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Identification of unusual *Chlamydia pecorum* genotypes in Victorian koalas (*Phascolarctos cinereus*) and clinical variables associated with infection

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Abstract

Chlamydia pecorum infection is a threat to the health of free-ranging koalas (*Phascolarctos cinereus*) in Australia. Utilising an extensive sample archive we determined the prevalence of *C. pecorum* in koalas within six regions of Victoria, Australia. The *ompA* genotypes of the detected *C. pecorum* were characterised to better understand the epidemiology of this pathogen in Victorian koalas. Despite many studies in northern Australia (i.e. Queensland and New South Wales), prior *Chlamydia* studies in Victorian koalas are limited. We detected *C. pecorum* in 125/820 (15%) urogenital swabs, but in only one ocular swab. Nucleotide sequencing of the molecular marker *C. pecorum ompA*, revealed that the majority (90/114) of *C. pecorum* samples typed were genotype B. This genotype has not been reported in northern koalas. In general, *Chlamydia* infection in Victorian koalas is associated with milder clinical signs compared to infection in koalas in northern populations. Although disease pathogenesis is likely to be multifactorial, the high prevalence of genotype B in Victoria may suggest it is less pathogenic. All but three koalas had *C. pecorum* genotypes unique to southern koala populations (i.e. Victoria and South Australia). These included a novel *C. pecorum ompA* genotype and two genotypes associated with livestock. Regression analysis determined that significant factors for the presence of *C. pecorum* infection were sex and geographic location. The presence of 'wet bottom' in males, and the presence of reproductive tract pathology in females, were significantly associated with *C. pecorum* infection suggesting variation in clinical disease manifestations between sexes.

Introduction

The koala (*Phascolarctos cinereus*) is an iconic arboreal marsupial, native to Australia. Koalas are considered a vulnerable species in parts of Australia due to rapid population decline. Whilst contraction of populations are primarily attributed to the impacts of

urbanisation (Lunney *et al.*, 2007; de Oliveira *et al.*, 2014), disease from pathogens such as *Chlamydia pecorum* may play an important role (Polkinghorne *et al.*, 2013).

C. pecorum, an intracellular bacteria of the *Chlamydiaceae* family, predominantly infects the urogenital tract of koalas (Brown *et al.*, 1984), although in northern populations (Queensland and New South Wales) it is sometimes associated with ocular pathology (Polkinghorne *et al.*, 2013). Urogenital infection with *C. pecorum* can be associated with 'wet bottom' or 'dirty tail', which refers to urine staining or scalding of the rump (Dickens, 1976), due to the presence of cystitis. In females, reproductive tract abnormalities such as paraovarian cysts are also associated with *C. pecorum* infection (Obendorf, 1981) and often cause irreversible infertility (McColl *et al.*, 1984). At a population level, such infertility can result in a rapid decline over a short time span, with introduction of *C. pecorum* in naïve koala populations reducing fecundity to zero in as little as 25 years (Martin & Handasyde, 1999).

Our previous study of clinical signs associated with *Chlamydia* infection in Victorian koalas suggested that disease is more mild than that seen in northern populations (Patterson *et al.*, 2015), although further studies of koala populations are needed to definitively confirm this. The reasons behind these apparently milder signs are unclear and may be due to less virulent *C. pecorum* and/or variation in the prevalence of different *Chlamydia* species, but could also be due to other factors such as lower rates of koala retrovirus in Victoria (Simmons *et al.*, 2012). Variation in host and environmental factors between southern and northern populations would also be expected to result in variation of disease expression (Patterson *et al.*, 2015). Previous studies assessing the diversity of *C. pecorum* in Australian koala populations have focused on populations in Queensland and New South Wales (Kollipara *et al.*, 2013). Comprehensive *C. pecorum* genotyping studies in Victorian koalas

are currently lacking, but would be useful in understanding the observed differences in disease syndromes between koala populations.

In *C. pecorum*, *ompA* encodes a major outer membrane protein associated with virulence (Fitch *et al.*, 1993). The nucleotide sequence of *ompA*, which has four variable domains, has been used frequently to genotype *C. pecorum* samples collected from koalas, leading to the detection of 11 koala associated genotypes, named A to K (Jackson *et al.*, 1997; Kollipara *et al.*, 2013). *C. pecorum* in livestock species such as cattle, pigs, sheep and goats have also been genotyped using *ompA* (Jackson *et al.*, 1997; Yousef Mohamad *et al.*, 2014). Although a number of different methods are now available to genotype *C. pecorum* samples (Marsh *et al.*, 2011; Jelocnik *et al.*, 2013), the existing catalogue of *ompA* sequences from northern koala populations makes it a useful tool to undertake comparisons with Victorian koala populations. Interestingly, differences in the presence of a chlamydial plasmid, a known virulence factor in some chlamydial species (O'Connell *et al.*, 2007), was also found to be a distinguishing feature of *C. pecorum* strains from northern populations compared with those from southern populations in South Australia (Jelocnik *et al.*, 2015). While some Victorian koala *C. pecorum* samples were found to be PCR positive for this plasmid (p*Cpec*), only a relatively small number of animals were sampled.

In this study, *C. pecorum ompA* molecular typing and p*Cpec* screening was performed on a large number of koala samples collected from different free-ranging populations across Victoria. Furthermore, using our newly collected data, reported here, combined with our previously reported data (Patterson *et al.*, 2015), we analysed clinical information and *C. pecorum* genome copy number to detect any significant associations between *C. pecorum* infection load and clinical disease, and to identify any significant associations between *C. pecorum* infection and a range of host-related variables.

Materials and Methods

Sample collection

Sample collection was approved by The University of Melbourne Animal Ethics Committee (approval number 1011687.1 & 1312813.2) and Parks Victoria (Research Permit 10004605, 10006948, 10005388). In total, urogenital swabs from 820 koalas and ocular swabs from 459 koalas were used in this study. These included 430 urogenital samples collected as a component of previously described research (Patterson *et al.*, 2015; Legione *et al.*, in press). Other samples were collected between 2010-2015, inclusive, during a variety of research field trips, management programs and post-mortem examinations. Of the ocular swabs, 456 came from koalas from which urogenital swabs were also collected. Samples were collected using either an aluminium or plastic shafted rayon swab (Copan Italia). Clinical examinations of live captured animals were performed by veterinarians, including assessment of body condition score (Patterson *et al.*, 2015), wet bottom score (Griffith, 2010) and, for a subsample of koalas, the presence or absence of urogenital tract pathology by ultrasound (Patterson *et al.*, 2015). All koalas also had a suite of other parameters recorded including tooth wear class (Martin, 1981) and the presence or absence of young. Gross pathology of the urogenital tract of any koalas that required euthanasia for health and welfare reasons was also recorded. All but six koalas used in the study were from free ranging populations, with five koalas held in care for more than one month prior to euthanasia and one koala raised in captivity.

DNA extraction from swab samples

Swabs were added to 1.5 mL tubes containing 800 µL of either phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4), Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 8.0) or RLT buffer (Qiagen) and mixed briefly using

an Xtron Vortex Mixer (Bartelt Instruments Pty Ltd) prior to processing. DNA extraction was then carried out using an X-tractor robot (Qiagen), utilising the Qiaxtractor® VX extraction kit as per manufacturer's instructions. Each extraction contained both positive and negative extraction controls. The positive control was a diluted broth of *E. coli* containing a pGEM®-T (Promega) plasmid, which in turn contained the *C. pecorum* 16S rRNA target, whilst the negative control was either sterile water or PBS.

Quantitative PCR for *Chlamydia* prevalence

Extracted DNA was tested for the presence of *Chlamydia* spp. using the *Chlamydia* 16SG qPCR first described by Robertson *et al.* (2009). The resulting melt curves from this qPCR, coupled with appropriate controls, can be used to distinguish detected *Chlamydia* species (Fig. S1). The koala house-keeping gene β-actin was utilised to standardise genome copy numbers detected in each sample, as described previously (Shojima *et al.*, 2013). Each sample was standardised based on the number of 16SG copies per β-actin copies in the extracted liquid sample. A standard curve was employed for each qPCR consisting of 10-fold dilutions, in triplicate, of purified plasmid containing either the 16SG or β-actin gene from 10^7 to 10^1 copies per reaction. Copy numbers were calculated using a Qubit 3.0 fluorometer (Invitrogen).

Genotyping of *C. pecorum* using *ompA*

C. pecorum positive DNA was used as template for conventional PCR to amplify *ompA*, as described previously (Kollipara *et al.*, 2013). PCR products of the predicted size (~1170 bp) were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced using BigDye® Terminator v3.1 (Life Technologies) using *ompA* PCR primers (Kollipara *et al.*, 2013), and internal primers 5' – AGAGCTACTTTTGATGCAGA - 3' and 5' – TTTGTGAACCACTCCGCATC - 3'. Geneious 7 software (Biomatters) was used for

150 sequence analysis and ClustalW (Thompson *et al.*, 1994) was used to align results with
151 published *ompA* sequences available in GenBank and in the literature (Jackson *et al.*, 1997).
152 Genotypes were assigned based on similarity to published genotypes. A difference of >1%
153 nucleotides across the ~1170 bp gene was used as the threshold for classifying a new
154 genotype, as described previously (Kollipara *et al.*, 2013).

155 **p*Cpec* screening in *C. pecorum* positive Victorian koala samples**

156 The prevalence of p*Cpec* across the *C. pecorum* positive samples identified in this
157 study was detected by use of p*Cpec*-specific conventional PCR, as described previously
158 (Jelocnik *et al.*, 2015).

159 **Assessment of factors associated with chlamydial infection**

160 Statistical analysis was conducted using Minitab 17 software (Minitab Inc). The
161 analysed parameters included sex, age (based on tooth wear), body condition score,
162 reproductive and urinary tract gross pathology, fecundity (based on the presence of back or
163 pouch young), wet bottom presence/absence, time of year and location collected. Not all
164 parameters were available for all animals. Koalas were classified as young (tooth wear <
165 class III), mature (tooth wear class III-V) or old (tooth wear > class V) (Patterson *et al.*,
166 2015). Specific location data was pooled into broader regional locations (Fig. 1).

167 Binomial logistical regression univariable analysis was performed on each variable in
168 relation to the presence or absence of *C. pecorum*. Variables with a P value ≤ 0.25 were
169 included in multivariable analysis. A backward elimination method was used to determine the
170 final multivariable logistic regression model to identify which variables were either
171 significant risk factors for, or useful predictors of, *C. pecorum* infection.

172 Standardised copy numbers were logarithmically transformed to normally distribute
173 the data. Data that were more than two standard deviations from the mean were considered
174 outliers and removed from the analysis. *C. pecorum* genome copies in each sample in relation

to clinical signs (body condition, wet bottom, urinary and reproductive tract pathology), as well as organism-specific variables such as *ompA* genotype and p*Cpec* presence, were compared using Student's t-test. Linear regression was used to determine which variable had the strongest effect on the amount of *C. pecorum* DNA detected via qPCR.

Results

The prevalence of *Chlamydia* infection in Victorian koalas varied between populations

We detected *C. pecorum* in 15.2% (125/820) of the urogenital tract samples tested (Fig. 1). Only 0.4% (2/459) of ocular swabs were positive for *Chlamydia*, with one case of *C. pneumoniae* detected in the South West Coast region, and one *C. pecorum*-positive ocular swab from the Greater Gippsland region. Substantial variation in prevalence was seen across the different geographical regions. The lowest prevalence of *C. pecorum* was 0.8% (2/237) in the French Island koala population and 7.1% (15/210) in the South West Coast region, which incorporates the Great Otway National Park and its surrounding coastline (Fig. 1). The highest prevalence was found in the greater Gippsland (36.7%, 11/30) and Mornington Peninsula (46.1%, 6/13) regions (Fig. 1).

Sequencing of *ompA* revealed that genotype B dominates in Victorian koalas, but also identified a novel genotype and two genotypes not previously associated with koalas

The *C. pecorum ompA* sequence was determined for 114 *C. pecorum* positive samples. A summary of the different genotypes detected, and their geographical distribution, is shown in Table 1. Genotype B predominated in Victorian koalas, occurring in 90/114 *ompA* genotyped urogenital cases (78.9%). Nine koalas were infected with genotype C and only three koalas, all from the Greater Gippsland region, were infected with genotype F. A novel genotype, designated "M", was detected in one urogenital sample from the Greater Gippsland

region. This genotype has 98.6% identity to a *C. pecorum ompA* sequence from a livestock isolate (strain: M14, Genbank: EU684920.1) (Yousef Mohamad *et al.*, 2008). The single detected case of ocular *C. pecorum* infection, also occurring in the Greater Gippsland region, was also genotype M. Nine koalas that tested positive for *C. pecorum* from the Great Otway National Park region on the South West Coast of Victoria were found to have a genotype not previously found in koalas. This genotype, which we designated "L", has 99.9% nucleotide identity to a livestock-associated *C. pecorum* (strain: DC49, GenBank GQ228195) (Yousef Mohamad *et al.*, 2014).

The *C. pecorum* plasmid p*Cpec* is present in 90% of Victorian samples

We screened the *C. pecorum* positive samples for the *C. pecorum* plasmid p*Cpec*. This revealed a p*Cpec* prevalence of 90% (113/125) (Table 1). This high prevalence precluded statistical evaluation of the contribution of the plasmid to the pathogenicity of *C. pecorum*, although it is interesting that in all cases where pCpec was absent, wet bottom was also absent. There was no significant relationship between p*Cpec* presence and the number of bacterial genome copies detected from swabs ($P = 0.326$). Univariable binomial logistic regression was conducted to test the relationship between p*Cpec* presence and gender or body condition score, but neither were significant factors ($P = 0.104$ and $P = 0.785$ respectively).

Male koalas had a higher likelihood of urogenital infection with *C. pecorum* than female koalas

Binary logistic regression analysis, with the presence or absence of *C. pecorum* infection as an outcome, was used to assess animal signalment and associated sampling information. Univariable analysis determined that sex, age of animal, season and region captured and the presence of back or pouch young (in females only) were significant

predictive factors for *C. pecorum* infection (Table 2). In the multivariable model (n = 614), after stepwise backwards elimination, only capture region was identified as a significant factor ($P < 0.001$). Removing capture region from the multivariable model to assess the variables at a population wide level, and repeating the stepwise elimination, identified sex as a significant factor ($P < 0.001$), with male koalas 2.7 times more likely (95% CI 1.60 - 4.45) to be positive for *C. pecorum*.

Urogenital *C. pecorum* infection was significantly associated with wet bottom in male koalas, and with reproductive tract pathology in female koalas

Assessment of clinical signs and pathological findings, through binary logistic regression analysis using the presence or absence of *C. pecorum* infection as an outcome, was conducted with male and female animals pooled, and with male and female animals separated. The results from this univariable analysis are summarised in Table S1 and Table 3 respectively. After separating data by sex, a multivariable analysis found that for male koalas, wet bottom was the only significant indicator of *C. pecorum* infection ($P < 0.001$). For females, reproductive tract pathology was the only significant indicator ($P < 0.001$).

***C. pecorum* genome copy numbers were significantly higher in swabs from koalas infected with Genotype F**

The geometric mean of bacterial genome copies for each *C. pecorum* genotype was determined (Table 4). Swabs collected from koalas infected with genotype F had a significantly higher mean genome copy number than swabs collected from koalas infected with genotypes B and L. Linear regression analysis with stepwise backwards elimination was conducted with genome copy number as an outcome and genotype, p*Cpec*, wet bottom

presence and gender as categorical predictors. This showed that genotype was the only significant predictive factor of genome load ($P = 0.034$).

Discussion

Our *Chlamydia* prevalence data incorporated three koala populations not previously investigated for these pathogens (Greater Gippsland and Mornington Peninsula in eastern Victoria, and the South West Coast in western Victoria). In addition we have expanded on previously published results for three populations (Mt Eccles (in the Far Western region), French Island and Raymond Island (Patterson *et al.*, 2015)). From Patterson *et al.* (2015) we included 288 koalas from which we previously reported prevalence, 286 from which the presence or absence of wet bottom was reported, and 117 koalas for which urogenital tract pathology was reported. This data was included to improve the power of our study, and increase the likelihood of accurate correlations being described. For similar reasons, here we also include the clinical data from two *Chlamydia*-positives koalas from French Island, whose *ompA* genotypes we recently reported (Legione *et al.*, 2016).

This is the largest molecular study of *Chlamydia* in free-ranging koala populations reported to date. Prevalence of *C. pecorum* varied substantially across the state, ranging from 1 – 46%, with the highest prevalence of *C. pecorum* occurring in the Mornington Peninsula and Greater Gippsland regions. All but two samples from these high prevalence regions were obtained post mortem from free-ranging koalas after euthanasia was required because of injury or disease (the other two samples, both *Chlamydia*-negative, were obtained from koalas that had been kept in captivity for up to one year). If euthanasia was performed due to disease related to *Chlamydia* infection, or resulted in immune suppression that enhanced bacterial shedding, the likelihood of detecting *Chlamydia* would increase, which may in part explain the higher prevalence in these populations. Easier access to anatomical sampling sites

during post mortem examination may also have increased detection rates. Our findings reflect previous investigations of *C. pecorum ompA* genotypes in northern koala populations (Kollipara *et al.*, 2013), which also found variable *C. pecorum* prevalence (28 - 61% in Queensland koalas, and 20 - 63% in New South Wales koalas), and noted that *C. pecorum* was more commonly detected in samples taken from sick or injured koalas. In contrast, the prevalence of *C. pneumoniae* in northern populations ranges from 4 – 23% (Polkinghorne *et al.*, 2013), but we detected *C. pneumoniae* in only one ocular swab. Interestingly, ocular pathology is commonly associated with *C. pecorum* infection in northern koalas (Polkinghorne *et al.*, 2013), but no koalas with ocular pathology in our study (n = 44) were infected with *Chlamydia,* suggesting other causes were responsible.

The existence of a large catalogue of *ompA* genotyped samples from northern koala populations make *ompA* genotyping a useful tool for undertaking comparisons between northern and southern populations. Recent research also suggests *ompA* is a valuable epidemiological marker for phylogenetic analysis (Marsh *et al.*, 2011), although it may overestimate whole genome evolution due to its exposure to strong selection pressure and recombination (Harris *et al.*, 2012). Here, we used *ompA* genotyping to reveal that, in comparison with the considerable heterogeneity seen in northern koala populations (Kollipara *et al.*, 2013), *C. pecorum ompA* genotype B dominates in samples collected from Victorian koalas. Genotype B has previously been found only in other southern koalas (Jackson *et al.*, 1997; Higgins *et al.*, 2012; Kollipara *et al.*, 2013). The lower diversity of *C. pecorum* genotypes in Victorian populations could be reflective of the translocation history of koalas in Victoria. Following a near extinction event in Victorian koalas around 1900 (Troughton, 1941), koalas from French Island and remnant Gippsland koala populations were used to repopulate Victoria (Martin, 1989). It is possible that koalas infected with *C. pecorum* were translocated from the Gippsland region, and from there the pathogen was moved into the

299 newly established Victorian populations (Martin & Handasyde, 1999). The observed
300 homogeneity provides support for this hypothesis, although we cannot rule out that these
301 infections arose due to separate transmission events.

302 Recorded *C. pecorum*-associated clinical signs are more severe in northern populations
303 than those observed to date in infected Victorian koalas, and we isolated *C. pecorum* from
304 only one ocular swab, which was not associated with clinical disease (Griffith 2010;
305 Polkinghorne *et al.*, 2013; Patterson *et al.*, 2015). Although *C. pecorum* disease in koalas is
306 no doubt multi-factorial, the dominant presence of genotype B across Victoria, and its
307 absence from northern populations, may suggest this *C. pecorum* genotype is less pathogenic
308 for koalas, particularly relating to the lack of ocular infection and/or pathology. However,
309 even amongst genotype B infected koalas we saw both asymptomatic and diseased animals,
310 with 38% (34/89) displaying wet bottom and 38% (13/34) with urogenital pathology such as
311 paraovarian cysts.

312 Genotype F, found in all northern populations previously tested (Kollipara *et al.*, 2013),
313 was only detected in Greater Gippsland, one of the remnant Victorian populations. This may
314 suggest it has been present in this population since before the near extinction event. Genotype
315 C, found previously only in koalas of Victorian origin (Jackson *et al.*, 1997; Higgins *et al.*,
316 2012), and the novel genotype M, were also found in the Greater Gippsland population. This
317 region appears to have greater genetic diversity than the populations in the rest of Victoria,
318 and additional future sampling from the Gippsland region would provide greater insight into
319 *C. pecorum* genetic variation in this population.

320 The South West Coast koala population suffers from severe overpopulation. *C.*
321 *pecorum* prevalence was significantly lower here when compared to other mainland
322 populations, which may explain why there is no apparent loss of fertility. The lower
323 prevalence may also suggest recent acquisition of *Chlamydia* by this population. The

presence of genotype L in this population, not previously detected in koalas and not found in other Victorian populations in this study, suggests this *C. pecorum* strain was not moved with the koalas when this population was established by translocation of koalas from French Island in the 1980s (Martin, 1989). The origin of genotypes L, M and N in koalas is unknown, although their close similarity to *C. pecorum ompA* sequences from livestock raises interesting questions over the potential for cross-host transmission from livestock, for which molecular evidence continues to grow (Jelocnik *et al.*, 2013; Bachmann *et al.*, 2015; Legione *et al.*, 2016). Future sampling of livestock that occupy ranges overlapping those of koala populations would be invaluable in investigating this hypothesis further.

In addition to analysing *ompA* genotype, we examined the presence of p*Cpec* in our samples. p*Cpec* is more commonly present in *C. pecorum* from koalas than in *C. pecorum* present in other animal species, and its prevalence in koala *C. pecorum* strains from South Australia appears to be much lower than those from northern populations (Jelocnik *et al.*, 2015). In contrast to the results observed in these populations, 90% of Victorian koala *C. pecorum* samples in this study were positive for p*Cpec*. This is the highest prevalence of the plasmid seen to date, and higher than previous studies in Victorian (79%) and northern (76%) populations (Jelocnik *et al.*, 2015). The high prevalence of p*Cpec* in our sample set made it difficult to identify significant effects on virulence, but analysis of genome copy numbers showed no significant difference between cases with or without p*Cpec*. The function of p*Cpec* in koala *C. pecorum* pathogenesis therefore remains unknown.

Previous studies in koalas and other species show that differences in clinical signs are not explained by *ompA* genotype alone (Higgins *et al.*, 2012; Yousef Mohamad *et al.*, 2014). Despite the predominance of genotype B, we observed variation in the presence of clinical signs in animals carrying this genotype. Finer detailed evolutionary analysis such as multi-locus sequence typing (Jelocnik *et al.*, 2013), which has been used to investigate pathogenesis

of *C. pecorum* infections in sheep (Jelocnik *et al.*, 2014), or newly-developed full genome sequencing techniques (Bachmann *et al.*, 2015) could be employed to further examine the relationship between *C. pecorum* strain type and the observed clinical signs. An analysis of host genetic factors, such as major histocompatibility complex class II variation (Lau *et al.*, 2014), would also be useful for understanding the host-pathogen interaction. The publication of the koala genome will accelerate efforts in this area (Johnson *et al.*, 2014).

Chlamydia in koalas has long been associated with clinical disease, primarily ocular and urogenital pathology (Jackson *et al.*, 1999). Our findings partially support this, but also suggest that these clinical signs are not pathognomonic of *Chlamydia* infection. This is an important factor for koala conservation, with recent research advocating the culling of infertile koalas and treatment of *Chlamydia*-infected koalas as a means to reverse population declines (Wilson *et al.*, 2015). In female koalas we found that reproductive tract pathology is a significant factor for predicting the presence of *C. pecorum.* However there were still a large number of koalas with evidence of reproductive tract pathology where qPCR did not detect *C. pecorum* (26/45), suggesting other aetiological agent(s) may be present that should also be considered in conservation programs. Signs of disease due to other pathogens such as koala herpesvirus (Stalder *et al.*, 2015) or koala retrovirus (Denner, 2014) could also overlap with *C. pecorum* infection. Comprehensive investigations into these and other pathogens may also further our understanding of the true significance of *C. pecorum* in Victorian koalas.

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519 **Figure 1.** Prevalence of *C. pecorum* infection in Victorian koala populations. **a.** Map of
520 Australia highlighting the state of Victoria (in box). **b.** Regional prevalence of *C. pecorum*
521 detected in Victorian koala urogenital swabs. Regions: 1st = Far Western Victoria, 2nd =
522 South West Coast, 3rd = Mornington Peninsula, 4th = French Island, 5th = Greater Gippsland,
523 6th = Raymond Island. Map not to scale.

524

525 **Table 1.** *C. pecorum ompA* genotype in urogenital swabs from Victorian koalas and

526 prevalence of *pCpec*

Region	*C. pecorum* positive	*ompA* genotype[#]							p*Cpec* positive
		B	C	F	L	M	N[†]	Unknown[*]	
1. Far Western Victoria	36	35	0	0	0	0	0	1	33
2. South West Coast	15	2	0	0	9	0	0	4	11
3. Mornington Peninsula	6	1	4	0	0	0	0	1	6
4. French Island	2	0	0	0	0	0	2	0	2
5. Greater Gippsland	11	1	3	3	0	1[+]	0	3	10
6. Raymond Island	50	49	0	0	0	0	0	1	47
Other	5	2	2	0	0	0	0	1	4
Total	125	90	9	3	9	1	2	11	113

527 [#] Examples of *ompA* genotypes submitted to GenBank under accession numbers KU214244
528 (genotype N), KU214245 (C), KU214246 (F), KU21427 (M), KU214248 (B), KU214249
529 (B), KU214250 (L) and KU214251 (B)

530 [†] This genotype was previously described in Legione *et al*, (in press), however was not
531 named as genotype N.

532 [*] Unknown genotypes were *C. pecorum* positives from which *ompA* was not able to be
533 amplified. All samples were confirmed to be *C. pecorum* via sequencing of the 16S rRNA
534 region and/or presence of *C. pecorum* plasmid *pCpec*

535 [+] The only ocular swab positive for *C. pecorum* was also genotype M from the Greater
536 Gippsland region

537

538

539 **Table 2.** Univariable analysis assessing select epidemiological variables relating to animal
540 signalment and sample collection as predictors for the presence of *Chlamydia* DNA

Variable	*Chlamydia* positive	Prevalence	Odds ratio*	95% CI	Co-efficient *P* value	Likelihood ratio *P* value^
Sex						**0.020**
Female	84/604	13.9%	1.00	-	-	
Male	39/184	21.2%	1.67	1.09 – 2.54	0.018	
Not recorded	2/32					
Age						**0.007**
Young	13/124	10.5%	0.75	0.40 – 1.41	0.400	
Mature adult	70/519	13.5%	1.00	-	-	
Old adult	16/55	29.1%	2.63	1.40 – 4.96	0.002	
Not recorded	27/122					
Season						**0.107**
Summer	1/7	14.3%	1.18	0.14 – 9.94	0.880	
Autumn	30/156	19.2%	1.68	1.05 – 2.70	0.030	
Winter	0/24	0%	-	-	-	
Spring	69/557	12.4%	1.00	-	-	
Not recorded	25/76					
Back/Pouch Young or lactation (females)						**< 0.001**
No	36/247	14.6%	1.00	-	-	
Yes	11/251	4.4%	0.27	0.13 – 0.54	< 0.001	
Not recorded	37/106					
Region[+]						**< 0.001**
Western Victoria	36/168	21.4%	3.55	1.87 – 6.73	< 0.001	
South west coast	15/210	7.1%	1.00	-	-	
Mornington Peninsula	6/13	46.2%	-	-	-	
French Island	2/237	0.8%	-	-	-	
Gippsland	11/30	36.7%	7.53	3.03 – 18.69	< 0.001	
Raymond Island	50/153	32.7%	6.31	3.38 – 11.78	< 0.001	
Other/Not recorded	5/9					

541 * Reference levels are indicated by odds ratio of 1.0.

542 ^ Results highlighted in bold (log likelihood $P \leq 0.25$) represent variables included in the
543 initial multivariable model, with the exception of the presence or absence of young/lactation,
544 as this correlated with sex and was thus excluded. In the final model, (n = 614, after stepwise
545 backwards elimination), only 'region' was identified as a significant factor ($P < 0.001$).

546 Removing ‘region’ from the multivariable model to assess the variables at a population wide
547 level and repeating the stepwise elimination identified sex as a significant factor ($P < 0.001$).

548 [+] Only regions with more than ten positive cases were included in the analysis.

549 **Table 3.** Univariable analysis assessing select epidemiological variables relating to animal
550 health and disease as predictors for the presence of *Chlamydia* DNA in koalas of each sex

Variable	*Chlamydia* positive	Prevalence	Odds ratio*	95% CI	Co-efficient *P* value	Likelihood ratio *P* value^
Female koalas						
Body condition score						0.734
≤ 2	8/80	10%	0.87	0.40 – 1.92	0.737	
≥ 3	53/470	11.3%	1.00	-	-	
Unknown	23/54					
Wet bottom						0.277
Absent	57/444	12.8%	1.00	-	-	
Present	26/159	16.4%	1.33	0.80 – 2.20	0.271	
Not recorded	1/1					
Urinary tract pathology						0.511
Absent	27/124	21.8%	1.00	-	-	
Present	9/33	27.3%	1.35	0.56 – 3.24	0.505	
Not recorded	48/447					
Reproductive tract pathology						**< 0.001**
Absent	16/110	14.5%	1.00	-	-	
Present	19/45	42.2%	4.29	1.94 – 9.50	< 0.001	
Not recorded	49/449					
Male koalas						
Body condition score						**0.008**
≤ 2	2/32	6.3%	0.19	0.04 – 0.85	0.030	
≥ 3	29/112	25.9%	1.00	-		
Unknown	8/40					
Wet bottom						**< 0.001**
Absent	24/155	15.5%	1.00	-	-	
Present	15/28	53.6%	6.30	2.66 – 14.90	< 0.001	
Not recorded	0/1					
Urinary tract pathology						0.644
Absent	13/44	29.5%	1.00	-	-	
Present	3/13	23.1%	0.72	0.17 – 3.03	0.649	
Not recorded	23/127					

551 * Reference levels are indicated by odds ratio of 1.0.

552 ^ Results highlighted in bold (log likelihood $P \leq 0.25$) represent variables included in the
553 initial multivariable model. For female koalas, only one variable was significant at the
554 univariable level so multivariable analysis was not conducted. For males in the final model (n
555 = 144), after stepwise backwards elimination, the only significant factor ($P < 0.001$) was the
556 presence or absence of wet bottom.

Table 4. Mean *Chlamydia pecorum* load for different *ompA* genotypes. Genome copies were quantitated from urogenital swabs via 16S rRNA qPCR and standardised using koala β-actin qPCR.

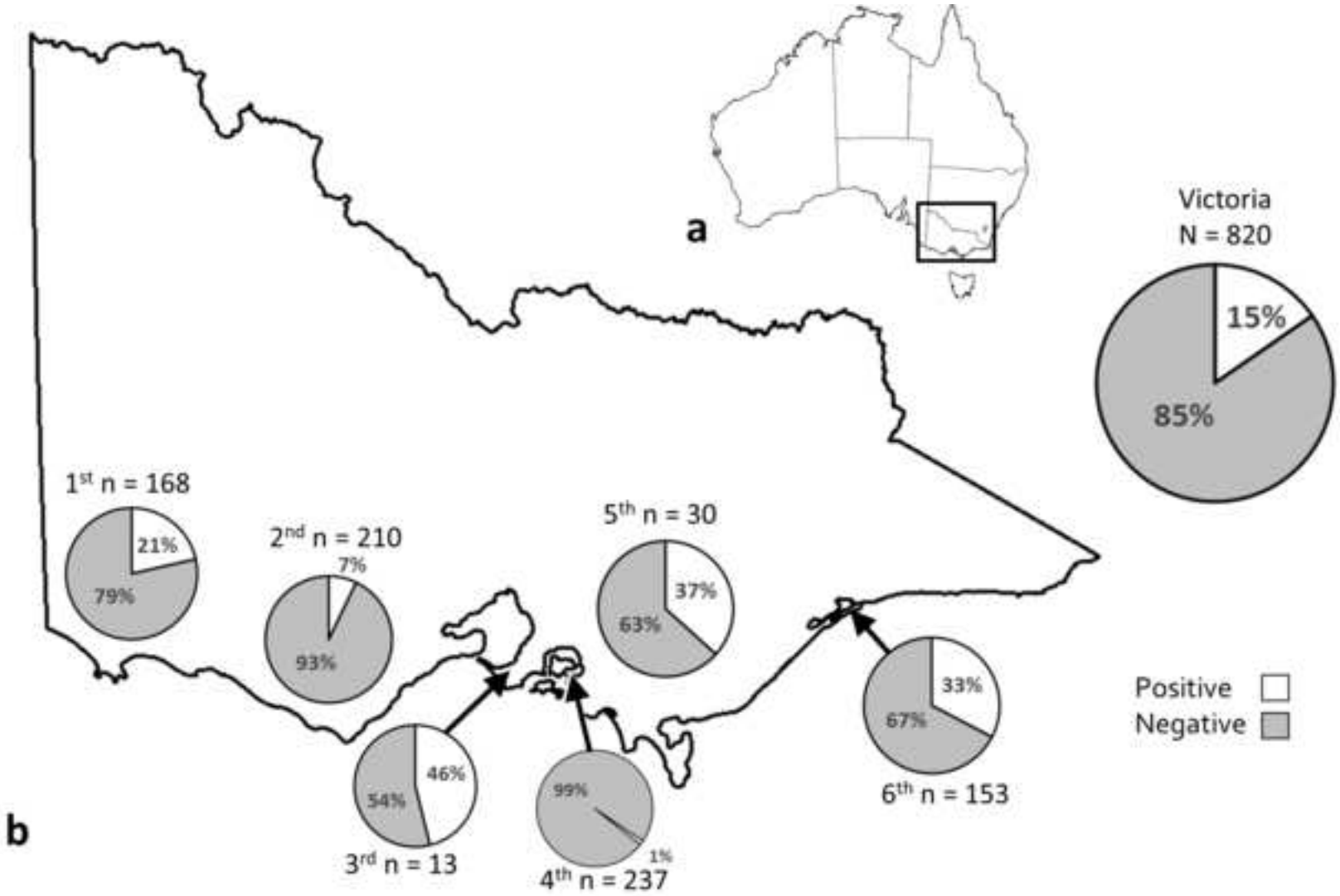
Genotype	Samples *	$\log_{10}$ 16SG:10^6 β-actin copies ± SD
B	87	2.52 ± 0.96 #
C	7	2.84 ± 1.07 #, ^
F	3	3.73 ± 0.13 ^
L	9	2.78 ± 0.74 #
M	1	1.95 +
N+	2	1.01 ± 0.20 +

* Values that fell 2 standard deviations from the mean were removed from the data set before analysis. These included 3 samples from genotype B and 2 from genotype C.

#, ^ Values with the same superscript symbol were not significantly different to each other using two sample Student's t-test ($P \geq 0.05$)

+ Analysis was not performed due to insufficient samples (that is, less than 3)

Figure 1

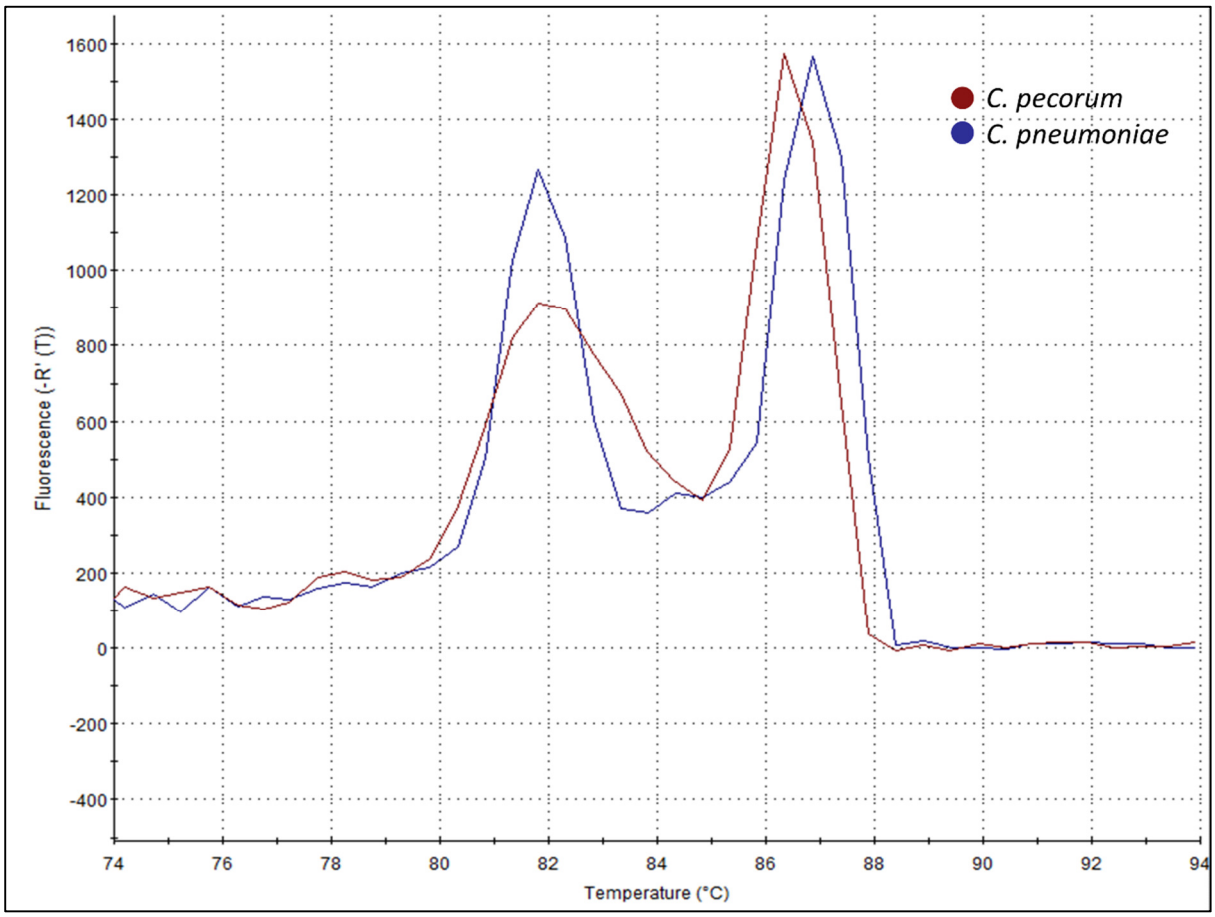


Supplementary information

Supplementary Table 1. Univariable analysis assessing select epidemiological variables relating to animal health and disease as predictors for the presence of *Chlamydia* DNA in both sexes of koala.

Variable	*Chlamydia* positive	Prevalence	Odds ratio*	95% CI	Co-efficient *P* value	Likelihood ratio *P* value^
Body condition score						**0.105**
≤ 2	10/113	8.8%	0.58	0.29 – 1.16	0.126	
≥ 3	84/589	14.3%	1.00	-	-	
Not recorded	31/118					
Wet bottom						**0.008**
Absent	83/608	13.7%	1.00	-	-	
Present	41/187	21.9%	1.78	1.17 – 2.69	0.007	
Not recorded	1/25					
Urinary tract pathology						0.736
Absent	40/169	23.7%	1.00	-	-	
Present	12/46	26.1%	1.14	0.54 – 2.40	0.734	
Not recorded	73/605					

* Reference levels are indicated by odds ratio of 1.0. ^ Results highlighted in bold (log likelihood $P \leq 0.25$) represent variables included in the initial multivariable model. In the final model (n = 702), after stepwise backwards elimination, only wet bottom was identified as a significant factor ($P = 0.002$).



Supplementary Figure 1. Melt curve of 16S rRNA region SYBR green qPCR (16SG, Robertson *et al.*, 2009). Melt curve generated at a resolution of 0.3°C. *C. pneumoniae* and *C. pecorum* are differentiated by the shape of the first peak and melting temperature of the second peak.