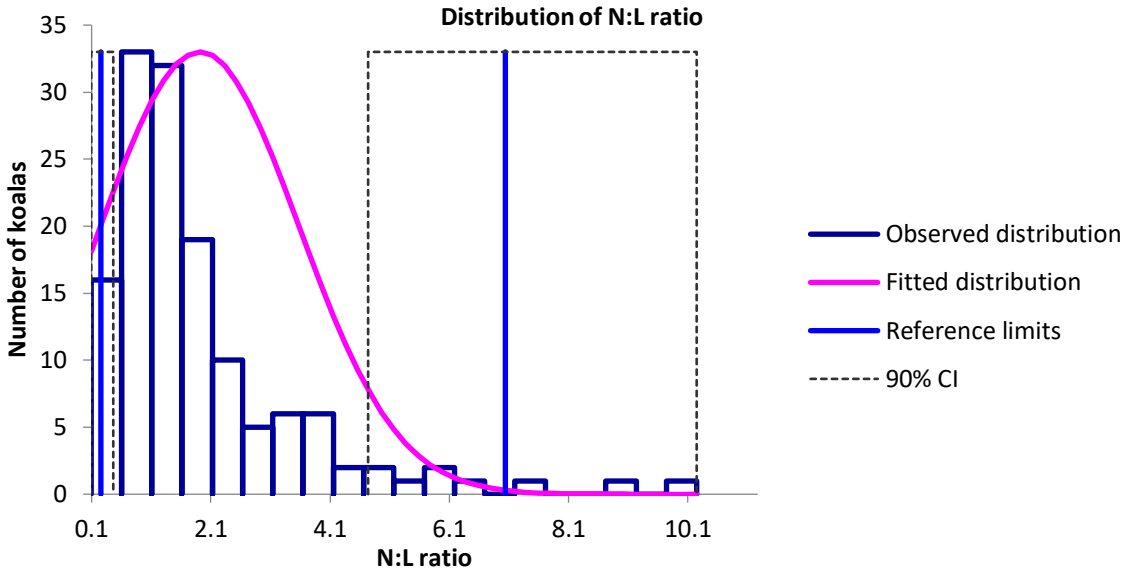
Neutrophil



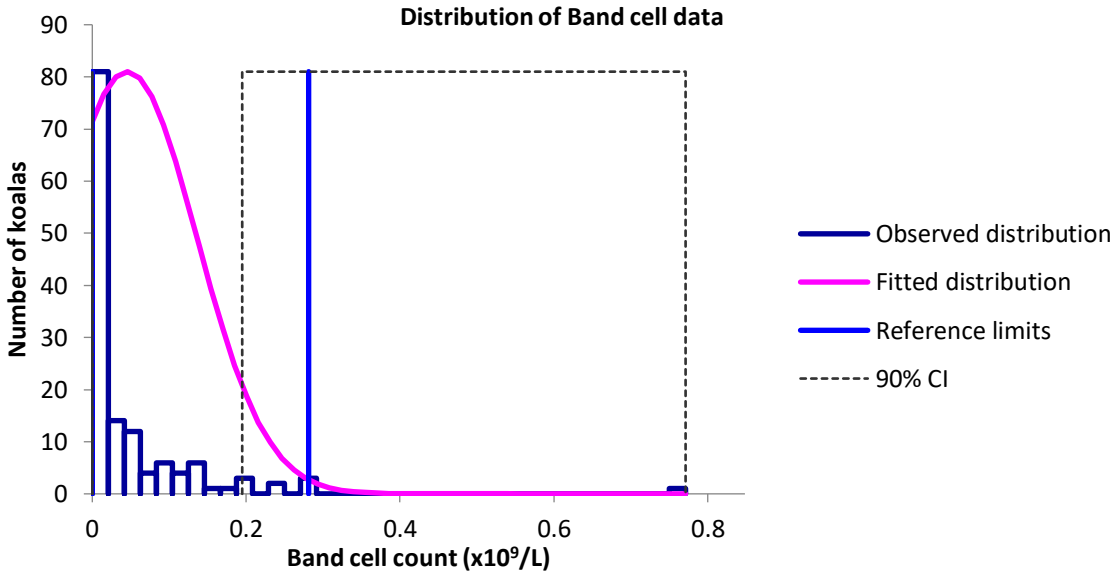
Lymphocyte



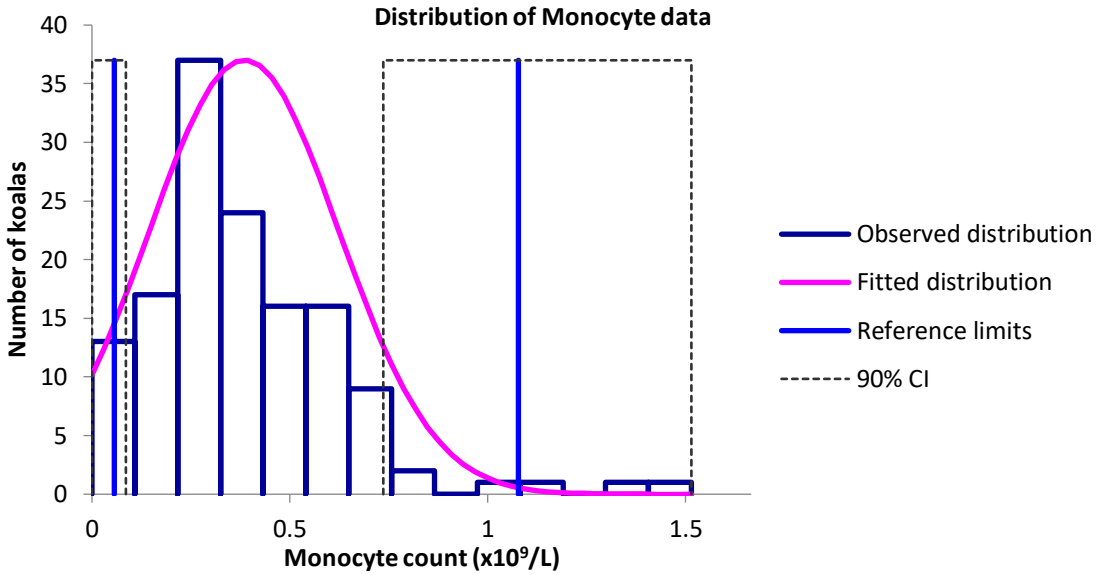
Neutrophil:lymphocyte (N:L) ratio



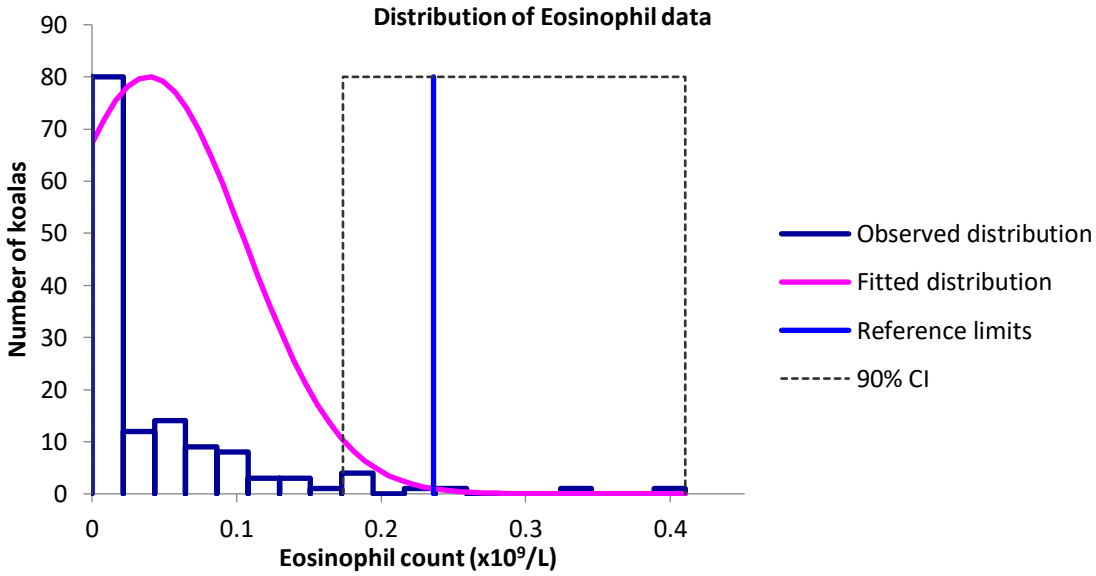
Band cell



Monocyte



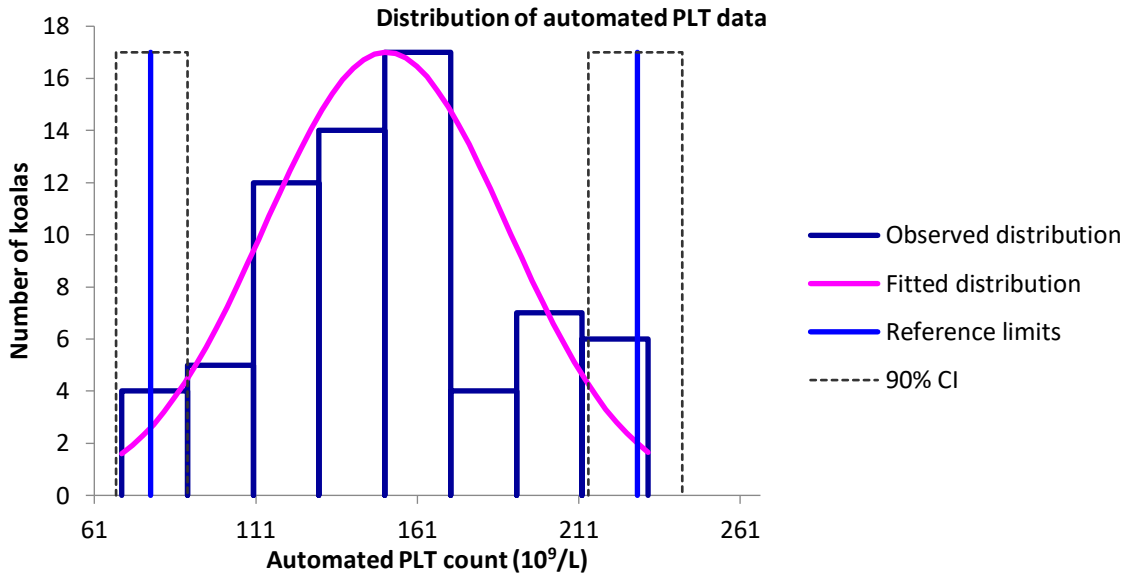
Eosinophil



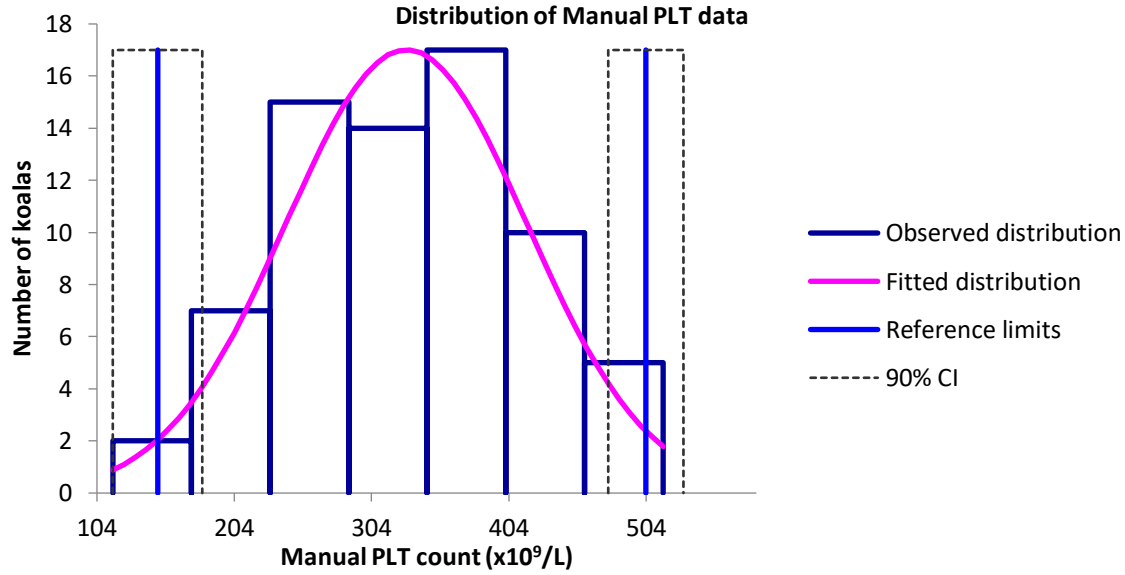
Basophil

A histogram was not generated for basophil count due to only 14/138 koalas having basophil counts above zero. The mean (range) basophil count for koalas with basophils present was 0.060 (0.025-0.148) x10^9/L.

Automated Platelet (PLT)



Manual PLT



Chapter 5

Pathological findings in koalas with *Chlamydia pecorum* and Koala retrovirus infections

Chapter 5.1.

Lymphoma, koala retrovirus infection and reproductive chlamydiosis in a koala (*Phascolarctos cinereus*)

Statement of Authorship

Title of Paper	Lymphoma, Koala retrovirus infection and reproductive chlamydiosis in a koala (*Phascolarctos cinereus*)
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	J Fabijan, L Woolford, S Lathe, G Simmons, F Hemmatzadeh, DJ Trott, N Speight Lymphoma, Koala retrovirus infection and reproductive chlamydiosis in a koala (*Phascolarctos cinereus*) Journal of Comparative Pathology, vol. 157, pp. 188-192, 2017

Principal Author

Name of Principal Author (Candidate)	Jessica Fabijan
Contribution to the Paper	Performed necropsy, sample collection for diagnostics and processing for histopathology Performed analysis and interpretation of data Wrote and edited manuscript and is corresponding author
Overall percentage (%)	85%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 6/9/18

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Lucy Woolford
Contribution to the Paper	Performed histopathology interpretation Helped to edit manuscript
Signature	Date 7/9/18

Name of Co-Author	Sheridan Lathe
Contribution to the Paper	Veterinary diagnosis and submission of koala for necropsy Helped to edit manuscript
Signature	Date 13/9/18

Name of Co-Author	Greg Simmons

Contribution to the Paper	Provided study funding Helped with KoRV diagnostic PCR Helped to edit manuscript
Signature	Date 12/9/2018

Name of Co-Author	Farhid Hemmatzadeh
Contribution to the Paper	Supervised study design Helped to edit manuscript
Signature	Date 19.11.18

Name of Co-Author	Darren J Trott
Contribution to the Paper	Supervised study design Helped to edit manuscript
Signature	Date 06/09/2018

Name of Co-Author	Natasha Speight
Contribution to the Paper	Supervised study design and development, helped with sample analysis and interpretation Helped to edit manuscript
Signature	Date 7/9/2018

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DISEASE IN WILDLIFE OR EXOTIC SPECIES

Lymphoma, Koala Retrovirus Infection and Reproductive Chlamydiosis in a Koala (*Phascolarctos cinereus*)

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** School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy, † The Adelaide Koala and Wildlife Hospital, Plympton, South Australia and ‡ The University of Queensland, Gatton, Queensland, Australia*

Summary

Koala retrovirus (KoRV) infection, thought to be associated with lymphoid neoplasia, and *Chlamydia pecorum*-related ocular and urogenital disease are both highly prevalent in eastern Australian koala (*Phascolarctos cinereus*) populations. However, in South Australian koalas, little is known about KoRV infection and *C. pecorum*-associated disease. We report the first South Australian case of lymphoma in a KoRV-A-positive female koala also affected by severe reproductive chlamydiosis. The koala was from the Mount Lofty Ranges population and was presented with hindlimb lameness. Clinical examination identified right stifle crepitus, enlarged superficial lymph nodes and paraovarian cysts. Necropsy examination revealed extensive cartilage degeneration and loss over the medial femoral condyle, solid femoral bone marrow, mesenteric and ovarian tumours, paraovarian cysts and purulent metritis. Histopathology confirmed lymphoma in the bone marrow, mesenteric lymph nodes and ovary, with infiltration and parenchymal effacement in the pancreas, adrenal glands and other tissues. Lymphoma, KoRV and chlamydiosis are being investigated further in this population.

Keywords: Chlamydia pecorum; koala; koala retrovirus; lymphoma

Lymphoid neoplasia is the most commonly reported cancer in north-eastern Australian koala populations (i.e. Queensland and New South Wales) (Connolly *et al.*, 1998). Koalas may develop either lymphoid leukaemia or lymphoma as a result of neoplastic bone marrow infiltration. Non-lymphoid tissues may also be affected, with diffuse infiltration or defined foci (Connolly *et al.*, 1998). Lymphoid neoplasia has been associated with high levels of koala retrovirus (KoRV) viraemia (Tarlinton *et al.*, 2005).

The KoRV, a gamma retrovirus, is the only known retrovirus to be undergoing the process of becoming an endogenous retrovirus inserted into the host genome (Tarlinton *et al.*, 2006). In Queensland koala populations, 100% of koalas are infected with endogenous KoRV-A; however, in South Australia the prevalence of KoRV has only been investigated in animals on Kangaroo Island and found to be low (15% in 2012), suggesting exogenous transmission of the virus (Simmons *et al.*, 2012). Queensland koalas have also been found to carry exogenous KoRV variants, types B to I (Chappell *et al.*, 2017). The KoRV prevalence and variants in the mainland Mount Lofty Ranges koala population in South Australia is currently unknown.

The variant KoRV-B was found in 25% ($n = 36$) of Queensland koalas and appears to be an exogenous infection that has been associated with the development of lymphoid neoplasia in captive Queensland koalas (Xu *et al.*, 2013). In addition, KoRV-B was recently found to predispose Queensland koalas to disease caused by infection with the opportunistic

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pathogen, *Chlamydia pecorum* (Waugh *et al.*, 2017), as has been suggested for KoRV (Tarlinton *et al.*, 2005). *Chlamydia pecorum* infects up to 100% of Queensland koalas and presents as ocular, urinary or reproductive disease, which is often severe in nature (Polkinghorne *et al.*, 2013). In a recent study, 88% of humanely destroyed Mount Lofty Ranges koalas were positive for *C. pecorum* by polymerase chain reaction (PCR); however, only a low incidence of ocular and urinary tract disease was found and this was of mild severity (Speight *et al.*, 2016).

This case study presents the first report of lymphoma, KoRV-A infection and severe reproductive chlamydiosis in a koala from the Mount Lofty Ranges population.

A wild female koala from the Mount Lofty Ranges population was admitted to the Adelaide Koala and Wildlife Hospital, Plympton, South Australia (July 2nd, 2014), with right hindlimb lameness. The koala weighed 6.6 kg, was in fair bodily condition and was approximately 10 years old based on tooth wear (Martin, 1981). For examination, the koala was sedated with alfaxalone (1–2 mg/kg by intramuscular injection; Jurox Ltd., Rutherford, Australia). Right stifle crepitus was identified and subsequent radiographs showed possible osteomyelitis or neoplasia, with lucent areas subjacent to the articular surfaces of the femoral condyles.

All superficial lymph nodes were found to be enlarged. A fine needle aspirate of the submandibular lymph node showed high numbers of medium to large atypical lymphocytes, with large round nuclei and scant cytoplasm. Large cystic structures were palpable in the left caudal abdomen, with ultrasound confirming large fluid-filled cystic structures in the region of the left ovary.

In light of the cytological diagnosis of presumptive lymphoma and the ultrasonographical evidence of reproductive chlamydiosis, the koala was humanely destroyed. Blood was collected for KoRV detection and haematological analysis prior to death.

A complete blood count was performed within 24 h of sample collection using a Cell-Dyn 3700 automated haematology analyser (Abbott Diagnostics Division, Ramsey, Minnesota, USA), with manually performed packed cell volume (Clements, Lidcombe, New South Wales, Australia) and total solid protein measured by spectrophotometer (Reichert VET 360 refractometer, Seefeld, Germany). These tests revealed normocytic normochromic anaemia and leucopenia due to neutropenia (Supplementary Table 1). The blood film showed medium to large atypical lymphocytes with cleaved nuclei and coarsely clumped chromatin. Mild polychromasia and anisocytosis was observed in erythrocytes. New methylene blue stain did not show increased reticulocytes in circulation, indicating a non-regenerative anaemia; however, a high number of nucleated red blood cells (RBCs) were observed, suggesting inappropriate metarubricytosis.

Post-mortem examination was performed 18 h after death at the School of Animal and Veterinary Sciences, University of Adelaide. The superficial lymph nodes (submandibular, cervical, axillary and inguinal) were enlarged. There was an increased amount of clear fluid within the abdominal cavity. A prominent nodular mass measuring 30 × 20 × 15 mm, in the region of the mesenteric lymph nodes, was firm, off-white in colour and lacked internal nodal architecture. Multiple small, white nodules were visible over the capsular and cut surfaces of the pancreas. The liver and spleen appeared normal. Both adrenal glands appeared enlarged, but were not measured. A second firm, white, oval mass (25 × 10 × 5 mm) was located in the position of the right ovary.

Two large fluid-filled cystic structures encapsulated the left ovary. Both were spherical to oval in shape, measuring approximately 70 × 50 mm and 30 × 30 mm, respectively, and contained red–brown, turbid fluid. The endometrial surfaces were inflamed and the left uterine horn was filled with purulent exudate. The lateral vaginae were also dilated with inflamed mucosal surfaces. The subcapsular surface of the kidney was pitted, while the bladder wall was thickened, with reddened mucosa.

The femoral bone marrow was solid and pale pink. Examination of the right stifle articular surfaces showed extensive cartilage degeneration, fibrillation and loss over the medial femoral condyle with exposure of subchondral bone (Fig. 1). Tissue samples

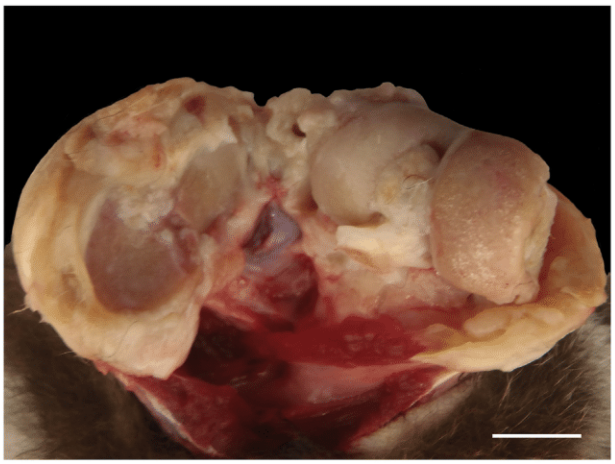

Fig. 1. Right stifle. Extensive cartilage degeneration and loss over the medial femoral condyle with exposure of subchondral bone (arrow). Bar, 1 cm.

were fixed in 10% neutral buffered formalin, processed routinely and embedded in paraffin wax.

The nodular mesenteric mass was confirmed as being the mesenteric lymph nodes. The nodal architecture was effaced and replaced by a diffuse infiltration of a monomorphic population of medium to large neoplastic round cells, characterized by scant basophilic cytoplasm, with central large round nuclei, vesicular to coarse chromatin, with prominent and sometimes multiple nucleoli. There was mild to moderate anisocytosis and anisokaryosis. Mitotic figures were rarely observed, ranging between 0 and 1 per 10 high-power fields (×400). Admixed with the neoplastic cells, there was pyknotic and karyorrhectic debris and numerous vacuolated macrophages with phagocytosed cellular and nuclear debris.

The subcutaneous lymph nodes largely retained normal architecture; however, there were occasional poorly demarcated, densely cellular aggregates of neoplastic cells as described above. There were no significant changes to splenic architecture, but there was mild neoplastic infiltration in the liver, myocardium and gastric mucosa, with marked infiltration and parenchymal effacement in the pancreas and adrenal glands.

The right ovarian mass was composed of diffuse sheets of neoplastic cells, which completely effaced ovarian tissue, apart from a remnant border of ovarian fimbriae along one margin of the tissue section. The paraovarian cysts were multiloculated fibrocollagenous cysts, lined by papilliform projections of hyperplastic epithelium. There was extensive erosion and ulceration of endometrium, with remnant mucosa infiltrated diffusely by small non-neoplastic lymphocytes and macrophages.

Femoral bone marrow was hypercellular with an approximate cell to marrow fat ratio of 85:15 (Fig. 2). Haemopoietic cells were largely replaced by neoplastic cells, admixed with scattered foci of necrotic debris (Fig. 3). The femoral condylar articular surface showed extensive loss of cartilage and erosion of subchondral bone, with replacement by immature and mature fibrocollagenous connective tissue and woven bone (Fig. 4). Subchondral bony trabeculae were thinned with increased osteoclastic activity evident along scalloped margins, and adjacent marrow spaces were frequently replaced by densely cellular infiltrates of neoplastic lymphocytes.

Both kidneys showed segmental corticomedullary tubular loss, with fibrocollagenous replacement and

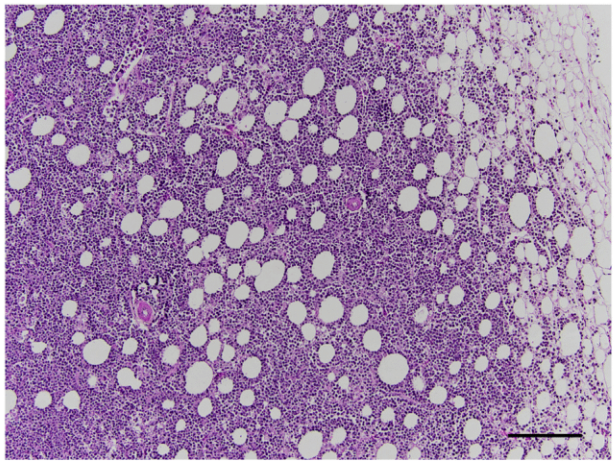

Fig. 2. Femoral bone marrow. Hypercellularity of bone marrow with cell to marrow fat ratio 85:15. HE. Bar, 200 µm.

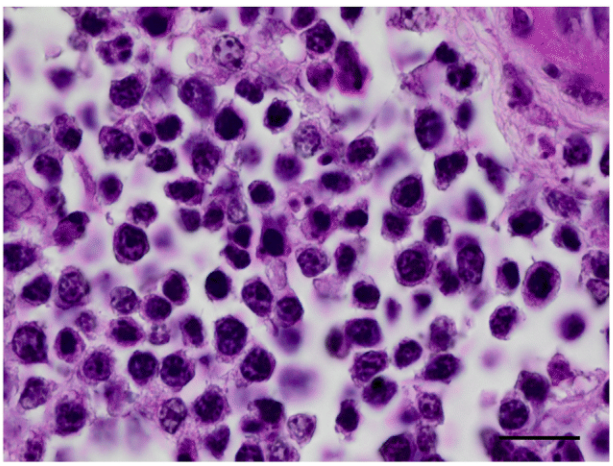

Fig. 3. Femoral bone marrow. Neoplastic cell morphology characterized by scant basophilic cytoplasm with central large round nuclei, vesicular to coarse chromatin. HE. Bar, 10 µm.

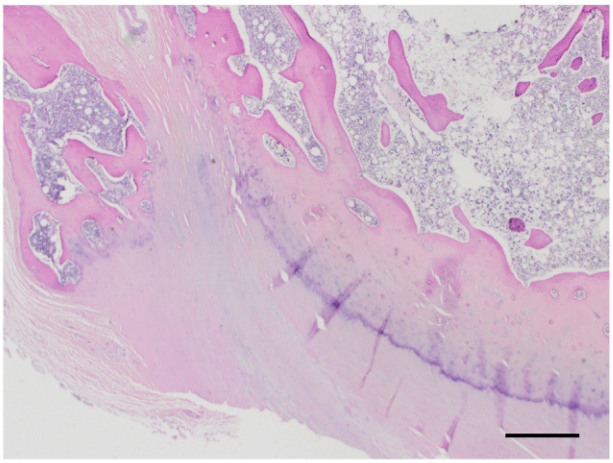

Fig. 4. Articular surface of right stifle. Degeneration of cartilage along femoral articular surface with erosion of subchondral bone and replacement by immature and mature fibrocollagenous connective tissue and woven bone. Marrow spaces subjacent to subchondral bone are replaced by densely cellular infiltrates of neoplastic lymphocytes. HE. Bar, 1 mm.

contraction, while remnant tubules were ectatic. Foci of medullary perivascular interstitial lymphoid aggregates were present, consistent with neoplastic infiltrates. The bladder showed extensive epithelial loss, with the submucosa focally expanded by a dense cellular infiltrate of the neoplastic cells.

Immunohistochemistry (IHC) was performed on the mesenteric mass, cervical lymph node, bone marrow and endometrium using standardized immunoperoxidase procedures to determine B or T-cell immunophenotype with CD3 (polyclonal rabbit anti-human, Dako, Glostrup, Denmark), CD79a (monoclonal mouse anti-human, Dako) and CD79b (monoclonal mouse anti-human, Dako) using 3,3′-diaminobenzidine as chromogen. As controls, both healthy koala lymph node and human lymph node were used.

Strong diffuse positive cytoplasmic and membranous CD3 labelling was observed in scattered light infiltrates of small non-neoplastic T cells within all tissues; however, the larger pleomorphic neoplastic lymphocytes were negative. The endometrium had multifocal moderate infiltrates of CD3-positive T cells admixed with fewer negative lymphocytes, suggesting inflammation rather than neoplastic infiltration. Weak and inconsistent labelling of large neoplastic lymphocytes with CD79a and CD79b was observed.

For KoRV detection, DNA was extracted from whole blood with QIAamp DNA mini-kit® (Qiagen, Hilden, Germany) for nested-PCR with KoRV polymerase-specific primers (Simmons *et al.*, 2012). KoRV variant type was determined by conventional PCR with KoRV-A and KoRV-B specific primers (Waugh *et al.*, 2017). For *C. pecorum* detection, DNA was extraction from dry aluminium shaft swabs (Copan Italia spa, Brescia, Italy) collected from the ocular and urogenital sites with QIAamp DNA mini-kit® for quantitative PCR utilizing *C. pecorum*-specific primers (Marsh *et al.*, 2011). As a control for DNA quality and PCR inhibition the koala β-actin gene was utilized for all assays. The koala was confirmed positive for KoRV-A, but negative for KoRV-B, and positive for *C. pecorum* infection at the urogenital site only.

This is the first case report of a KoRV-A-infected koala from the Mount Lofty Ranges population, South Australia with lymphoma. The atypical clinical presentation of hindlimb lameness was caused by extensive cartilage destruction and subchondral bone resorption and remodelling in the stifle joint associated with infiltration by neoplastic lymphocytes in subjacent bone marrow, and has not been described previously. Although erosive osteoarthritis due to other aetiology cannot be excluded, it is considered less likely due to the close association between neoplastic infiltrates and osteoclastic subchondral bone resorption. Neoplastic cells were also observed in the mesenteric lymph nodes and right ovary, with infiltration into the superficial lymph nodes, myocardium, liver, pancreas, adrenal glands, kidneys, bladder and gastric mucosa.

IHC indicated that the lymphoma was not of T-cell origin; B-cell labelling was inconclusive, which may be as a result of altered antigen quality due to autolysis or other processing artefact. Therefore, we were unable to distinguish between B-cell or null-cell type origins, which are both possible. In eastern koalas, lymphomas have been reported as predominantly of the T-cell immunophenotype (51%, 26/51), while B-cell immunophenotype is also common (24%, 12/51), with no predilection of immunophenotype for lymphoma involving alimentary lymphoid tissues. Immunophenotype may also be undetermined (25%, 13/51) (Connolly *et al.*, 1998).

This koala also presented with severe reproductive chlamydiosis due to *C. pecorum* infection, consistent with that described in female koalas from northern Australian koala populations (Obendorf, 1981). Previously, only mild reproductive disease due to *C. pecorum* infection has been reported at low prevalence in the Mount Lofty Ranges koala population (Speight *et al.*, 2016). In northern koalas, KoRV-B has recently been found to predispose koalas to the development of clinical chlamydiosis (Waugh *et al.*, 2017), while KoRV-B has not yet been identified in southern koala populations in Victoria (Legione *et al.*, 2017). This case of KoRV-A infection with overt reproductive chlamydiosis in a second southern koala population may highlight the potential pathogenicity of KoRV-A, warranting further investigation into the role of KoRV variants and immunosuppression in koalas.

With a low prevalence of clinical chlamydial disease in the Mount Lofty Ranges, and an unknown prevalence of KoRV infection, this South Australian population may shed further light on the relationship between KoRV and *Chlamydia* infections. This is the first report of lymphoma in a KoRV-A-positive female koala with severe reproductive chlamydiosis from the Mount Lofty Ranges koala population. Given that South Australia is thought to harbour some of the last KoRV-free koalas, this case supports the need for further investigation of KoRV and *C. pecorum* infections in these populations.

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Conflict of Interest Statement

The authors declare no conflicts of interest with respect to the publication of this manuscript.

Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jcpa.2017.07.011.

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Supplementary Table 1

Haematological data from the koala

Haematological data			Canfield et al. (1989) Reference interval*
Erythrocyte count	2.1	$\times 10^{12}$/l	2.58–4.17
Haemoglobin	77.4	g/l	88–139
Haematocrit	0.244	l/l	0.28–0 45
Mean cell volume	116	fl	90–116
Mean cell haemoglobin	36.9	pg	
Mean cell haemoglobin concentration	318	g/l	289–332
Platelets	Clumped and adequate		
Platelet count	303	$\times 10^{9}$/l	222–558
Reticulocytes (%)	0.4		
Nucleated erythrocytes	44	/100 leucocytes	0–20
Leucocyte count	2.63	$\times 10^{9}$/l	3.1–10
Neutrophil	0.74	$\times 10^{9}$/l	0.9–6.6
Band	0.03	$\times 10^{9}$/l	
Lymphocyte	1.74	$\times 10^{9}$/l	0.7–7.3
Monocyte	0.132	$\times 10^{9}$/l	0.0–0.9
Eosinophil	0	$\times 10^{9}$/l	0.0–1.4
Basophil	0	$\times 10^{9}$/l	0
Packed cell volume	0.25	l/l	0.28–0.45
Total protein	47	g/l	58–88

Chapter 5.2.

Pathological findings in koala retrovirus-positive koalas (*Phascolarctos cinereus*) from northern and southern Australia

Statement of Authorship

Title of Paper	Pathological findings in koala retrovirus positive northern and southern koalas (*Phascolarctos cinereus*): a comparative study
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Jessica Fabijan, Nishat Sarker, Natasha Speight, Helen Owen, Joanne Meers, Greg Simmons, Jennifer M Seddon, Richard D Emes, Rachael Tarlinton, Farhid Hemmatzadeh, Lucy Woolford, Darren J Trott Manuscript submitted to the *Journal of Comparative Pathology* on the 24th of October 2019 The manuscript was accepted by *J Comp Pathol* on 6th February 2020

Principal Author

Name of Principal Author (Candidate)	Jessica Fabijan
Contribution to the Paper	Contributed to study design and development, organised and coordinated the findings of this pathological study Coordinated sample collection from South Australian koalas, performed routine necropsy examinations and sample collection for koala retrovirus pathogenesis project Processed South Australian koala tissues for histological examination and immunohistochemistry Classified South Australian and Queensland koala pathology categories Classified chlamydial disease severity Performed koala haematology for South Australian koalas Performed splenic morphometrics Interpreted and performed statistical analysis comparing South Australian and Queensland koala pathology, haematology and splenic morphometrics Wrote manuscript
Overall percentage (%)	75%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date: 24.10.19

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Nishat Sarker		
Contribution to the Paper	Facilitated and coordinated sample collection from Queensland koalas Performed DNA extraction on koala blood samples and KoRV proviral and viral detection by qPCR for all South Australian and Queensland koalas Assisted in editing the manuscript		
Signature		Date	09.08.2019

Name of Co-Author	Natasha Speight		
Contribution to the Paper	Contributed to study design Facilitated South Australian koala sample collection Assisted in classifying South Australian and Queensland koala pathology categories Assisted in statistical analysis and interpretation for pathology, haematology and splenic morphometrics Edited the manuscript		
Signature		Date	13/9/19

Name of Co-Author	Helen Owen		
Contribution to the Paper	Contributed to study design Facilitated and coordinated sample collection from Queensland koalas Performed necropsy examination and pathology of necropsied Queensland koalas Assisted in editing the manuscript		
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Contribution to the Paper	Contributed to study design Contributed to KoRV qPCR design and analysis Assisted in editing the manuscript		
Signature		Date	14/10/2019

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Contribution to the Paper	Chief investigator on research grant Contributed to study design Assisted in editing the manuscript
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Name of Co-Author	Jennifer M Seddon
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Contribution to the Paper	Contributed to study design Assisted in editing the manuscript
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Name of Co-Author	Lucy Woolford
Contribution to the Paper	Performed histopathology of necropsied South Australian koalas Assisted in classifying South Australian and Queensland koala pathology categories Assisted haematology analyses for South Australian koalas Assisted with splenic morphometric analysis Edited the manuscript
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Name of Co-Author	Darren J Trott		
Contribution to the Paper	Contributed to study design Coordinated funding and collaboration between Universities Assisted in editing the manuscript		
Signature		Date	13/09/2019

DISEASE IN WILDLIFE OR EXOTIC SPECIES

Short Title: Pathology in Retrovirus-positive Koalas

Pathological Findings in Koala retrovirus-positive Koalas (*Phascolarctos cinereus*) From Northern and Southern Australia

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Summary

Koala retrovirus (KoRV) infection shows differences in prevalence and load between northern and southern Australian koala populations; however, the effect of this on diseases such as lymphoma and chlamydial disease is unclear. This study compared clinicopathological findings, haematology and splenic lymphoid area of KoRV-positive koalas from northern (Queensland [Qld], *n* = 67) and southern (South Australia [SA], *n* = 92) populations in order to provide further insight into KoRV pathogenesis. Blood was collected for routine haematology and for measurement of KoRV proviral load by quantitative polymerase chain reaction (qPCR). Plasma samples were assessed for KoRV viral load by reverse transcriptase qPCR and conjunctival and cloacal swabs were collected for measurement of the load of *Chlamydia pecorum* (qPCR). During necropsy examination, spleen was collected for lymphoid area analysis. Lymphoma was morphologically similar between the populations and occurred in koalas with the highest KoRV proviral and viral loads. Severe ocular chlamydial disease was observed in both populations, but urinary tract disease was more severe in Qld, despite similar *C. pecorum* loads. No associations between KoRV and chlamydial disease severity or load were observed, except in SA where viral load correlated positively with chlamydial disease severity. In both populations, proviral and viral loads correlated positively with lymphocyte and metarubricyte counts and correlated negatively with erythrocyte and neutrophil counts. Splenic lymphoid area was correlated positively with viral load. This study has shown further evidence for KoRV-induced oncogenesis and highlighted that lymphocytes and splenic lymphoid tissue may be key sites for KoRV replication. However, KoRV infection appears to be highly complex and continued investigation is required to fully understand its pathogenesis.

Keywords: koala; koala retrovirus; Chlamydia; neoplasia

Introduction

Koala (*Phascolarctos cinereus*) populations are distributed down the eastern and south-eastern coast of Australia and are broadly separated into two groups, northern koalas in the states of Queensland (Qld) and New South Wales, and southern koalas in South Australia (SA) and Victoria (DSEWPC, 2012). Between the regions, koalas differ in genetics, conservation status and disease prevalence. Recent studies have shown that while koalas across Australia are one species, there are different genetic lineages between northern and southern regions (Kjeldsen *et al.*, 2016; Neaves *et al.*, 2016). Additionally, southern koalas are less genetically diverse than northern koalas (Neaves *et al.*, 2016), which likely arose from historical translocation conservation efforts post-European settlement (Robinson, 1978). These translocations may have altered the prevalence of disease in southern populations. Oxalate nephrosis is a prevalent disease of southern koalas compared with rare reports in northern koalas and is thought to have a genetic basis due to the bottlenecks that occurred as a result of the translocations (Speight *et al.*, 2013). Compared with southern koalas, northern populations are recognised as vulnerable to extinction, as these populations are declining at a considerable rate, unlike southern populations, where koalas were introduced into previously unoccupied areas and are considered overabundant (DSEWPC, 2012). This is due partly to differences in the prevalence of disease between the two regions. *Chlamydia pecorum* and koala retrovirus (KoRV) are highly prevalent and have been associated with a high prevalence of disease in northern populations, while in southern populations there is a lower prevalence of infection and disease (Tarlinton *et al.*, 2005; Polkinghorne *et al.*, 2013; Quigley *et al.*, 2018a).

Overt chlamydial disease develops as ocular disease (conjunctivitis and keratitis) (Wan *et al.*, 2011), respiratory tract infections (Mackie *et al.*, 2016), urinary tract infections (urethritis, cystitis and nephritis) (Canfield, 1989) and reproductive tract disease in females (vaginitis, metritis and paraovarian cysts) (Obendorf, 1981) and males (prostatitis, orchitis and

epididymitis) (Johnston *et al.*, 2015). *C. pecorum* infection may present subclinically, with no outward signs of infection or develop into overt disease. In northern populations, the prevalence of *C. pecorum* infection has been reported to be as high as 90% (Polkinghorne *et al.*, 2013), while in southern koalas the prevalence of *C. pecorum* was lower, up to 46% in Victoria (Legione *et al.*, 2016) and 47% in mainland SA (Fabijan *et al.*, 2019a). Northern koalas have also shown a higher prevalence of severe, overt chlamydial disease (Wan *et al.*, 2011; Polkinghorne *et al.*, 2013), with 52% of hospitalized Qld koalas presenting with chlamydiosis (Gonzalez-Astudillo *et al.*, 2017). In southern koalas, there appears to be a lower prevalence of overt disease with reduced disease severity, but in wild-caught Victorian koalas, only mild 'wet-bottom disease' was observed in 41.6% of koalas and ocular disease was not observed (Patterson *et al.*, 2015), and in SA, a low prevalence (21%) of mild ocular and urinary tract disease was reported in koalas subjected to necropsy examination (Speight *et al.*, 2016). These differences in prevalence and severity of *C. pecorum* infection between the northern and southern populations suggests that there may be koala population differences that facilitate or inhibit chlamydial disease development.

KoRV, a gammaretrovirus, could be considered the most important pathogen to infect koalas due to the oncogenic and immunosuppressive potential of retroviral infections. In northern populations, KoRV-A is 100% prevalent (Chappell *et al.*, 2017; Sarker *et al.*, 2019b) and is an active endogenous infection (Greenwood *et al.*, 2017; Hobbs *et al.*, 2017), while KoRV-B, which differs to KoRV-A in the *env* gene (Xu *et al.*, 2013), is presumed to be only an exogenous infection (Quigley *et al.*, 2018b). KoRV-B has been associated with the development of lymphoid neoplasia (Xu *et al.*, 2013), which is the most commonly reported neoplasia in northern koalas (Canfield, 1990; Gillett, 2014). KoRV-B has also been associated with the development of overt chlamydial disease (Waugh *et al.*, 2017; Quigley *et al.*, 2018a), where KoRV may modulate the immune system and predispose koalas to chlamydial disease development from *C. pecorum* infection. In southern koalas, KoRV is less

prevalent, is predominantly KoRV-A infection (Legione *et al.*, 2017; Fabijan *et al.*, 2019b) and is thought to transmit exogenously (Simmons *et al.*, 2012), which may account for the reduced prevalence of chlamydial disease compared with northern koalas.

This study is part of the Koala Retrovirus Pathogenesis Project, a collaborative study which aimed to investigate the differences in disease development between northern (Qld) and southern (SA) koala populations based on *C. pecorum* and KoRV infection status, based on proviral DNA loads (KoRV infection burden) and viral RNA loads (KoRV replication activity). Previous studies from this collaboration have reported on KoRV proviral and viral loads (Sarker *et al.*, 2019a) and KoRV *env* variants (Sarker *et al.*, 2019b). Presented here are the detailed clinicopathological findings of these koalas; the current study aimed to compare: (1) disease prevalence and severity, (2) haematology values, (3) splenic lymphoid area between the populations, and (4) to determine whether disease severity, haematology and splenic lymphoid area differed based on KoRV proviral or viral load between the populations.

Materials and Methods

Sample Collection

Routine necropsy examinations were performed on wild, rescued koalas that had been humanely destroyed on welfare grounds between February 2014 and December 2016. Thirty-two northern koalas from south-east Brisbane, Qld, were subjected to necropsy examination at the School of Veterinary Science, the University of Queensland, Qld, Australia, and for comparison, 97 southern koalas from the Mount Lofty Ranges, SA, were subjected to necropsy examination at the School of Animal and Veterinary Sciences, the University of Adelaide, SA. In Qld, an additional 18 wild, rescued koalas were sampled at local wildlife hospitals, and 21 koalas from captive populations were sampled during routine health examination. Four SA and four Qld koalas were excluded due to inadequate records and one

SA koala with oxalate nephrosis was removed as it was the only KoRV-negative koala (by polymerase chain reaction [PCR]) in the study.

Whole blood was collected via the cephalic vein prior to humane destruction or at clinical examination into EDTA (Becton Dickinson, New Jersey, New Jersey, USA) for haematology, KoRV proviral (DNA) and viral (RNA) loads. Dry aluminum-shaft cotton tipped swabs (Copan Italia, Brasica, Italy) of the left and right conjunctiva (ocular site) and of the cloaca of females and urethra of males (urogenital site) were taken for *Chlamydia pecorum* detection by methods previously described (Blanshard and Bodley, 2008). Age was assessed and classified by the amount of wear of the upper premolar (tooth wear class [TWC]: I, 1–2 years; II, 2–3 years; III, 4 years; IV, 5–6 years; V, 8–9 years; VI, 12+ years) (Martin and Handasyde, 1999) or from captive records. Body condition score was assessed by the degree of musculature of the scapula (Blanshard and Bodley, 2008). Tissue samples were collected into 10% neutral buffered formalin for histopathological examination. This research was approved by the animal ethics and state government research permits issued by the University of Adelaide Animal Ethics Committee S-2013-198, the University of Queensland Animal Ethics Committee, ANFRA/SVS/461/12 and ANRFA/SVS/445/15, the South Australian Government Department of Environment, Water and Natural Resources Scientific Research Permit Y26054 and the Queensland Government Department of Environment and Heritage Protection permit WISP11989112.

Haematological Examination

When fresh blood was available, routine haematology was performed using a Cell-Dyn 3700 automated haematology analyser (Abbott Diagnostics Division, Santa Clara, California, USA). Blood parameters determined by the analyser included erythrocyte (RBC) count, haemoglobin (Hb) concentration, haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration

(MCHC) and leucocyte (white blood cell [WBC]) count. Blood smears were reviewed to perform manual leucocyte differential counts and estimate nucleated RBCs (nRBCs) per 100 WBCs. For nRBC counts >5 per 100 WBCs, the analyser WBC count was corrected and absolute nRBC count determined. Blood smears were also used to perform manual platelet counts. Packed cell volume (PCV) was determined following centrifugation of microhaematocrit tubes at 2,800 g for 5 min. Haematological results were assessed against population specific reference intervals, where Qld koalas were assessed against established reference intervals in northern populations (Canfield *et al.*, 1989b) and SA koalas were assessed against southern koala reference intervals (Fabijan *et al.*, 2020).

Histopathology, Immunohistochemistry and Disease Category

Lymph nodes (submandibular, axillary, inguinal and mesenteric), spleen, liver, kidney, bladder and reproductive organs, where available, were processed routinely for histopathological examination. Sections (4 µm) were stained with haematoxylin and eosin (HE). Lymph nodes affected by lymphoid neoplasia were subjected to immunohistochemistry (IHC) with markers for T- and B-lymphocytes at the Koala Health Hub, University of Sydney, Sydney, NSW, as described previously (Connolly *et al.*, 1998). Lymph nodes were labelled with the T-cell marker CD3 (polyclonal rabbit anti-human, Dako, Glostrup, Denmark) and the B-cell marker CD79b (monoclonal mouse anti-human, Dako) and appropriate horseradish peroxidase-conjugated secondary reagents. Labelling was 'visualized' using 3, 3'-diaminobenzidine as a chromogen. Healthy koala and human lymph node sections were used as controls. The primary antibody was omitted for negative control tissues.

Koalas were classified into four disease categories based on their primary disease finding: neoplasia, chlamydial disease, miscellaneous diseases (including, but not limited to, oxalate nephrosis, cardiovascular, respiratory and gastrointestinal tract diseases) and disease-free

(including clinically healthy captive koalas and koalas humanely destroyed due to vehicle trauma, predation or musculoskeletal abnormalities such as scoliosis and kyphosis, but with no other abnormalities).

Splenic Lymphoid Area Analysis

One randomly selected section of spleen from 10 Qld and 31 SA koalas was examined histologically to compare lymphoid follicle and periarteriolar lymphoid sheath (PALS) size in order to assess changes due to antigenic stimulation or lymphoid depletion (Woolford *et al*., 2015). Splenic morphology was compared between koalas with chlamydial disease (Qld, *n* = 8; SA, *n* = 15), miscellaneous diseases (Qld, *n* = 2; SA, *n* = 5) and disease-free koalas (SA, *n* = 11). The area of each follicle and PALS were measured using the area tool (□m^2) using LabSens^{TM} software (Olympus, Shinjuku-ku, Tokyo, Japan). The mean lymphoid area (mean of total follicle and PALSs) was determined for each koala.

C. pecorum *Detection by Quantitative Polymerase Chain Reaction and Chlamydial Disease Classification*

Assessment of *C. pecorum* positivity (all genotypes) and load (copies/µl) was outsourced to researchers in the Faculty of Science, Health, Education and Engineering, the University of the Sunshine Coast, Qld, Australia (Marsh *et al.*, 2011). Briefly, *C. pecorum* DNA was detected from swabs collected from the ocular and urogenital sites by quantitative (q) PCR. DNA was extracted from each swab using a Qiagen DNA Mini kit (Qiagen, Hilden, Germany) and stored at –80ºC. Detection of *C. pecorum* was performed using qPCR (Marsh *et al.*, 2011).

Overt ocular and urinary tract diseases were graded on a three-point scale (1, mild; 2, moderate; 3, severe) as described previously (Wan *et al.*, 2011). Briefly, ocular disease was

observed as: grade 1, acute conjunctival inflammation; grade 2, chronic conjunctival hyperplasia; and grade 3, chronic, active conjunctivitis with exudation. Urinary disease was observed as: grade 1, mild cystitis (acute/subacute infection including histologically detected only) or cloacal discharge; grade 2, chronic inactive cystitis and/or paraovarian cysts; and grade 3, chronic active cystitis ± nephritis, and/or active reproductive tract infection (Wan *et al.*, 2011).

Koala Retrovirus Detection and Viral Load Quantification

The PCR protocols and results have been previously reported (Sarker *et al.*, 2019a). Briefly, KoRV DNA was detected and proviral load determined from whole blood using qPCR. Plasma collected into RNALater™ (Qiagen), was used to determine the KoRV RNA viral load using a two-step reverse transcription qPCR. Both the proviral and viral qPCR protocols utilized primers that amplified a portion of the *pol* gene conserved to all KoRV *env* variants (Sarker *et al.*, 2019a).

Statistical Analysis

Multivariate logistic regression analysis for the Koala Retrovirus Pathogenesis Project has been reported previously (Sarker *et al.*, 2019a). The present study compared Qld and SA koalas based on haematological parameters, splenic lymphoid area, chlamydial loads and KoRV loads and associated clinicopathological findings. For statistical analysis, SPSS version 24 (IBM, New York, United States) was utilised. Normality was determined for the continuous variables; KoRV proviral loads, KoRV viral loads, chlamydial loads, haematological parameters and splenic lymphoid area by the Shapiro–Wilk test. KoRV proviral load, KoRV viral loads, chlamydial load, splenic lymphoid area, eosinophil count and basophil count were not normally distributed and were compared using non-parametric Mann–Whitney or Kruskal–Wallis tests. All other haematological parameters were normally

distributed and were compared using a univariate general linear model with type III sums of squares modelled with population, disease category and population nested within disease category. Binomial explanatory variables included population (Qld or SA) and sex (female or male). Ordinal explanatory variables including disease category (neoplasia, chlamydial disease, miscellaneous disease and disease-free), KoRV group (based on log transformation of viral load: none, low and high), chlamydial disease severity (mild, moderate and severe), age group (young, TWC I and II; adult, TWC III and IV; senior, TWC V and VI), BCS (poor, BCS 1 and 2; fair, BCS 3; excellent, BCS 4 and 5). A Chi-squared test of proportions was used to compare between binomial and ordinal variables. In order to determine if any correlations existed between continuous variables, a Spearman's rho (ρ) correlation coefficient and statistical significance was determined if monotonic relationships existed. A 5% level of significance was used to define significant relationships.

Results

Clinical and Pathological Findings

Details of all of the koalas are given in Table 1. Neoplasia was detected in 13.4% of Qld koalas (9/67) and 5.4% of SA koalas (5/92). In Qld, five koalas presented with lymphoid neoplasia (7.5%, two with lymphoma and three with lymphoid leukaemia), one with mesothelioma (1.5%) and three with osteochondroma (4.5%; two craniofacial and one costal). In SA, four koalas presented with lymphoma (4.3%) and one with craniofacial osteochondroma (1.1%).

Both Qld and three SA koalas with lymphoma affecting lymph nodes, thymus and spleen also had involvement of non-lymphoid bone marrow, gastrointestinal tract, liver, pancreas, heart, lungs, kidney, bladder (Fig. 1), adrenal gland and/or brain. A single young male koala from SA presented with lymph node involvement only. Neoplastic lymphocytes were

characterized as intermediate to large round cells with minimal cytoplasm with up to a four-fold anisocytosis and anisokaryosis. Nuclei were round to oval with coarsely granular chromatin, with single to several nucleoli. In the SA koalas with lymphoma, the median mitotic rate was 4 (range 2–15) mitotic figures per 10 ×400 high-power fields (HPFs), but mitotic rate was not evaluated in the affected Qld koalas. One Qld koala and two SA koalas had B-cell lymphoma of the lymph node as determined by IHC (Fig. 2), while phenotype for the remaining tumours was undetermined.

Chlamydial disease was observed in 46.3% (31/67) of Qld and 35.9% (33/92) of SA koalas. In Qld, nine koalas (29.0%) presented with *C. pecorum* disease confirmed by PCR, 16 (51.6%) with *Chlamydia*-like disease (PCR negative) and six (19.4%) with *Chlamydia*-like disease with unknown *C. pecorum* status, as they were not tested for *C. pecorum*. In SA, 15 koalas (16.3%) presented with *C. pecorum* disease confirmed by PCR, 14 (15.2%) presented with *Chlamydia*-like disease (PCR negative) and four (4.3%) with *Chlamydia*-like disease with unknown *C. pecorum* status.

Conjunctivitis, pneumonia, urinary and reproductive tract disease was observed in both females and males. Chlamydial disease was observed at one site only in 61.3% (19/31) of Qld and 54.5% (18/33) of SA koalas and the remaining koalas had disease at two or more sites (Table 2). In Qld, all cases of chlamydial disease were identified during necropsy or clinical examinations. In SA, 70% (35/50) of chlamydial disease was identified as gross lesions during necropsy examination (75% of ocular disease cases [3/4], 74% of urinary tract disease [17/23] and 65% of reproductive tract disease [15/23]); the remaining 30% of lesions (15/50) were detected during histological examination.

Grossly apparent ocular lesions (Fig. 3) were observed in both populations from grade 1 to 3 in severity (Table 3). The mean ocular chlamydial load of Qld koalas with confirmed chlamydial ocular disease (n = 2) was 1,105 copies/µl (range 796–1,413) and in SA (n = 3) was only 30 copies/µl (range 30–100). Qld koalas were significantly more likely to present

with ocular disease than SA koalas (P <0.001) and had significantly higher ocular chlamydial load (P <0.001). There was no difference in chlamydial disease severity between the populations in the proportion of koalas (P = 0.894) or chlamydial load (P = 0.076) for those with severe ocular disease.

Qld koalas were significantly more likely to present with grade 3 urinary tract disease, while SA koalas were more likely to present with grade 1 disease (P <0.001) (Fig. 4). No SA koalas were observed with grade 3 urinary tract disease (Table 3). The mean urogenital chlamydial load of Qld koalas (n = 4) was 1,224 copies/µl (range 290–19,127) and in SA (n = 9) was 1,660 copies/µl (range 35–320,000). There was no difference in the occurrence of urinary tract disease between the populations (P = 0.479) or the urogenital load of *C. pecorum* positive koalas (P = 0.903).

Reproductive tract disease was observed in both female and male koalas from SA, while in Qld disease was only recorded as being present in four female koalas at necropsy examination. The prevalence is difficult to compare between the two populations as the reproductive tracts of the other Qld koalas were not available for histological examination (Table 3). The SA koalas with reproductive tract disease only were predominantly female (8/9), and of all the female koalas with reproductive tract disease (n = 13), six had concurrent endometritis and paraovarian cysts. Most lesions in females were seen grossly at necropsy examination (11/13). Reproductive tract disease in male koalas (n = 10) was mild in nature, and orchitis and epididymitis were not observed.

Comorbidities with chlamydial disease were observed commonly. Miscellaneous comorbidities (including trauma, scoliosis and kyphosis, renal, cardiac and respiratory disease) were observed in 12 Qld and 16 SA koalas. Koalas with neoplasia were also often seen with concurrent chlamydial disease, including five Qld and two SA koalas.

Subclinical chlamydial infection (no associated lesions) was identified at the ocular and urogenital sites in both Qld and SA koalas. The median ocular chlamydial load of Qld koalas

with subclinical *C. pecorum* ocular infection (n = 4) was 270 copies/µl (range 120–32,942) and in SA (n = 5) was 1,500 copies/µl (range 513–7,469). The urogenital load of the single Qld koala with subclinical urogenital infection was 793 copies/µl, and the urogenital chlamydial load of SA koalas with subclinical urogenital infection (n = 6) was 1,725 copies/µl (range 180–19,400).

Miscellaneous diseases were recorded in 6.0% (4/67) of Qld and 33.7% (31/92) of SA koalas. Miscellaneous diseases observed in Qld koalas included respiratory disease (1.5%, 1/67), hepatic disease (1.5%, 1/67) and poor condition (3.0%, 2/67). No cases of oxalate nephrosis were observed in Qld. In SA, miscellaneous diseases included oxalate nephrosis (18.5%, 17/92), infected traumatic injuries (5.4%, 5/92), respiratory disease (3.2%, 3/92), gastric torsion (2.2%, 2/92), thromboembolic disease (1.1%, 1/92) and no significant findings at necropsy examination (4.4%, 4/92).

Haematology and Disease Category

Koalas with neoplasia (SA and Qld combined) had significantly lower RBC, Hb, PCV, WBC, neutrophil and lymphocyte counts, and the highest nRBC counts compared with the other disease categories (Fig. 5). All koalas with neoplasia had severe anaemia (normocytic and normochromic non-regenerative anaemia based on red cell indices, as reticulocyte counts were not performed). Most WBC indices were below reference intervals; three animals had moderate leucopenia and neutropenia, and one koala had moderate neutropenia and lymphocytosis. There were no differences in haematological values between koalas from Qld and SA with neoplasia.

Koalas with chlamydial disease had lower Hb and monocyte counts and higher WBC and neutrophil counts than the other disease categories (Fig. 5). Koalas with ocular chlamydial disease had elevated nRBC counts compared with koalas with urinary tract and reproductive

tract disease ($P = 0.006$). Koalas with urinary tract disease had haematological parameters that were within reference intervals. Koalas with reproductive tract infections were significantly more likely to present with leucocytosis than koalas with ocular and urinary tract diseases ($P = 0.011$) and neutrophilia was commonly observed but was not significant overall ($P = 0.068$). The only parameter that differed between Qld and SA koalas with chlamydial disease was a higher nRBC count in the former group ($P = 0.002$).

Koalas with miscellaneous diseases had significantly higher PCVs and monocyte counts and low neutrophil counts, while disease-free koalas had the highest Hb, PCV, WBC and lymphocyte counts and the lowest nRBC and monocyte counts (Fig. 5).

Splenic Lymphoid Area and Disease Category

Splenic morphological examination in koalas from both populations with lymphoid neoplasia showed mild to severe lymphoproliferation and/or neoplastic transformation, without defined follicles or PALSs. Qld and SA koalas with chlamydial disease showed lymphoid atrophy ($n = 2$), mild lymphoid hyperplasia ($n = 2$) or non-specific findings (NSF) ($n = 19$). All koalas with miscellaneous diseases had NSFs ($n = 7$). The disease-free SA koalas showed congestion (n = 6), contraction ($n = 1$) or NSFs ($n = 2$). No splenic tissue samples were available for disease-free Qld koalas.

A higher number of PALSs than follicles were observed per section of spleen examined at random. When disease categories were compared (with populations combined), the median number of PALSs within a mean spleen section of 2.45×10^7 ($\pm$ SD 1.60×10^7) $\square m^2$, was significantly higher in koalas with chlamydial disease compared with miscellaneous diseases ($P = 0.002$). Qld koalas with chlamydial disease had significantly ($P = 0.045$) larger median PALSs at 129,227 μm^2 (range 40,466–530,077) than SA koalas, 57,564 μm^2 (range 18,201–230,588).

Koala Retrovirus Status

KoRV loads for koalas within the Koala Retrovirus Pathogenesis Project have been reported separately (Sarker *et al.*, 2019a). A multivariate logistic regression analysis showed that Qld koalas had overall higher KoRV proviral and viral loads compared with SA koalas, and that koalas with neoplasia had significantly higher proviral and viral loads than koalas from the other disease categories. Additionally, all KoRV genes (LTR, *gag*, *pol* and *env*) were detected at the proviral and viral level in all Qld koalas, but only five (5.4%) SA koalas were positive for all proviral and viral genes (Sarker *et al.*, 2019a).

Of the koalas in this study, the median KoRV proviral load of the Qld koalas (n = 67) was 5.40×10^4 copies/10^3 β-actin copies (range 1.04×10^4–5.90×10^5) and in SA (n = 92) was 2.67×10^3 copies/10^3 β-actin copies (range 10.6–4.32x10^5). All Qld koalas had KoRV viraemia, where the median KoRV viral load was 4.03×10^8 copies/ml of plasma (7.76×10^6–7.58×10^{11}); however, only 52.6% of the SA koalas (20/38) were KoRV viraemic; the viral loads of active KoRV infections in SA was 2.22×10^5 copies/ml of plasma (2.28×10^4–4.34×10^{10}).

Comparison of Koala Retrovirus Between Queensland and South Australian Koalas Within Each Disease Category

To further investigate the role of KoRV infection in Qld and SA koalas, KoRV proviral and viral loads were compared between Qld and SA koalas within each disease category (Table 5). Proviral load (P = 0.947) and viral load (P = 0.758) were similar between Qld and SA koalas with neoplasia, while Qld koalas in the chlamydial disease, miscellaneous disease and disease-free categories all had significantly higher proviral and viral loads than the SA koalas in the same groups (P <0.01).

All koalas were grouped into three KoRV activity groups based on the log transformed KoRV viral load; high KoRV activity (log_{10} viral load >6.5), low KoRV activity (log_{10} viral load <6.5) and no KoRV activity (viraemia negative) (Fig. 6). All Qld koalas were in the high KoRV activity group; the low KoRV and no KoRV activity groups consisted of SA koalas only. Of the SA koalas in the high KoRV activity group, three had neoplasia, one had chlamydial disease and one had a miscellaneous disease; there were no disease-free koalas in the high KoRV group. In SA, koalas with neoplasia were significantly more likely to be in the high KoRV activity group (P = 0.013) than koalas without neoplasia. Only one koala with neoplasia (osteochondroma) fell into the low KoRV activity group, while all koalas with lymphoma were in the high KoRV activity group. KoRV proviral load was significantly different between all three KoRV activity groups (P = 0.001), while the median proviral load increased with increasing KoRV activity. The median proviral loads of SA koalas with no, low and high KoRV activity were 1.55×10^3 copies/10^3 β-actin copies (range 1.11×10^2–1.65×10^4), 6.35×10^3 copies/10^3 β-actin copies (range 5.29×10^2–5.05×10^4) and 2.14×10^5 copies/10^3 β-actin copies (range 6.26×10^3–4.32×10^5).

Koala Retrovirus and Chlamydial Disease

There was no correlation between chlamydial disease severity and proviral and viral loads for Qld koalas (P = 0.060 and P = 0.850, respectively) or proviral load in SA (P = 0.401); however, in SA there was a positive correlation between increasing chlamydial disease severity and increasing KoRV viral load (ρ = 0.745; P = 0.031). In SA, there were no differences between chlamydial disease severity and KoRV groups (high, low, no) (P = 0.390). There was no correlation between ocular or urogenital chlamydial loads and KoRV proviral or viral loads in Qld (P >0.1) or SA koalas (P >0.05).

Koala Retrovirus and Haematology

Significant, negative correlations were observed between proviral load and RBC (ρ= -0.297; $P = 0.003$), PCV (ρ = -0.257; $P = 0.012$) and neutrophil counts (ρ = -0.331; $P = 0.001$), and positive correlations were observed with absolute nRBCs (ρ = 0.264; $P = 0.014$) and lymphocyte counts (ρ = 0.239; $P = 0.018$). Significant, negative correlations were also observed between viral load and RBC (ρ = -0.279; $P = 0.018$) and neutrophil counts (ρ = -0.280; $P = 0.018$), and positive correlations were observed with absolute nRBC (ρ = 0.318; $P = 0.012$) and lymphocyte counts (ρ = 0.301; $P = 0.011$). Koalas in the high KoRV activity group had significantly ($P = 0.001$) higher median lymphocyte counts (2.76×10^9/l ; range 0.05–5.94) than koalas in the low KoRV activity group (1.04×10^9/l; range 0.41–5.10) and in the no KoRV activity group (0.79×10^9/l; range 0.26–3.82).

Koala Retrovirus and Splenic Lymphoid Area

There were no significant correlations between number of follicles or PALSs and proviral and viral load ($P >0.2$) or for follicle size ($P >0.1$). There was no correlation between PALS size and proviral load ($P = 0.213$); however, there was a positive correlation between PALS size and increasing viral load (ρ = 0.438; $P = 0.025$). Koalas with no follicles were significantly more likely to be in the high KoRV activity group than koalas with follicles ($P = 0.030$), but there was no difference in the presence or absence of PALSs and KoRV group ($P = 0.078$).

Discussion

This study compared the pathological findings of KoRV-positive northern (Qld) and southern (SA) koalas in order to determine whether differences in disease prevalence and severity, haematology and splenic lymphoid area occurred, and whether these were affected by KoRV proviral and viral loads. Koalas were grouped into four disease categories: neoplasia,

chlamydial disease, miscellaneous disease and disease-free, based on the known association of KoRV with lymphoid neoplasia and the proposed link of KoRV to chlamydial disease (Tarlinton *et al.*, 2005). Key findings were: (1) SA koalas developed lymphoma with similar gross, histopathological and immunophenotypic presentations as Qld koalas and, in both populations, lymphoma only occurred in koalas with high KoRV loads, (2) severe ocular chlamydial disease was observed in both populations, but was less prevalent in SA and in SA clinical disease was only seen in koalas with high KoRV viral loads, (3) urogenital tract chlamydial disease was common in both populations, but was less severe in SA koalas despite similar urogenital chlamydial loads, (4) no association was found between KoRV proviral and viral loads and chlamydial disease severity, and (5) correlation between KoRV loads and peripheral blood lymphocyte count and splenic lymphoid area suggested that lymphocytes may be key sites for KoRV viral replication.

Lymphoid neoplasia was the most common neoplasm observed in koalas from both Qld and SA populations. There were a number of similarities in lymphoma observed between Qld and SA koalas; all neoplastic cells had the same morphological appearance, being intermediate to large neoplastic cells, and B-cells were identified as the neoplastic cell of origin in both populations, which is similar to other cases of lymphoma described in northern koalas (Connolly *et al.*, 1998; Spencer and Canfield, 1996). All koalas also had multiple organ involvement except for one young, 1- to 2-year-old (TWC I) male SA koala where lymphoma was detected only in lymph nodes. Lymphoid neoplasia has been well described in northern koalas since the first reports in 1961 (Backhouse and Bolliger, 1961; Heuschele and Hayes, 1961; Spencer and Canfield, 1996; Connolly *et al.*, 1998). The prevalence of lymphoid neoplasia in Qld (7.5%) was similar to findings in other studies in northern koalas, such as a study of wild koalas from New South Wales that reported neoplasia in 7% (11/162) of koalas, and lymphoma was the most common tumour (Canfield, 1990). In contrast, lymphoma was only reported recently in a single SA female koala (Fabijan *et al.*, 2017), with no previous

reports in wild, rescued SA koalas (Speight *et al.*, 2018) or captive southern koalas from Victoria or SA (Gillett, 2014). In addition to the histopathological similarities, these koalas also had the highest KoRV proviral and viral loads (Sarker *et al.*, 2019a). This was consistent with a previous study of northern koalas where koalas with lymphoid neoplasia had the highest KoRV proviral and viral loads (Tarlinton *et al.*, 2005). Notably, the SA koalas with neoplasia had proviral and viral loads at the same high level as the Qld koalas with neoplasia, which were significantly higher than all other SA koalas. The increased prevalence of lymphoid neoplasia in SA koalas may reflect a possible increase in the prevalence of KoRV within the population. Recently, the prevalence of KoRV in the SA Kangaroo Island population was shown to have increased from 15% in 2012 (Simmons *et al.*, 2012) to 43% in 2017 (Fabijan *et al.*, 2019b). The KoRV *env* variant infections of the koalas in this study have been previously reported (Sarker *et al*., 2019b), and no association between KoRV variant infections and neoplasia was found. The findings of the current study may then suggest that total KoRV burden, rather than KoRV variants, may be more important in the development of lymphoma.

Osteochondroma was the second most common tumour observed, which was similarly reported in a study of koalas from New South Wales (Canfield, 1990). This is also the first report of this tumour affecting SA koalas. It is unknown whether KoRV plays a role in the development of osteochondroma. In this study, Qld koalas with osteochondroma had high proviral and viral loads, while the single SA koala had a high proviral load and low viral load. Previous studies have also hypothesized the role of KoRV in the development of osteochondroma in the koala (Hanger and Loader, 2014) as this tumour is known to develop in association with feline leukaemia virus (FeLV) infection in cats (Hartmann, 2012).

Differences in chlamydial disease severity were observed between Qld and SA koalas, despite some limitations in comparing disease. Ocular disease was more prevalent in Qld (58.1%) koalas compared with animals from SA (12.1%), but severe cases were observed in both

locations with comparable chlamydial loads. A low prevalence of mild ocular disease has been previously reported in SA koalas (Speight *et al.*, 2016), despite the first reports of chlamydiosis in SA describing three koalas with severe conjunctivitis (Funnell *et al.*, 2013). The prevalence of urinary tract disease and the urogenital chlamydial load of koalas with disease were similar between the populations; however, the urinary tract disease observed in SA koalas had reduced severity. As the reproductive tracts of the Qld koalas were not available for examination, comparison of chlamydial reproductive tract disease between the populations was limited. This study found a higher prevalence of reproductive tract disease in SA koalas than a previously necropsy study that reported mild reproductive lesions in 9.2% (6/65) of koalas (Speight *et al.*, 2016). These differences in disease severity may be due to chlamydial factors, such as different *C. pecorum* genotypes present within the populations (Kollipara *et al.*, 2013) and the absence of the chlamydial plasmid, pCpec, in SA isolates (Jelocnik *et al.*, 2015) which may carry virulence factors (Phillips *et al.*, 2018). Koala factors may also account for the disease differences, as northern and southern koalas fall into separate genetic lineages (Kjeldsen *et al.*, 2016), and there may be variation in koala immunity that results in different disease severity (Mathew *et al.*, 2014).

Compared with previous studies of *C. pecorum* prevalence in SA and Qld, there was a low PCR detection rate of *C. pecorum*, similar to other studies of chlamydial disease in Qld and Victorian koalas that reported a low detection rate of *C. pecorum* where not all koalas with chlamydial disease were *C. pecorum* positive (Wan *et al*., 2011; Patterson et al., 2015; Legione *et al*., 2016; Nyari *et al.*, 2017). Koalas with chlamydial disease that were *C. pecorum*-negative may cease shedding of the bacteria where the disease has not resolved (Nyari *et al*., 2017). The koalas with *Chlamydia*-like disease may also have been infected with *C. pneumoniae*; however, based on previous studies that showed *C. pecorum* to be a more common and virulent pathogen (Polkinghorne *et al*., 2013), *C. pneumoniae* was not tested in the present study.

It has been hypothesized that KoRV may cause immune suppression, predisposing koalas to developing chlamydial disease. This theory was thought to explain the differences in chlamydial disease observed between northern and southern koalas, where southern koalas may have a lower KoRV prevalence and resulting lower prevalence of chlamydial disease. Recent studies in northern koala populations found an association between KoRV-B infection and chlamydial disease (Waugh *et al.*, 2017; Quigley *et al.*, 2018a) and in Victorian koalas KoRV infection was associated with wet-bottom disease (Legione *et al.*, 2017). In this collaborative study, no clear association was observed between KoRV proviral load, viral load or variant type and chlamydial disease in either population (Sarker *et al.*, 2019a, b), although a strong, positive correlation between increasing chlamydial disease severity and increasing KoRV viral load was found in SA koalas. As the development of chlamydial disease is likely to be highly complex, studies with larger numbers of koalas may find clear associations between KoRV and chlamydial disease severity, particularly in SA.

Comparison of haematological variables between the four disease categories has highlighted some key diagnostic indicators for disease. In both populations, non-regenerative anaemia and inappropriate metarubricytosis were strongly associated with neoplasia, while neutrophilia was most commonly observed in koalas with chlamydial disease. Previous haematological studies have found similar indicators of these diseases in northern koalas (Obendorf, 1983; Canfield *et al.*, 1989a); however, this has not been reported previously for southern koalas, in which lymphoid neoplasia and chlamydial disease are being observed more frequently.

Splenic lymphoid area analysis suggested lymphoid hyperplasia in koalas with chlamydial disease. While there were considerably more PALSs than follicles for all koalas, PALSs were significantly more numerous in koalas with chlamydial disease. A higher number of PALSs than follicles has been reported previously in the koala (Backhouse and Bolliger, 1961; Hemsley, 1996) and IHC was used to demonstrate that large populations of T-cells reside in the PALS and B-cells in follicles (Hemsley, 1996). If PALSs are more common in

chlamydial infection, this may suggest increased cell-mediated immunity in these koalas; cytotoxic T cells in particular are key in the immune response to intracellular pathogens in other species (Clerici and Shearer, 1993).

Comparison of haematology and splenic lymphoid area with KoRV proviral and viral loads highlighted that lymphocytes may be a key site of KoRV replication. In the current study, increased lymphocyte counts (lymphocytosis), anaemia (non-regenerative normocytic normochromic), and increasing inappropriate metarubricytosis were all correlated with increasing KoRV proviral and viral loads. Additionally, splenic lymphoid area was correlated positively with KoRV viral load. These findings in koalas may mirror retroviral infections in other species, where retroviral infection of haemopoietic stem cells can disrupt haemopoietic stem cell differentiation (Hartmann, 2012). Lymphocytosis may arise in response to retroviral infection such as in people with human immunodeficiency virus infection (Mellors *et al.*, 1997) and cats with feline immunodeficiency virus (FIV) infection (Powers *et al.*, 2018). Mild to severe normocytic normochromic anaemia and inappropriate rubricytosis in cats is associated with FeLV infection (Stockham and Scott, 2008; Gleich and Hartmann, 2009) and inappropriate metarubricytosis may also occur in domestic species with viral infection (Stockham and Scott, 2008). However, it should be noted that lymphocytosis, non-regenerative anaemia and inappropriate metarubricytosis may also occur under other circumstances, such as with bone marrow injury, altered splenic function, heat stroke and dyserythropoiesis and for unknown reasons (Stockham and Scott, 2008). Future studies should investigate the role of lymphocytes, bone marrow, spleen, lymph nodes and other lymphoid tissues in KoRV infection and replication, and the implications of this on koala health.

Qld koalas had high levels of KoRV viral activity, while the SA koalas were more variable, falling into one of three distinct groups based on KoRV viral load: koalas with high, low and no KoRV activity. There are two possible hypotheses that may explain these population

differences in KoRV viral activity: (1) KoRV is an exogenous infection in SA koalas which is being suppressed by the koala's immune response, or (2) SA koalas have defective KoRV proviral inserts that do not produce KoRV viral particles. The differences in KoRV activity in SA koalas is reminiscent of exogenous FeLV infections in domestic cats (Hartmann *et al.*, 2012). The cat's initial immune response to FeLV dictates disease progression; cats that mount a rapid immune response develop latent FeLV infections and may be asymptomatic, while a delayed or deficient immune response leads to progressive infections observed as persistent high FeLV viraemia and neoplasia development (Hartmann, 2012). If KoRV is transmitted exogenously between SA koalas, koalas with no or low KoRV activity may have mounted sufficient immune responses to suppress KoRV replication and harbour latent infections, while koalas with high KoRV activity may have progressive infections and be at higher risk of developing lymphoid neoplasia, as was observed in this study. The koala's immune response to KoRV infection has only recently been investigated and studies are yet to show clear immune responses to KoRV infection (Maher and Higgins, 2016; Waugh *et al.*, 2016; Olagoke *et al.*, 2018, 2019; Maher *et al.*, 2019).

Alternatively, SA koalas with no or low KoRV activity may have truncated or defective proviral inserts that are not transcribed to make virus particles. Evidence for this has been observed as part of this collaborative project. Transcriptomic analysis of submandibular lymph nodes from Qld (n = 10) and SA (n = 19) koalas showed that all genes of KoRV-A, KoRV-B and other *env* variants are highly expressed in Qld koalas, while in SA only the LTR and partial *gag* gene were expressed in all koalas, and the expression of the *pol* and *env* genes were significantly reduced in 14 koalas and no expression was detected in five koalas (Tarlinton *et al.*, 2017). The low level or lack of expression of the *pol* and *env* genes occurred at the same RNA base pairs for all koalas, which suggests that transcription of the provirus is inhibited at this site and that the provirus may be truncated (Tarlinton *et al.*, 2017). The KoRV pathogenesis project further investigated the possibility of truncated proviruses in SA

koalas and showed all Qld koalas possessed all KoRV genes (LTR, *gag*, *pol* and *env*), but only 79% (77/97) of SA koalas were positive for all proviral genes (Sarker *et al.*, 2019a). Further investigation is required to understand the differences in KoRV infection between Qld and SA koalas, to understand if KoRV is exogenously transmitted or if KoRV is a defunct, endogenous retrovirus in some SA koalas.

The finding that SA koalas develop lymphoma with the same neoplastic morphology, site predilections and high KoRV loads as Qld koalas, suggests similar oncogenic mechanisms of KoRV may be occurring in both populations. The high proviral load in these koalas may support the idea of lymphoma developing via insertional mutagenesis (Tarlinton *et al.*, 2005) or via upregulation of adjacent genes (Xu *et al.*, 2013). KoRV variants may also be involved. A previous study showed that KoRV-B infection, thought to be exogenously transmitted, was associated with the development of lymphoid neoplasia in captive northern koalas (Xu *et al.*, 2013). In the current collaborative project, no clear associations between neoplasia and KoRV variants were observed (Sarker *et al.*, 2019b); however, this study was based on a small number of koalas from SA and further investigation with more koalas may shed more light on the role of KoRV variants and disease development. While continued investigation is required to understand the mechanisms of KoRV oncogenesis, this study has provided evidence to suggest similar mechanisms are occurring in geographically separate koala populations where differences in koala genetics (Kjeldsen *et al.*, 2016) and KoRV variants (Sarker *et al.*, 2019b) appear to have little effect on lymphoma development.

This study has provided further information on the pathogenesis of KoRV infection by comparing the aetiology, haematology and splenic lymphoid area of Qld and SA koalas. It is becoming more apparent that there are significant differences in KoRV infection between Qld and SA koalas, including prevalence, KoRV proviral load differences, range of KoRV variants and viral activity. In Qld koalas, KoRV is an active endogenous virus, KoRV proviral and viral loads are high in all koalas and all koalas have many concurrent KoRV

variant infections, while in SA, proviral loads are lower, not all koalas had active KoRV infections and KoRV-A was the most prevalent variant. The reasons behind KoRV inactivity in SA koalas may be due to the immune response to exogenous infections or to a truncated KoRV, theories currently under investigation. Despite these population differences, lymphoid neoplasia was found to develop in both northern and southern koalas with the same pathological features, which suggests the same basic KoRV-induced oncogenic pathway is occurring in both populations. Additionally, haematology and splenic investigations highlighted that in both northern and southern koalas, lymphocytes and lymphoid tissue may be key sites where KoRV replication occurs, and haematological changes in viraemic koalas may mirror those of regressive or progressive FeLV infection in cats. Therefore, in SA koalas, high KoRV activity may be a useful prognostic indicator for the development of lymphoma and chlamydial disease. KoRV infection appears to be highly complex, therefore continued investigation is required to fully understand the pathogenesis of KoRV and implications of infection for koala health.

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Conflict of Interest Statement

The authors declare no conflict of interest with respect to publication of this manuscript.

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Table 1

Summary of koalas from South Australia (*n* = 92) and Queensland (*n* = 67) for four pathological categories

Category	*SA Wild*	*Qld Wild*[*]	*Qld Captive*
Neoplasia	5	9	0
Female	3	2	-
Male	2	7	-
Mean TWC (± SEM)	2.75 ± 0.85	4.22 ± 0.32	-
Mean BCS (± SEM)	3.00 ± 0.00	1.67 ± 0.24	-
Chlamydial disease	33	31	0
Female	20	12	-
Male	13	19	-
Mean TWC (± SEM)	3.73 ± 0.21	4.74 ± 0.32	-
Mean BCS (± SEM)	3.13 ± 0.17	1.80 ± 0.14	-
Miscellaneous disease	31	4	0
Female	12	2	-
Male	19	2	-
Mean TWC (± SEM)	3.07 ± 0.22	6.00 ± 1.00	-
Mean BCS (± SEM)	3.10 ± 0.18	1.00 ± 0.00	-
Disease-free	23	2	21
Female	7	1	12
Male	16	1	9
Mean TWC (± SEM)	3.19 ± 0.25	4.00 ± 1.00	3.00 ± 0.40
Mean BCS (± SEM)	4.00 ± 0.22	4.00 ± 0.50	0.51 ± 0.11

[*]Includes wild necropsied (*n* = 28) and wild, rescued (*n* = 18) koalas sampled during clinical examination

SA, South Australia; Qld, Queensland

Table 2

Number of koalas observed with chlamydial disease at one or more sites from Queensland (*n* = 31) and South Australia (*n* = 33)

Chlamydial disease site	*Qld*[*] n *(%)*	*SA* n *(%)*
Ocular disease only	10 (32.3)	1 (3.0)
Respiratory disease only	0 (0)	1 (3.0)
Urinary tract disease only	8 (25.8)	7 (21.2)
Reproductive tract disease only[†]	1 (3.2)	9 (27.3)
Disease at two or more sites	12 (38.7)	15 (45.5)

[*]Not all tissues were examined histologically from all koalas

[†]Reproductive tracts were not available for examination from all Qld koalas

SA, South Australia; Qld, Queensland

Table 3

Summary of histopathological changes in koalas with chlamydial disease (PCR positive and negative for Chlamydia pecorum infection) from Queensland (n = 31) and South Australia (n = 33) submitted for necropsy examination*

Site	*Queensland*	*South Australia*
Ocular lesions†	18 (58.1%) (4 female, 14 male) Grossly apparent lesions Grade 1 (*n* = 2) Grade 2 (*n* = 2) Bilateral conjunctivitis with corneal opacity Grade 3 (*n* = 7) Bilateral, chronic active conjunctivitis, with or without keratitis Not graded (*n* = 7)	4 (12.1%) (1 female, 3 male) Grossly inapparent lesions Grade 1 (*n* = 1) Minimal to mild non-suppurative conjunctivitis Grossly apparent lesions Grade 2 (*n* = 1) Bilateral, minimal to mild non-suppurative conjunctivitis Grade 3 Bilateral, mild to marked proliferative, chronic active mixed neutrophilic and lymphoplasmacytic conjunctivitis (*n* = 2) and mixed keratitis (*n* = 1)
Urinary lesions	19 (58.1%) (8 female, 11 male) **Kidney (*n* = 2)** Nephritis (*n* = 2) Chronic, non-suppurative or granulomatous, with fibrosis **Bladder (*n* = 18)** Grade 1 (*n* = 3) Grade 2 (*n* = 2) Superficial, mild to moderate, non-suppurative cystitis Grade 3 (*n* = 7) Chronic, moderate, active mixed cystitis	23 (69.7%) (10 female, 13 male) **Kidney (*n* = 9)** Interstitial fibrosis (*n* = 3) Nephritis Non-suppurative, mild to moderate (*n* = 4) Pyelonephritis (*n* = 2) Mild to moderate, lymphoplasmacytic, neutrophilic, or mixed, with segmental tubular degeneration, loss and fibrosis **Bladder (*n* = 18)** Grade 1 (*n* = 14) Superficial, mild to moderate, non-suppurative cystitis Grade 2 (*n* = 4)

	Not graded (*n* = 6) Penile and/or prostatic urethra not examined	Chronic, moderate, active lymphoplasmacytic or mixed cystitis, pericloacal urine staining Penile and/or prostatic urethritis (*n* = 10)
Reproductive female lesions[†]	4 (12.9%) (4 female) **Ovary** Paraovarian cyst (*n* = 3) **Vagina** Vaginitis (*n* = 1)	23 (69.7%) (13 female, 10 male) **Ovary** Paraovarian cyst (*n* = 10) Hyperplastic, fibrocollagenous cyst, non-suppurative **Uterus** Mild, chronic, non-suppurative endometritis (*n* = 6) Mild to moderate, mixed, active endometritis (*n* = 2) Necrosuppurative endometritis (*n* = 1) **Vagina** Moderate to severe, non-suppurative ulcerative vaginitis (*n* = 1)
Male lesions[†]	Not examined	**Testis** Mild interstitial fibrosis (*n* = 6) Sperm granuloma (*n* = 1) **Epididymis** Sperm granuloma (*n* = 2) **Prostate** Non-suppurative prostatitis (*n* = 1) Moderate to severe, chronic, active mixed periurethral or glandular prostatitis, may have microabscessation (*n* = 6)

[*]Some koalas presented with disease at multiple sites
[†]Histopathology was not performed routinely on tissues from Qld koalas

Table 4

Median number of lymphoid follicles and periarteriolar lymphoid sheaths and lymphoid area in spleen histological sections collected from necropsied koalas from South Australia and Queensland

Category	n =	*Median number of follicles (range)*	*Median number of PALSs (range)*	*Median follicle size (range) (µm²)*	*Median PALS size (range) (µm²)*
Population					
SA	31	0 (0–4)	3 (0–13)	6,955 (0–216,678)	71,754 (0–303,866)
Qld	10	1 (0–2)	4.5 (1–11)	46,365 (0–215,330)	108,408 (40,466–530,077)
Disease category					
Chlamydial	23	1 (0–3)	**5 (1–13)**	40,697 (0–215,330)	90,303 (18,201–530,077)
Miscellaneous	7	0 (0–3)	**1 (0–3)**	0 (0–157,264)	64,551 (0–210,788)
Disease-free	11	0 (0–4)	**3 (0–6)**	57,273 (0–216,678)	128,150 (303,866–127,920)
Sex					
Female	20	0.5 (0–2)	4 (1–11)	35,142 (0–215,330)	81,028 (18,202–230,588)
Male	21	1 (0–4)	3 (0–13)	23,027 (0–216,678)	97,050 (0–530,077)
BCS					
Excellent	12	1 (0–4)	2.5 (1–6)	**90,935 (0–216,678)**	121111 (42,242–303,866)
Fair	12	0 (0–2)	3 (0–6)	**0 (0–76,960)**	46,314 (0–210,788)
Poor	13	1 (0–2)	5 (1–11)	**40,697 (0–215,330)**	98,836 (40,466–530,077)

Follicle numbers and area were compared by non-parametric Mann–Whitney test. Bold values indicate the variables are significantly different from each other (P <0.05).
SA, South Australia; Qld, Queensland.

Table 5

Median KoRV proviral and viral loads of koalas from Queensland and South Australia for four disease categories

Category	n	*Median proviral load (range) (copies KoRV DNA/10^3 β-actin copies)*	n	*Median viral load (range) (copies KoRV RNA/ml of plasma)*
Neoplasia				
Qld	9	5.25×10^4 (3.38×10^4–4.78×10^5)	9	3.15×10^9 (2.68×10^7–7.58×10^{11})
SA	5	2.14×10^5 (6.71×10^3–4.32×10^5)	4	5.26×10^9 (6.19×10^5–4.34×10^{10})
Chlamydial				
Qld	31	5.83×10^4 (2.55×10^4–5.91×10^5)	30	2.18×10^9 (2.16×10^7–1.06×10^{11})
SA	33	4.26×10^3 (2.2×10^1–4.08×10^4)	8	6.39×10^4 (2.28×10^4–1.38×10^9)
Miscellaneous				
Qld	4	6.62×10^4 (2.67×10^3–7.88×10^4)	4	4.12×10^7 (5.79×10^7–5.88×10^{10})
SA	31	2.16×10^3 (1.1×10^1–1.82×10^5)	5	2.55×10^5 (8.48×10^4–1.64×10^9)
Disease-free				
Qld	23	4.46×10^4 (1.04×10^4–7.92×10^4)	23	7.43×10^7 (7.76×10^6–6.59×10^8)
SA	23	2.51×10^3 (2.5×10^1–5.05×10^4)	3	8.54×10^4 (4.38×10^4-1.85×10^5)

SA, South Australia; Qld, Queensland

Figures

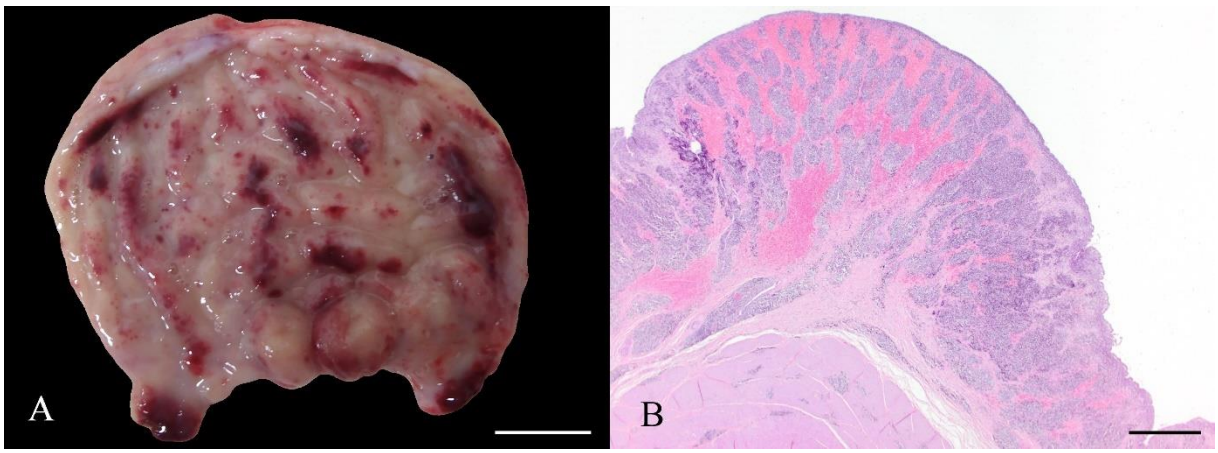

Fig. 1. Lymphoma. Infiltration of the bladder mucosal layer in a 4-year-old (TWC III) male South Australian koala. (A) Bladder wall is thickened with irregular reddened and pale mucosal surface. Bar, 1 cm. (B) Lymphoma of the bladder mucosa and submucosa, with neoplastic infiltration and loss of normal architecture. Note neoplastic cellular infiltration into muscularis (arrow). HE. Bar, 1 mm.

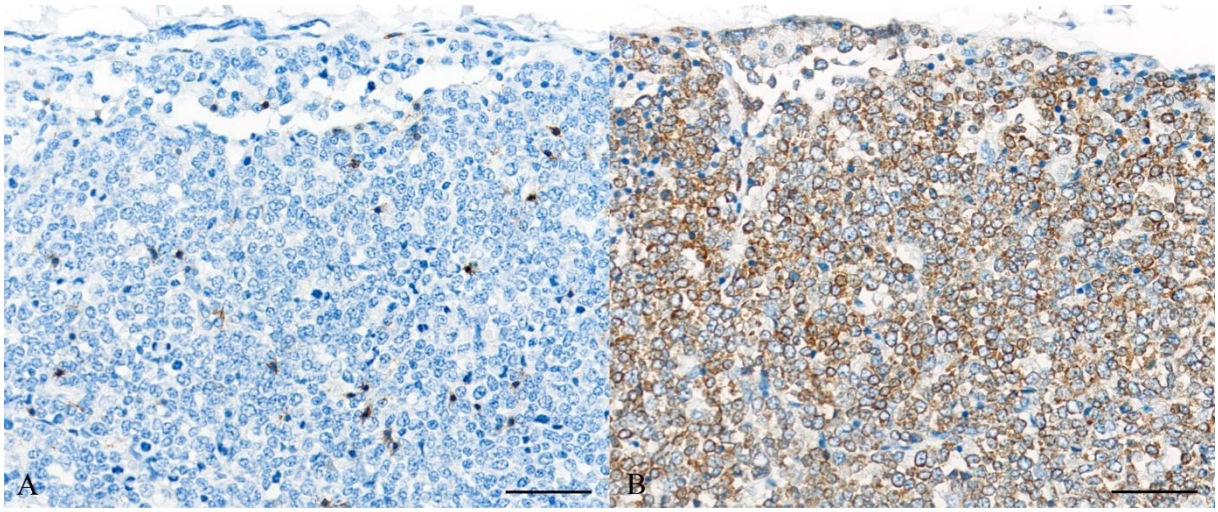

Fig. 2. Lymphoma. Infiltration of the submandibular lymph node of a 1- to 2-year-old (TWC I) male South Australian koala. (A) Scant T cells within the affected submandibular lymph node. IHC. Bar, 50 µm. (B) Neoplastic B cells efface normal nodal architecture. IHC. Bar, 50 µm.

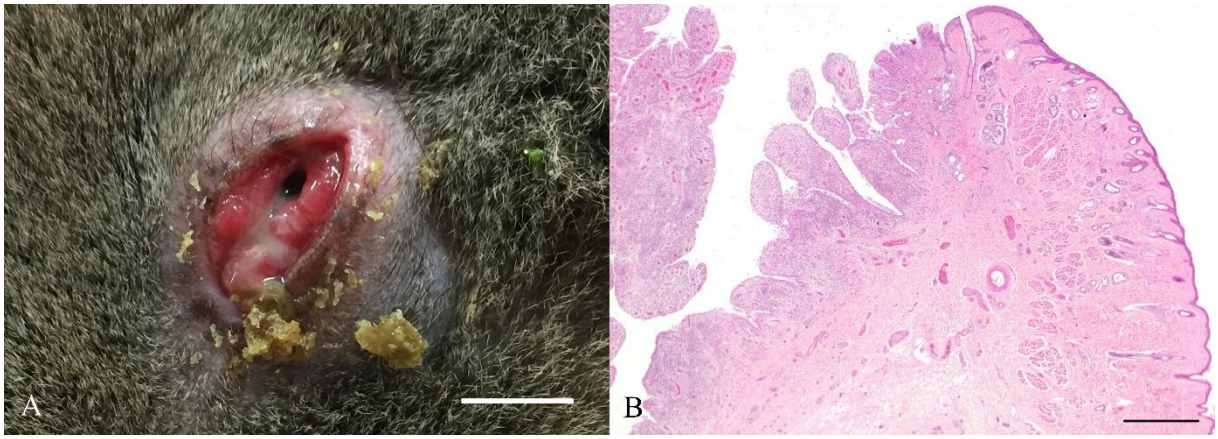

Fig. 3. Conjunctivitis. Grade 3 conjunctivitis of the left eye in a 2- to 3-year-old (TWC II) male koala from South Australia infected with Chlamydia pecorum. (A) Conjunctival hyperplasia and purulent exudate. Bar, 1 cm. (B) Marked proliferative chronic, active ulcerative neutrophilic, histiocytic and lymphoplasmacytic conjunctivitis. HE. Bar, 1 mm.

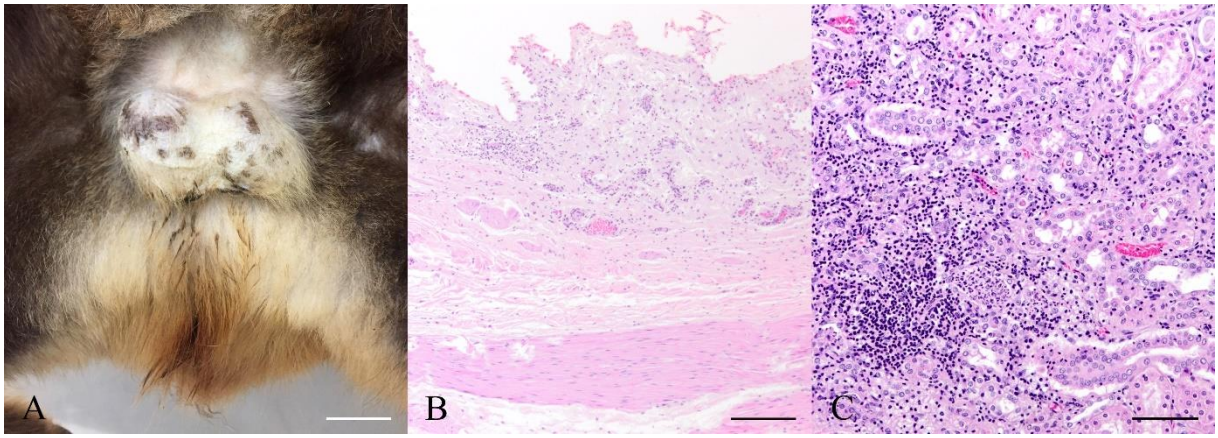

Fig. 4. Urinary tract disease. Grade 2 urinary tract disease in a 5- to 6-year-old (TWC IV) male koala from South Australia infected with Chlamydia pecorum. (A) Pericloacal urinary staining. Bar, 1 cm. (B) Mild to moderate non-suppurative cystitis. HE. Bar, 1 mm. (C) Multifocal chronic active neutrophilic, histiocytic and lymphoplasmacytic interstitial pyelonephritis. HE. Bar, 50 µm.

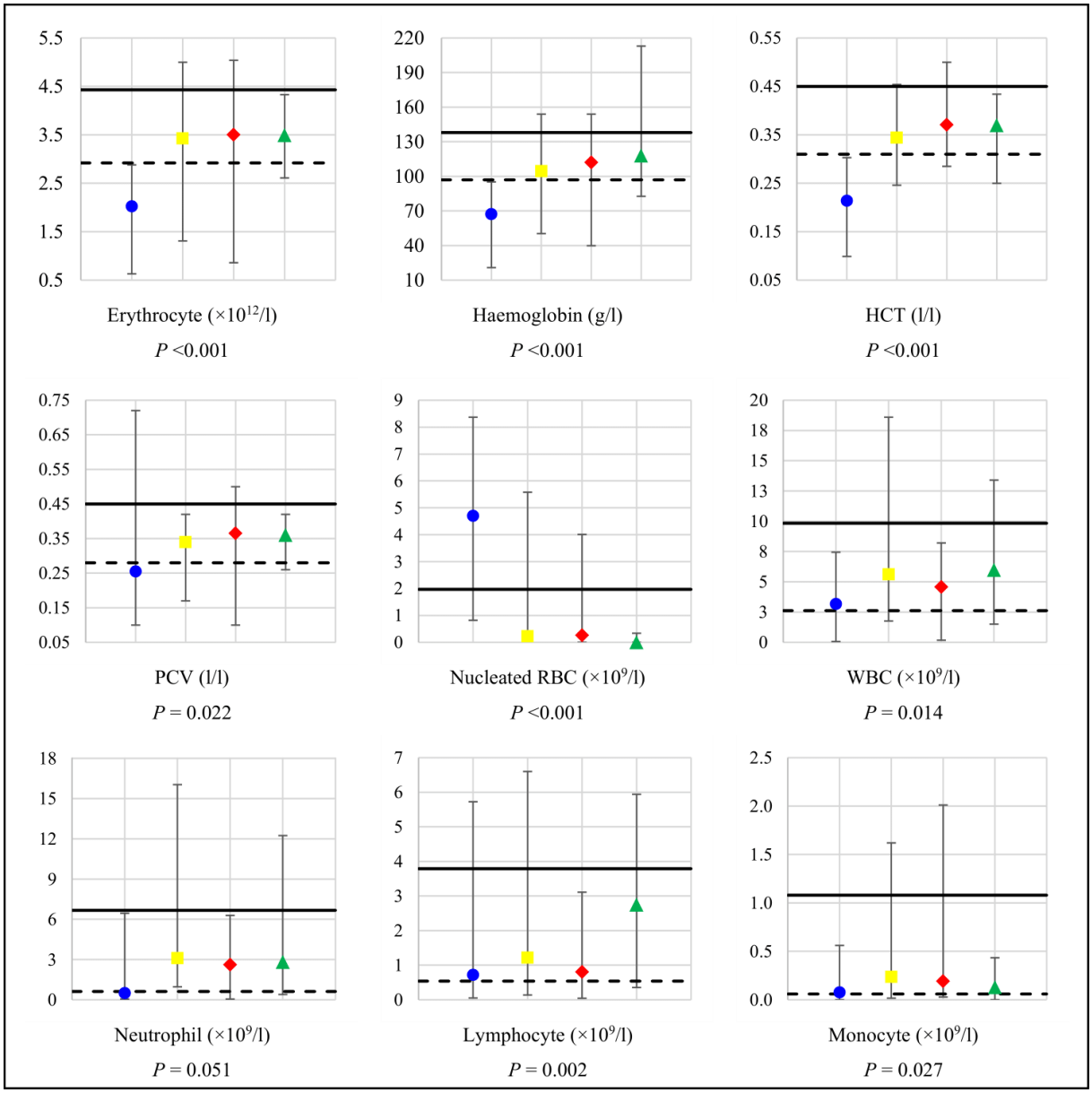

Fig. 5. Comparison of mean ± SD haematological values of koalas between disease categories; neoplasia (n = 8; blue circle), chlamydial disease (n = 38; yellow square), miscellaneous disease (n = 17; red diamond) and disease-free (n = 35; green triangle), compared with koala haematological reference intervals for northern (upper and lower intervals, broken lines) (Canfield et al., 1989b) and southern koalas (upper and lower intervals, solid lines) (Fabijan et al., 2020).

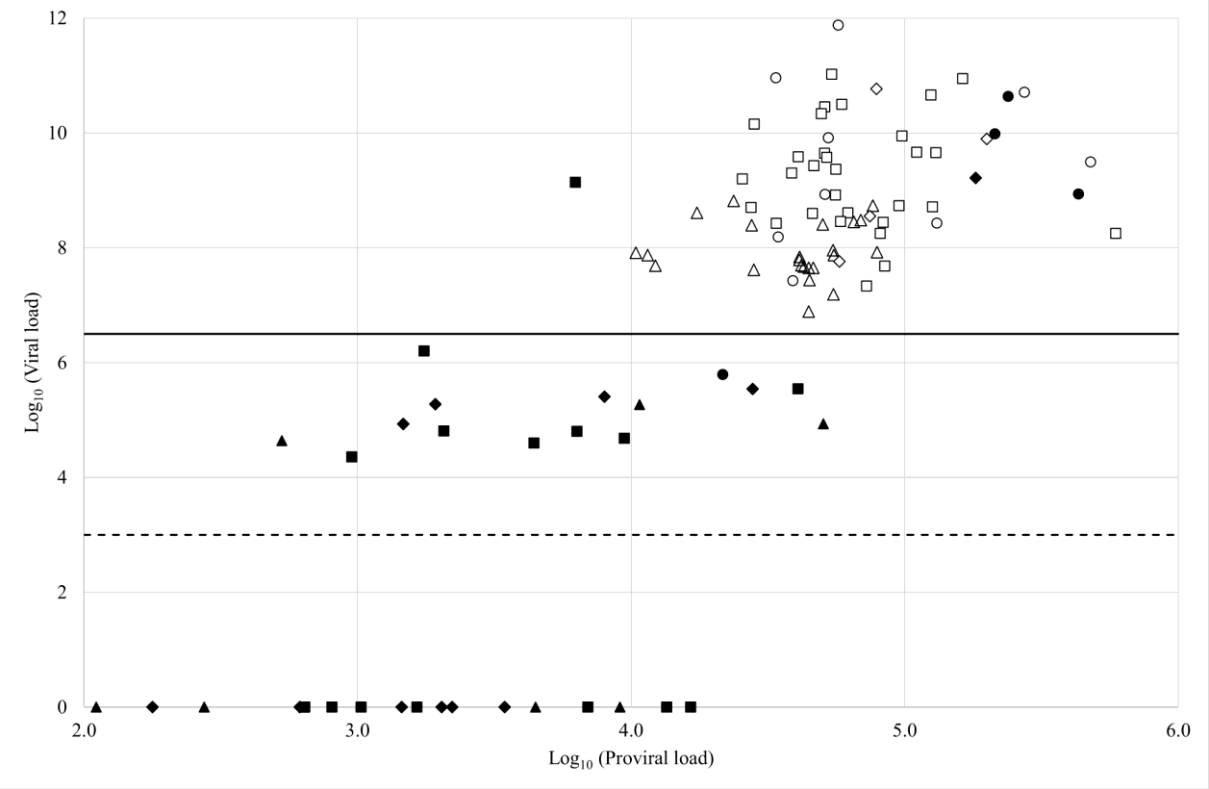

Fig. 6. Relationship of log transformed koala retrovirus (KoRV) proviral load (copies KoRV DNA/10^3 β-actin copies) and viral load (copies KoRV RNA/ml plasma) of Queensland (n = 66) (open) and South Australian (n = 38) (solid) koalas from three distinct KoRV activity groups; high KoRV activity (log_{10} viral load >6.5, solid line), low KoRV activity (log_{10} viral load <6.5) and no KoRV activity (log_{10} viral load = 0). Disease categories of each koala are presented; neoplasia (circle), chlamydial disease (square), miscellaneous disease (diamond) and disease-free (triangle).

Chapter 6

General discussion

6.1. General Summary

Chlamydia pecorum and koala retrovirus (KoRV) are two key pathogens of koalas in northern Australia (Queensland [Qld] and New South Wales) where infection can cause severe disease and mortality. In northern populations, *C. pecorum* disease is contributing to the significant population declines in these regions (Rhodes *et al.*, 2011) and KoRV has been associated with lymphoid neoplasia (Tarlinton *et al.*, 2005), the most common cancers of northern koalas (Canfield, 1990; Gillett, 2014). Evidence is building to suggest KoRV may also modulate the koala's immune response, predisposing koalas to developing *C. pecorum* disease (Tarlinton *et al.*, 2005; Waugh *et al.*, 2017). In South Australia there was a considerable lack of knowledge regarding the prevalence and pathology of these pathogens. This thesis focuses on characterising *C. pecorum* and KoRV infections in South Australian populations and determining if an association between *C. pecorum* disease and KoRV infection occurs in southern koalas.

The prevalence of *C. pecorum* (Chapter 2) and KoRV (Chapter 3) was determined in the South Australian Kangaroo Island (KI; n=170) and Mount Lofty Ranges (MLR; n=75) koala populations. These studies revealed that the KI population was *C. pecorum*-free, but KoRV prevalence was 42.4%, and in the MLR population the prevalence of both *C. pecorum* and KoRV was high, at 46.7% and 65.3%, respectively. Only 4% (3/75) of MLR koalas were observed with chlamydial disease at the time of sampling, while a further five koalas developed chlamydial disease in the following two years. Urogenital tract infections were more common than ocular infections, and urogenital tract *C. pecorum* infection in female koalas was significantly associated with reproductive inactivity. The median KoRV proviral load was low in the KI and MLR populations, with a median load of 113 (range: 2- 12,641) copies/10^3 *β*-actin copies and 35 (range: 1- 574) copies/10^3 *β*-actin copies, respectively. In both populations, only

KoRV-A and not KoRV-B were detected by PCR and 4.8% (3/62) of KI and 20.5% (8/39) of MLR KoRV-positive koalas were negative for both KoRV-A and KoRV-B proviral *env* genes. There was no association between *C. pecorum* infection or disease and KoRV, however koalas with concurrent infections had 3 times the odds of developing chlamydial disease. Subclinical *C. pecorum* and KoRV infections were found to have no effect on haematology parameters, allowing for the development of the first southern koala haematology reference intervals (n=138) (Chapter 4).

Pathological findings from necropsied KoRV infected koalas from the MLR were presented in Chapter 5. In Chapter 5.1 the first South Australian koala with severe reproductive chlamydial disease, lymphosarcoma and KoRV infection was reported. Chapter 5.2 presented an extensive comparative pathological investigation of disease in KoRV-positive koalas from the MLR (n=92) and from south-east Brisbane, Qld (n=67) forming part of the koala retrovirus pathogenesis project conducted in collaboration with the University of Queensland. In MLR koalas, lymphosarcoma and chlamydial disease were observed in 4.3% (4/92) and 35.9% (33/92) of koalas, respectively, and in Qld both diseases were more prevalent, at 7.5% (5/67) and 46.3% (31/67), respectively. The lymphosarcomas of koalas from both populations had the same morphological features and were observed in MLR and Qld koalas with similar high KoRV proviral and viral loads, suggesting that the same basic oncogenic pathways occur in both populations. Severe conjunctivitis was observed in both populations but was less prevalent in MLR koalas, and urinary tract disease was prevalent in both populations but was less severe in MLR koalas, despite the same high *C. pecorum* load. No association was found between *C. pecorum* disease, chlamydial disease severity or chlamydial load with KoRV proviral load or viral load in koalas from Qld, but in MLR there was a significant, positive correlation between chlamydial disease severity and KoRV proviral and viral loads. These findings have

furthered our understanding of diseases that affect MLR koalas and shown that both the prevalence and severity of *C. pecorum* and KoRV is lower than that in Qld koalas. These differences may be due to *C. pecorum* or KoRV transmission and virulence variability, but it is also likely that the mechanisms involved in the development of chlamydial disease and lymphoid neoplasia in the koala host are complex and may vary across Australia.

6.2. Major findings

6.2.1. Kangaroo Island koalas are *C. pecorum*-free and may be an important population for koala conservation

The KI population was found with 95% confidence to be *C. pecorum*-free (Chapter 2). Only two other island populations have been considered to be *C. pecorum*-free; Magnetic island, Qld and French Island, Victoria (Polkinghorne *et al.*, 2013), however, ocular disease has been reported to effect Magnetic Island koalas (Hirst *et al.*, 1992) and recently two infected koalas from French Island were detected (Legione *et al.*, 2016a). This leaves KI as the last large, isolated *C. pecorum*-free population.

A population free from *C. pecorum* infection is of great importance to koala conservation, as *C. pecorum* disease is contributing to significant population declines in northern Australia. Habitat fragmentation due to urbanisation was considered the key factor causing koala populations to decline (McAlpine *et al.*, 2006), until a population modelling study based in south-east Qld showed that chlamydial disease was the most important factor (Rhodes *et al.*, 2011). Another modelling study predicted that the populations which are currently declining would begin to increase over a seven-year period if koalas with chlamydial disease were actively removed from the population (Wilson *et al.*, 2015). Consequently, considerable efforts are being made to reduce the impacts of *C. pecorum* on these populations, including the development of

a *C. pecorum* vaccine (Carey *et al.*, 2010; Khan *et al.*, 2016; Nyari *et al.*, 2019). Given the significant impacts of *C. pecorum* in northern populations, the *C. pecorum*-free KI koala population could act as a possible insurance population for the species in the future.

6.2.2. *C. pecorum* infection in Mount Lofty Ranges koalas is changing

6.2.2.1. The prevalence of *C. pecorum* infection remains high

The prevalence of *C. pecorum* infection in the wild MLR population was found to be high (47%). However, the majority of infected koalas were clinically healthy with only three koalas (4%) observed with overt chlamydial disease (Chapter 2). A previous study undertaken around the year 2000 reported 88% of wild MLR koalas (n=17) to be infected with *C. pecorum* (Polkinghorne *et al.*, 2013). Whilst this previous study showed that *C. pecorum* infection has been present in the MLR for the past 20 years, the larger cohort in the current study increases the accuracy of the prevalence estimate. The prevalence of *C. pecorum* in the MLR population was higher than a recent investigation of wild-caught koalas in Victoria, where only 15% (125/820) of koalas were infected (Legione *et al.*, 2016b) but similarly a low prevalence of mild chlamydial disease was observed (Patterson *et al.*, 2015). In comparison, although the prevalence of *C. pecorum* infection in wild-caught Qld koalas was also lower at 31%, the prevalence of clinical disease was much higher, where chlamydial disease was observed in 28% of koalas, however not all koalas with disease were *C. pecorum* positive by qPCR (Nyari *et al.*, 2017).

6.2.2.2. Urogenital tract *C. pecorum* infections were common and affected female koala infertility

C. pecorum urogenital tract infections were more common than ocular infections in both wild caught (Chapter 2) and necropsied MLR koalas (Chapter 5.2). More wild MLR koalas were positive at the urogenital site (97.1%, 34/35) and had

higher chlamydial loads (median, range: 170, 10–30,600 copies/µL) than at the ocular site (8.6%, 3/35; median, range: 30, 17–2,020 copies/µL). In necropsied MLR koalas, urinary tract disease was also observed more commonly than ocular disease. This infection pattern differed from a previous study in necropsied MLR koalas where more koalas were positive for *C. pecorum* at the ocular site (88%, 50/57) than the urogenital site (70%, 40/57) and mild chlamydial disease was equally observed at both sites (Speight *et al.*, 2016), despite the first cases of chlamydial disease reported in the MLR being three cases of conjunctivitis (Funnell *et al.*, 2013). However, this infection pattern was consistent with recent reports in other koala populations, where *C. pecorum* infection was more commonly detected at the urogenital site in Victorian (Legione *et al.*, 2016b) and Qld (Nyari *et al.*, 2017) koalas.

Urogenital *C. pecorum* infection in wild female MLR koalas with no signs of infection, was significantly associated with reproductive inactivity. No *C. pecorum* infected female koalas were reproductively active at the time of sampling, while all active females were *C. pecorum*-negative (Chapter 2). The reproductive success of wild female MLR koalas was 37.2% (16/43), which was similar to another *C. pecorum* infected population, Mount Eccles National Park, Victoria where 39.2% (47/120) of females were reproductively active (Patterson *et al.*, 2015). In comparison, the reproductive rate of the *C. pecorum*-free KI population was double that of the MLR population, where 79.2% (118/149) of female KI koalas were reproductively active (Chapter 2). This finding in MLR koalas suggests that *C. pecorum* infection in apparently healthy female koalas may cause infertility, however there may have been underlying changes to the reproductive tracts that were not detected during examination. Continued surveillance on the impacts of *C. pecorum* infection in the MLR population is needed to monitor how this infection may affect the population.

6.2.2.3. *C. pecorum* disease severity is increasing in the Mount Lofty Ranges population

The severity of chlamydial disease observed in rescued, necropsied MLR koalas has increased compared to a previous study conducted in the same population between 2012-13. In the current study, of the necropsied koalas with chlamydial disease, 70% of lesions (35/50) [75% (3/4) of ocular disease, 74% (17/23) of urinary tract disease and 65% (15/23) of reproductive tract disease cases] were moderate to severe in nature and consequently were identified grossly at necropsy examination. The remaining cases were diagnosed microscopically during histological examination of tissues (Chapter 5.2). In the previous study of MLR koalas euthanased in 2012-13, chlamydial disease was also observed in PCR positive (n=41) and negative (n=7) koalas, however, only 43.8% (21/48) of koalas had overt disease identified grossly during necropsy and the disease was predominantly mild in severity. The remaining koalas had microscopic lesions only (Speight *et al.*, 2016). Both the current and previous study utilised the *C. pecorum* disease classifications described by Wan *et al*., (2011). Most notably were the observations of grade 3 ocular disease, kidney disease and severe reproductive tract disease in the current study (Chapter 5.2) that weren't reported in the previous study (Speight *et al.*, 2016). The previous study also reported more koalas with subclinical *C. pecorum* carriage, where 28% (16/57) of *C. pecorum* infected koalas had no chlamydial lesions (Speight *et al.*, 2016), compared to the current study where only 12.0% (11/92) of *C. pecorum* infected koalas had no chlamydial lesions. The comparison of koalas sampled in this study (2014-2016) to the previous study (2012-13) show an increase in the severity of chlamydial disease.

The reason for the increase in disease severity in MLR koalas in a short period of time could be due to a number of bacterial and host factors. High chlamydial loads have previously been associated with severe disease in Qld koalas (Wan *et al.*, 2011) however this was not observed in the current study. In necropsied MLR koalas,

chlamydial load was not associated with disease severity; the chlamydial load of koalas with ocular disease (n=3; median, range: 30, 30-100 copies/µL) was lower than koalas without ocular disease (n=5; median, range: 1,500, 513-7,469 copies/µL). There was also no difference between the urogenital chlamydial loads of koalas with disease (n=9; median, range: 1,660, 5-320,000 copies/µL) or without disease (n=6; median, range: 1,725, 180-19,400 copies/µL). A limitation of the current study was that 42.4% (14/33) of koalas with chlamydial disease were negative for *C. pecorum* by qPCR which may have influenced the chlamydial load findings. A recent study of wild-caught Qld koalas also observed lower chlamydial loads in koalas with disease compared to koalas without disease and reported *Chlamydia*-like disease in PCR negative koalas (Nyari *et al.*, 2017). The authors of the Qld study proposed that negative koalas with *Chlamydia*-like disease may have resolved the *C. pecorum* infection (Nyari *et al.*, 2017) or the physical changes caused by disease may have inhibited the collection of infected epithelial cells during sampling. Future studies could investigate other methods of *C. pecorum* detection, such as serology to correctly identify *C. pecorum* exposure in PCR negative koalas with *Chlamydia*-like disease.

Another possible explanation for the increase in chlamydial disease severity may be due to the introduction of new *C. pecorum* genotypes into the population, or an increase in the prevalence of a plasmid, *pCpec*, which is thought to contain virulence factors. Previously, differences in the *C. pecorum ompA* (major outer membrane protein) genotypes, which has been associated with chlamydial virulence factors (Fitch *et al.*, 1993) have been found in different koala populations. Genotype B was the only genotype detected in the MLR population (Kollipara *et al.*, 2013) that was similar to Victorian koalas, where genotype B was the most common and genotypes C, F, L, M and N were detected at low levels (Legione *et al.*, 2016b). In comparison, genotype F and G were the most common in Qld and New South Wales koala

populations (Kollipara *et al.*, 2013). *C. pecorum* genotype B may therefore be less pathogenic than other genotypes present in northern koala populations and may have changed in MLR since the 2013 study to a new genotype that is more virulent and resulting in the increased disease severity. Alternatively, the lower disease severity observed in southern koalas may be due to a lower prevalence of the chlamydial plasmid, *pCpec*. The prevalence of *pCpec* in koala *C. pecorum* isolates from Victoria, New South Wales and Qld was high, at 79%, 84% and 73%, respectively, but was shown to be lower in the MLR population, where only 11% (4/36) of isolates collected between 2012-2013 harboured *pCpec* (Jelocnik *et al.*, 2015). Recently, a study detected *pCpec* in all Qld koalas with urogenital tract disease (Phillips *et al.*, 2018) highlighting the potential pathogenicity of *C. pecorum* carrying this plasmid. The prevalence of *pCpec* may have increased in the MLR population which may explain the increase in disease severity. Future studies should investigate the prevalence of *C. pecorum* genotypes and *pCpec* in MLR koalas and the possible role these factors may have on disease severity.

The increasing chlamydial disease severity may also be a result of reduced immunity in some koalas due to stress. The MLR population is considered to be growing and overabundant (Sequeira *et al.*, 2014) and the increase in population size has been coinciding with the increase in chlamydial disease severity. The necropsied MLR koalas in this study were commonly rescued in suburban areas (unpublished data), on the fringe of their habitat, and this would likely cause considerable physiological stress, as has been shown in Qld koalas (Davies *et al.*, 2013). High physiological stress could consequently reduce the immune function of some koalas (Munck *et al.*, 1984) and may lead to chlamydial disease development in subclinically infected koalas or increased disease severity. A recent study of faecal glucocorticoid (cortisol) metabolites found increased levels in koalas with chlamydial disease (median

24.5 ng/dry weight) compared to healthy female and male koalas, 4.7 and 9.5 ng/dry matter, respectively (Narayan, 2019), suggesting increased physiological stress levels in koalas with chlamydial disease, however, whether the increased concentration of glucocorticoids causes *C. pecorum* disease to develop, or whether *C. pecorum* disease increases glucocorticoid concentration is unclear. Alternatively, the increase in population size may have simply resulted in increased koala contact and the increased transmission of *C. pecorum* which has also been observed in fragmented habitat with high koala densities in Qld (McAlpine *et al.*, 2017). Future studies should investigate the possible role of stress in the development of chlamydial disease in overabundant koala populations.

6.2.3. KoRV infection differs between southern and northern Australian populations

6.2.3.1. Southern koalas have lower KoRV proviral loads than northern koalas

The prevalence of KoRV was found to be higher than expected in both the KI and MLR populations (Chapter 3). In the KI population, the prevalence of KoRV has increased to 42.4% from the prevalence of 14.8% (24/162) reported in 2012 (Simmons *et al.*, 2012) and 30-35% reported in 2013 (Denner and Young, 2013). In the MLR population, the prevalence of KoRV had not previously been investigated, and was shown to be 65.3%. The median proviral load of both populations was low; 113 (range: 2- 12,641) copies/10^3 *ϐ*-actin copies in the KI population and 35 (range: 1- 574) copies/10^3 *ϐ*-actin copies in the MLR population (Chapter 3). The proviral loads of wild MLR koalas was similar to that of wild Victorian koalas, where the median proviral load of Victorian koalas was 10 (range: 0.1-398) copies/10^3 *ϐ*-actin copies (Legione *et al.*, 2017). This was to be expected as Victorian and MLR koalas are genetically related (Kjeldsen *et al.*, 2016) as the MLR were populated with koalas that originated from Victoria (Robinson, 1978). In necropsied MLR koalas from the current study, the

median proviral load of disease-free koalas (euthanased due to trauma or musculoskeletal disorders but otherwise healthy) was 2,510 (range: 25-50,500) copies/10^3 *β*-actin copies, which was slightly higher than the wild MLR koalas, but the PCR method differed between the studies (Fabijan *et al.*, 2019; Sarker *et al.*, 2020). In contrast the median proviral load of disease-free Qld koalas in the current study was considerably higher, at 44,600 (range: 10,400-79,200) copies/10^3 *β*-actin copies (Sarker *et al.*, 2020). These differences may be due to differences in KoRV transmission, where Qld koalas have an active endogenous infection (Hobbs *et al.*, 2017) compared to MLR koalas which are presumed to have an exogenous infection (Simmons *et al.*, 2012), further discussed in section 6.2.3.5.

6.2.3.2. Many KoRV provirus-positive South Australian koalas had inactive infections

All Qld koalas have been shown to have highly active KoRV infections with high circulating viral loads indicative of ongoing replication (Sarker *et al.*, 2020; Tarlinton *et al.*, 2005). In contrast, the current collaborative study found that not all KoRV provirus-positive MLR koalas had active KoRV infections. At the time of sampling, only 51.2% (21/41) of necropsied MLR koalas had KoRV viraemia detected by RT-qPCR, and the rest were recorded with a viral load of zero, indicating an inactive infection (Sarker *et al.*, 2020). In necropsied MLR koalas, three distinct groupings were observed based on KoRV viral load; koalas with no KoRV activity (provirus positive and virus negative), koalas with low KoRV activity (<10^6 copies/ml plasma) and those with highly active KoRV infections (>10^6 copies/ml plasma) (Chapter 5.2). To further investigate KoRV viral activity, transcriptomic analysis of submandibular lymph nodes from KoRV positive koalas was undertaken and showed a unique viral expression pattern in some MLR koalas (Tarlinton *et al.* 2017). Transcripts of the complete KoRV genome were detected for all Qld koalas (n=10) but only for 73.7% (14/19) of MLR koalas. For the remaining MLR koalas (n=5), only the LTR and partial *gag* and *env*

genes were detected and the *pol* and remaining *env* genes were not expressed (Tarlinton *et al.* 2017). The difference between no/low KoRV activity and high KoRV activity in MLR koalas may be due to the immune response to exogenous infection, or as a result of an endogenous, truncated KoRV, which is further discussed in section 6.2.3.5.

6.2.3.3. The proportion of KoRV variants differs between southern and northern populations

In South Australian koalas, while KoRV-A was shown to be the dominant variant, not all koalas were KoRV-A positive and other variants were detected at low levels. Of the wild-caught South Australian koalas in this study, only KoRV-A and not KoRV-B was detected in both populations by PCR, and for 4.8% (3/62) of KI and 20.5% (8/39) of MLR koalas, the KoRV-A and KoRV-B proviral *env* gene were not detected (Chapter 3). This was consistent with a Victorian study which also only detected KoRV-A and not KoRV-B in 88% (141/160) of KoRV provirus-positive koalas, and for the remaining 19 koalas neither gene was detected (Legione *et al.*, 2017). However, in a subset of the necropsied MLR koalas in the collaborative study, KoRV-B and other KoRV variants were detected. Illumina sequencing performed on DNA showed KoRV-A to be the most dominant variant in MLR koalas (n=28) and accounted for more than 90% of all KoRV proviruses, while KoRV-B, KoRV-D (including multiple subtypes) and KoRV-I were detected at low levels (Sarker *et al.*, 2019). Transcriptomic investigation of koala submandibular lymph node tissue identified two MLR koalas to be KoRV-A negative and KoRV-E positive, where these are the only two koalas reported with this infection pattern in the literature (Tarlinton *et al.*, 2017). Another previous study also detected a sequence similar to KoRV-C in a koala from KI (Young, 2014). These findings suggest that the number of KoRV-B proviral inserts in South Australia koalas is low and likely falls below the detection limits for conventional PCR, resulting in the lack of KoRV-B detection in the wild-caught koalas. The koalas that were negative for

KoRV-A and KoRV-B may have been infected with a low level of KoRV-B, KoRV-E, KoRV-C or another variant but these proviral loads would also likely be low in southern koalas and fall below the detection limits of PCR. Future studies to investigate KoRV variants in southern koalas should therefore utilise more advanced techniques for variant detection, such as Illumina sequencing utilised by Sarker *et al.* (2019).

Studies to utilise advanced techniques for KoRV variant detection in Qld koalas have shown all koalas to be concurrently infected with KoRV-A and multiple other variants. In the collaborative study, Illumina sequencing of Qld koala DNA (n=33) detected KoRV-A in all koalas, where the proportion of KoRV-A was variable, accounting for 30-90% of KoRV sequences, and the proportion of KoRV-D (all subtypes), KoRV-B and KoRV-I concurrent infections were at higher levels than in MLR koalas (Sarker *et al.*, 2019). This finding has also been reported by similar studies of Qld koalas, that showed all koalas to be infected with KoRV-A and multiple other variants, including KoRV-B to KoRV-J (Chappell *et al.*, 2017; Hobbs *et al.*, 2017; Quigley *et al.*, 2019; Waugh *et al.*, 2017; Xu *et al.*, 2015). The number of proviral inserts of these other variants is also higher, and consequently KoRV-B is detectable by PCR in Qld koalas (Quigley *et al.*, 2018b; Waugh *et al.*, 2017).

6.2.3.4. Koala lymphocytes and lymphoid tissues may be key sites of KoRV replications

Routine haematology and splenic lymphoid area analysis of necropsied MLR and Qld koalas showed that lymphocytes and lymphoid tissues, such as bone marrow and spleen, may be key sites of KoRV replication (Chapter 5.2). In necropsied MLR koalas, KoRV proviral load and viral load were significantly, positively correlated with lymphocyte counts which suggests that KoRV may increase lymphocyte proliferation. Cattle infected with bovine leukaemia virus (BLV) infection also develop lymphocytosis due to retroviral infection (Aida *et al.*, 2013). KoRV proviral and viral loads were also positively correlated with metarubricyte count and negatively correlated with

erythrocyte and neutrophil counts. This may indicate that KoRV can infect haematopoietic stem cells in bone marrow and may disrupt myeloid progenitor cellular differentiation, resulting in low numbers of neutrophils and erythrocytes, and a higher frequency of metarubricytes (inappropriate metarubricytosis) within the blood. MLR koalas were shown to have a normocytic normochromic anaemia which is commonly observed in cats with feline leukaemia virus (FeLV) infection (Stockham and Scott, 2008). FeLV has been shown to infect the bone marrow of cats (Hartmann, 2012) and feline immunodeficiency virus (FIV) also replicates within other lymphoid tissues such as bone marrow, lymph nodes and spleen (Eckstrand *et al.*, 2017). In MLR koalas, splenic lymphoid area was significantly, positively correlated with KoRV viral load, which suggests that KoRV may be replicating in lymphocytes within the spleen. These haematology and splenic changes may be more likely to be observed in necropsied koalas with increased KoRV proviral load as no changes were observed in haematological values due to KoRV proviral infection in the clinically healthy wild-caught South Australian koalas (Chapter 4).

6.2.3.5. In southern koalas KoRV may be an infectious exogenous virus or a defective endogenous virus preventing superinfection

The differences in KoRV proviral load, viral load and variants between MLR and Qld koalas may reflect differences in KoRV pathogenesis between the populations. In Qld koalas, KoRV-A has been shown to be an active endogenous infection (Greenwood *et al.*, 2017; Hobbs *et al.*, 2017) while in southern koalas, KoRV is presumed to be an exogenous infection, based on koalas free from infection (Tarlinton *et al.*, 2006) and low KoRV proviral loads (Simmons *et al.*, 2012). However, the findings in the present studies of southern koalas (Chapters 3 and 5.2) and the collaborative project (Sarker *et al.*, 2020; Sarker *et al.*, 2019; Tarlinton *et al.*, 2017) reflect both exogenous and endogenous retroviral infections in other species.

Southern koalas may be infected exogenously with KoRV, resulting in low proviral loads, inactive infections (no KoRV viraemia) and less KoRV variant diversity. In contrast, the high proviral loads in northern koalas may be due to the active endogenous infections, where nearly half of the proviral inserts (58/133) in a Qld koala were shown to be endogenous KoRV elements and the remaining provirus inserts were exogenously acquired (Hobbs *et al.*, 2017). Further evidence for an exogenous infection comes from the three KoRV viral activity groups observed in necropsied MLR koalas (Chapter 5.2). This pattern in MLR koalas is reflective of FeLV infection in cats, which is an exogenous gammaretroviral infection. The early immune response of cats to FeLV dictates FeLV viraemia; some cats can clear FeLV infection and are never viraemic, some cats develop a regressive/latent infection that reactivates after a length of time and they become viraemic, and other cats fail to mount an immune response and become persistently infected with highly active FeLV infections; cats in the latter group develop lymphoid neoplasia (Hartmann, 2012). The median proviral load of cats with regressive infection was $4x10^3$ copies/1000 cells and the load in cats with progressive infection was $5x10^6$ copies/1000 cells (Powers *et al.*, 2018). KoRV activity may be similar to this whereby koalas with no and low KoRV activity may have a regressive infection, as the median proviral load of koalas with no and low KoRV activity was $2.1x10^3$ copies/10^3 *β*-actin copies, and koalas with highly active KoRV infections (median proviral load, $2.1x10^5$ copies/10^3 *β*-actin copies) (Chapter 5.2) may have failed to mount an immune response, were persistently infected with KoRV and developed lymphosarcoma, similar to cats persistently infected with FeLV (Hartmann, 2012).

If koalas are able to mount an effective immune response to exogenous KoRV infection, this may explain the low or no expression of KoRV in koala submandibular lymph nodes (Tarlinton *et al.*, 2017). Other species have been shown to have latent

retrovirus infections, where the animals are provirus positive and viraemia negative, such as cats with FIV (Eckstrand *et al.*, 2016), cattle with BLV infection (Lagarias and Radke, 1989) and humans with HIV infection (Mellors *et al.*, 1997). Additionally, lymph nodes have been shown to be a site for FIV replication in cats during the latent phase of infection (Eckstrand *et al.*, 2017), and if lymph nodes are also a site for KoRV replication during a regressive/latent infection, this may account for the low level of expression from submandibular lymph nodes where the immune system is suppressing viral replication. This theory of KoRV replication in lymphoid tissues is supported by the findings of altered haematology and splenic lymphoid area in necropsied MLR koalas (Chapter 5.2). The response of the koala immune system to KoRV infection has only recently been shown by the fact that both southern and northern koalas develop antibodies post-vaccination (Olagoke *et al.*, 2018; Olagoke *et al.*, 2019; Waugh *et al.*, 2016), and that cytokine expression is altered due to KoRV infection in New South Wales (Maher and Higgins, 2016) and Victorian koalas (Maher *et al.*, 2019). An immune response to KoRV could explain the low expression of the *pol* and *env* genes in the lymph nodes of some MLR koalas, however, in the same koalas there was high expression of the LTR and partial *gag* and *env* genes that was not expected and may not be explained by an immune response theory (Tarlinton *et al.*, 2017). The expression of the outer KoRV genes closely resembles and may be attributed to the recently described recombinant KoRV (recKoRV) (Lober *et al.*, 2018). RecKoRV was identified in KoRV positive koalas across Australia, including the KI and MLR populations, and has arisen by recombination of KoRV with an endogenous virus, *Phascolarctos* retrovirus (PhRV) which has replaced the *pol* and *env* genes from KoRV with PhRV genes. It is unknown if recKoRV is actively transcribed by the koala but this may explain the high level of expression of the outer KoRV genes.

The differences in KoRV variants between MLR and Qld koalas may be due to exogenous KoRV-A having a high transmission rate. Whilst all Qld koalas have endogenous KoRV-A, they also have exogenous KoRV-A showing that this virus is still active within the population (Hobbs *et al.*, 2017), but due to the ubiquitous presence of endogenous KoRV-A, it is difficult to determine the transmission rate of exogenous KoRV-A. A recent study in Qld that monitored koalas longitudinally reported the transmission rate of KoRV-B to be 3% per year (Quigley *et al.*, 2018b). The finding of KoRV-E (Tarlinton *et al.*, 2017) and KoRV-C (Young, 2014) and not KoRV-A in a few South Australian koalas suggests that exogenous transmission of other variants also occurs. However, as KoRV-A was the most dominant variant type present in South Australian koalas (Fabijan *et al.*, 2019; Sarker *et al.*, 2019), this would suggest a high transmission rate.

Alternatively, the lack of KoRV activity in MLR koalas may be due to an endogenous, defective, truncated KoRV provirus that is blocking koala cells from superinfection, resulting in lower proviral loads, lower viral loads and less KoRV variant diversity. Expression of the *env* gene from defective endogenous viruses can cause a blockade effect, where these defective *env* proteins block the host cell surface receptors, preventing entry of exogenous retroviral infections. Examples of this are observed in chickens with avian leucosis virus (Hunt *et al.*, 2008) and sheep infected with jaagsiekte sheep retrovirus (Spencer *et al.*, 2003). This theory is supported by the expression of the outer regions of KoRV in MLR koalas in the transcriptomics study (Tarlinton *et al.*, 2017). This expression profile may be of a truncated KoRV provirus where inner KoRV genes were lost during proviral insertion into the cell. The partial *env* genes being expressed may transcribe defective proteins which block the koala cellular receptors used by other KoRV variants. This theory is supported by the high proportion of KoRV-A in MLR koalas, as KoRV-A utilises a sodium dependent

phosphate transporter (PiT1) (Oliveira *et al.*, 2006) to gain entry into koala cells compared to KoRV-B that utilises a thiamine transporter protein (Xu *et al.*, 2013). The defective proteins translated from a truncated KoRV provirus may block the thiamine transporter protein, or other receptors for other exogenous KoRV variants, preventing superinfection and contributing to the abundance of KoRV-A in these southern populations. This theory is further supported by the findings of the transcriptomics study, where no Qld koalas had high expression of the outer genes (Tarlinton *et al.*, 2017), which suggests that Qld koalas do not transcribe defunct *env* proteins and are vulnerable to KoRV superinfection. Evidence of KoRV superinfection in Qld koalas was shown by Sarker et al., (2020) where Qld koalas had highly active KoRV infections, with high proviral and viral loads observed. Additionally, if there is no blockage of host receptors, this would allow for the large diversity of KoRV variants observed in northern koalas (Chappell *et al.*, 2017; Sarker *et al.*, 2019).

Regardless of the mode of transmission, it is clear that diagnosing KoRV infection in southern koalas may not be as simple as positive or negative results based on detection of KoRV provirus, as has previously occurred. In positive koalas, detection of KoRV viral RNA, to reflect the viral activity in the koala, may be a more accurate prognostic indicator to highlight whether these koalas are infectious or at risk of developing disease. Sequencing of genomic DNA of individual koalas may further suggest that some koalas possess complete KoRV proviral genomes and that others possess a truncated, defective KoRV provirus. Investigation of KoRV viral RNA activity in these koalas may suggest whether these koalas have regressive/latent infections or whether KoRV is defunct and unlikely to be infectious. Further studies are required to understand KoRV infection in southern koalas, to determine if KoRV is exogenous or endogenous, and to determine whether koalas are able to mount an immune response to other KoRV variants.

6.2.4. High KoRV proviral and viral loads are strongly associated with the development of neoplasia in the koala

High KoRV proviral and viral loads were associated with neoplasia in MLR and Qld koalas. Koalas with lymphosarcoma from both the MLR and Qld populations showed identical morphological appearance of neoplastic B-cells and similar distribution of infiltration in lymph nodes and extranodal sites (Chapter 5). The KoRV proviral and viral loads in MLR koalas with lymphoid neoplasia were as high as in the Qld koalas with lymphoid neoplasia, and this group of koalas had higher loads than all other Qld and MLR koalas suggesting highly active KoRV infections. No association was found between KoRV variants and neoplasia (Sarker *et al.*, 2019), as was suggested by a previous study that found an association between KoRV-B and lymphosarcoma in captive northern koalas (Xu *et al.*, 2013), however the study by Sarker *et al.* (2019) was performed on low numbers of koalas from MLR and Qld. The high KoRV proviral and viral loads in the MLR and Qld koalas with lymphoid neoplasia suggests that the same basic oncogenic pathways occur in all KoRV infected koalas, and that high viral load may be more important than the KoRV variants present. The high activity of KoRV in these koalas suggests that KoRV may cause lymphoid neoplasia to develop through insertional mutagenesis, where a KoRV provirus has inserted near a koala cellular oncogene and caused unregulated proliferation of the infected host cell (Tarlinton *et al.*, 2005; Xu *et al.*, 2013).

Retroviruses are known to cause lymphoid neoplasia in their respective hosts, such as BLV in domestic cattle, human T-cell leukaemia virus (HTLV) in humans (Aida *et al.*, 2013; Gillet *et al.*, 2007), murine leukaemia viruses in wild and inbred laboratory mice (Kozak, 2015) and FeLV in domestic cats (Hartmann, 2012). BLV and HTLV are deltaretroviruses which possess a genomic pX region in the *env* gene that encodes an oncoprotein, Tax (Aida *et al.*, 2013). This protein has known oncogenic potential and

plays a role in immortalizing B-cells in cattle and T-cells in humans resulting in persistent lymphocytosis, but the infected cells do not become neoplastic without the aid of host factors (Aida *et al.*, 2013). In infected cattle, mutations in the tumour suppressor gene (p53), changes in amino acid sequence of the bovine leukocyte antigen and upregulation of the cytokine tumour necrosis factor alpha (TNFα) have been linked to the development of lymphoid neoplasia (Gillet *et al.*, 2007). FeLV causes lymphoid neoplasia to develop in domestic cats through insertional mutagenesis, usually within T-cells but also in B-cells, were FeLV provirus is usually inserted near a cellular oncogene, *myc*, that causes unregulated expression of this gene (Hartmann, 2012). FeLV proviral and viral loads are also strongly correlated with the development of lymphosarcoma (Hartmann, 2017), as is observed in koalas with KoRV infection. As FeLV and KoRV are gammaretroviruses, an oncogenic process similar to that caused by FeLV may also lead to the development of lymphosarcoma in the koala. While only B-cell lymphosarcomas were identified in this study, T-cell lymphosarcomas have been documented in the koala (Connolly *et al.*, 1998). There have been a few studies to investigate KoRV proviral insertion sites in koalas from Qld, New South Wales and Victoria, where few insertion sites were shared between koalas (Hobbs *et al.*, 2017; Ishida *et al.*, 2015; Johnson *et al.*, 2018; Tsangaras *et al.*, 2014). However, these koalas did not have lymphoid neoplasia, so future studies should investigate the KoRV proviral insertion sites in koalas with lymphoid neoplasia and may provide evidence for the mechanisms of oncogenesis in KoRV infected koalas.

6.2.5. No definitive association between *C. pecorum* disease and KoRV infection was identified

No strong association was identified between *C. pecorum* infection or disease and KoRV in wild-caught MLR koalas, however koalas with concurrent *C. pecorum* and KoRV infections were over 3 times more likely to develop chlamydial disease. In the

necropsied MLR koalas there was no association found between chlamydial disease, chlamydial disease severity or chlamydial load and KoRV proviral load, however there was a strong, positive association between chlamydial disease severity and KoRV viral load. While KoRV-B has been associated with the development of chlamydial disease in Qld koalas (Quigley *et al.*, 2018a; Waugh *et al.*, 2017) there was no association between KoRV proviral variants and chlamydial disease in the subset of necropsied MLR koalas analysed, however viral variants were not compared to disease as there were too few koalas analysed (Sarker *et al.*, 2019). The ubiquitous nature of KoRV in the necropsied MLR koalas makes drawing any definitive conclusions difficult. Additionally, as both the wild and necropsied MLR koala studies were based on low numbers of koalas with chlamydial disease, larger studies may identify associations between *C. pecorum* disease severity and KoRV infection. If a relationship between *C. pecorum* and KoRV infections exists, it is likely to be highly complex and will involve different koala, *C. pecorum* and KoRV factors.

6.3. Implications of findings and future work

C. pecorum and KoRV infections cause significant mortality in northern Australian koala populations through the development of chlamydial disease and lymphoid neoplasia, which are contributing to declining population numbers (Gonzalez-Astudillo *et al.*, 2017; Rhodes *et al.*, 2011). While the situation for northern koalas is growing increasingly dire, this study has shown South Australian koala populations to be less affected by *C. pecorum* and KoRV infections. The KI population was shown to be *C. pecorum*-free and this population may be the last large, isolated *C. pecorum*-free population in Australia. While the prevalence of *C. pecorum* infection is high in the MLR population, the prevalence of disease was low, and the vast majority of the population were clinically healthy. Koalas from South Australia could therefore act as insurance populations to ensure the longevity of the species.

KoRV infections in South Australian koala populations have been shown to be extraordinarily complex, with a higher prevalence than expected but vast differences between individual koalas in their proviral load, viral load and KoRV variant infections. Necropsied MLR koalas with the highest KoRV proviral and viral loads developed lymphosarcoma while some KoRV provirus positive koalas were not viraemic and were clinically healthy. If KoRV is exogenously transmitted in South Australia, koalas may overcome exogenous infections immunologically, suppress infections into a latent phase or if unable to do so may be at risk of developing lymphosarcoma. Some koalas may inherit endogenous, defective KoRV proviral segments that may protect from superinfection. Once there is a deeper understanding of KoRV transmission, we may be able to determine the role of KoRV in disease development and to further investigate if an association between *C. pecorum* disease and KoRV exists. Furthermore, investigating the immune response of southern koalas that are not viraemic may shed light on the koala's immune system and may have implications for KoRV vaccine development (Olagoke *et al.*, 2018; Olagoke *et al.*, 2019; Waugh *et al.*, 2016).

KoRV-free South Australian koalas may be key for koala conservation through the establishment of disease-free breeding colonies to eliminate the negative effects of both KoRV and *C. pecorum* infections. Although South Australian koalas may ensure the survival of the species, there are a number of considerations which need to be addressed prior to the translocation of southern koalas to northern Australia, which include reducing the risk of disease exposure, adapting to dietary changes, coping with climate differences and improving genetic diversity. Southern koalas which have had no prior exposure to *C. pecorum* infection may be highly susceptible to developing *C. pecorum* disease. To prevent this, these koalas would be key candidates for the *C. pecorum* vaccine currently being developed (Carey *et al.*, 2010; Nyari *et al.*,

2019). There is considerable diversity in eucalypt species across Australia and southern koalas may not have encountered species in northern Australia (Phillips, 1990). Koalas would have to be gradually introduced to new eucalypt species in their diet to avoid stress and malnutrition prior to translocation into northern Australia. They would also need to be acclimatised to their new location as there are also considerable phenotypic differences between southern and northern koalas (Phillips, 1990), and southern koalas would likely struggle to cope with the climate in northern Australia. Southern koalas, which reside in a temperate climate, are considerably larger (up to 13 kg), and have thicker and darker fur than their northern counterparts, which reside in a subtropical climate, are smaller (up to 9 kg) and have light grey fur (Phillips, 1990). To overcome these issues and to introduce southern koalas into northern Australia, an extensive breeding program would be required, to acclimatise southern koalas to northern Australian food species and climate and to increase their genetic diversity, which is currently restricted, to ensure the survival of koalas into the future.

6.4. Conclusions

This study has provided crucial information for our understanding of *C. pecorum* and KoRV infections in wild South Australian koala populations and how they differ from those in northern Australia. In the KI koala population, this study has shown that despite the prevalence of KoRV increasing, that this is the largest isolated healthy *Chlamydia*-free koala population. In the MLR population, the majority of *C. pecorum* and KoRV infected koalas were clinically healthy, and the prevalence of chlamydial disease and lymphosarcoma was low. These populations, particularly on KI, may be key for the conservation of the species and therefore warrant continued surveillance of *C. pecorum* and KoRV.

This study has also contributed further to our understanding of KoRV infection. There are key differences, but also some similarities, in KoRV infection between

southern and northern Australian koalas. Lymphosarcoma developed in necropsied MLR and Qld koalas with the highest KoRV proviral and viral loads. Some KoRV provirus positive MLR koalas do not have active viral infections, which may reflect an immunologically controlled exogenous KoRV infection, or is the result of a truncated, defective endogenous provirus protecting southern koalas from KoRV superinfection. Therefore, the simple diagnostic tests for KoRV proviral DNA currently in use may not provide a complete picture of KoRV infectivity in positive koalas, and further testing for KoRV viraemia may be more beneficial to determine if a koala has a non-active KoRV infection or whether the koala is persistently infected and at risk of developing lymphosarcoma.

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