Veterinary Microbiology 165 (2013) 214–223

Contents lists available at SciVerse ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Review

Recent advances in understanding the biology, epidemiology and control of chlamydial infections in koalas

Adam Polkinghorne [a,*], Jon Hanger [b], Peter Timms [a]

[a] Institute of Health and Biomedical Innovation, Queensland University of Technology, 60 Musk Avenue, Kelvin Grove, Brisbane 4059, Australia
[b] Endeavour Veterinary Ecology Pty Ltd, 1695 Pumicestone Road, Toorbul 4510, Australia

ARTICLE INFO

Article history:
Received 23 October 2012
Accepted 24 February 2013

Keywords:
Chlamydia
Koala
Vaccine
Diagnostics
Antibiotics
Evolution

ABSTRACT

The koala (*Phascolarctos cinereus*) is recognised as a threatened wildlife species in various parts of Australia. A major contributing factor to the decline and long-term viability of affected populations is disease caused by the obligate intracellular bacteria, *Chlamydia*. Two chlamydial species infect the koala, *Chlamydia pecorum* and *Chlamydia pneumoniae*, and have been reported in nearly all mainland koala populations. Chlamydial infections of koalas are associated with ocular infections leading to blindness and genital tract infections linked to infertility, among other serious clinical manifestations. Diagnosis can be based on clinical presentation alone, however, it is complicated by the observation that many koala chlamydial infections occur with no overt signs of clinical disease. Instead, accurate diagnosis requires detailed clinical assessment and confirmatory testing by a range of PCR-based assays. Antibiotic treatment for koala chlamydial infection is possible, however, results on its success are mixed. A more practical solution for the protection of diseased populations is the application of a koala *Chlamydia* vaccine, with recent trials indicating promising results. Interestingly, molecular epidemiology studies of koala *C. pecorum* infections and recent comparative genomic analyses of koala *C. pneumoniae* have revealed potential differences in their origin that will have wider ramifications for our understanding of human chlamydial infections and host adaptation of the chlamydiae. This review summarises changes to the taxonomy of koala chlamydial infections and recent advances in our understanding of the epidemiology, diagnosis, treatment, control and evolution of *Chlamydia* infections in this iconic wildlife species.

Contents

* Corresponding author at: Adam Polkinghorne, Institute of Health and Biomedical Innovation, Queensland University of Technology, 60 Musk Avenue, Kelvin Grove, 4059, Australia. Tel.: +61 7 3138 6259; fax: +61 7 3138 6030.
E-mail address: a.polkinghorne@qut.edu.au (A. Polkinghorne).

0378-1135/$ – see front matter
http://dx.doi.org/10.1016/j.vetmic.2013.02.026

1. Koalas: a declining national icon of Australia's biodiversity

The koala (*Phascolarctos cinereus*) is an arboreal herbivorous marsupial and, as the last surviving member of the family *Phascolarctidae*, is an international icon of the rich biodiversity found on the Australian continent. Despite the esteem that is held for this wildlife species, it is generally acknowledged that koala numbers are in a decline across the majority of free-living populations in mainland Australia. Prior to European settlement, the koala's natural range was thought to extend from the eucalyptus forests of North-Eastern Queensland to those of the southern coast of South Australia. Koala populations are now highly fragmented across this range and appear to be declining rapidly in many areas, with one population in South-East Queensland experiencing a 64% decline in the last 10 years and a 51% decline in the last three years (Department of Environment and Resource Management, 2009). Translocation of animals by humans has also led to the establishment of koala populations on islands off the southern and eastern coasts of Australia.

The decline of koala populations across Australia, particularly in previously densely populated habitats along Australia's eastern seaboard, has been attributed to a range of naturally occurring and anthropogenic factors. While bushfire (Lunney et al., 2007) and other factors can impact on koala numbers leading to localised extinctions, the encroachment of human settlement into koala habitats has been estimated to have the most dramatic effect on koala declines. These factors include a loss of habitat due to land clearing (Melzer et al., 2000), motor vehicle traumas (Dique et al., 2003) and dog attacks (Lunney et al., 2007). Of the multiple threatening processes that have been linked to koala declines in peri-urban populations, however, recent modelling has suggested that control of disease is the most important in terms of introducing strategies to bring wild koala populations back to stability (Rhodes et al., 2011). While other pathogens have been reported in the koala, including an endogenous Koala retrovirus (KoRV; Tarlinton et al., 2005; Simmons et al., 2012), and a series of koala and marsupial-specific trypanosomes (McInnes et al., 2011), the most important pathogen of this wildlife species is *Chlamydia*.

Species in the genus *Chlamydia*, like other members of the order *Chlamydiales*, share a biphasic developmental cycle and require growth in a unique host cell membrane-bound inclusion. Paradoxically, despite this unique evolutionary niche, these bacteria are ubiquitous and have adapted to infect and cause a range of significant diseases in domesticated animals including livestock species such as cattle, sheep and pigs and household animals such as guinea pigs and cats, as well as humans. Chlamydial infections of the koala are easily the most intensively studied of any wildlife species and, apart from avian and zoonotic infections caused by *C. psittaci*, of any animal host altogether. As such, koala chlamydial infections serve as an important model for understanding the biology of the host-pathogen interaction, the epidemiology and the impact of chlamydial disease on a native species. Despite this, only a limited number of reviews have been published on this topic (Brown et al., 1987; Whittington, 2001). Recent years have seen major advances in our understanding of the taxonomy and evolution, diagnosis, treatment, control and epidemiology of *Chlamydia* in koalas and these topics and the remaining challenges and future directions in the conservation of these iconic species from chlamydial disease are the subject of this review.

2. The devastating effects of chlamydial disease

Early records indicate that lesions resembling chlamydiosis had been observed in koalas as early as the late 1800s (Mackenzie, 1919, Pratt, 1934, Troughton, 1941). These authors referred to epidemics of disease late in the 1800s and early 1900s, including cystic reproductive tract disease, leading to infertility, and also "ophthalmic disease and periostitis of the skull" (Troughton, 1941). Gordon and McGreevy (1978) suggested that "epidemic disease" was the primary cause of koala population decline in Queensland following the last open season in 1927. Chlamydiae were reported to have been isolated from kerato-conjunctivitis lesions in 1974 (Cockram and Jackson), and from various sites in koalas with kerato-conjunctivitis, rhinitis/pneumonia complex, urinary tract infection, and ascending genital tract infection in female koalas (Brown and Grice, 1984). Although the full range of clinical conditions and syndromes caused by chlamydial infection in koalas has not been comprehensively investigated, the "classical" diseases are relatively well described (Blanshard and Bodley, 2008). These are kerato-conjunctivitis, urinary tract disease, reproductive tract disease, and the rhinitis/pneumonia complex (Fig. 1).

Infection of the mucosal surfaces of the eye results in inflammation, characterised in the early stages by serous discharge, blepharospasm and hyperaemia of the conjunctiva and sclera, progressing to purulent discharge, conjunctival hyperplasia and fibrosis. One or both eyes may be affected. In some severe and chronic cases, the cornea is affected by opacity caused by oedema and pannus with or without pigmentation, and severe end-stage cases may have rupture and collapse of the globe. Clinical presentations are highly variable in severity and chronicity between koalas, and lesions may be active, with copious exudate, or inactive, with no exudate and mature scarring (Wan et al., 2011). Blindness may occur because of the physical barrier to sight caused by bulging and hyperplasia of the conjunctiva, by chronic pathological changes affecting the cornea, such as oedema, scarring and pannus, and in rare cases, by severe ophthalmitis and rupture of the globe. Mild and acute cases respond well to treatment, but the therapeutic efficacy in chronic and severe cases is dependent upon the degree of fibrosis affecting the cornea and conjunctiva. Extensive and advanced fibrosis of the conjunctiva leads to reduction in the size of the palpebral fissure and sometimes entropion, and scarring of the cornea may lead to severe impairment of sight, or blindness. Acute and chronic active cases of kerato-conjunctivitis tend to have higher levels of shedding of chlamydial organisms than chronic inactive cases,

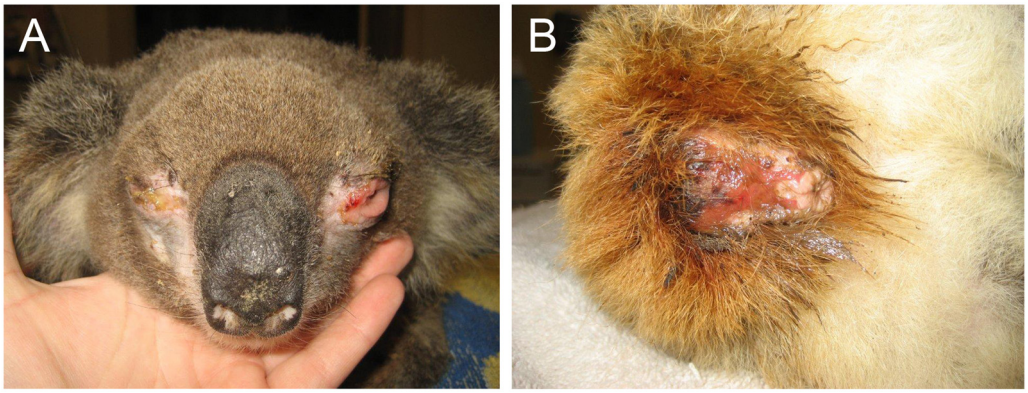

Fig. 1. Classical signs of koala chlamydiosis, including (A) bilateral keratoconjunctivitis and (B) an example of koala "wet-bottom" or "dirty-tail", including discolouration of the fur around the koala's rump and tail and ulceration. The latter signs are a result of chlamydial cystitis leading to incontinence and loss of bladder function.

although shedding is highly variable between koalas (Wan et al., 2011).

Inflammation of the urinary tract often manifests overtly as brown urine staining and wetness of the fur of the rump and tail. This occurs as a result of cystitis leading to incontinence and loss of bladder function; hence, the colloquial terms "wet-bottom" and "dirty-tail". Koalas with severe cystitis show evidence of discomfort on urination, which is sometimes sufficiently painful to cause vocalisation or crying, and there may be gross haematuria (Blanshard and Bodley, 2008). In severe, chronic cases there may be alopecia, urine scalding and erythema, skin erosions and ulceration, secondary bacterial and yeast dermatitis of the skin around the common opening, and oedema and protrusion of the mucosa of the common vestibule. Caseous exudate and pseudomembrane material often shows heavy infection with yeasts, cytologically. Sonographic changes in affected koalas include mild to marked thickening and irregularity of the bladder wall, reduction in lumen diameter, presence of urinary casts and flocculence of the luminal echo. Scarring of the bladder wall may be evident as increased echogenicity. In a proportion of chronic, severe cases, there is marked dilatation of the ureter and renal pelvis (hydroureter and hydronephrosis) as a consequence of outflow obstruction, presumably caused by fibrosis around the intramural ureter as it passes through the bladder wall. Urethritis, prostatitis, ureteritis and nephritis are also reported in association with chlamydial infection (Brown et al., 1987; Canfield, 1989). Severe, chronic cystitis cases are often associated with extensive loss of bladder mucosa, marked loss of functional capacity and chronic haematuria.

Reproductive tract disease associated with chlamydial infection is common in both male and female koalas. Reproductive disease is rarely apparent overtly, unless associated with concurrent urinary tract infection and/or general debility, and, in many female koalas, failure to produce joeys may be the only indication of reproductive tract disease. Female reproductive tract disease commonly results in gross pathological changes that are easily detected by sonography and/or caudal abdominal palpation. Such changes include unilateral or, more commonly, bilateral cystic dilatation of the ovarian bursae, oviducts and uteri, pyometron, metritis, vaginitis and urogenital sinusitis (Blanshard and Bodley, 2008). Acute reproductive tract disease in females may be associated with muco-purulent exudate from the common opening, but frequently there is no external evidence of infection. More chronic disease is indicated by cystic changes in the oviducts and ovarian bursae and sometimes thickening of the uterine walls, however, these changes may not always be present in cases of chronic reproductive tract disease. In contrast, male reproductive tract disease is infrequently diagnosed clinically because sonographic changes are uncommon (Loader, 2010). Prostatic abscesses and other sonographic lesions have been reported in koalas, but generally definitive diagnosis of reproductive disease in male koalas relies upon histological examination of tissues collected during necropsy examination (Loader, 2010).

Reproductive disease caused by chlamydial infection is a very common cause of infertility and reproductive loss in koala populations, and may cause significant detriment to the viability of many koala populations. Recent studies on disease prevalence in some South East Queensland (SEQ) wild koala populations showed reproductive disease prevalence in sexually mature female koalas of between 31% and 57% across three populations. In each population, fecundity rates were consistently somewhat less than the proportion of female koalas without detectable reproductive tract disease, suggesting that a proportion of these koalas had reproductive disease that was not detected sonographically (Loader, 2010).

3. A history and update on the taxonomy of koala chlamydiae

The previous and current taxonomy of chlamydial infections of the koala is summarised in Fig. 2. The taxonomy of members of the order *Chlamydiales* has undergone significant revisions since *Chlamydia* was first isolated and implicated in disease in the mid 1970s

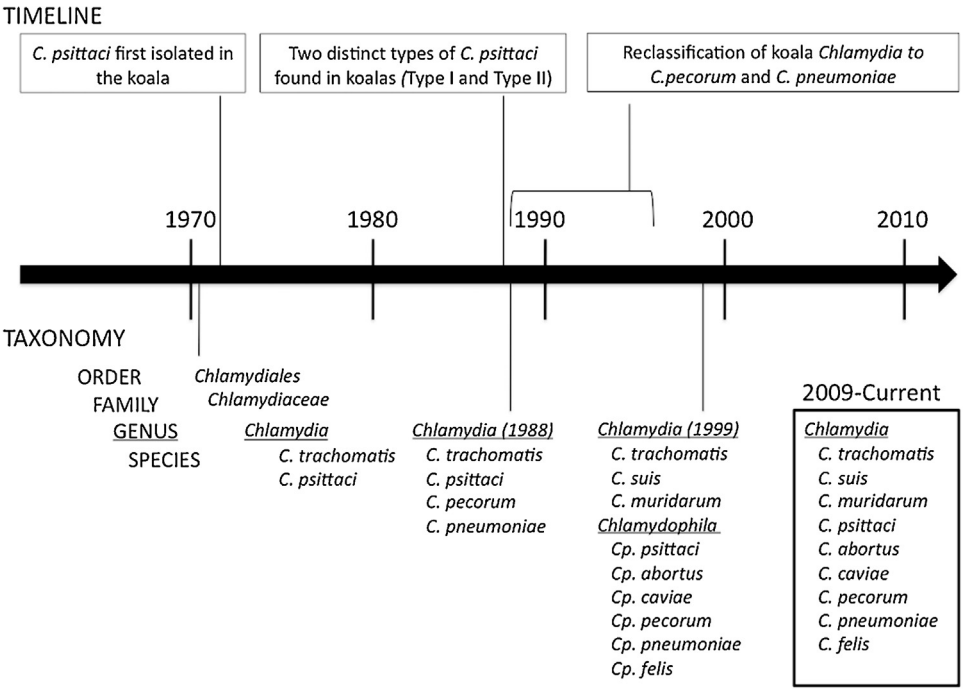

Fig. 2. A timeline and summary of the identification and classification of chlamydial species infecting the koala against the taxonomic changes to the order *Chlamydiales* since the 1970s.

(Cockram and Jackson, 1974). At that time, the causative agent was classified as *C. psittaci* based on cell culture observations. With advances in fundamental molecular biology techniques 10 years later, it was revealed that there were indeed two distinct types (designed Type I and Type II) of *C. psittaci* in the koala (Girjes et al., 1988). These types were subsequently reclassified to *C. pneumoniae* and *C. pecorum* respectively, when nucleotide sequence analysis of *ompA*, the gene encoding the chlamydial major outer membrane protein (MOMP), demonstrated there was substantial sequence homology between the type strains for these species and those previously identified in the koala (Timms et al., 1988; Girjes et al., 1994; Glassick et al., 1996). In 1999, using sequence analysis of the 16S and 23S rRNA genes of members in the order *Chlamydiales*, a taxonomic revision was proposed that saw the two species infecting koalas, *C. pneumoniae* and *C. pecorum*, moved into a new genus, *Chlamydophila*, alongside seven other species (Everett et al., 1999). The splitting of the genus *Chlamydia*, however, was not widely adopted and a compromise was reached by the merging of all nine species in the family *Chlamydiaceae* back into a single genus, *Chlamydia* (Stephens et al., 2009). For the remainder of this review, the two main chlamydial pathogens of the koala will be referred to as *Chlamydia pecorum* and *C. pneumoniae*, according to this currently accepted nomenclature.

New families incorporating a range of novel *Chlamydia*-like organisms sharing bi-phasic developmental cycles, characteristic of the traditional chlamydiae, were also proposed during the 1999 revision. In 2003, Devereaux et al. revealed molecular evidence to suggest that novel *Chlamydia*-like bacteria also existed in the koala, although these bacteria remain uncultured and have, hence, not been formally classified.

4. Epidemiology of chlamydial infections in the koala

Even though a variety of approaches have been used to detect and analyse chlamydial infections in koalas over the last 15 or more years, they all indicate that these organisms are common in virtually all of Australia's wild (and captive) koala populations. While early studies used serological approaches, these are unreliable. It is really only molecular methods involving PCR that have provided accurate data on the prevalence of chlamydial infections, in wild populations in particular. A summary of both published and unpublished epidemiological data is provided in Table 1. The geographic distribution of the tested populations is shown in Fig. 3. Infection levels range from zero in some island populations, up to very high levels (*e.g.* 72–100%) in those populations which generally show high prevalences of both ocular and urogenital tract disease. Interestingly, populations in the northern regions of Australia (*e.g.* Queensland and northern New South Wales in particular) generally show higher levels of chlamydial infection as well as more evidence of clinical disease. Several small "isolated" populations have been reported to have no chlamydial infections: Magnetic Island, French Island, Kangaroo Island. These are all artificial island populations, which were started by translocation of presumably healthy, *Chlamydia*-free animals, which have subsequently flourished in the reported absence of chlamydial infections.

Table 1
Summary of cross-sectional population and wildlife carer studies of chlamydial infections in koalas throughout Australia, separated by Australian state.

Location	Method used	Animals sampled	Observations on disease	*Chlamydia* positivity (%)	*C. pecorum* positivity (%)	*C. pneumoniae* positivity (%)	Reference
Queensland							
Magnetic Island[a]	Serology	70	Outwardly healthy population	0%	NT	NT	Hirst et al. (1992)
Brendale	qPCR	16	Ocular and urogenital tract disease observed	50	50	NT	Unpublished
Narangba	qPCR	22	Ocular and urogenital tract disease observed	50	50	NT	Unpublished
Mutdapilly	PCR	33	Significant ocular and urogenital tract disease observed	85%	73%	21%	Jackson et al. (1999)
Coombabah	PCR	20	High prevalence of clinical disease	10%	10%	10%	Jackson et al. (1999)
Koala Coast	PCR	23	High prevalence of clinical disease	87%	87%	4%	Unpublished
East Coomera	qPCR	80	Ocular and urogenital tract disease common	33%	33%	NT	Unpublished
New South Wales							
Koala Beach	PCR	29	Ocular and urogenital tract disease present	67%	67%	10%	Unpublished
Pine Creek State Forest	PCR	25	Mainly ocular disease observed	72% [b]	52%	12%	Devereaux et al. (2003)
Pilliga State Forest	PCR	26	Mainly ocular disease observed	12%	8%	8%	Unpublished
Port Macquarie	PCR	13	Ocular and urogenital tract disease observed	45%	38%	23%	Unpublished
Victoria							
Framlingham	PCR	10	No clinical disease observed	0%	0%	0%	Unpublished
Ballarat	PCR	10	Urogenital tract disease observed	100%	90%	20%	Unpublished
French Island[a]	PCR	5	No clinical disease observed	0%	0%	0%	Unpublished
South Australia							
Kangaroo Island[a]	PCR	10	No clinical disease observed	0%	0%	0%	Unpublished
Cleland Wildlife Sanctuary	PCR	28	No clinical disease observed	18%	NT	NT	Unpublished
Mt Lofty Ranges	PCR	17	Low prevalence of clinical disease observed	90%	90%	53%	Unpublished

NT = not tested.
[a] Translocated island populations.
[b] Detection of chlamydiae in this study included novel *Chlamydia*-like bacteria.

A common approach to chlamydial epidemiological studies has been to use a Family-specific PCR assay initially to detect "all" *Chlamydia* present and then to sequence either the 16SrRNA PCR product or the *ompA* gene product to speciate the *Chlamydia* present (Devereaux et al., 2003). This approach has shown that *C.pecorum* is always the dominant species present (10% to 90%; averaging 57%), with *C. pneumoniae* usually present only at low levels (8% to 53%; averaging 18%). *C. pecorum* is not only the most dominant species infecting the koala, it is also present at the highest infection level. Jackson et al. (1999) showed that while low grade infections (1+ infectious load in their study) were recorded for both *C. pecorum* and *C. pneumoniae* (at both ocular and urogenital tract sites), only *C. pecorum* produced high grade infections (3+ or 4+ infectious load scores). They also showed that the infectious load was significantly higher in the female genital tract site than in the eyes. More recently, Wan et al. (2011) confirmed these observations using a highly sensitive quantitative PCR approach targeting *C. pecorum*. Perhaps not surprisingly, they found that animals with active clinical disease were shedding the highest levels of *C. pecorum* organisms/DNA. These animals therefore might be a significant source of transmission of infections in wild populations. In contrast, koalas with chronic, but inactive disease tended to shed organisms at significantly lower levels. While no studies have been reported that directly tested the method of transmission of chlamydiae between koalas, clinical observations would suggest that it is primarily transmitted sexually, and from mother to joey. Females often have high levels of organisms detectable in swabs collected from the vaginal tract and swabs collected from the male penile urethra equally show the presence of chlamydial organisms. Jackson et al. (1999) conducted a study on the Mutdapilly wild population, examining infection levels in animals at various age groups (as determined by the animal's tooth ware class). In the case of *C. pecorum* infections, they found that 9/17 young koalas (tooth ware classes 1 and 2) had chlamydial infections, whereas 100% (12/12) of the older animals (tooth ware classes 3 to 10) were infected. This suggests that animals become infected as they become more sexually active and remain infected, or become re-infected, as they age. While only 4/17 young animals had *C. pneumoniae* infections (which may simply represent the lower infection levels for this species), by comparison to *C. pecorum*, only 3/12 older animals had *C. pneumoniae* infections. While sexual transmission is clearly a major mechanism of transmission for koala chlamydiae, the data of Jackson et al. (1999) also suggest that young animals, prior to sexual contact, are

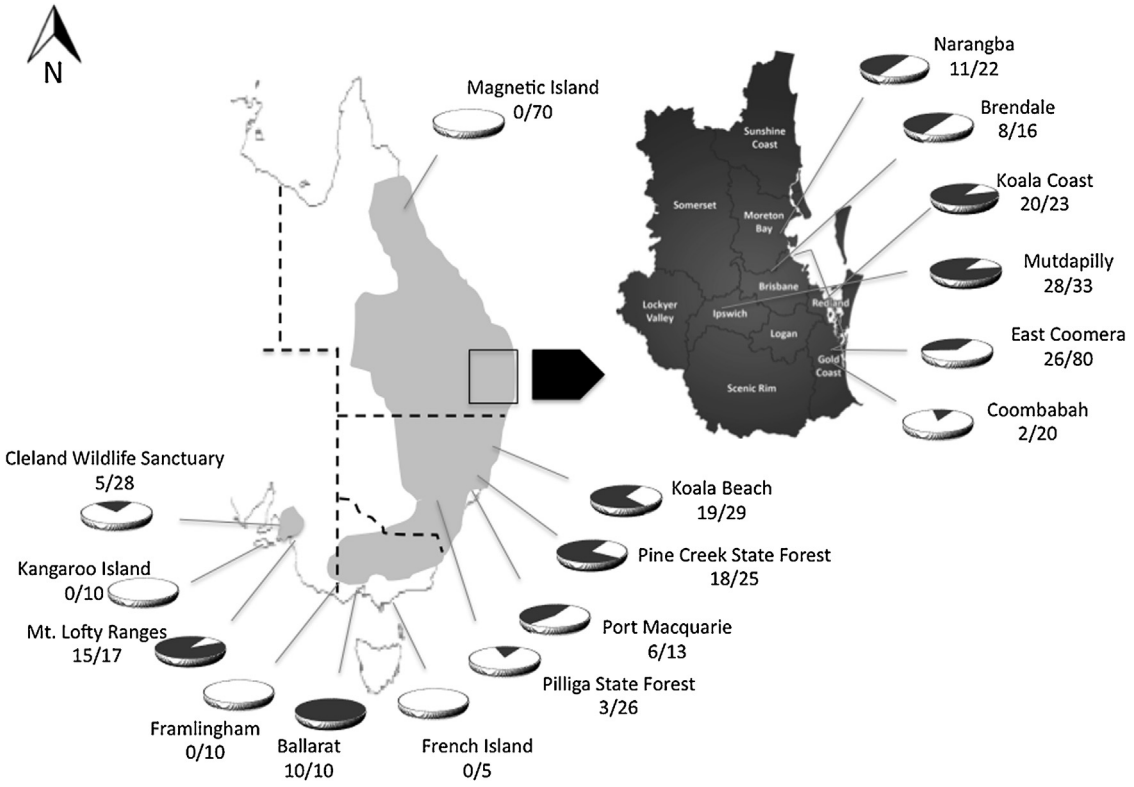

Fig. 3. A map of the eastern half of Australia illustrating the known geographic range of the koala (grey shading; https://www.savethekoala.com/about-koalas/distribution) and the geographic location and results of known koala *Chlamydia* prevalence studies to date (Table 1).

infected from their mothers; 7 out of 12 animals in the lowest tooth ware class were infected with *C. pecorum*. This is perhaps not surprising as koalas engage in pap feeding of their young, to ensure that their gut is colonised by the unique microbial flora required for digestion of eucalyptus leaves.

5. Diagnosis of chlamydial infections in the koala

Chlamydial infections may be apparent as (i) overt or obvious disease; (ii) inapparent overtly, but detectable using veterinary techniques; or (iii) unassociated with any detectable clinical signs or changes (Loader, 2010; Wan et al., 2011). Current veterinary diagnosis of chlamydial disease in koalas in the wildlife hospital setting relies upon a thorough veterinary clinical examination, the use of in-house diagnostic aids, and ultrasonography of the urinary and reproductive tracts. Thorough reviews of clinical findings and diagnosis of chlamydial disease are provided by Whittington (2001) and Blanshard and Bodley (2008). The definitive diagnosis of chlamydial aetiology is complicated, however, by the variable shedding of organisms from mucosal sites affected by disease, both between koalas and individually over the course of infection and clinical disease progression. Furthermore, shedding of organisms is commonly demonstrated in koalas without any detectable evidence of inflammation or disease. Hence, the clinical diagnosis of chlamydial aetiology is generally presumptive and based on typical clinical findings, and often made independently of the results of antigen detection tests. Diagnosis of chlamydial infections and disease in the field is challenging, and arguably cannot effectively be conducted without experienced veterinary input. Failure to use a standardised veterinary assessment, including diagnostic aids, such as ultrasonography and cytology, inevitably leads to some cases of clinically significant disease being missed, and disease prevalences in wild koala populations underestimated (Loader, 2010).

For laboratory diagnosis of chlamydial infections of the koala, swab sampling of the mucosal sites has proven to be a reliable and mostly non-invasive method for sampling the conjunctiva and lower genital and urinary tract sites, since infected animals shed moderate to high levels of chlamydial bodies that can be detected by PCR methods (Wan et al., 2011). While cell culture methods were initially used to diagnose and describe the chlamydial pathogens of koalas (Cockram and Jackson, 1974), rapid DNA and antigen-based methods have revolutionised the diagnosis and description of *Chlamydia* in humans, koalas and other animals. The modern gold standard for *Chlamydia* detection in both human and animal speciments is PCR (Sachse et al., 2009). The first population survey for chlamydial infection in koalas, utilising molecular methods, screened for chlamydial DNA in swab samples collected from the conjunctival sac and urogenital tract (Jackson et al., 1999). This assay utilised a *Chlamydia* genus-specific PCR targeting the *ompB* gene and DNA hybridisation using *C. pecorum* or *C. pneumoniae*-species

specific DNA probes for speciation of positive samples. Order, genus and species-specific conventional or nested PCR assays, targeting a range of gene targets including the *16S rRNA* gene and *ompA* genes, have been subsequently employed to screen swab samples, blood and semen collected from infected koalas for the presence of *C. pecorum* and/or *C. pneumoniae* DNA (Bodetti and Timms, 2000; Bodetti et al., 2002; Devereaux et al., 2003). For order- and genus-specific PCR assays, speciation involved either DNA sequencing of the PCR product or the tandem use of species-specific PCR assays on positive samples. While DNA sequencing is more laborious, the pan-*Chlamydiales* PCR trialled in Devereaux et al. (2003) and targeting a conserved region of the 16S rRNA gene (Everett et al., 1999), also led to the first description and detection of *Chlamydia*-like organisms in the koala, which could not be detected in other species-specific assays. More recently, quantitative genus or species-specific PCR methods targeting the 16S rRNA gene (Markey et al., 2007; Wan et al., 2011) have emerged and been utilised not only for detection and speciation of the chlamydial species present but also to measure chlamydial shedding over time (Markey et al., 2007) as well as to correlate the chlamydial infectious load with clinical disease presentation in koalas (Wan et al., 2011).

While PCR-based methods have proven to be the most sensitive and reliable method for screening chlamydial infections in the koala, these methods are not suitable for point-of-care field testing of captured koalas or where molecular testing might not be otherwise available. In koalas, a series of *Chlamydia*-genus specific enzyme-linked immunosorbent assays and direct fluorescent-antibody kits were earlier trialled for detection of chlamydial infections in the koala with mixed results (Wood and Timms, 1992). This field analysis revealed that one qualitative, solid phase direct antigen detection kit, the Clearview *Chlamydia* test, was sensitive and specific for chlamydial species infecting the koala but that otherwise, these tests performed poorly for both sensitivity and specificity. More recently, Hanger et al. (2012) showed that the Clearview assay, while not as sensitive as qPCR-based methods, is potentially a suitable diagnostic tool for the rapid diagnosis of active chlamydial infections by field workers (Hanger et al., 2012).

6. Advances in the treatment and control of koala chlamydial infections

Current therapeutic regimens aim to eliminate infection using antimicrobial drugs, relieve discomfort, and reduce immune-mediated tissue and organ damage. Systemic antimicrobial drugs used for chlamydial infections at koala treatment facilities include enrofloxacin (Baycox® Bayer Healthcare AG, Lerverkusen, Germany), and chloramphenicol (Chloramphenicol 150 Ceva Delvet, Seven Hill, Australia; Blanshard and Bodley, 2008). Only chloramphenicol administered at 60 mg/kg subcutaneously once per day for 45 days has been demonstrated to result in cessation of shedding of *Chlamydia* during and at 2 weeks after the completion of treatment using both quantitative PCR and Clearview *Chlamydia* assays (Markey et al., 2007). Griffith (2010) reported a failure of systemic treatment with enrofloxacin to affect microbial cure in most treated koalas, which demonstrated rebound shedding of organisms after cessation of antibiotic therapy. There are significant issues associated with the use in koalas of some antibiotics commonly used to treat chlamydial infections in humans and other species, such as the tetracycline and macrolide antibiotics (Markey et al., 2007; Griffith et al., 2010). These primarily relate to the potentially fatal effect of these antibiotics on the microflora of the koala gastrointestinal tract, and apparent limitations on the complete clearance of chlamydial infections of the lower genital tract by antibiotics such as enrofloxacin.

A vaccine to control chlamydial infections in the koala is currently under development and showing promise. The initial trial in this programme immunised healthy female koalas with a multi-subunit vaccine, consisting of three antigens including MOMP (Carey et al., 2010). Subsequent analyses revealed that vaccinated animals were capable of producing strong antigen-specific peripheral blood mononuclear cell (PBMC) proliferation responses to the vaccine antigens, lasting over one year, and sustained plasma antibody levels in immunised animals (Carey et al., 2010). *In vitro* experiments using sera from vaccinated animals were also able to show that koala IgG antibodies were functionally active since they could neutralise a chlamydial infection of McCoy cells.

The efficacy of this multi-subunit chlamydial vaccine approach in the koala was further examined by vaccination of captive healthy koalas and koalas with clinical signs of chlamydial disease (Kollipara et al., 2012). The multi-subunit antigen preparation used in this study included a recombinant *C. pecorum* MOMP and a conserved protein, NrdB, both cloned from sequences amplified from a koala *C. pecorum* isolate from South-East Queensland. Importantly, in light of studies in other animal models showing an increase in immunopathogenic consequences following exposure to chlamydial antigens (Cappello et al., 2009), no worsening in clinical disease signs was observed in any of the vaccinated koalas. Lasting vaccine antigen-specific IgG antibodies could be detected in healthy vaccinated animals in plasma and ocular secretions and, to the author's surprise, vaccination also appeared to enhance the baseline immune response of koalas with existing chlamydial infection and disease, despite the fact that these koalas were infected with a *C. pecorum* strain with a genetically distinct MOMP. Unlike the initial study, *in vitro* neutralisation assays confirmed that serum IgGs in vaccinated koalas could neutralise *C. pecorum* infections.

While the data from these initial vaccine studies are encouraging, particularly in stimulating a *C. pecorum*-specific humoral immune response, there is still no proof that vaccination will actually protect against live challenge infection to immunised animals. These measures will continue to be difficult without completion of traditional challenge experiments or a further ability to measure the Type I immune response to chlamydial infection in these animals.

7. The origin and evolution of *Chlamydia* in koalas?

The predicted divergence of marsupials from placental mammals approximately 145 million years ago (Bininda-Emonds et al., 2007) and the concurrent geographic separation of Australia from Gondwana has raised fascinating questions about the origin of chlamydial infections in extant marsupials such as the koala, the answers of which may give us insight into the evolution and adaptation of these pathogens in other hosts, including humans.

The recent sequencing of the koala *C. pneumoniae* genome has created the first opportunities to explore this further (Myers et al., 2009). Prior to this, sequencing of four human *C. pneumoniae* respiratory tract strain genomes found that they were effectively clonal, with less than 200 single nucleotide polymorphisms identified between each of the isolates. Comparative analysis of the koala *C. pneumoniae* genome, as the only current example of an animal *C. pneumoniae strain*, surprisingly revealed that it appears basal to these human strains. This conclusion was supported by the identification of several genes in the koala *C. pneumoniae* genome that were intact compared to their human *C. pneumoniae* gene homologues that were either fragmented, truncated or in the process of gene decay. Subsequent analysis of 23 distinct genetic loci in *C. pneumoniae* isolates from humans, including indigenous Australian isolates, and of animal origins from around the world, including isolates from a horse, reptiles (snakes, chameleon), amphibians (frogs) and marsupials (koalas, potoroos, bandicoots) revealed that there are at least five distinct *C. pneumoniae* genovars circulating worldwide (Mitchell et al., 2010). In this mixture, the koala strain was most similar to human indigenous Australian isolates identified from two distinct geographic regions leading to the suggestion that at least one of these genovars was endemic to Australia and that zoonotic transmission had occurred in the evolutionary history of the koala strain of *C. pneumoniae*. Interestingly, these zoonotic events may prove to be more regular than thought since molecular evidence also exists for the presence of koala-like *C. pneumoniae* strains in the carotid artery plaques of humans with atherosclerotic disease (Cochrane et al., 2005).

The origin and evolution of *C. pecorum*, the most pathogenic of the two species infecting koalas is less clear. The first evidence to point to an origin for this pathogen in the koala came from *ompA* genotyping studies of koala *C. pecorum* strains in comparison to strains from cattle and sheep (Jackson et al., 1997). This analysis found significant sequence diversity at variable domain IV of *ompA* (29.5% nucleotide sequence dissimilarity) within geographically-distinct koala *C. pecorum* strains analysed but, interestingly, in several instances, koala *C. pecorum* sequences were identified that were identical or more closely similar to Australian sheep and cattle isolates than they were to each other. This was a surprising outcome from this analysis and the combination of (a) high sequence diversity; and the (b) presence of certain koala *C. pecorum* sequences that distinctly clustered with cattle and sheep strains over other koala strains was used to conclude that koala *C. pecorum* strains had originated in koalas from multiple cross-host transmission events from cattle and sheep (Jackson et al., 1997). The presumption in this case is that these strains were transmitted upon the introduction of cattle and sheep into Australia upon European colonisation in the late 18th century, although there is no evidence to support this yet.

With question marks emerging over whether *ompA* genotyping does indeed reflect the phylogeny of *Chlamydia* (Brunelle & Sensabaugh, 2006), a more recent molecular analyses has raised questions over this earlier hypothesis on the origin of *C. pecorum* in koalas. Using a multi-locus sequence analysis approach, including *ompA* and several novel molecular typing genes, it was found that koala *C. pecorum* strains examined formed a monophyletic clustering that was obviously distinct from *C. pecorum* strains from livestock, however, no Australian livestock strains were included in this analysis (Marsh et al., 2011). If a link between *C. pecorum* strains infecting koalas, sheep and cattle does indeed exist, then these data would suggest that rapid diversification in the koala has occurred, possibly supporting isolated cross-host transmission and selection in this new host - consistent with the high pathogenic potential of this bacterium in the koala, compared to *C. pneumoniae*.

Set against the ongoing efforts to understand the evolution of chlamydial infections in koalas is the repeated observation that these pathogens also infect and cause primarily ocular disease in several other Australian marsupials including Greater Gliders (*Petauroides volans)*, Brushtail Possums (*Trichosurus caninus*) and Western-barred bandicoots (*Perameles bougainville*) (Bodetti et al., 2003; Warren et al., 2005). Less is known about the epidemiology of these diseases in their hosts but further analysis of these isolates, alongside chlamydial strains infecting domesticated Australian animals, will be central to efforts to further elucidate the origin of these chlamydial pathogens in koalas.

8. Future directions

The future of research into chlamydial diseases in the koala offers hope for efforts to conserve this Australian wildlife species but will also face continued difficulties. A primary challenge will be the lack of fundamental knowledge of the marsupial and koala immune system, and a paucity of reagents to measure the koala chlamydial immune response. A balanced adaptive immune response appears to be important for a successful response to chlamydial infection, highlighted by the significant level of immunopathology evident in chlamydial infection of the female lower genital tract in other host species (Hafner et al., 2008). In humans and other animal models, chronic inflammation of the female upper reproductive tract is driven by persistent or recurrent infections, and nothing is known about this in the koala due to the absence of (a) immunological reagents to measure systemic and localised immune responses to infection; and (b) time course data observing the outcome of individual koalas infected with *C. pecorum* or *C. pneumoniae*. While the recent sequencing of genomes of related Australian marsupials (Renfree et al., 2011) should assist with the design of koala-specific

immune reagents, these aspects of koala immunology will need attention if we are to understand the relationship between chlamydial infection and disease in this host more clearly.

The interactions between the koala immune response to chlamydial infection and the potential effects of exogenous and endogenous KoRV infections are quite unknown. KoRV appears to be endemic in northern Australia but less so in koalas in Australia's southern states (Simmons et al., 2012). KoRV is associated with lymphoid neoplasia and there are limited data to suggest that there is a link between KoRV infection and susceptibility to chlamydial disease (Tarlinton et al., 2005). Given the prevalence of this retrovirus in Australian koalas, this link is obviously concerning, but more research is required to establish both a causal link between KoRV and immunosuppressive diseases and the relationship to chlamydial infection.

Our review of the limited set of epidemiology data highlights the reliance on cross-sectional data and small studies, not necessarily involving studies of complete populations, for understanding the prevalence and outcomes of chlamydial infection – a limitation that means that the true impact of chlamydial infections on affected populations is largely unknown. Although logistically challenging, an important focus of future koala chlamydial research efforts should be the incorporation of longitudinal studies of koala populations, and of individual animals within a population, to form a greater understanding of the outcomes and factors that may influence progression of chlamydial infection and disease.

Koala chlamydial research efforts also hold tantalising possibilities for a wider understanding of chlamydial infections in other host species. Comparative genomic analysis of koala *C. pneumoniae* has already shed important insights into the origin of the human *C. pneumoniae* (Myers et al., 2009; Mitchell et al., 2010) but similar analysis of *C. pecorum* in strains from koalas, Australian livestock and livestock strains from the rest of the world should be similarly rewarding. The geographic isolation of Australian animals from the rest of the world gives researchers a unique opportunity in the chlamydial research world to identify genetic changes that may be associated with host adaptation and the opportunity to place a timeline on these events, depending on the origin of this pathogen in the koala. This and related research efforts, combined with the tireless efforts of wildlife carers, veterinarians and other researchers will contribute importantly to preserving this iconic native species for the enjoyment of future generations.

Acknowledgements

We would like to thank Joanne Loader, Courtney Waugh and Eileen Roulis for assistance in preparation of the figures for this review. AP was supported by a 2010/2011 Australian Academy of Science "Margaret Middleton Fund for Endangered Wildlife" Early Career Research Award. This work was financially supported by an Australian Research Council Linkage Grant awarded to PT and the Queensland State Government NIRAP scheme.

References

Bininda-Emonds, O.R., Cardillo, M., Jones, K.E., MacPhee, R.D., Beck, R.M., Grenyer, R., Price, S.A., Vos, R.A., Gittleman, J.L., Purvis, A., 2007. The delayed rise of present-day mammals. Nature 446, 507–512.

Blanshard, W., Bodley, K., 2008. Koalas. In: Vogelnest, L., Woods, R. (Eds.), Medicine of Australian Mammals. CSIRO Publishing, Victoria, Australia, pp. 227–327.

Bodetti, T.J., Viggers, K., Warren, K., Swan, R., Conaghty, S., Sims, C., Timms, P., 2003. Wide range of *Chlamydiales* types detected in native Australian mammals. Vet. Microbiol. 96, 177–187.

Bodetti, T.J., Johnston, S., Pospischil, A., Knox, C., Timms, P., 2002. Screening semen from koalas (*Phascolarctos cinereus*) for *Chlamydia* species by PCR. Vet. Rec. 151, 147–149.

Bodetti, T.J., Timms, P., 2000. Detection of *Chlamydia pneumoniae* DNA and antigen in the circulating mononuclear cell fractions of humans and koalas. 68, 2744–2747.

Brown, A.S., Girjes, A.A., Lavin, M.F., Timms, P., Woolcock, J.B., 1987. Chlamydial disease in koalas. 64, 346–350.

Brown, A.S., Grice, R.G., 1984. Isolation of *Chlamydia psittaci* from koalas (*Phascolarctos cinereus*). Aust. Vet. J. 61, 413.

Brunelle, B., Sensabaugh, G., 2006. The *ompA* gene in *Chlamydia trachomatis* differs in phylogeny and rate of evolution from other regions of the genome. Infect. Immun. 74, 578.

Canfield, P.J., 1989. A survey of urinary tract disease in New South Wales koalas. Aust. Vet. J. 66, 103–106.

Cappello, F., Conway de Macario, E., Di Felice, V., Zummo, G., Macario, A.J., 2009. *Chlamydia trachomatis* infection and anti-Hsp60 immunity: the two sides of the same coin. PLoS Pathog. 5, 1000552.

Carey, A., Timms, P., Rawlinson, G., Brumm, J., Nilsson, K., Harris, J.M., Beagley, K.W., 2010. A multi-subunit chlamydial vaccine induces antibody and cell-mediated immunity in immunized koalas (*Phascolarctos cinereus*): comparison of three different adjuvants. Am. J. Reprod. Immunol. 63, 161–172.

Cochrane, M., Walker, P., Gibbs, H., Timms, P., 2005. Multiple genotypes of *Chlamydia pneumoniae* identified in human carotid plaque. Microbiology 151, 2285–2290.

Cockram, F.A., Jackson, A.R.B., 1974. Isolation of a *Chlamydia* from cases of keratoconjunctivitis in koalas. Aust. Vet. J. 50, 82–83.

Department of Environment and Resource Management, 2009. Decline of the Koala Coast Koala Population: Population Status in 2008. Queensland Government, Brisbane, Australia.

Devereaux, L.N., Polkinghorne, A., Meijer, A., Timms, P., 2003. Molecular evidence for novel chlamydial infections in the koala (*Phascolarctos cinereus*). Syst. Appl. Microbiol. 26, 245–253.

Dique, D., Thompson, J., Preece, H.J., Penfold, G.C., de Villiers, D.L., Leslie, R.S., 2003. Koala mortality on roads in south-east Queensland: the koala speed-zone trial. Wildl. Res. 30, 419–426.

Everett, K.D., Bush, R.M., Andersen, A., 1999. Emended description of the order *Chlamydiales*, proposal of *Parachlamydiaceae* fam. nov. and *Simkaniaceae* fam. nov., each containing one monotypic genus, revised taxonomy of the family *Chlamydiaceae*, including a new genus and five new species, and standards for the identification of organisms. Int. J. Syst. Bacteriol. 2, 415–440.

Hirst, L.W., Brown, A.S., Kempster, R., Hall, J., Woolcock, J.B., et al., 1992. Keratitis in free-ranging koalas (*Phascolarctos cinereus*) on Magnetic Island, Townsville. J. Wildl. Dis. 28, 424–427.

Girjes, A.A., Carrick, F.N., Lavin, M.F., 1994. Remarkable sequence relatedness in the DNA encoding the major outer membrane protein of *Chlamydia psittaci* (koala type I) and *Chlamydia pneumoniae*. Gene 138, 139–142.

Girjes, A.A., Hugall, A.F., Timms, P., Lavin, M.F., 1988. Two distinct forms of *Chlamydia psittaci* associated with disease and infertility in *Phascolarctos cinereus* (koala). Infect. Immun. 56, 1897–1900.

Glassick, T.V., Giffard, P., Timms, P., 1996. Outer membrane protein 2 gene sequences indicate that two chlamydial species, *Chlamydia pecorum* and *Chlamydia pneumoniae* cause infections in koalas. Syst. Appl. Microbiol. 19, 457–464.

Gordon, G., McGreevy, D.G., 1978. The status of the koala in Queensland. In: Bergin, T.J. (Ed.), The Koala: Proceedings of the Taronga Symposium. Zoological Parks Board of N.S.W., Sydney, pp. 125–131.

Griffith, J.E., Higgins, D.P., Li, K.M., Krockenberger, M.B., Govendir, M., 2010. Absorption of enrofloxacin and marbofloxacin after oral and subcutaneous administration in diseased koalas (*Phascolarctos cinereus*). J. Vet. Pharmacol. Ther. 33, 595–604.

Hafner, L.M., Beagley, K.W., Timms, P., 2008. *Chlamydia trachomatis* infection: host immune responses and potential vaccines. Mucosal Immunol. 1, 116–130.

Hanger, J., Loader, J., Wan, C., Beagley, K.W., Timms, P., Polkinghorne, A., 2012. Comparison of antigen detection and quantitative PCR in the

detection of chlamydial infection in koalas (*Phascolarctos cinereus*). Vet. J., http://dx.doi.org/10.1016/j.tvjl.2012.07.024, [epub ahead of print].

Jackson, M., White, N., Giffard, P., Timms, P., 1999. Epizootiology of *Chlamydia* infections in two free-range koala populations. Vet. Microbiol. 65, 255–264.

Jackson, M., Giffard, P., Timms, P., 1997. Outer membrane protein A gene sequencing demonstrates the polphyletic nature of koala *Chlamydia pecorum* isolates. Syst. Appl. Microbiol. 20, 187–200.

Kollipara, A., George, C., Hanger, J., Loader, J., Polkinghorne, A., Beagley, K., Timms, P., 2012. Vaccination of healthy and diseased koalas (*Phascolarctos cinereus*) with a *Chlamydia pecorum* multi-subunit vaccine: Evaluation of immunity and pathology. Vaccine 30, 1875–1885.

Loader, J., 2010. An investigation of the health of Wild Koala populations in South-East Queensland. Honours Thesis. The University of Queensland, St. Lucia, Brisbane, Australia.

Lunney, D., Gresser, S., O'Neil, L.E., Matthews, A., Rohdes, J.R., 2007. The impact of fire and dogs on koalas at Port Stephens, New South Wales, using population viability analysis. Pacific Conserv. Biol. 13, 189–201.

Mackenzie, C., 1919. The Comparative Anatomy of Australian Mammals. Jenkin Buxton, Melbourne.

Markey, B., Wan, C., Hanger, J., Phillips, C., Timms, P., 2007. Use of quantitative real-time PCR to monitor the shedding and treatment of chlamydiae in the koala (*Phascolarctos cinereus*). Vet. Microbiol. 120, 334–342.

Marsh, J., Kollipara, A., Timms, P., Polkinghorne, A., 2011. Novel molecular markers of *Chlamydia pecorum* genetic diversity in the koala (*Phascolarctos cinereus*). BMC Microbiol. 11, 77.

McInnes, L.M., Gillett, A., Hanger, J., Reid, S.A., Ryan, U.M., 2011. The potential impact of native Australian trypanosome infections on the health of koalas (*Phascolarctos cinereus*). Parasitology 27, 1–11.

Melzer, A., Carrick, F., Menkhorst, P., Lunney, D., John, B.S., 2000. Overview, critical assessment, and conservation implications of koala distribution and abundance. Conserv. Biol. 14, 619–628.

Mitchell, C.M., Hutton, S., Myers, G.S., Brunham, R., Timms, P., 2010. *Chlamydia pneumoniae* is genetically diverse in animals and appears to have crossed the host barrier to humans on (at least) two occasions. PLoS Pathog. 6, e1000903.

Myers, G.S., Mathew, S.A., Eppinger, M., Mitchell, C., O'Brien, K.K., White, O.R., Benahmed, F., Brunham, R.C., Read, T.D., Ravel, J., Bavoil, P.M., Timms, P., 2009. Evidence that human Chlamydia pneumoniae was zoonotically acquired. J Bacteriol. 191, 7225–7233.

Pratt, A., 1934. Koala fiseases. In: The Call of the Koala. Robertson and Mullen, Melbourne, Australia, pp. 96–99.

Renfree, M.M., Papenfuss, A.T., Deakin, J.E., Lindsay, J., Heider, T., Belov, K., Rens, W., Waters, P.D., Pharo, E.A., Shaw, G., Wong, E.S., Lefèvre, C.M., Nicholas, K.R., Kuroki, Y., Wakefield, M.J., Zenger, K.R., Wang, C., Ferguson-Smith, M., Nicholas, F.W., Hickford, D., Yu, H., Short, K.R., Siddle, H.V., Frankenberg, S.R., Chew, K.Y., Menzies, B.R., Stringer, J.M., Suzuki, S., Hore, T.A., Delbridge, M.L., Patel, H.R., Mohammadi, A., Schneider, N.Y., Hu, Y., O'Hara, W., Al Nadaf, S., Wu, C., Feng, Z.P., Cocks, B.G., Wang, J., Flicek, P., Searle, S.M., Fairley, S., Beal, K., Herrero, J., Carone, D.M., Suzuki, Y., Sugano, S., Toyoda, A., Sakaki, Y., Kondo, S., Nishida, Y., Tatsumoto, S., Mandiou, I., Hsu, A., McColl, K.A., Lansdell, B., Weinstock, G., Kuczek, E., McGrath, A., Wilson, P., Men, A., Hazar-Rethinam, M., Hall, A., Davis, J., Wood, D., Williams, S., Sundaravadanam, Y., Muzny, D.M., Jhangiani, S.N., Lewis, L.R., Morgan, M.D., Okwuonu, G.O., Ruiz, S.J., Santibanez, J., Nazareth, L., Cree, A., Fowler, G., Kovar, C.L., Dinh, H.H., Joshi, V., Jing, C., Lara, F., Thornton, R., Chen, L., Deng, J., Liu, Y., Shen, J.Y., Song, X.Z., Edson, J., Troon, C., Thomas, D., Stephens, A., Yapa, L., Levchenko, T., Gibbs, R.A., Cooper, D.W., Speed, T.P., Fujiyama, A., Graves, J.A., O'Neill, R.J., Pask, A.J., Forrest, S.M., Worley, K.C., 2011. Genome sequence of an Australian kangaroo, *Macropus eugenii*, provides insight into the evolution of mammalian reproduction and development. Genome Biol. 12, R81.

Rhodes, J.R., Chooi, F.N., de Villiers, D.L., Preece, H.J., McAlpine, C.A., Possingham, H.P., 2011. Using integrated population modelling to quantify the implications of multiple threatening processes for a rapidly declining population. Biol. Conserv. 144, 1081–1088.

Sachse, K., Vretou, E., Livingstone, M., Borel, N., Pospischil, A., Longbottom, D., 2009. Recent developments in the laboratory diagnosis of chlamydial infections. Vet. Microbiol. 135, 2–21.

Simmons, G.S., Young, P.R., Hanger, J.J., Jones, K., Clarke, D., McKee, J.J., Meers, J., 2012. Prevalence of koala retrovirus in geographically diverse populations in Australia. Aust. Vet. J. 90, 404–409.

Stephens, R.S., Myers, G., Eppinger, M., Bavoil, P.M., 2009. Divergence without difference: phylogenetics and taxonomy of *Chlamydia* resolved. FEMS Immunol. Med. Microbiol. 55, 115–119.

Tarlinton, R., Meers, J., Hanger, J., Young, P., 2005. Real-time reverse transcriptase PCR for the endogenous koala retrovirus reveals an association between plasma viral load and neoplastic disease in koalas. J. Gen. Virol. 86, 1–5.

Timms, P., Eaves, F.W., Girjes, A.A., Lavin, M.F., 1988. Comparison of *Chlamydia psittaci* isolates by restriction endonuclease and DNA probe analyses. Infect. Immun. 56, 287–290.

Troughton, E.L.G., 1941. Furred Animals of Australia. Angus & Robertson, Sydney.

Wan, C., Loader, J., Hanger, J., Beagley, K., Timms, P., Polkinghorne, A., 2011. Using quantitative polymerase chain reaction to correlate *Chlamydia pecorum* infectious load with ocular, urinary and reproductive tract disease in the koala (*Phascolarctos cinereus*). Aust. Vet. J. 89, 409–412.

Warren, K., Swan, R., Bodetti, T., Friend, T., Hill, S., Timms, P., 2005. Ocular *Chlamydiales* infections of western barred bandicoots (*Perameles bougainville*) in Western Australia. J. Zoo Wildl. Med. 36, 100–102.

Whittington, R., 2001. Chlamydiosis of koalas. In: Williams, E.S., Barker, I.K. (Eds.), Infectious Diseases of Wild Mammals. Blackwell Publishing, United States, pp. 423–434.

Wood, M.M., Timms, P., 1992. Comparison of nine antigen detection kits for diagnosis of urogenital infections due to *Chlamydia psittaci* in koalas. J. Clin. Microbiol. 30, 3200–3205.