Koala retrovirus genotyping analyses reveal a low prevalence of KoRV-A in Victorian koalas and an association with clinical disease

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Abstract

Purpose. Koala retrovirus (KoRV) is undergoing endogenization into the genome of koalas in Australia, providing an opportunity to assess the effect of retrovirus infection on the health of a population. The prevalence of KoRV in north-eastern Australia (Queensland and New South Wales) is 100%, whereas previous preliminary investigations in south-eastern Australia (Victoria) suggested KoRV is present at a lower prevalence, although the values have varied widely. Here, we describe a large study of free-ranging koalas in Victoria to estimate the prevalence of KoRV and assess the clinical significance of KoRV infection in wild koalas.

Methodology. Blood or spleen samples from 648 koalas were tested for KoRV provirus, and subsequently genotyped, using PCRs to detect the pol and env genes respectively. Clinical data was also recorded where possible and analysed in comparison to infection status.

Results. The prevalence of KoRV was 24.7% (160/648). KoRV-A was detected in 141/160 cases, but KoRV-B, a genotype associated with neoplasia in captive koalas, was not detected. The genotype in 19 cases could not be determined. Genomic differences between KoRV in Victoria and type strains may have impacted genotyping. Factors associated with KoRV infection, based on multivariable analysis, were low body condition score, region sampled, and ‘wet bottom’ (a staining of the fur around the rump associated with chronic urinary incontinence). Koalas with wet bottom were nearly twice as likely to have KoRV provirus detected than those without wet bottom (odds ratio=1.90, 95% confidence interval 1.21, 2.98).

Conclusion. Our findings have important implications for the conservation of this iconic species, particularly regarding translocation potential of Victorian koalas.

INTRODUCTION

The current process of koala retrovirus (KoRV) endogenization into the koala genome makes KoRV a gammaretrovirus of significant general interest [1]. While the virus was first observed using electron microscopy over 20 years ago [2] and the first full genome published just over a decade later [3], the impact of KoRV on koala health, and the role of viral genotypes in affecting immune function or causing disease, is still limited. Although early studies suggested that leukaemia in koalas may be linked to KoRV [4], this was based on the observation that other gammaretroviruses (gibbon ape leukaemia virus, murine leukaemia virus, feline leukaemia virus) are associated with leukaemia, but no causal relationship between KoRV and leukaemia was established at the time. Later studies linked increased levels of circulating virus in plasma, but not provirus in white blood cells, with development of neoplasia [5]. The detection of a neoplasia-associated genotype (KoRV-B) in captive koalas [6] allowed for the absence of clinical signs in some koalas infected with KoRV to be better understood. KoRV-B differs from KoRV-A (the original virus sequenced) in the envelope
protein-encoding env gene. This change in the envelope protein alters the host receptor used for virus attachment. Differences also exist in the long terminal repeats, with KoRV-B containing four 18-nucleotide long tandem repeats and KoRV-A containing only a single copy. More recently, a number of other KoRV genotypes (based on the env nucleotide sequence) have been described, namely KoRV-C and -D [7] and KoRV-E and -F [8], as well as variants within the genotypes [9, 10], but the clinical significance of these remains unclear.

In Australian koala populations, it appears that KoRV is undergoing endogenization in a southward direction [1]. This hypothesis is based on investigations of KoRV prevalence variance in wild populations, which ranges from 100% in northern populations (Queensland and New South Wales (NSW)) to, on average, 15 and 51% in southern states (South Australia and Victoria, respectively) [11]. Investigations utilizing museum specimens detected KoRV infection in Queensland koalas as early as the late 1800s [9]. However, only two specimens from NSW and none from Victoria or South Australia were used in that investigation, making it difficult to draw conclusions about the presence of the infection across the rest of the country throughout history. It has been shown that in Victorian koalas, the number of proviral copies per genomic unit is significantly less than that seen in northern populations, suggesting an exogenous infection [11, 12]. Information on KoRV genotypes present in modern wild populations is limited. Five unique museum specimens (of wild origin) have been used to investigate genomic variation and no historical evidence of KoRV-B and a low number of single nucleotide polymorphisms across the entire genome were found [9, 13]. KoRV-B has been detected in the transcriptome of a wild koala from Queensland [14] and KoRV-C-like virus detected in a South Australian koala [15], but there are no published large-scale studies of the prevalence of different genotypes. Here, we focus on Victorian populations, of particular interest due to the variation in KoRV infection levels in previous studies [1, 11, 12]. To better estimate the prevalence of KoRV in Victorian koalas, we undertook a rigorous investigation of the KoRV prevalence in a large number of individual koalas in multiple populations. We also aimed to estimate the prevalence of both KoRV-A and KoRV-B in Victorian koalas, and combine this with clinical data collected from free-ranging koalas to assess the clinical significance of KoRV infection. In the absence of a laboratory animal model to study KoRV infection, and with experimental studies in the natural host impracticable, these field-based studies represent the best approach to assessing the clinical significance of KoRV infection.

METHODS

Sample collection

Sample collection was approved by The University of Melbourne Animal Ethics Committee (approval numbers 1011687.1 and 1312813.2) and Parks Victoria (Research Permits 10004605, 10006948 and 10005388). Blood samples were collected from 530 live koalas and spleen samples were collected from 135 necropsied koalas, with a total of 648 koalas sampled. A small number of live koalas were later euthanized and necropsied (n=17), thus providing both blood and spleen samples. All of the necropsied animals were euthanized for health or welfare reasons unrelated to this study. Koala samples used in this study were collected between 2010 and 2016. Live koalas were sampled either as part of previous research efforts focusing on three regions in Victoria (n=271) [16] or as part of management operations (n=259). Blood samples were collected from anaesthetized koalas and separated (where possible) intouffy coat and plasma fractions. Both fractions were added to RNAlater stabilization solution (ThermoFisher Scientific). Spleen tissue was stored in 1.5 ml tubes at −80°C until processing for extraction. Clinical examinations of live captured animals were performed by veterinarians, including assessment of body condition score (BCS) (1 to 5) [16], wet bottom presence/absence [17] and, for a subsample of koalas, the presence/absence of urogenital tract disease by ultrasound [16, 18]. Wet bottom is a clinical sign of disease characterized by a soiling of fur, or scalding, around the rump, as a result of urinary incontinence [19]. In contrast, urogenital tract disease represents gross changes to the urogenital tract, such as reproductive cysts. Other parameters that were also recorded included tooth wear class, as a measure of age [20], and the presence or absence of young, as a measure of fecundity. Lymph node enlargement was recorded as a proxy for potential lymphoid leukaemia. Previous data on detecting Chlamydia pecorum prevalence were utilized here [16, 21].

DNA extraction

DNA was extracted from either whole blood or separateduffy coat fractions. Spleen tissue was first vigorously homogenized using a plastic shafted rayon swab (Cohan Italia), which was then added to 800 μl of PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.4) and vortexed briefly. DNA was extracted from 200 μl of the blood or spleen samples using an X-tractor robot (Qiagen), utilizing the Qiaxtractor VX extraction kit as per manufacturer’s instructions. Each bulk extraction contained both positive and negative extraction controls. The positive control was a diluted liquid culture of E. coli containing a portion of pol inserted into pGEM-T (Promega) plasmid, and the negative control was sterile PBS.

Detection of KoRV provirus and env genotypes

Extracted DNA was tested for KoRV provirus using the KoRV pol qPCR primers described previously [5], but replacing the probe with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). The housekeeping gene β-actin was utilized to standardize the genome copy numbers detected in each sample, as described previously [22]. Results were reported as the number of KoRV pol copies per β-actin copies in the extracted liquid sample.
Differentiation of KoRV-A and KoRV-B was conducted using genotype-specific primers as described previously [6], but replacing the genotype-specific probes with Platinum SYBR Green qPCR SuperMix-UDG. Our protocol also used ROX as a reference dye. Reactions were performed using an MX3000P real-time PCR machine (Stratagene). A melt curve with a 0.3°C resolution from 75 to 95°C was used to determine the amplification of the correct product, with comparison to positive controls.

A standard curve was employed for each qPCR consisting of 10-fold dilutions, in triplicate, of purified plasmid containing one of either KoRV pol, β-actin, KoRV-A env or KoRV-B env target genes from $10^0$ to $10^1$ copies per reaction. Copy numbers were calculated using a Qubit 3.0 fluorometer (Invitrogen) and dilutions prepared using a QIAgility robot (Qiagen).

**Statistical analyses**

Univariable and multivariable statistical analyses to estimate associations with KoRV detection were conducted using Stata 14.1 (StataCorp) software. Explanatory variables included sex, age (based on tooth wear), BCS (1 to 5), reproductive and urinary tract disease (presence/absence), fecundity in females (based on the presence/absence of back or pouch young), wet bottom presence/absence, lymph node enlargement, C. pecorum presence/absence and geographic region of sampled koalas. Not all parameters were available for all animals. Koalas were classified as young (tooth wear<class III), mature adult (tooth wear between classes III and V, inclusive) or old adult (tooth wear>class V) [16]. Specific location data was pooled into seven broader regional locations (Fig. 1). As C. pecorum genotypes in Victoria are largely homogeneous and have no known pathogenic variation [21], only the presence or absence of the organism was considered. Univariable logistic regression was performed to estimate the association of each explanatory variable with an outcome variable representing the presence or absence of KoRV. Multivariable logistic regression models were fitted to the data using a manual backwards stepwise approach [23]. Variables with a $P$ value $>0.25$ in univariable analysis were considered for the multivariable model as well as potential confounders. At each step, all eligible candidate variables were individually tested and removed from the model if they had a $P$ value $>0.10$ based on the likelihood ratio test. First-order biologically plausible interaction terms were added to the final main-effects model and tested for statistical

![Fig. 1. Prevalence of koala retrovirus (KoRV) in sampled Victorian koala populations. (A) Map of Australia highlighting the state of Victoria. (B) Regional prevalence of KoRV detected in Victorian koala blood and spleen samples. Regions: (a) Far Western Victoria, (b) South West Coast, (c) Mornington Peninsula, (d) French Island, (e) Greater Gippsland, (f) Raymond Island, (g) Far North Victoria. Map not to scale. Regions not recorded for 10 koalas.](https://www.microbiologyresearch.org/)

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significance at $P < 0.05$. Standard regression diagnostics were performed, including assessment of goodness-of-fit using the Hosmer–Lemeshow technique [23]. To assess the effects of clustering of observations from the same region, we refitted the final model using multilevel mixed-effects logistic regression, with a regional cluster-level random effects term.

Standardized KoRV copy numbers were logarithmically transformed but remained severely skewed, so comparison of KoRV provirus copies in each sample in relation to associated factors (sex, region, BCS, wet bottom presence/absence, urinary and reproductive tract disease presence/absence, lymph node enlargement and $C. pecorum$ detection) was undertaken using non-parametric (Kruskal–Wallis) statistical tests. Outliers (determined using Tukey’s outlier test) were removed from the dataset.

RESULTS

Prevalence of KoRV in Victorian koala populations

The prevalence of KoRV in each specific Victorian region ranged from 17 to 40 % of tested koalas, with an overall prevalence of KoRV in Victorian koalas of 24.7 % (95 % CI: 21.4, 28.2 %) (160/648) (Fig. 1). KoRV was significantly more likely to be detected in samples collected from koalas in Raymond Island, Far Western and Far Northern Victoria than in samples from koalas in the South West Coast region (Table 1). The South West Coast region was selected for use as the reference region as it had the largest number of samples available for comparison. The proportion of spleen samples from which KoRV was detected (36/135, 26.7 %) was comparable to the prevalence in blood samples (127/530, 24.0 %). KoRV-B was not detected in any of the 160 KoRV-positive koalas, whereas KoRV-A was detected in 141 koalas. In 19 pol-positive cases, env could not be amplified using either the KoRV-A or KoRV-B specific primers, and attempts to amplify the entire gene using previously published primers [24] were unsuccessful.

The prevalence of KoRV in koalas sampled from four regional populations could be compared across two time points, separated by either 2 years (South West Coast) or 3 years (French Island, Raymond Island and Far Western Victoria). In all four cases, there was no clear trend in the proportion of KoRV detections over time (Table 2).

Relationship between KoRV infection and clinical observations

Univariable analyses revealed that koalas with wet bottom had 2.20 (95 % CI: 1.48, 3.27) times the likelihood of being

<table>
<thead>
<tr>
<th>Variable</th>
<th>KoRV positive/n</th>
<th>Prevalence (%)</th>
<th>Odds ratio*</th>
<th>95 % CI</th>
<th>$P$ value†</th>
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<td>Sex</td>
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<tr>
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<td>1.00</td>
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<td>–</td>
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<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
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<td>Young</td>
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<td>26.1</td>
<td>1.14</td>
<td>0.71, 1.83</td>
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<td>Mature adult</td>
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<td>Old adult</td>
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<td>28.9</td>
<td>1.32</td>
<td>0.67, 2.60</td>
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</tr>
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<td>Back/pouch young or lactation (females only)</td>
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<td>1.00</td>
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<td></td>
</tr>
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<td>Region (west to east)</td>
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<td></td>
<td></td>
<td>0.070</td>
</tr>
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<td>Far Western Victoria</td>
<td>51/167</td>
<td>30.5</td>
<td>2.08</td>
<td>1.25, 3.47</td>
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<td>17.4</td>
<td>1.00</td>
<td></td>
<td></td>
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<td>Far North Victoria</td>
<td>6/15</td>
<td>40.0</td>
<td>3.16</td>
<td>1.05, 9.53</td>
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<td>Mornington Peninsula</td>
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<td>26.7</td>
<td>1.72</td>
<td>0.52, 5.77</td>
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<td>French Island</td>
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<td>1.54</td>
<td>0.84, 2.83</td>
<td></td>
</tr>
<tr>
<td>Greater Gippsland</td>
<td>6/33</td>
<td>18.2</td>
<td>1.05</td>
<td>0.40, 2.77</td>
<td></td>
</tr>
<tr>
<td>Raymond Island</td>
<td>38/136</td>
<td>27.9</td>
<td>1.84</td>
<td>1.07, 3.15</td>
<td></td>
</tr>
<tr>
<td>Other/not recorded</td>
<td>1/10</td>
<td>–</td>
<td>–</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Reference levels are indicated by odds ratios of 1.0. An interpretation of the odds ratio here would be that the odds of male koalas testing positive for KoRV DNA were 24 % less than those of female koalas. CI, confidence interval; –, not measured.

†$P$ values estimated using the likelihood ratio test statistic. Results highlighted in bold ($P \leq 0.25$) represent variables included in the initial multivariable model.
KoRV positive (Table 3). Koalas with the lowest BCS (a score of 1 out of 5) were 7.04 (95 % CI 1.98–25.00) times more likely to be KoRV positive than the reference population (a BCS of 3) (Table 3). We did not detect an association between KoRV positivity and the detection of C. pecorum (P=0.84). In the final multivariable logistic regression model (Table 4), the variables significantly associated with KoRV detection included the presence/absence of wet bottom, low BCS (<2) and region.

Repeating the multivariable analysis treating urogenital tract disease (commonly associated with C. pecorum) in female koalas as the outcome variable showed that KoRV was not statistically significantly associated with urogenital tract disease on its own (P=0.21). Although not statistically significant in this analysis, there was a clinically important increase in the odds of urogenital tract disease when C. pecorum and KoRV were both detected (odds ratio=20.5; 95 % CI: 0.97, 433.7) compared to the odds of urogenital tract disease when only C. pecorum was detected (odds ratio=5.71; 95 % CI: 1.04, 31.3); see Table S1 (available in the online Supplementary Material).

Kruskal–Wallis tests comparing log-transformed KoRV pol copies per β-actin copies found that median normalized proviral copy numbers were highly comparable in most categories (sex, region, BCS, wet bottom presence/absence, C. pecorum presence/absence, lymph node enlargement); however, a statistically significant increase in detected copy numbers was identified for female koalas with urogenital tract disease (Table 5). The median normalized KoRV proviral copy number of all samples was 10 KoRV copies per 1000 β-actin copies (range: 0.1 to 398 KoRV copies per 1000 β-actin copies).

**DISCUSSION**

The detection of KoRV in a relatively low proportion of Victorian koalas is of significance for the future conservation of the koala across Australia. Previous research suggested that the prevalence in Victorian populations such as in Gippsland and the Strzelecki Ranges was as high as 63 % (29/46), while an assortment of samples from mainland Victoria had a detection rate of 84 % (36/43) [11]. Our research, which incorporates a larger number of test samples than in previous studies, from multiple regions across the state, suggests that the overall prevalence (for our sampled regions) is much lower than previously suspected. The sampling methodology utilized may have played a role in our reduced prevalence finding, with random sampling of free-ranging individuals less likely to be biased towards diseased individuals. The median normalized KoRV provirus detected fell within the range seen in a previous study of Victorian koalas that compared tissue and faecal samples [12].

Despite the current hypothesis of a north-to-south transmission of infection [1], there is no strong evidence in regard to the history of the virus in Victorian populations aside from its presence on French Island, as also detected previously [11]. French Island is a closed population that was established around 1900 with very few individuals [25]. Based on detailed records, the island has not had new individuals added since this initial translocation [26]. Therefore, the presence of KoRV within the French Island koala population suggests the virus was present in the founding animals and yet it has not managed to reach a high prevalence in this small, closed population. Historical museum specimens have shown that KoRV could not be detected in an individual NSW koala from 1899 (a similar time point to when the French Island population was established) but was detected in an individual NSW koala from 1971 [9]. We have shown, on a much shorter time scale, that there is no significant change in the prevalence of the infection when populations were sampled up to 3 years apart. While establishing correct information from small sample sizes (in the historical cases) or narrow time lines (in our case) has limitations, it may inform future modelling of viral spread in southern populations. Longer time-course studies of select populations and assessment of historical samples from Victorian origin could help to establish a trend of KoRV prevalence over time, to determine whether the proportion of infected koalas is increasing, decreasing or static. Such studies could be aided by recent findings that KoRV can be detected in faecal samples [12], making it easier to

<table>
<thead>
<tr>
<th>Region</th>
<th>Year sampled</th>
<th>KoRV positive/n</th>
<th>Prevalence (%)</th>
<th>95 % CI</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Far Western Victoria</td>
<td>2010</td>
<td>39/120</td>
<td>32.5</td>
<td>24.2, 41.7</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>2013</td>
<td>11/44</td>
<td>25.0</td>
<td>13.2, 40.3</td>
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<tr>
<td>South West Coast</td>
<td>2013</td>
<td>11/56</td>
<td>19.6</td>
<td>10.2, 32.4</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>2015</td>
<td>15/99</td>
<td>15.2</td>
<td>8.7, 23.8</td>
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<tr>
<td>French Island</td>
<td>2010</td>
<td>15/63</td>
<td>23.8</td>
<td>14.0, 36.2</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>2013</td>
<td>8/30</td>
<td>26.7</td>
<td>12.3, 45.9</td>
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</tr>
<tr>
<td>Raymond Island</td>
<td>2010</td>
<td>25/88</td>
<td>28.4</td>
<td>19.3, 39.0</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>2013</td>
<td>11/36</td>
<td>30.6</td>
<td>16.3, 48.1</td>
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</tr>
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</table>

CI, confidence interval.

*P value estimated using Fisher’s exact method (significant where P<0.05).
accumulate the sample sizes required to assess a population. This finding would be particularly beneficial in regions of northeastern Victoria and southern NSW, where KoRV testing has been limited.

The absence of KoRV in a large proportion of koalas in Victoria also allows us to investigate links between clinical disease and KoRV presence, which is not possible in northern koalas where KoRV prevalence is 100%. Interestingly, we found that koalas with KoRV were almost twice as likely to be recorded as having wet bottom, which is commonly associated with *C. pecorum* infection. Recently, we showed that a large number of Victorian koalas exhibiting mild wet bottom signs were negative for *C. pecorum* [16], and our subsequent studies demonstrated that the association between wet bottom and *C. pecorum* detection was only significant in male koalas [21]. KoRV may be directly contributing to wet bottom through a yet unknown mechanism or indirectly by causing immunosuppression that allows opportunistic pathogens to cause urogenital infections.

Our results did not show a significant link between the presence of *C. pecorum* and KoRV in Victorian koalas, nor did the inclusion of KoRV improve a multivariable model assessing associations between urogenital tract disease, koala age, wet bottom and *C. pecorum* in female koalas. However, there was a near significant interaction between infection with both KoRV and *C. pecorum*, and the detection of urogenital tract disease. Our findings disagree with a hypothesis that immunosuppression due to KoRV infection would increase the likelihood of subsequent infection with *C. pecorum* [27]. However, collection of more data could more accurately determine whether there is an increase in the development of the clinical signs associated with *C. pecorum* as a result of concurrent KoRV infection [28].

### Table 3. Outputs of univariable logistic regression assessing clinical variables for associations with the presence of KoRV DNA in 648 koalas from Victoria, Australia, sampled between 2010 and 2016

<table>
<thead>
<tr>
<th>Variable</th>
<th>KoRV positive/n</th>
<th>Prevalence (%)</th>
<th>Odds ratio*</th>
<th>95% CI</th>
<th>P value†</th>
</tr>
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<tbody>
<tr>
<td>Body condition score (BCS)</td>
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<td></td>
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</tr>
<tr>
<td>BCS 1 – Emaciated</td>
<td>7/11</td>
<td>63.6</td>
<td>7.04</td>
<td>1.98, 25.0</td>
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<tr>
<td>BCS 2</td>
<td>16/75</td>
<td>21.3</td>
<td>1.09</td>
<td>0.58, 2.06</td>
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<tr>
<td>BCS 3 – Healthy</td>
<td>49/246</td>
<td>19.9</td>
<td>1.00</td>
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<td>BCS 4</td>
<td>52/186</td>
<td>28.0</td>
<td>1.56</td>
<td>1.00, 2.44</td>
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<tr>
<td>BCS 5 – Obese</td>
<td>5/12</td>
<td>41.7</td>
<td>2.87</td>
<td>0.87, 9.44</td>
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<tr>
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<td>31/118</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
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<tr>
<td>Wet bottom</td>
<td></td>
<td></td>
<td></td>
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<td>&lt;0.001</td>
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<tr>
<td>Present</td>
<td>56/153</td>
<td>36.6</td>
<td>2.20</td>
<td>1.48, 3.27</td>
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<tr>
<td>Absent</td>
<td>97/466</td>
<td>20.8</td>
<td>1.00</td>
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<tr>
<td>Not recorded</td>
<td>7/29</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>Urinary tract disease (females only)</td>
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<td>Present</td>
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<td>1.06</td>
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<tr>
<td><em>Chlamydia pecorum</em></td>
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<td>0.25‡</td>
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CI, confidence interval; –, not measured.

*Reference levels are indicated by odds ratios of 1.0.

†P values estimated using the likelihood ratio test statistic. Results highlighted in bold (P<0.25) represent variables included in the initial multivariable model.

‡Excluded from multivariable model due to >20% missing data.
The absence of KoRV-B in our samples is significant given recent suggestions that KoRV-B may be associated with the development of neoplasia. With a sample size of 160 KoRV pol koalas, our study would have been expected, with 95% confidence, to have detected KoRV-B if it was present at a rate of 2% or higher [29]. However, the methodology used to detect KoRV-B in this project may have not detected variants within this genotype should the variants contain nucleotide changes in the primer binding region, nor can it determine within this genotype should the variants contain nucleotide changes in regions of the KoRV env gene outside the qPCR target, as has previously been demonstrated [10]. A recent publication used deep sequencing on 18 individual koalas from South East Queensland and noted a large number of variants, which were grouped into nine different genotypes of KoRV (A to I) [30]. Further analysis utilizing similar deep-sequencing approaches should be considered in future studies of Victorian koalas. This would allow the detection of all genotypes that may be present (and variants within those genotypes) both in the individuals where KoRV-A was detected as well as in the cases from which pol was amplified successfully but env was not.

While the potentially clinically important KoRV-B has now been detected in wild northern koalas [30], a prevalence survey has not been undertaken. In the initial findings in Californian zoos, KoRV-B was detected in 46% (6/13) of captive koalas at Los Angeles Zoo, all of which were koalas originating from northern Australia [6]. In that study, the authors determined that KoRV-B was transmitted from dam to offspring. It is possible that the high percentage of koalas found to have KoRV-B in this population was influenced by captive breeding, resulting in amplification of a relatively rare genotype. This may also explain the high rates of neoplasia seen in captive koalas in zoos in northern Australia, with 60 to 80% of deaths in captivity attributed to leukaemia and lymphoma [3, 5], while these diseases were detected in only 2% of free-ranging koalas admitted to one Queensland animal hospital [28]. There was no association in our study between lymph node enlargement, a potential sign of lymphoid leukaemia [28], and the detection of KoRV provirus. Other pathogens may also be influencing the prevalence of neoplasia detected in some koala populations. For example, gammaherpesviruses, two of which have been detected in koalas [31, 32], can cause lymphoma in other species including mice [33] and humans [34]. In Victoria, herpesviruses were detected in 33/99 koalas sampled in one study, and a significant association was found between koalas with herpesvirus and those detected with C. pecorum [35]. A broader epidemiological assessment of herpesvirus in koalas Australia-wide would be worthwhile.

The presence of KoRV in only a quarter of koalas tested, and the apparent absence of the neoplasia-associated KoRV-B genotype in sampled Victorian koalas (based on methodology used), is of importance to koala conservation efforts. Despite lower genetic diversity in Victorian koalas compared to northern koalas [36, 37] and current research suggesting that the genetic diversity of koalas across Australia is impacted by their historical geographic barriers, koalas still belong to a single evolutionary significant unit [38]. It, therefore, follows that individuals could be translocated between populations without mixing of subspecies. A lower risk of the presence of KoRV and Chlamydia infection [21] may make Victorian koalas good candidates for future translocation programmes to establish or re-establish populations in northern regions of Australia, particularly in

### Table 4. Outputs of multivariable logistic regression assessing clinical and demographic variables for associations with the presence of KoRV DNA in 521 koalas from Victoria, Australia, sampled between 2010 and 2016

<table>
<thead>
<tr>
<th>Variable</th>
<th>OR*</th>
<th>SE (OR)</th>
<th>95% CI</th>
<th>P value†</th>
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<tr>
<td>Present</td>
<td>1.90</td>
<td>0.44</td>
<td>1.21, 2.98</td>
<td>0.005</td>
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<td>Absent</td>
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<tr>
<td>Body condition score (BCS)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCS 1 – Emaciated</td>
<td>8.06</td>
<td>7.02</td>
<td>1.46, 44.4</td>
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<tr>
<td>BCS 2</td>
<td>1.39</td>
<td>0.47</td>
<td>0.71, 2.70</td>
<td>0.34</td>
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<tr>
<td>BCS 3 – Healthy</td>
<td>1.00</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>BCS 4</td>
<td>1.20</td>
<td>0.31</td>
<td>0.72, 2.00</td>
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<tr>
<td>BCS 5 – Obese</td>
<td>2.51</td>
<td>1.57</td>
<td>0.73, 8.58</td>
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<tr>
<td>Region (west to east)</td>
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<td></td>
</tr>
<tr>
<td>Far Western Victoria</td>
<td>2.16</td>
<td>0.70</td>
<td>1.14, 4.09</td>
<td>0.018</td>
</tr>
<tr>
<td>South West Coast</td>
<td>1.00</td>
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<td>–</td>
<td>–</td>
</tr>
<tr>
<td>French Island</td>
<td>1.79</td>
<td>0.62</td>
<td>0.91, 3.52</td>
<td>0.090</td>
</tr>
<tr>
<td>Raymond Island</td>
<td>1.53</td>
<td>0.52</td>
<td>0.79, 2.98</td>
<td>0.21</td>
</tr>
<tr>
<td>Constant</td>
<td>0.14</td>
<td>0.62</td>
<td>0.08, 2.03</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

n=521; log likelihood=−274.91906; P<0.001; d.f.=9; Akaike information criterion=567.838; Hosmer–Lemeshow goodness-of-fit test (P=0.83).

*OR, odds ratio; SE, standard error; –, not measured. Reference levels are indicated by odds ratios of 1.0.

†P values estimated using the likelihood ratio test statistic. Results highlighted in bold (P<0.05).
NSW. Furthermore, it indicates they are a potentially more suitable population from which to derive animals for captive populations in Australia and internationally. A key barrier to this translocation of Victorian koalas is the current lack of accurate, rapid diagnostic tests for the detection of KoRV and *Chlamydia*, without which animals would be required to undergo lengthy holding periods before introduction into their new environment. Future research to develop such tests is warranted.

**Funding information**

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**Acknowledgements**

We gratefully acknowledge all of the assistance we have received with collection of samples and clinical information by staff and students of The University of Melbourne, Wildlife Health Surveillance Victoria, Zoos Victoria, Victorian Department of Environment, Land, Water and

![Table 5. Comparisons of median KoRV provirus copies per 1000 β-actin copies within variable groups for up to 146 koals](image_url)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Samples</th>
<th>Median</th>
<th>Interquartile range (Q1, Q3)</th>
<th>P value*</th>
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<td></td>
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<tr>
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<td>31</td>
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<td><strong>Region (west to east)</strong></td>
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<tr>
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<td>Greater Gippsland</td>
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*P values estimated using the Kruskal–Wallis test for the equality of medians. Significant differences (P<0.05) are in bold.
Planning, and Parks Victoria. We thank Damien Higgins, Iona Maher and Andrea Casteriano of the Koala Health Hub at The University of Sydney for assistance with the KoRV-env qPCRs. Alistair Legione is supported by an Australian Postgraduate Award.

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

References