DISEASE IN WILDLIFE OR EXOTIC SPECIES

Short Title: Pathology in Retrovirus-positive Koalas

Pathological Findings in 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 aluminium-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 5,000 rpm 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 (μm^2) using LabSens^{TM} software ( ). 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 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 ( ) 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 GLM 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 (± SD 1.60×10^7) μm^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 ($\rho = 0.745$; $P = 0.031$). 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 ($\rho = -0.297$; $P = 0.003$), PCV ($\rho = -0.257$; $P = 0.012$) and neutrophil counts ($\rho = -0.331$; $P = 0.001$), and positive correlations were observed with absolute nRBCs ($\rho = 0.264$; $P = 0.014$) and lymphocyte counts ($\rho = 0.239$; $P = 0.018$). Significant, negative correlations were also observed between viral load and RBC ($\rho = -0.279$; $P = 0.018$) and neutrophil counts ($\rho = -0.280$; $P = 0.018$), and positive correlations were observed with absolute nRBC ($\rho = 0.318$; $P = 0.012$) and lymphocyte counts ($\rho = 0.301$; $P = 0.011$). 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 ($\rho = 0.438$; $P = 0.025$). 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

Figure Legends

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).