

**STUDIES INTO THE DIAGNOSIS,
TREATMENT AND MANAGEMENT OF
CHLAMYDIOSIS IN KOALAS**

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**A thesis submitted in fulfilment of the requirements for
the degree of Doctor of Philosophy**

Faculty of Veterinary Science

The University of Sydney

2010

STATEMENT OF ORIGINALITY

Apart from assistance acknowledged, this thesis represents the unaided work of the author.

The text of this thesis contains no material previously published or written unless due reference to this material is made. This work has neither been presented nor is currently being presented for any other degree.

Joanna Griffith

March 2010

ACKNOWLEDGEMENT OF THE CONTRIBUTION TO RESEARCH WORK AND/OR AUTHORSHIP

This thesis includes one original paper published in a peer-reviewed journal (Chapter 7).

The core theme of the thesis is the diagnosis, treatment and management of chlamydiosis in koalas.

The ideas, development and writing of all the chapters and papers in this thesis were the principal responsibility of the candidate, working independently within the Faculty of Veterinary Science under the supervision of Damien Higgins (principal supervisor), Merran Govendir (associate supervisor), Mark Krockenberger (associate supervisor), Paul Canfield (associate supervisor), Sue Hemsley (associate supervisor) and Richard Malik (associate supervisor).

The inclusion of co-authors in the authorship of Chapter 7 reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

ACKNOWLEDGEMENTS

If the production of a thesis is a journey, then there are many fellow passengers who have travelled alongside me for some, or all, of the trip.

Firstly I would like to thank my supervisors, in particular my principal supervisor, Dr Damien Higgins. Without his unfailing support, diligent hard work beyond expectation and sage advice, this research project would have foundered at the first post. My associate supervisors also deserve heartfelt thanks; each has contributed in their own way. I thank Dr Merran Govendir for expert advice in planning the pharmacology work, organising access to appropriate equipment for HPLC, unflagging support in the face of technical difficulties and reading more drafts than should be humanly possible. Dr Mark Krockenberger was fundamental in formulating and refining the project in the early stages, was tremendously supportive, and always found time to discuss aspects of the work. Prof Paul Canfield provided immense inspiration, astute advice and incredible help in bringing this thesis together as one body of work. Drs Sue Hemsley and Richard Malik both provided assistance particularly in the early stages in refining this work.

Special mention must be made of the invaluable help and advice provided by Dr Kong Li (University of Sydney) with pharmacology method development and Prof Andrew McLachlan (University of Sydney) with calculation of pharmacokinetic parameters. Dr Ross Harley (Bristol University) and Dr Mike Bunce (Murdoch University) were instrumental in providing indispensable and timely advice on real-time PCR primer and method development from far away. Dr Navneet Dhand (University of Sydney) was invaluable in providing help with statistical analysis, assisted by reading drafts and always exhibited tireless patience and good cheer. Dr Peter Holz (Zoos Victoria) graciously collected and stored samples from koalas housed at Healesville Sanctuary in Victoria.

This work comprised a large amount of time spent in the field, which would not have been possible without help. Cheyne Flanagan (Koala Preservation Society of NSW) was instrumental in providing field work assistance, training in all things “koala” and insight into the rehabilitation of these animals, and was unflagging in her support of research in koalas. Without Cheyne’s efforts to bring together researchers and wildlife rehabilitators, this investigation would not have been possible. Andrea Reiss, Bronwyn Fancourt, Sarah Jobbins, Kim Skogvold, Alison Stewart and Jade Norris also provided essential assistance with field work. In particular, Bronwyn Fancourt put in many hours of unpaid volunteer labour in the field and provided friendship and support throughout. I would like to thank the executive committee (2005-2009), the team leaders, associate supervisors, koala rescuers, maintenance team, leaf collectors and data-enterers of the Koala Preservation Society of NSW for their invaluable help during my time at the Koala Hospital. Finally, many of the Koala Preservation Society of NSW’s volunteers have worked tirelessly to help with this and past research projects: their ongoing support and enthusiasm for koala research over many years has immensely advanced knowledge of diseases affecting koalas and is to be applauded.

Within the faculty I thank: Karen Barnes, Elaine Chew, Svetlana Patoka, Denise Wigney, Benjamin Kimble, Angelika Trube and Dr Tiziana Beninati for their generous help with various laboratory techniques. Many other people too numerous to name have supported my work in large and small ways: I am sincerely grateful to all for their generosity.

Thanks go to my fellow PhD students Sarah Jobbins, Nathan Saul, Elissa Kluger, Jo White, Michelle Lepherd, Quintin Lau, Julie Irish, Georgiana Sheridan and Alison Stewart, who shared the moments of triumph, and of anguish, and helped shoulder the load.

I thank my family and friends, who have tolerated physical and mental absences on so many occasions. My parents and many of my extended family provided inspiration for a career in science. Finally, the most indispensable person to the whole adventure was, and is, my partner, Simon Craig, who has supported me in so many ways and whose patient encouragement, personal sacrifice, tolerance and faith in my ability to finish this process were crucial.

This study was supported by the Australian Research Council, The Koala Preservation Society of NSW, Australian Koala Foundation, Symbion Vetnostics, Pfizer Inc and the Wildlife Information Rescue and Education Service (ARC Linkage Grant LP0560572). Pfizer Inc provided the analytical grade marbofloxacin, Vetoquinol donated injectable marbofloxacin, Schering Plough donated analytical grade orbifloxacin and Ulco Medical donated equipment. Professional proofreading services were provided by Dr Julie Irish. The author was supported by funds provided by an Australian Postgraduate Award, the University of Sydney Postgraduate Research Support Scheme and the Eric Horatio Maclean Scholarship scheme. This study was approved by The University of Sydney Animal Care and Ethics Committee - N00/4-2005/3/4088 and carried out under permit (S10171) issued by the National Parks and Wildlife Service of NSW.

SUMMARY OF THE THESIS

Koalas are an iconic Australian marsupial species that attract much public sympathy and support. Despite several thousand koalas being presented to wildlife rehabilitation facilities annually for treatment of traumatic injuries (primarily motor vehicle strikes and dog predation) and disease (principally chlamydiosis), little information exists regarding the success of treatments or whether rehabilitated animals survive in the wild after release. This thesis examines several aspects of the diagnosis, treatment and management of the most important infectious disease of koalas, chlamydiosis, and provides an evidence base for rational diagnostic and treatment decisions in the rehabilitation setting.

Experimental work commences in Chapter 2 with a study of the admission records of a large koala rehabilitation facility (the Koala Hospital of the Koala Preservation Society of NSW) showing that traumatic presentations and those relating to clinical chlamydiosis were most common, with motor vehicle collisions apparently a significant and increasing threat to survival of the local koala population. The implications of these findings are discussed with reference to measures aimed at maintaining a viable population of wild koalas in Port Macquarie and for logistic planning at the Koala Hospital.

Initial studies in this thesis confirmed that koalas with chlamydiosis are frequently treated at wildlife rehabilitation facilities. Despite the commonness of this disease, there is a lack of rigorous scientific studies examining frequently used treatments. Chapter 3, a retrospective review of medical records of a cohort of koalas admitted for treatment for chlamydiosis, revealed that diagnostic and treatment decisions were frequently based on clinical signs alone and treatment choices and durations were inconsistent with those used to successfully treat chlamydiosis in other species. Despite this, treated animals were frequently released and many survived in the wild.

Chapter 4 outlines general methods common to the clinical work undertaken in Chapters 5, 7 and 9.

Antibiotic treatment with drugs commonly used to treat chlamydiosis in other species (erythromycin, oxytetracycline) has led to wasting and death in koalas. A pilot study, presented in Chapter 5, found that, similarly, more modern forms of these drugs (doxycycline and azithromycin) cannot be used safely in koalas, leading to the author's decision to investigate, in detail, the efficacy of the less conventional anti-chlamydial drugs, the fluoroquinolones.

Studies of marsupial pharmacokinetics are uncommon and, prior to this thesis, there were no published studies of pharmacokinetics in koalas. The author's investigations, using a modified agar diffusion assay (Chapter 6) and high performance liquid chromatography (Chapter 7), found the absorption of enrofloxacin and marbofloxacin by the oral route in koalas was extremely poor and suggested absorption rate limited disposition pharmacokinetics. In combination with plasma protein binding of approximately 50%, the concentrations of enrofloxacin and marbofloxacin achieved in plasma were not considered likely to inhibit the growth of chlamydial pathogens *in vivo*.

In Chapter 8 the author explored the apparent contradiction between the failure to achieve appropriate plasma concentrations of fluoroquinolones to treat chlamydiosis and the apparent efficacy of these drugs reported in historical medical records. Methods to monitor clinical signs by clinical scoring and chlamydial load using real-time polymerase chain reaction were developed during the study. The results of these studies showed that clinical signs were poorly sensitive in determining the presence of chlamydial organisms in koalas; all fluoroquinolone treatment regimes led to a dramatic reduction in *Chlamydomphila pecorum* load during treatment; and clinical signs improved in many animals. Importantly,

however, pathogen load rebounded after withdrawal of treatment, indicating that most animals failed to clear infections. These findings have implications for the diagnosis, and treatment of chlamydial disease in koalas and for the subsequent return of fluoroquinolone treated animals to the wild.

The findings and limitations of these studies are presented in general terms in Chapter 9 and recommendations for future studies are proposed.

GLOSSARY

ALKP	alkaline phosphatase
ALT	alanine transaminase
ANOVA	analysis of variance
AST	aspartate aminotransferase
ATP	adenosine triphosphate
AUC	area under the curve
BID	twice daily
BIW	twice weekly
bp	base pairs
BSP	bromosulphalein
cAMP	cyclic adenosine monophosphate
chi-sq	chi-square test for goodness of fit
CI	confidence interval
CK	creatinine kinase
C_{\max}	maximum plasma concentration
C_T	cycle threshold (real-time polymerase chain reaction)
Ct	last measurable concentration above level of quantification (high performance liquid chromatography)
d.f.	degrees of freedom

EDTA	ethylene diamine tetra-acetic acid
ELISA	enzyme linked immunosorbent assay
HPLC	high performance liquid chromatography
i.m.	intramuscular injection
K_{el}	elimination constant
Koala Hospital	Koala Preservation Society of New South Wale's Koala Hospital in Port Macquarie, New South Wales, Australia
LOD	limit of detection
LOQ	limit of quantification
MVA	motor vehicle accident
NSW	New South Wales, Australia
OR	odds ratio
PCR	polymerase chain reaction
p.o.	per os
Qld	Queensland, Australia
qPCR	real-time polymerase chain reaction
REML	restricted maximum likelihood procedure
s.c.	subcutaneous injection
SD	standard deviation
SEM	standard error of the mean

SID	once daily
SIW	once weekly
T _{max}	maximal plasma concentration
t _{1/2}	half life
TWC	tooth wear class
Vic	Victoria, Australia
vs.	versus

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CHAPTER 1 - GENERAL INTRODUCTION, LITERATURE REVIEW AND AIMS OF THE STUDY

The koala (*Phascolarctos cinereus*), a popular and iconic animal in Australia, faces many threats to survival (Department of the Environment, Water, Heritage and the Arts, 2009). One effort to mitigate threats to wildlife is rehabilitation of injured, diseased, displaced and orphaned wildlife; and such efforts may contribute to maintenance of some wildlife populations (Tribe & Brown, 2000). Koalas appear particularly suitable for rehabilitation. In studies to date post-release survival of rehabilitated animals appears to be high: 70 - 100% of rehabilitated koalas survived over 2 - 11 months monitoring (Ellis, White *et al.*, 1990; Tribe, Hanger *et al.*, 2005; Jones, 2008) and survival rates were no different to that of wild controls (Lunney, Gresser *et al.*, 2004). In contrast, post-release survival of other rehabilitated wildlife in Australia and overseas is often poor¹.

¹ Hand-reared ringtail possums (*Pseudocheirus peregrinus*) survived a median 31 days c.f. median 169 days of wild controls (Augee, M.L., Smith, B. & Rose, S. (1996) Survival of wild and hand-reared ringtail possums (*Pseudocheirus peregrinus*) in bushland near Sydney. *Wildlife Research*, **23**(1), 99-108.);

70% of healthy translocated wild brushtail possums (*Trichosurus vulpecula*) died within a week (Pietsch, R. (1994) The fate of urban common brushtail possums translocated to sclerophyll forest. In *Reintroduction biology of Australian and New Zealand fauna*. Ed Serena, M., Surrey Beatty, Chipping Norton, NSW. pp 236-246.);

44-59% survival of rehabilitated little penguins (*Eudyptula minor*) c.f. 50-77% of wild controls at 20 months (Goldsworthy, S., Giese, M., Gales, R., Brothers, N. & Hamill, J. (2000) Effects of the Iron Baron oil spill on little penguins (*Eudyptula minor*). II. Post-release survival of rehabilitated oiled birds. *Wildlife Research*, **27**(6), 573-582.);

Rehabilitated barn owls (*Tyto alba*) survived mean 77.9 days c.f. 365.7 days survival of wild controls (Fajardo, I., Babiloni, G. & Miranda, Y. (2000) Rehabilitated and wild barn owls (*Tyto alba*) dispersal, life expectancy and mortality in Spain. *Biological Conservation*, **94**(3), 287-295.);

Hand-reared fox cubs (*Vulpes vulpes*) survived a mean 94 days c.f. 466 days of wild controls (Robertson, C.P.J. & Harris, S. (1995) The condition and survival after release of captive-reared fox cubs. *Animal Welfare*, **4**(4), 281-294.);

55% of river otters (*Lontra canadensis*) were alive at 100 days compared to 80% of wild controls (Ben-David, M., Blundell, G. & Blake, J. (2002) Post-release survival of river otters: effects of exposure to crude oil and captivity. *Journal of Wildlife Management*, **66**(4), 1208-1223.);

52% of hand-reared white tailed deer fawns (*Odocoileus virginianus*) died within 30 days of release (Beringer, J., Mabry, P., Meyer, T., Wallendorf, M. & Eddleman, W. (2004) Post-release survival of rehabilitated white-tailed deer fawns in Missouri. *Wildlife Society Bulletin*, **32**(3), 732-738.).

Despite the treatment of many thousands of koalas annually in wildlife rehabilitation facilities, little information exists regarding which treatments are most successful in returning koalas to the wild (Blanshard, 1994). Chlamydiosis is the most common infectious disease of koalas with up to 85% of some populations affected (Jackson, White *et al.*, 1999), and treatment of this disease in the wildlife rehabilitation setting is common despite equivocal results as to its success (Cockram & Jackson, 1976; Bodetti, Johnston *et al.*, 2002; Markey, Wan *et al.*, 2007) and the unknown effect of rehabilitated animals on wild populations.

This introductory chapter first examines the popularity and uniqueness of koalas as a motivation for their preservation and as a driving force for wildlife carers. Threats to the survival of koalas are considered, with particular reference to disease caused by chlamydial organisms. The ethics and success of wildlife rehabilitation, in particular the ethics of treating infectious disease, are discussed. The clinical signs and pathology of chlamydiosis in koalas are summarised, and literature describing diagnostic tests and past treatments examined. The background literature suggesting the potential for unusual pharmacokinetics in koalas is discussed. Finally, the key gaps in the body of knowledge are highlighted to identify future directions of research and the aims of the thesis are outlined.

1.1 WHY CARE FOR KOALAS?

Koalas elicit enormous public interest and sympathy, and feature frequently in the media around the world. Their annual economic contribution to the tourist industry has been estimated to be worth several billion dollars (Hundloe & Hamilton, 1997). They are one of few wild Australian animals with multiple rehabilitation centres solely dedicated to their care and a number of non- governmental organisations dedicated to preservation of the

species². Clearly, there is huge concern as to the plight of this iconic and charismatic animal.

Although the public may be interested in preserving the koala for its own sake, the loss of koalas as a species would be a tragedy on a number of levels. Koalas, the only living representative of the family Phascolarctidae, have unique anatomical (Snipes, Snipes *et al.*, 1993), physiological (Cork & Dawson, 1983) and behavioural (Nagy & Martin, 1985) adaptations to life as an obligate eucalypt folivore and occupy an unusual ecological niche, most analogous to that of the neotropical sloth, *Bradypus variegates* (Nagy & Martin, 1985) of Central and South America. Thus, study of these animals provides unique opportunities to learn about Australian evolutionary history and adaptation of marsupials to difficult environments. Secondly, koalas may be useful to medical research by providing a naturally occurring biological model for diseases such as cryptococcosis and chlamydiosis in other species, including people (Krockenberger, Canfield *et al.*, 2002; Higgins, Hemsley *et al.*, 2005b). Finally, loss of biodiversity may have wide reaching effects including changes to the balance of ecosystems, and wider social, political and economic effects (Clark, Schuyler *et al.*, 2002). Current proposed threats to the survival of koalas include loss of habitat (Melzer, Carrick *et al.*, 2000), predation by dogs (Caneris & Jones, 2004; Lunney, Gresser *et al.*, 2007), collision with motor vehicles (Dique, Thompson *et al.*, 2003; Caneris & Jones, 2004), loss of genetic diversity (Sherwin, Timms *et al.*, 2000), bushfire (Lunney, Gresser *et al.*, 2004; Lunney, Gresser *et al.*, 2007), climate change

² Friends of the Koala, Lismore NSW; Friends of the Koalas Inc, Cowes, Vic; Hunter Koala Preservation Society, Hunter Valley, NSW; Ipswich Koala Protection Society, Ipswich, Qld; The Koala Preservation Society of NSW (the Koala Hospital), Port Macquarie, NSW; Koala Squad, Eumundi, Qld; Koalas In Care Inc, Taree, NSW; Moggill Koala Hospital Association, Toowoomba, Qld; Moreton Bay Koala Rescue Inc, Moreton Bay, Qld; Pine Rivers Koala Care Association Inc, Strathpine, Qld; Australian Koala Foundation, Brisbane, Qld; Friends of Local Koalas, Land and Wildlife, Somers, Vic; Koala Action Pine Rivers Inc, Warner, Qld.

(Clifton, Ellis *et al.*, 2007; Department of the Environment, Water, Heritage and the Arts, 2009) and diseases (Canfield, 1987; Tarlinton, Meers *et al.*, 2005; Department of the Environment, Water, Heritage and the Arts, 2009), the most prevalent and important of which is chlamydiosis (Obendorf, 1983; Canfield, 1987; Weigler, Girjes *et al.*, 1988; Canfield, 1989; Jackson, White *et al.*, 1999; McLean, 2003; Stalder, 2003). The significance of these threats to the population of koalas living within and in the surrounds of the Port Macquarie is investigated in Chapter 2 through analysis of admission data from the Koala Hospital of the Koala Preservation Society of NSW.

1.2 THE CHLAMYDIACEAE AND CHLAMYDIOSIS IN KOALAS

The Chlamydiaceae are a group of obligate intracellular bacterial parasites of eukaryotic cells characterised by a biphasic replication cycle illustrated in Figure 1-1. The lifecycle involves an infectious, extracellular, metabolically inert “spore-like” stage (the elementary body) that becomes attached to host cells, is endocytosed, and then differentiates into the metabolically active form (the reticulate body) within the phagocytic vacuole (phagosome), avoiding lysis by inhibiting lysosome fusion. Reticulate bodies multiply by binary fission using host cell ATP, glucose, and nucleotides and eventually differentiate back into elementary bodies. The life cycle begins again after the vacuole ruptures, killing the host cell and allowing infective elementary bodies to disperse (Brooks, Butel *et al.*, 2007). An important factor in the pathogenesis of chlamydial disease is the ability of chlamydial organisms to exist perpetually within host cells in an atypical metabolically inert state in response to stressors such as nutrient deficiency; host cytokines (principally interferon γ); antimicrobial agents, particularly in sub-inhibitory concentrations; and various inhibitors of normal cellular metabolism (cAMP, calcium antagonists, inhibitors of peptidyl-prolyl isomerase) (Beatty, Morrison *et al.*, 1994). This form is difficult to

eliminate by normal immunological mechanisms and, as organisms are metabolically inert, by antibiosis; thus disease may recur after stressors are removed (Mpiga & Ravaoarino, 2006).

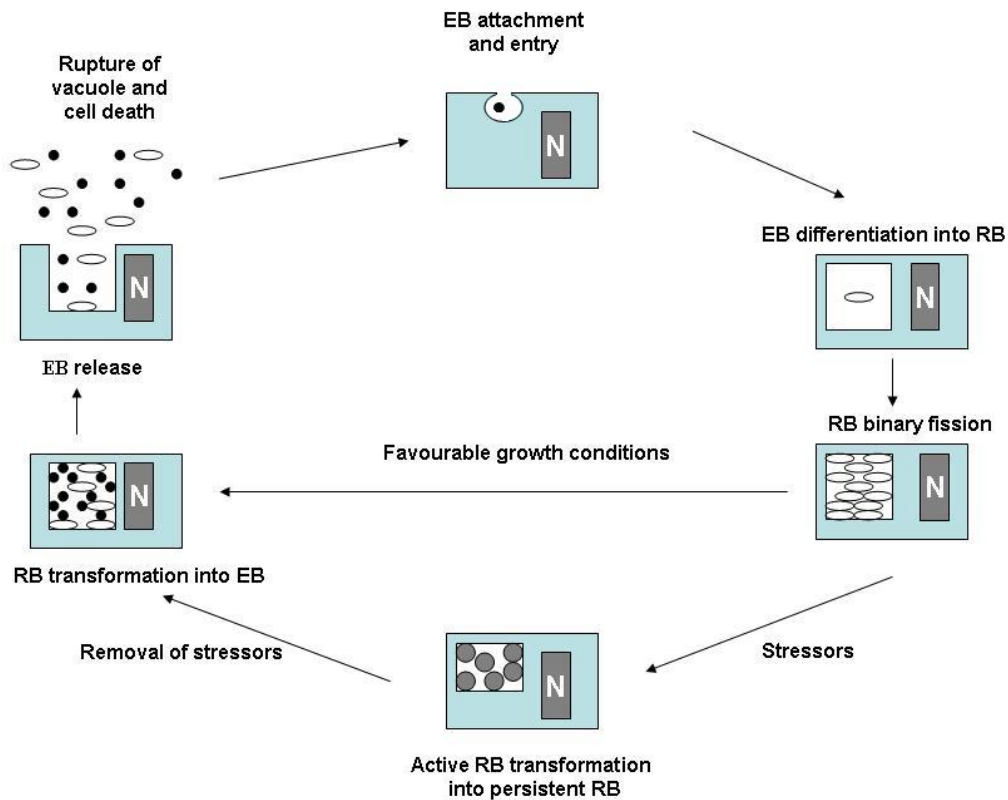


Figure 1-1. Chlamydial life cycle.

N: nucleus; EB: elementary body; RB: reticulate body (Mpiga & Ravaoarino, 2006).

Chlamydiaceae parasitise a wide range of hosts including humans, mammals, birds, reptiles, amphibians, fish, invertebrates and amoebae and generally cause a spectrum of inflammatory disease of the conjunctiva, urinary, reproductive, gastrointestinal and respiratory systems (Nigg, 1942; Kazdan, Schachter *et al.*, 1967; Storz, 1971; Gaillard, Hargis *et al.*, 1984; Grimes & Clark, 1986; Storz, 1988; Harrison, 1989; Fukushi & Hirai, 1992; Fox, Stills *et al.*, 1993; Grimes, 1994; Mare, 1994; Carrasco, Segales *et al.*, 2000; Donati, Piva *et al.*, 2005). Marsupial species in which Chlamydiales (*C. pecorum*, *C.*

pneumoniae and novel uncultured Chlamydiales strains) have been detected in the ocular, urogenital or respiratory tract include the greater glider (*Petauroides volans*), mountain brushtail possum (*Trichosurus caninus*), western barred bandicoot (*Perameles bouganville*), greater bilby (*Macrotis lagotis*) and Gilbert's potoroo (*Potorous gilbertii*) (Bodetti, Viggers *et al.*, 2003; Warren, Swan *et al.* 2005; Kutlin, Roblin *et al.*, 2007). Both clinical disease (commonly conjunctivitis) and subclinical disease have been reported in these species (Bodetti, Viggers *et al.*, 2003; Warren, Swan *et al.* 2005; Kutlin, Roblin *et al.*, 2007) with a particularly strong association between ocular Chlamydiales infection and clinical disease observed in bandicoots and gliders in one study (Bodetti, Viggers *et al.*, 2003). In koalas, Chlamydiaceae have been detected in conjunctivae and throughout the urogenital tract (Brown & Grice, 1984; McColl, Martin *et al.*, 1984; Obendorf & Handasyde, 1990; Girjes, Hugall *et al.*, 1993; Hemsley & Canfield, 1997; Jackson, White *et al.*, 1999; Devereaux, Polkinghorne *et al.*, 2003), in the rectum (Brown & Grice, 1986; Hemsley & Canfield, 1996), pulmonary alveolar macrophages or epithelial cells, and hepatic and splenic macrophages (Higgins, Hemsley *et al.*, 2005b) and peripheral blood mononuclear cells (Bodetti & Timms, 2000).

The pioneering work of Girjes *et al.* (1988) demonstrated that chlamydial infection in koalas is caused by two species, now reclassified as *Chlamydophila pneumoniae* and *Chlamydophila pecorum* (Everett, Bush *et al.*, 1999). Prior to this work, chlamydial infections in koalas were considered to be one species (*Chlamydia psittaci*) (Cockram & Jackson, 1976; McColl, Martin *et al.*, 1984; Brown & Grice, 1986; Brown, 1986; Weigler, Girjes *et al.*, 1988; Canfield, Love *et al.*, 1991a). Even more modern studies have failed to speciate infections, possibly due to a lack of species-specific diagnostic tools (White & Timms, 1994; Hemsley & Canfield, 1996; Kempster, Hall *et al.*, 1996; Martin, 1996; Higgins, Hemsley *et al.*, 2005a; Higgins, Hemsley *et al.*, 2005b; Markey, Wan *et al.*,

2007). The lack of speciation in these works limits interpretation with regard to pathogenesis of chlamydiosis in koalas. In people and swine, infections by multiple Chlamydiaceae species have been associated with more severe clinical signs of ocular disease and abortion, respectively, but the role of mixed infections in pathogenesis is poorly understood (Schiller, Koesters *et al.*, 1997; Dean, Kandel *et al.*, 2008). In koalas, the situation is potentially complicated further by the possible co-infection by at least nine “*Chlamydia*-like” bacteria, although they have not yet been identified as primary aetiological agents of clinical disease (Devereaux, Polkinghorne *et al.*, 2003). Based on field studies of wild animals, Jackson *et al.* (1999) suggested *C. pecorum* is more pathogenic than *C. pneumoniae* and that *C. pneumoniae* has a predilection for ocular sites. This last assertion has more recently been disputed (Timms, 2001). Little work has been done to expand the original findings of Jackson *et al.* (1999), with the exception of a study in five animals that demonstrated *C. pecorum* was present more commonly than *C. pneumoniae* within diseased tissues (Devereaux, Polkinghorne *et al.*, 2003). In particular, detailed studies describing clinical disease in relation to the presence of specific chlamydial organisms are lacking and further clarification of the possible site bias of chlamydial species is required.

There is strong evidence supporting venereal transmission as the most frequent mode of chlamydial transmission in koalas (Handasyde, 1986; Girjes, Hugall *et al.*, 1988; Martin & Handasyde, 1999), but the common finding of chlamydial infection of prepubertal subadults (up to 58% of some populations) is supportive of a second, non-venereal mechanism of transmission (Weigler, Girjes *et al.*, 1988; Jackson, White *et al.*, 1999; Santamaria, 2001). The process of koala joeys ingesting caecal contents directly from the mother’s cloaca (Thompson, 1987; Osawa, Blanshard *et al.*, 1993) could provide a non-venereal route of transmission, as the rectum may be infected with Chlamydiaceae in

koalas (Brown & Grice, 1986; Hemsley & Canfield, 1996; Hemsley & Canfield, 1997) and intestinal infections and faecal shedding are common in other species (ruminants, birds) (Shewen, 1980). Alternatively, neonates could be infected during parturition, as the urogenital sinus may be infected with Chlamydiaceae (Hemsley & Canfield, 1997) and infection during parturition occurs in people (Schachter, Grossman *et al.*, 1979) and cats (Shewen, Povey *et al.*, 1978).

Clinical signs of chlamydial urogenital tract disease in koalas include dysuria, haematuria, incontinence, cloacal eversion and the secondary effects of urine scald, such as ulceration and cutaneous myiasis (Obendorf, 1983; Booth & Blanshard, 1999; Connolly, 1999).

Animals are often in poor condition (Obendorf, 1983; McLean, 2003) and females are frequently presumed infertile due to no evidence of recent breeding (Brown, Carrick *et al.*, 1984; McColl, Martin *et al.*, 1984; Handasyde, 1986; Santamaria, 2001; McLean, 2003).

The dark brown discolouration of rump pelage as a result of chronic incontinence lends this syndrome its common names “wet bottom” or “dirty tail” (Blanshard, 1994) (Figure 1-2). Clinical signs of chlamydial conjunctivitis include serous to mucopurulent ocular discharge, conjunctival hyperaemia, chemosis and papillary hypertrophy, corneal oedema and neovascularisation and periocular alopecia (Kempster, Hall *et al.*, 1996) (Figure 1-3).

Chlamydomphila pneumoniae has also been implicated in severe respiratory disease in captive koalas (Wardrop, Fowler *et al.*, 1999; Nicolson, 2002).

Gross pathological changes to the urogenital tract include: distortions, enlargements, and thickening of the uteri and oviducts; accumulation of purulent material within the uterine lumens, cystic dilations of the oviducts and ovarian bursae; adhesions between reproductive tract tissues and adjacent organs; and thickening of the wall and reddening of the mucosa of the bladder (Figure 1-4) (Obendorf, 1981; Canfield, Oxenford *et al.*, 1983; Obendorf & Handasyde, 1990).

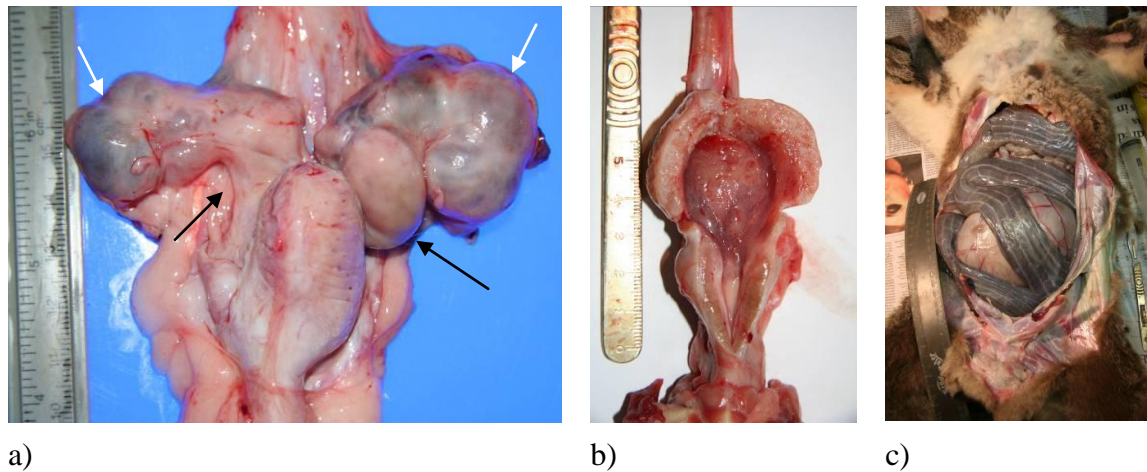


Figure 1-4. Examples of gross pathology associated with urogenital chlamydiosis.

a) Bilateral thickening and distension of both uteri (pyometra) (black arrows), bilateral oviduct and ovarian bursal cysts (white arrows); b) diffuse severe reddening of bladder mucosa and thickening of bladder wall (severe chronic diffuse fibrotic cystitis); c) gross distension of pus-filled ovarian bursal cyst with multiple areas of perforation, caecal adhesions and peritonitis.

1.3 EPIDEMIOLOGY OF CHLAMYDIOSIS IN WILD KOALAS

The ultimate expression of clinical disease is the result of a complex interaction between the host, pathogen and environment, so clinical disease might be expected to be variable between individuals and populations. In individual koalas chlamydiosis may be deleterious, causing infertility, blindness or death (Brown & Grice, 1986; Weigler, Booth *et al.*, 1987; Canfield, 1988) or act as a co-morbidity factor, potentially making animals more susceptible to other disease or predation (Canfield, 1987; McLean, 2003). In others, infection and associated structural disease may be subclinical (Weigler, Girjes *et al.*, 1988; White & Timms, 1994; Jackson, White *et al.*, 1999; Devereaux, Polkinghorne *et al.*, 2003; McLean, 2003). Such infections appear to affect animals little, as body condition, longevity and even fertility may be maintained (Handasyde, 1986; Santamaria, 2001; McLean, 2003). Similarly, both clinical and subclinical chlamydial disease are frequent in other species, including birds (Longbottom & Coulter, 2003), calves (Jaeger, Liebler-Tenorio *et al.*, 2007), cats (Sykes, 2005) and people (Solomon, Peeling *et al.*, 2004): and

have been reported at low prevalence in other marsupials (the greater glider, mountain brushtail possum, western barred bandicoot, greater bilby and Gilbert's potoroo (Bodetti, Viggers *et al.*, 2003).

The threat of extinction of koalas as a direct result of chlamydial disease is considered unlikely by many authors (Handasyde, 1986; White & Timms, 1994; Caneris & Jones, 2004; Department of the Environment, Water, Heritage and the Arts, 2009) and traditionally, disease alone has not been regarded as a major driver of extinction (de Castro & Bolker, 2005). This view is changing: recently documented extinctions as a direct result of infectious disease have included a captive species of land snail (*Partula turgida*) (Cunningham & Daszak, 1998) and a species of wild frog (*Taudactylus acutirostris*) (Schloegel, Hero *et al.*, 2006). In koalas, a model designed to investigate the impact of Chlamydiaceae on population dynamics did not predict extinction of koalas by Chlamydiaceae under most circumstances (Augustine, 1998). A number of assumptions underlying this model suggest its conclusions require review, particularly given the changing population dynamics of koalas in recent years. The model assumed a relatively stable population density and thus is not applicable to populations where koalas are declining, such as south-eastern Queensland (Department of Environment and Resource Management, 2009); or over-abundant, such as some populations on mainland Victoria (McLean, 2003). It assumed non-clinical animals were always fertile and that a different species of *Chlamydia* (now known to be *Chlamydophila*) (Everett, Bush *et al.*, 1999) infected the eyes to that infecting the urogenital tract. These suppositions are not supported by more recent large scale epidemiological studies of fertility and clinical signs (McLean, 2003) and the demonstration of both species of *Chlamydophila* at ocular and urogenital sites (Jackson, White *et al.*, 1999; Devereaux, Polkinghorne *et al.*, 2003). Finally, as the koala's mating system was unknown in the 1990s, the model assumed polygyny, yet a

recent study of the koala mating system has suggested evidence of transitory males and polyandry (Ellis, Hale *et al.*, 2002). Given the improved body of knowledge regarding chlamydiosis and the behavioural ecology of koalas, an updated epidemiological model would be useful to more accurately describe the effects of chlamydiosis on koala populations. This work aimed to provide baseline data that might be useful for the refinement of future epidemiological models examining the currently unknown impact of rehabilitated animals on local koala populations.

In the field, several authors have attributed individual population decline to the effects of chlamydiosis (Handasyde, 1986; Martin & Handasyde, 1999), however most infected populations in Victoria and Queensland have fertility rates greater than that considered adequate to maintain numbers (Weigler, Girjes *et al.*, 1988; Gordon, McGreevy *et al.*, 1990; White & Kunst, 1990; Martin & Handasyde, 1999) and have even become over-abundant in some areas of Victoria (Department of the Environment, Water, Heritage and the Arts, 2009). Several authors have argued the major role of chlamydiosis in extinction of koalas is as a contributory factor in populations made vulnerable for other reasons (Weigler, Girjes *et al.*, 1988; Martin & Handasyde, 1999). Although a stable host-pathogen balance may currently exist, the koala faces multiple potential threats that could alter this balance. For example, a recently described retrovirus in koalas (Canfield, Sabine *et al.*, 1988) has been hypothesised to contribute to immunosuppression and clinical expression of chlamydial disease (Tarlinton, Meers *et al.*, 2005). Alternatively, increased deforestation and fragmentation of habitat, as is occurring in Queensland (Department of Environment and Resource Management, 2009), could contribute to crowding and increased transmission rates. Finally, koalas could potentially acquire new more pathogenic species or strains of Chlamydiaceae, as there is genetic evidence *C. pecorum* has been acquired multiple times by koalas from ruminants (Glassick, Giffard *et al.*, 1996). Given the

difficulty of controlling an infectious disease in wildlife (Artois, Delahay *et al.*, 2001), research into understanding chlamydiosis in koalas whilst a stable host-pathogen balance exists is prudent.

1.4 SHOULD WE TREAT INFECTIOUS DISEASE IN WILD ANIMALS?

Given the high profile of koalas it is perhaps not surprising that a number of organisations are solely dedicated to the preservation of this species and many of these provide rehabilitation facilities for sick or injured koalas (see footnote 2, page 3). Wildlife rehabilitation frequently has poor outcomes (Robertson & Harris, 1995; Anderson, Gress *et al.*, 1996; Augee, Smith *et al.*, 1996; Fajardo, Babiloni *et al.*, 2000; Tribe & Brown, 2000; Ben-David, Blundell *et al.*, 2002; Beringer, Mabry *et al.*, 2004) and the treatment of wild animals with infectious disease is perhaps the most controversial area (Kirkwood & Sainsbury, 1996). Diseased animals harbour potentially infectious organisms that may spread within wildlife rehabilitation facilities (Menzano, Rambozzi *et al.*, 2008) or to animals in the wild (Jacobson, Gaskin *et al.*, 1991; Cunningham, 1996; Chipman, Slate *et al.*, 2008) or to wildlife carers (Allworth, Murray *et al.*, 1996). Some authors have argued that rehabilitation of diseased animals could in theory deleteriously alter selection pressures for the development of disease resistance, but that it might be justifiable in individuals on grounds of welfare, where recovery will not occur without treatment and may be accomplished without undue stress (Kirkwood & Sainsbury, 1996). Ultimately, the effect of rehabilitation of animals with infectious disease on wild populations is usually unknown. Whilst the merits of rehabilitation of wild animals with infectious disease will continue to be debated (Kirkwood & Sainsbury, 1996; Estes, 1998; Tribe & Brown, 2000), in the absence of direct evidence of harm, the treatment of chlamydiosis in koalas is likely to continue.

Currently, hundreds of koalas per year are treated for chlamydiosis (Jones, 2008) despite lack of information regarding whether treatment reduces suffering or time spent in care, whether recovery may occur without treatment, whether animals are released still harbouring organisms, and the rates of post-release survival and breeding success of such animals. Only one small study using modern molecular diagnostics has investigated the presence of pathogens after treatment in four rehabilitated koalas and this only followed animals for two weeks after treatment end (Markey, Wan *et al.*, 2007). Only two studies have examined post-release survival of animals treated for chlamydiosis (Tribe, Hanger *et al.*, 2005; Jones, 2008). In all three studies numbers of animals were fewer than six. In this thesis, the author aims to examine survival of treated animals by a reviewing the survival of treated animals based on historical clinical records (Chapter 3); and by monitoring animals for four weeks after treatment end, identifying whether treatments result in microbial cure (Chapter 8).

1.5 DIAGNOSIS AND TREATMENT OF CHLAMYDIAL DISEASE IN KOALAS

Historically, chlamydial infections in koalas have been diagnosed using cell culture, demonstration of organisms in cells or tissues by immunofluorescence or immunohistochemistry, DNA slot hybridisation, immuno-slot blot analyses and PCR, complement fixation tests, antibody ELISAs, clinical signs, contrast radiology and ultrasound imaging (reviewed in Blanshard, 1994; Blanshard & Bodley, 2008). The wide variety of diagnostic tests employed reflects the poor sensitivity and specificity of some tests (e.g. complement fixation), technical difficulty (e.g. cell culture), requirement for specialist equipment (e.g. immunofluorescence), poor availability outside a research environment, and the recent evolution of simpler more sensitive molecular biological techniques, such as PCR. In recent years PCR has been used widely to detect

Chlamydiaceae in koalas, as in other species (Ellis, Girjes *et al.*, 1993; Girjes, Hugall *et al.*, 1993; Martin, Alexander *et al.*, 1995; Bodetti & Timms, 2000; Timms, 2000; Santamaria, 2001; Bodetti, Johnston *et al.*, 2002; Nicolson, 2002; Amin, 2003; Bodetti, Viggers *et al.*, 2003; Helps, Reeves *et al.*, 2003; Higgins, Hemsley *et al.*, 2005a; Markey, Wan *et al.*, 2007). It is particularly useful in being able to distinguish between chlamydial species (Girjes, Hugall *et al.*, 1993; Jackson, White *et al.*, 1999), is regarded as more sensitive than other diagnostic tests (Ostergaard, Birkelund *et al.*, 1990; Sykes, Studdert *et al.*, 1999; Amin, 2003) and is the current diagnostic test of choice for chlamydial infections in people (Harindra, Underhill *et al.*, 2003).

Despite treatment of chlamydiosis in koalas since the 1970s (Cockram & Jackson, 1976; Dickens, 1978), few studies have systematically described symptoms throughout treatment or examined treatment success. Most treatment regimes have been based on “trial and error” or personal experience (Blanshard & Bodley, 2008). An early study, examining topical treatments for chlamydial conjunctivitis, stated a two week course of oxytetracycline ointment and powder was required to elicit a cure as determined by cell culture (Cockram & Jackson, 1976) and there are subsequent anecdotal reports of resolution of clinical signs using tetracycline or chloramphenicol eye ointments, although the treatment length was not reported (Robinson, 1978; Wood, 1978; Canfield, Love *et al.*, 1991a). The effectiveness of topical therapy in achieving microbial cure in koalas requires re-examination. The original study determined treatment success using chlamydial culture (Cockram & Jackson, 1976), which is at best approximately 75% sensitive (Harindra, Underhill *et al.*, 2003) and when more sensitive molecular diagnostics (real-time PCR) have been utilised, topical treatment with chloramphenicol and corticosteroids has been ineffective in reducing pathogen load in one animal (Markey, Wan *et al.*, 2007).

Chlamydial infections in koalas may be systemic (Bodetti & Timms, 2000; Higgins,

Hemsley *et al.*, 2005b) and, in such cases, topical therapy would be inappropriate. Such factors have contributed to the recommendation against use of topical monotherapy to treat chlamydiosis in people and cats, due to poor efficacy and the prolonged courses required to achieve microbial cure (Sparkes, Caney *et al.*, 1999; Chiu & Amsden, 2002; Donati, Piva *et al.*, 2005).

Historically, systemic antibiotic treatment of chlamydiosis in koalas has proved to be problematic. Use of oxytetracycline or erythromycin has led to emaciation and death, attributed to gut flora disturbances, within two and six weeks, respectively (Brown, Wood *et al.*, 1984; Brown, Woolcock *et al.*, 1986; Handasyde, 1986; Osawa & Carrick, 1990; Osawa, Bird *et al.*, 1993). The poor track record of oxytetracycline was improved through utilising a concurrently administered soya based infant formula nutritional supplement (Osawa & Carrick, 1990), but this regime has not gained wide acceptance. Although past treatments have included penicillins and potentiated sulpha drugs (Canfield, Love *et al.*, 1991a), weekly intramuscular doxycycline HCl (Blanshard, 1994) and tropospectomycin sulphate (Brown, Woolcock *et al.*, 1986), systemic treatment regimes currently employed in the three major koala hospitals use enrofloxacin (most frequently dosed orally) or chloramphenicol (oral or intramuscular administration) (Blanshard & Bodley, 2008). Treatment of chlamydial conjunctivitis varies between facilities, but generally includes topical oxytetracycline HCl or chloramphenicol (Blanshard & Bodley, 2008).

These treatment regimes are difficult to evaluate. Reports are usually anecdotal and resolution of clinical signs, rather than diagnostic testing, usually determines end points (Blanshard & Bodley, 2008). As clinical signs of chlamydial disease may resolve without treatment (Kempster, Hall *et al.*, 1996), there is little evidence as to which treatments are effective. Only two studies have used molecular diagnostic testing to determine whether koalas are microbially negative during (Bodetti, Johnston *et al.*, 2002) and after treatment

(Markey, Wan *et al.*, 2007) and these are contradictory. In one study, seven of the eight koalas with clinical signs of cystitis were PCR-positive for *Chlamydia* during treatment with chloramphenicol or enrofloxacin (frequency and route not reported), despite treatment of up to six months, suggesting these treatments were not effective in curing animals of chlamydial infections (Bodetti, Johnston *et al.*, 2002). In direct contrast, three koalas of three treated with intramuscular chloramphenicol had no chlamydial DNA detectable by real-time PCR by day 34 of treatment, suggesting chloramphenicol was an effective treatment (Markey, Wan *et al.*, 2007). The treatment failure documented in the first study might be a result of poor bioavailability or inadequate dose regimes if pharmacokinetics in koalas differ from other species. Alternatively, the latter study may have failed to detect latent infections as animals were only monitored for two weeks following treatment. Studies in other species have found chlamydial pathogen load may be undetectable during treatment, and then recur after treatment withdrawal, possibly due to chlamydial persistence in other sites or a false negative result (Dean, Harley *et al.*, 2005). Given this poor and contradictory evidence base, it is not surprising that there is no standardised method of examining koalas, describing clinical signs and prescribing effective treatments for chlamydiosis. This study sought to redress this obvious gap in the literature in Chapter 8.

1.6 PHARMACOKINETICS IN KOALAS

Pharmacokinetic studies in koalas are well overdue, and have the potential to shed light not only on the efficacy of treatments, but also the adaptive mechanisms associated with metabolism of other xenobiotics, such as eucalypt toxins. Pharmacokinetics in koalas might be expected to be different to eutherians: koalas have an increased hepatic excretory capacity as evidenced by increased bromosulphalein clearance in comparison with sheep and various macropods (*Macropus giganteus*, *M. rufus*, *M. rufogriseus*) (Pass & Brown, 1990). Many authors have speculated that the koala must have unique metabolic pathways allowing survival on a diet almost exclusively of eucalypts, which would be toxic to other species (McLean & Foley, 1997; Stupans, Jones *et al.*, 2001). Such adaptations may allow koalas to metabolise drugs faster than expected by metabolic scaling (Booth & Blanshard, 1999). Further, the koala's small intestinal transit time is short (particulate matter: 6 min; soluble material: 60 min) (Cork & Warner, 1983), and their caecal surface area is the largest of any animal (Snipes, Snipes *et al.*, 1993). Both of these could alter the absorption of orally administered drugs in comparison with domestic species. Given the unique anatomical and physiologic adaptations of koalas that might be expected to alter pharmacokinetics, the large number of animals treated annually, and the gap in the knowledge base of any pharmacokinetics in this species, a study of pharmacokinetics of commonly used drugs would prove extremely useful. Such work could provide guidelines for appropriate and timely medication, minimise wastage and reduce the potential for selection of resistant microbes in animals inappropriately medicated with antibiotics. This is addressed in Chapter 7.

1.7 SPECIFIC AIMS OF THIS THESIS

Despite the high public anxiety as to the plight of koalas, and the huge effort involved in the treatment and rehabilitation of this species, little evidence exists regarding efficacy of treatments, and pharmacokinetic studies remain to be done. This thesis aims to provide the necessary evidence base for effective treatment of chlamydiosis in koalas by addressing the diverse facets of diagnosis, treatment and management of koalas with chlamydiosis.

Aim 1: Identify threats to wild koalas in the Port Macquarie area and describe demographics of koalas entering a rehabilitation facility.

The study detailed in Chapter 2 adds to previous work by providing location-specific data, and utilises longitudinal data over an unusually long period (30 years) to identify changing threats to koalas in this area and help the Koala Hospital plan logistics.

Aim 2: Describe the diagnostic and treatment regimes and determine the outcomes of koalas treated for chlamydiosis at the Koala Hospital between 1995 and 2005.

This aim is addressed in Chapter 3 by analysis of medical records to identify prognostic indicators and treatment regimes associated with successful rehabilitation and survival in the wild. Several hypotheses that emerged from this study are addressed in Chapter 7 and Chapter 8.

Aim 3: Determine whether the anti-chlamydial antibiotics, azithromycin and doxycycline, may be safely used to treat chlamydiosis in koalas.

The aim of Chapter 5 was to identify a safe and effective treatment for chlamydiosis in koalas, given the history of theoretically unsuccessful treatments in common use found in Chapter 3. As this study did not identify such a treatment, the focus changed to examining the efficacy of fluoroquinolones in treating chlamydiosis - treatments that appeared to be

safe in koalas and had been reported as historically successful in treating this disease in Chapter 3.

Aim 4: Describe the pharmacokinetics of enrofloxacin and marbofloxacin in koalas following oral and subcutaneous administration and report any identifiable adverse effects.

As described in 1.6, pharmacokinetics of antibiotics in koalas have not been studied but might be expected to be unusual. Chapter 6 and Chapter 7 aimed to characterise the absorption of two fluoroquinolones to allow generation of pharmacokinetic indices that may be used to predict treatment success. Initial studies were by modified agar diffusion bioassay in a pilot study (Chapter 6) and then by the more sensitive method, high performance liquid chromatography (Chapter 7).

Aim 5: Determine whether clinical signs reflect the presence of chlamydial organisms in koalas and the intensity of *Chlamydomphila pecorum* shedding; and whether treatment with fluoroquinolones eliminates *C. pecorum* and *C. pneumoniae* from koalas.

As discussed, clinical signs have been used frequently to diagnose chlamydiosis and as an end point of treatment, yet their sensitivity has been reported to be poor. Chapter 8 examined the link between clinical signs and presence and load of chlamydial organisms by use of quantitative real-time PCR. The final aim of this study was to examine whether fluoroquinolones eliminated chlamydial infection from animals.

CHAPTER 2 - ADMISSION TRENDS OF KOALAS AT A WILDLIFE REHABILITATION FACILITY

2.1 INTRODUCTION

The study of wildlife disease is an area of growing interest and importance to the scientific community. Recent studies have demonstrated that disease may threaten wild species' survival or drive local populations or species to extinction and thus threaten biodiversity (Cunningham & Daszak, 1998; Schloegel, Hero *et al.*, 2006). Wildlife may provide an important sylvatic reservoir, or act as sentinels, for emerging infectious diseases that may threaten human, domestic and wild animal health (Daszak, Cunningham *et al.*, 2000). The study of wildlife health may indicate ecosystem health and provides opportunities to study the host-parasite interaction, changes in the ecology of which may contribute to disease emergence (Daszak, Cunningham *et al.*, 2000; Daszak, Cunningham *et al.*, 2001). Wildlife disease is likely to become an ever more important area of study, given rapidly changing ecosystems in the face of rising human populations, shrinking and fragmentation of wildlife habitat, introduction of exotic species and pathogens and implications of climate change on ecosystems. Recent initiatives such as the "One Health" project have recognised the importance of integrated human, domestic, wildlife and ecosystem health (Zinsstag, Schelling *et al.*, 2005; King, Anderson *et al.*, 2008; One Health Initiative Task Force, 2008).

Despite the recognised importance of such work, the study of disease in wild populations may be logistically difficult and expensive. This difficulty may be overcome, in part, through studies utilising the records of wildlife rehabilitation centres (Shine & Koenig, 2001; Trocini, Pacioni *et al.*, 2008). Although these data have strong inherent biases towards the type of animals entering the rehabilitation system, and extrapolating results to

wild populations must be done with care, study of such records has significant advantages. Record keeping is often a compulsory requirement of licensing for wildlife rehabilitators, and such records frequently comprise a significant data set. Useful information can be gathered regarding which animals present and why, the treatment regimes used and the ultimate fate of such animals (Hartup, 1996; Beckmen, Lowenstine *et al.*, 1997; Deem, Terrell *et al.*, 1998; Shine & Koenig, 2001; Koenig, Shine *et al.*, 2002; Dique, Preece *et al.*, 2003; Lander & Gulland, 2003; Mazaris, Mamakis *et al.*, 2008; Neese, Seitz *et al.*, 2008; Trocini, Pacioni *et al.*, 2008; Dau, Gilardi *et al.*, 2009; Kalpakis, Mazaris *et al.*, 2009). Such information may be made available at minimal cost, extend over several decades and can often be the only practical method of gathering longitudinal information. Study of such data may identify threats to local populations and may thus prove useful to local planning authorities when making urban planning decisions that might facilitate survival of local wildlife. Within rehabilitation centres, information can be used to refine management and treatment protocols and identify the success rates of rehabilitation.

There have been no detailed studies of the data collected by the Koala Preservation Society of NSW, which has been treating and rehabilitating koalas in the Port Macquarie district since 1973. This study examines the computerised records of animal signalment (age class based on tooth wear, and sex) and reasons for presentation to the Koala Preservation Society of NSW's Koala Hospital from January 1st 1975 to the 31st of December 2004. Examination of data seeks to provide useful information on which to base management decisions regarding deployment of volunteer time and resources, and the building of infrastructure within the hospital. More broadly, recommendations are made regarding the timing and type of seasonal educational campaigns required to reduce risk of animals coming into care. By relating these data to the population structure of the local wild population, threats to the local population are explored. Longitudinal changes in the

reasons animals present suggest evidence of emerging threats to the local population, and provide a sound basis for planning decisions by local authorities.

2.2 AIMS

The aims of this study were to:

- 1/ describe why koalas present to the Koala Hospital,
- 2/ determine the characteristics of groups of koalas presenting for different reasons to the Koala Hospital,
- 3/ describe the effect of season and time on reasons koalas are admitted, to identify changes that might indicate specific threats to the survival of the local wild koala population and help the Koala Hospital plan logistics, publicity campaigns and allocation of resources on a seasonal basis and for the future.

2.3 METHODS

2.3.1 Admission data

Members of the public report sick, injured and dead koalas to the Koala Hospital.

Volunteers then retrieve koalas, if necessary using flagging methods to encourage koalas down from the tree (Blanshard, 1994). Koalas are then placed into canvas bags and transported to the Koala Hospital. Morphometric measurements, signalment (age class and sex) and clinical observations are determined by the Koala Hospital supervisor and/or visiting veterinarians and recorded in paper medical records. Age class is based on assessment of tooth wear (Martin, 1981) and modified by previous admission records.

Animals are classed as joeys (< 1 year), juvenile (1 - 2 years), young adult (2 - 5 years), mature adult (5 - 8 years) or aged adult (8+ years), corresponding to tooth wear classes I, II, III, IV, V – VII, respectively (Martin, 1981).

Prior to release, all koalas are permanently identified with ear tags and subcutaneous passive transponder microchips (Microchips Australia, Keysborough, Vic, Australia). Thus, should an animal re-present, previous medical records are identified and up-dated. A reason for admission is added to the medical records based on the supervisor's and/or veterinarians' assessment (Appendix I). Weekly, between one and three individuals enter signalment (sex and age class), date and reason for admission to a computer database (Microsoft Access® 2003, Microsoft® Corporation, Redmond, Washington, USA).

2.3.2 Data management

Data were compiled on sex, age class, date of admission and reason for admission. Admission reasons were reclassified into 10 codes to combine analogous terms (Appendix I). Age class followed that described above, with the modification that joeys and juveniles were combined into one age class (juvenile). Admissions prior to 1975 were few (10 of 3951), comprising 0.25% of the total admissions, and were therefore excluded from further analysis. Admissions after the 31st of December 2004 were not included due to the possibility that commencement of research at the hospital confounded the data. Date of admission was reclassified into season (spring, summer, autumn, winter) and year, and then grouped into five-year intervals (1975 - 1979, 1980 - 1984, 1985 - 1989, 1990 - 1994, 1995 - 1999 and 2000 - 2004). Tables from this database were imported into SAS® statistical software (release 9.1 © 2002 - 2003, SAS Institute Inc., Cary, North Carolina, USA) or GenStat for Windows (2008, 11th ed., VSN International Ltd., Hemel Hempstead, Hertfordshire, UK) for further analyses.

2.3.3 Statistical analysis

Variables

The outcome with 10 categories represented different reasons for admission: motor vehicle accident (MVA), dog attack, healthy, wet bottom, eye disease, debilitated, fire, undiagnosed, joey and all other. Explanatory variables were sex, age class, season and five-year interval, as discussed above.

Descriptive analyses

A contingency table was created to assess the distribution of explanatory variables and their associations with the multicategory outcome (Table 2-1, Results). The percentage of the total data set for each explanatory variable was plotted using Graphpad Prism® 5 for Windows (Graphpad Software, La Jolla, California, USA).

Statistical models examining risk factors in comparison with the hospital population

Univariable multinomial logistic regression analyses were performed for each explanatory variable to examine their unadjusted association with the outcome using the SAS LOGISTIC procedure (Stokes, Davis *et al.* 2000). Variables significant in the univariable analyses ($p < 0.10$) were used to build a multivariable multinomial logistic regression model by a manual forward stepwise approach to quantify risk factors for admission reason after adjusting for each other. Decision on inclusion or exclusion of a variable at each step was based on the individual contribution of each variable to the model using a likelihood-ratio chi-square test retaining variables with $p \leq 0.05$. Results are shown as adjusted odds ratios (OR) with 95% confidence intervals. Probabilities for each outcome category were calculated using SAS statistical program and plotted using Graphpad Prism 5 for Windows. Arbitrarily chosen reference groups for explanatory variables were: female,

aged adults, autumn and 1975 - 1979 and the arbitrarily chosen reference group for the outcome variable was eye disease.

Identification of at-risk cohorts in comparison with the wild population

A population structure of a 1:1 sex ratio and age class percentages of 6.3% juveniles, 43.8% young adults, 40.6% mature adults and 9.4% aged adults were assumed for normal wild koala populations of Port Macquarie. This assumption was based on combined results from two field studies of wild koalas (n = 33) conducted in Thrumpster (Biolink, 2008) and the Innes Peninsula (Biolink, 2005) prior to their development as new suburbs of Port Macquarie. Chi-square goodness of fit tests were used to determine whether the population structure of hospital admissions approximated that of the wild population in each admission category. Statistical significance was concluded at $p \leq 0.05$.

2.4 RESULTS

2.4.1 Descriptive statistics of koalas admitted to the Koala Hospital

There were 3781 individual admissions from 2674 individual koalas between 1st January 1975 and 31st December 2004. Table 2-1 outlines the explanatory variables for cohorts according to admission category. The mean number of admissions per individual was 1.41 ± 1.04 (SD). Forty-one per cent of admissions were for trauma (MVA, dog attack), 20.5% of koalas were admitted with clinical signs associated with chlamydial disease (wet bottom, eye disease), and 15.8% were admitted healthy (Figure 2-1). Animals were most frequently admitted in spring and least likely to be admitted in autumn (chi-sq = 264.3, d.f. = 3, $p < 0.0001$; Figure 2-2). Admissions per five-year interval increased significantly between 1980 and 1994 (1980 - 1984 versus 1985 - 1989: chi-sq = 52.3, d.f. = 1, $p < 0.0001$; 1985 - 1989 versus 1990 - 1994: chi-sq = 34.2, d.f. = 1, $p < 0.0001$) but have not changed significantly since 1995 (Figure 2-3).

Table 2-1. Contingency table of explanatory variables (number (%)) for cohorts according to admission category for koalas admitted to the Koala Hospital from 1st January 1975 to 31st December 2004.

Outcome variables

		Dog attack	MVA	Fire	Eye disease	Wet bottom	Debilitated	Healthy	Joey	Undiagnosed	All other	Total	
Explanatory variables	Age												
		Juvenile	138 (3.5)	138 (3.5)	44 (1.1)	15 (0.4)	21 (0.5)	41 (1.0)	116 (2.9)	64 (1.6)	44 (1.1)	14 (0.4)	579 (14.7)
		Young	327 (8.3)	327 (8.3)	44 (1.1)	85 (2.2)	137 (3.5)	50 (1.3)	215 (5.5)	6 (0.2)	82 (2.1)	16 (0.4)	1248 (31.7)
		Mature	201 (5.1)	201 (5.1)	62 (1.6)	182 (4.6)	184 (4.7)	90 (2.3)	179 (4.5)	2 (0.1)	117 (3.0)	28 (0.7)	1380 (35.0)
		Aged	79 (2.0)	79 (2.0)	7 (0.2)	66 (1.7)	78 (2.0)	72 (1.8)	77 (2.0)	0 (0.0)	54 (1.4)	3 (0.1)	512 (13.0)
		Sex											
		F	373 (9.5)	373 (9.5)	90 (2.3)	121 (3.1)	289 (7.3)	132 (3.4)	278 (7.1)	28 (0.7)	127 (3.2)	26 (0.7)	1752 (44.5)
		Season											
		Autumn	88 (2.2)	88 (2.2)	2 (0.1)	52 (1.3)	96 (2.4)	53 (1.3)	49 (1.2)	6 (0.2)	53 (1.3)	5 (0.1)	524 (13.3)
		Winter	170 (4.3)	170 (4.3)	6 (0.2)	64 (1.6)	100 (2.5)	48 (1.2)	130 (3.3)	13 (0.3)	76 (1.9)	16 (0.4)	903 (22.9)
		Summer	124 (3.2)	124 (3.2)	43 (1.1)	113 (2.9)	132 (3.4)	87 (2.2)	130 (3.3)	32 (0.8)	75 (1.9)	17 (0.4)	1523 (38.7)
		Spring	367 (9.3)	367 (9.3)	108 (2.7)	121 (3.1)	94 (2.4)	71 (1.8)	289 (7.3)	21 (0.5)	103 (2.6)	25 (0.6)	831 (21.1)
		5-year interval											
		1974 - 1979	45 (1.1)	45 (1.1)	4 (0.1)	26 (0.7)	23 (0.6)	24 (0.6)	54 (1.4)	7 (0.2)	27 (0.7)	1 (0.0)	235 (6.0)
		1980 - 1984	50 (1.3)	50 (1.3)	2 (0.1)	26 (0.7)	28 (0.7)	25 (0.6)	65 (1.7)	7 (0.2)	26 (0.7)	1 (0.0)	270 (6.9)
		1985 - 1989	113 (2.9)	113 (2.9)	12 (0.3)	49 (1.2)	92 (2.3)	29 (0.7)	85 (2.2)	9 (0.2)	64 (1.6)	3 (0.1)	560 (14.2)
		1990 - 1994	157 (4.0)	157 (4.0)	106 (2.7)	75 (1.9)	90 (2.3)	51 (1.3)	150 (3.8)	9 (0.2)	69 (1.8)	6 (0.2)	871 (22.1)
		1995 - 1999	219 (5.6)	219 (5.56)	1 (0.03)	80 (2.03)	85 (2.16)	51 (1.29)	144 (3.65)	8 (0.20)	55 (1.40)	14 (0.36)	869 (22.1)
		2000 - 2004	165 (4.2)	165 (4.19)	34 (0.86)	94 (2.39)	104 (2.6)	79 (2.00)	100 (2.54)	32(0.81)	66 (1.67)	38 (0.96)	976 (24.8)
	Total	749 (19.8)	802 (21.2)	159 (4.2)	350 (9.2)	422 (11.2)	259 (6.9)	598 (15.8)	72 (1.9)	307 (8.1)	63 (1.7)	3781 (100)	

F: female, M: male, MVA: motor vehicle accident. Age classed using tooth wear class (TWC) (Martin, 1981) where Juvenile: TWC I - II; Young: TWC III; Mature: TWC IV; Aged: TWC V-VII. Data missing from 14 (0.4%) records of gender; 62 (1.6%) records of age class.

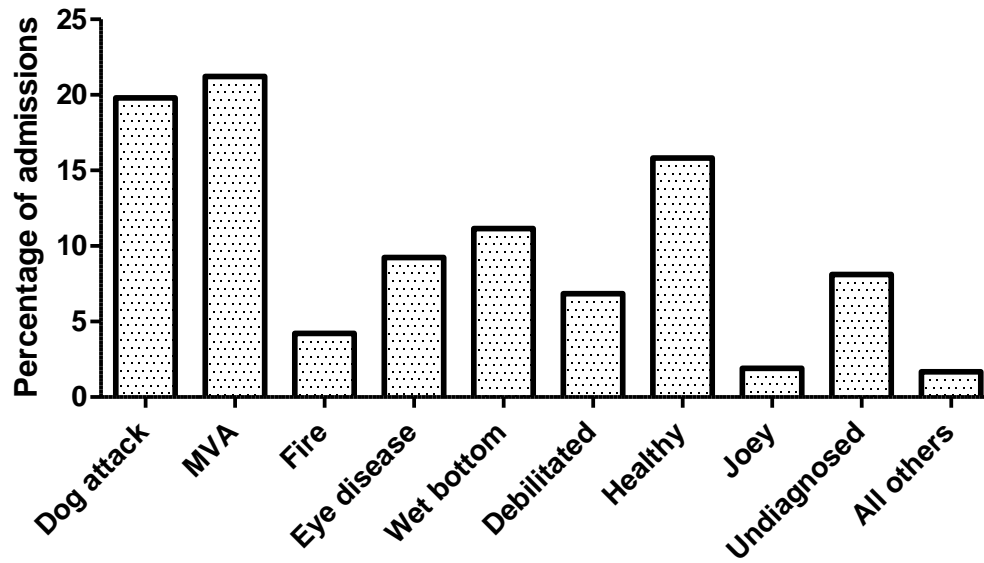


Figure 2-1. Percentage of 3781 admissions to the Koala Hospital from 1st January 1975 to 31st December 2004 in each re-coded admission category.

MVA: motor vehicle accident.

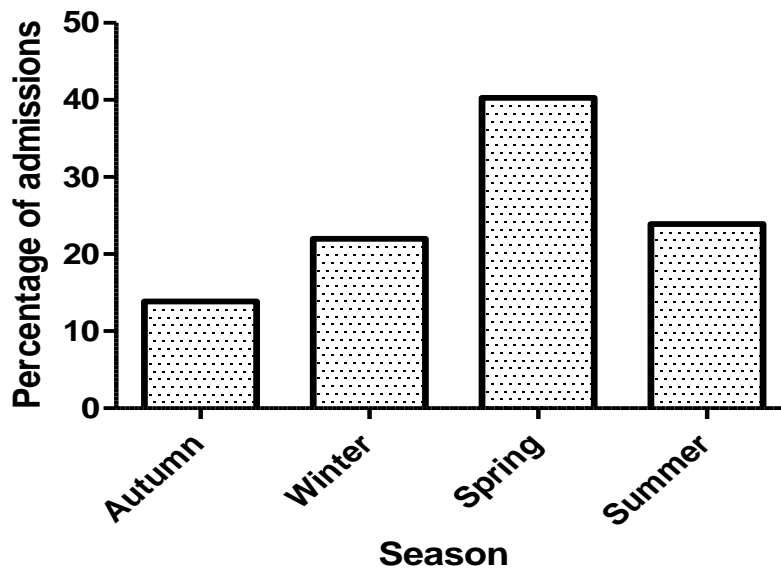


Figure 2-2. Percentage of admissions by season for 3781 admissions to the Koala Hospital from 1st January 1975 to 31st December 2004.

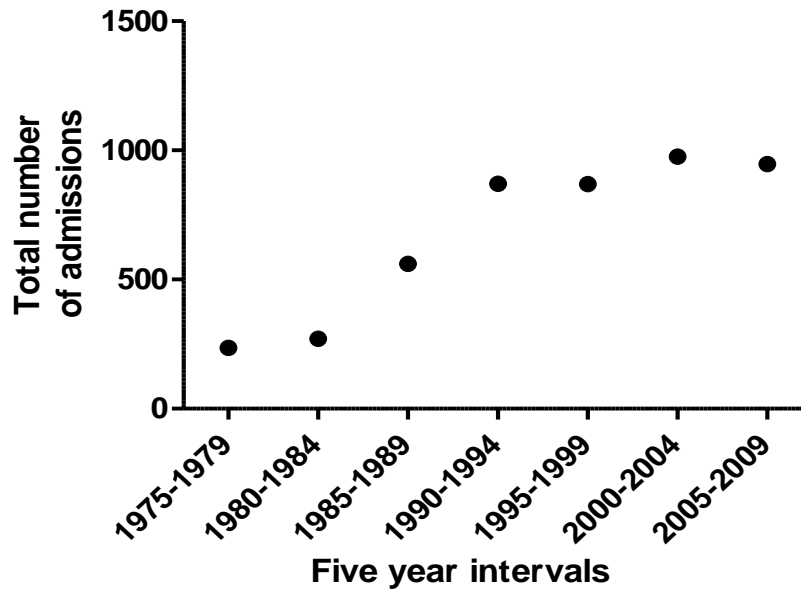


Figure 2-3. Total number of 4728 admissions per five-year interval to the Koala Hospital from 1st January 1975 to 31st December 2009.

Data from 1st January 2005 to 31st December 2009 not included in statistical analyses.

2.4.2 At-risk cohorts in comparison with the wild population

Analysis of all admissions in comparison with the assumed wild population structure found that males were at higher risk of presenting than females, aged and juvenile animals were over-represented and young and mature animals were under-represented in presentations (Table 2-2). Important findings were that young age groups were over-represented in categories associated with trauma, predation or misadventure (dog attack, fire, motor vehicle accidents, all other) and as healthy admissions; and older age groups were over-represented in admission categories associated with disease (eye disease, wet bottom, debilitated). Finally, females were over-represented for wet bottom and males for admission as motor vehicle accident or eye disease.

Table 2-2. Comparison of admissions between the Koala Hospital population and the wild reference population (chi-sq analysis).

	Age				Chi-sq	d.f.	p	Sex			Chi-sq	d.f.	p
	Juvenile	Young	Mature	Aged				Female	Male				
Dog attack	O (22.5)	P (0.002)	O (10.3)	P (0.3)	65.9	3	< 0.0001	P (0)	P (0)	0	1	1	
MVA	O (4.2)	U (2.4)	P (0.3)	P (0.03)	13.8	3	0.003	U (8.9)	O (6.8)	31.5	1	< 0.0001	
Fire	O (10.8)	O (2.7)	P (0.01)	P (1.4)	29.9	3	< 0.0001	P (0.4)	P (0.4)	1.5	1	0.215	
Eye disease	P (0.7)	U (9.5)	P (2.6)	O (5.5)	36.5	3	< 0.0001	U (4.9)	O (3.6)	16.8	1	< 0.0001	
Wet bottom	P (0.3)	U (3.4)	P (0.2)	O (6.5)	20.9	3	< 0.0001	O (6.1)	U (8.8)	29.9	1	< 0.0001	
Debilitated	O (5.5)	U (11.5)	P (0.4)	O (12.1)	58.9	3	< 0.0001	P (0.01)	P (0.01)	0.05	1	0.826	
Healthy	O (20.4)	P (1.9)	U (4.1)	P (1.8)	56.5	3	< 0.0001	P (0.3)	P (0.3)	1.3	1	0.246	
Joey	O (31.8)	U (7.7)	U (15.3)	U (3.3)	108.4	3	< 0.0001	P (0.5)	P (0.4)	1.8	1	0.180	
Undiagnosed	O (5.0)	U (5.4)	P (0.02)	O (4.2)	29.1	3	< 0.0001	U (1.2)	O (0.9)	4.3	1	0.038	
All other	O (2.9)	O (1.3)	P (0.1)	P (0.5)	9.5	3	0.023	P (0.2)	P (0.2)	0.8	1	0.368	
Total	O (74.2)	U (25.0)	U (3.0)	O (15.4)	235.2	3	< 0.0001	U (2.4)	O (2.2)	9.2	1	0.002	

Figures in brackets are contribution of each explanatory variable to the chi-square. d.f: degrees of freedom. O: over-represented, U: under represented, P: on par; compared with wild reference population assumed to have a 1:1 sex ratio, and age class percentages of: 6.3% juveniles, 43.75% young adults, 40.63% mature adults and 9.4% aged adults (Biolink, 2005; Biolink, 2008). Age classes based on tooth wear class (TWC) according to Martin (1981), where Juvenile: TWC I - II; Young: TWC III; Mature: TWC IV; Aged: TWC V - VII. MVA: motor vehicle accident.

2.4.3 Statistical models of the Koala Hospital population

Findings from the multivariable multinomial logistic regression model are presented as probabilities in Figure 2-4 through Figure 2-6 and as odds ratios and 95% confidence intervals in Table 2-3 through Table 2-6. All the explanatory variables were significant (age class, $p = 0.0001$; sex, $p = 0.0066$; season, $p < 0.0001$; five-year interval, $p < 0.0001$). In addition, an interaction between season and sex was significant, meaning that the effect of sex on admission categories varied by season ($p = 0.0361$) and data, presented as probabilities, are thus presented for both sex and season in one figure (Figure 2-5).

Table 2-3. The odds ratios (95% confidence intervals) calculated using logistic regression for the relationships between age class and the reason for admission.

Outcome	Juvenile	p	Young	p	Mature	p
Eye disease	1		1		1	
Dog attack	7.43 (3.95 - 14.0)	< 0.0001	3.09 (2.05 - 4.66)	< 0.0001	1.12 (0.75 - 1.67)	0.584
MVA	4.8 (2.6 - 9.3)	< 0.0001	2.73 (1.81 - 4.14)	< 0.0001	0.46 (0.3 - 0.71)	0.0004
Fire	21.9 (8.1 - 59.1)	< 0.0001	4.56 (1.90 - 10.9)	0.0007	1.59 (1.08 - 2.33)	0.019
Wet bottom	1.3 (0.6 - 2.8)	0.4896	1.47 (0.95 - 2.27)	0.0837	0.98 (0.67 - 1.46)	0.937
Debilitated	2.62 (1.32 - 5.19)	0.0058	0.56 (0.34 - 0.9)	0.0181	3.49 (1.49 - 8.16)	0.0039
Healthy	6.1 (3.2 - 11.5)	< 0.0001	2.14 (1.4 - 3.26)	0.0004	0.87 (0.59 - 1.3)	0.507
Joey	> 999.99 (<0.001 - > 999.9)	0.9175	> 999.99 (<0.001 - >999.9)	0.9398	> 999.99 (<0.001 - > 999.9)	0.950
Undiagnosed	3.81 (1.9 - 7.6)	0.0002	1.24 (0.77 - 2.0)	0.3672	0.86 (0.55 - 1.33)	0.489
All other	22.4 (5.7 - 88.8)	< 0.0001	3.45 (0.96 - 12.4)	0.0585	2.8 (0.82 - 9.66)	0.100

Age estimated according to tooth wear class (TWC) (Martin, 1981): Juvenile: TWC I – II; Young: TWC III; Mature: TWC IV. MVA: motor vehicle accident. Reference groups: Aged (TWC V- VII) and eye disease.

Table 2-4. The odds ratios (95% confidence intervals) calculated using logistic regression for the relationship between sex and the reason for admission.

Outcome	Male	p
Eye disease (ref)	1	
Dog attack	0.48 (0.23 - 1.03)	0.059
MVA	0.49 (0.24 - 1.01)	0.054
Fire	0.47 (0.027 - 8.12)	0.60
Wet bottom	0.2 (0.09 - 0.43)	< 0.001
Debilitated	0.44 (0.19 - 1.0)	0.049
Healthy	0.72 (0.3 - 1.69)	0.443
Joey	0.57 (0.09 - 3.5)	0.551
Undiagnosed	0.44 (0.19 - 1.01)	0.053
All other	0.26 (0.038 - 1.74)	0.164

MVA: motor vehicle accident. Reference groups: female and eye disease.

Table 2-5. The odds ratios (95% confidence intervals) calculated using logistic regression for the relationships between season and the reason for admission.

	Winter	p	Spring	p	Summer	p
Eye disease	1		1		1	
Dog attack	1.55 (0.67 - 3.6)	0.3086	1.44 (0.72 - 2.89)	0.3025	0.42 (0.2 - 0.86)	0.018
MVA	1.43 (0.62 - 3.25)	0.4000	0.59 (0.29 - 1.18)	0.1392	0.27 (0.13 - 0.56)	0.0004
Fire	3.81 (0.37 - 39.0)	0.2588	16.7 (2.1 - 132.9)	0.0079	7.3 (0.90 - 59.8)	0.063
Wet bottom	0.93 (0.41 - 2.12)	0.8625	0.32 (0.16 - 6.4)	0.0013	0.49 (0.25 - 0.95)	0.036
Debilitated	0.911 (0.36 - 2.34)	0.8463	0.44 (0.199 - 0.98)	0.0433	0.48 (0.22 - 1.1)	0.067
Healthy	2.71 (1.08 - 6.85)	0.0340	2.45 (1.12 - 5.35)	0.0248	0.91 (0.41 - 2.02)	0.812
Joey	2.4 (0.47 - 12.67)	0.2928	0.68 (0.15 - 3.19)	0.6262	0.61 (0.14 - 2.8)	0.531
Undiagnosed	1.17 (0.47 - 2.96)	0.7329	0.47 (0.21 - 1.03)	0.0603	0.39 (0.17 - 0.87)	0.021
All other	1.6 (0.32 - 8.22)	0.5620	0.87 (0.2 - 3.73)	0.8554	0.61 (0.14 - 2.67)	0.519

MVA: motor vehicle accident. Reference groups: autumn and eye disease.

Table 2-6. The odds ratios (95% confidence intervals) calculated using logistic regression for the relationships between five-year interval and the reasons for admission.

Outcome	1980 - 1984	p	1985 - 1989	p	1990 - 1994	p	1995 - 1999	p	2000 - 2004	p
Eye disease (ref)	1		1		1		1		1	
Dog attack	1.09 (0.55 - 2.2)	0.79	1.13 (0.61 - 2.08)	0.69	0.99 (0.56 - 1.76)	0.97	1.42 (0.80 - 2.51)	0.23	1.03 (0.59 - 1.8)	0.91
MVA	1.55 (0.7 - 3.3)	0.25	2.14 (1.1 - 4.16)	0.02	1.95 (1.04 - 3.66)	0.04	2.58 (1.38 - 4.82)	0.0029	2.75 (1.49 - 5.1)	0.0012
Fire	0.488 (0.08 - 2.94)	0.43	1.57 (0.45 - 5.46)	0.48	8.08 (2.67 - 24.48)	0.0002	0.08 (0.01 - 0.76)	0.03	2.43 (0.78 - 7.6)	0.13
Wet bottom	1.19 (0.54 - 2.6)	0.67	1.9 (0.96 - 3.75)	0.06	1.19 (0.6 - 2.29)	0.61	1.17 (0.6 - 2.26)	0.64	1.21 (0.64 - 2.3)	0.56
Debilitated	0.96 (0.43 - 2.11)	0.91	0.53 (0.25 - 1.10)	0.09	0.65 (0.33 - 1.27)	0.21	0.63 (0.32 - 1.23)	0.17	0.89 (0.47 - 1.7)	0.73
Healthy	1.16 (0.59 - 2.28)	0.66	0.74 (0.4 - 1.35)	0.33	0.825 (0.47 - 1.45)	0.50	0.79 (0.45 - 1.39)	0.43	0.49 (0.28 - 0.88)	0.02
Joey	0.88 (0.24 - 3.19)	0.84	0.6 (0.19 - 2.06)	0.44	0.38 (0.12 - 1.25)	0.11	0.33 (0.10 - 1.10)	0.07	2.29 (0.81 - 6.48)	0.12
Undiagnosed	0.89 (0.411 - 1.94)	0.78	1.12 (0.57 - 2.18)	0.74	0.77 (0.41 - 1.47)	0.43	0.58 (0.30 - 1.11)	0.10	0.62 (0.33 - 1.17)	0.14
All other	0.93 (0.054 - 15.7)	0.96	1.58 (0.16 - 16.1)	0.70	1.89 (0.21 - 16.23)	0.58	4.04 (0.50 - 32.6)	0.19	11.04 (1.4 - 84.9)	0.02

MVA: motor vehicle accident. Reference groups: 1975 - 1979 and eye disease.

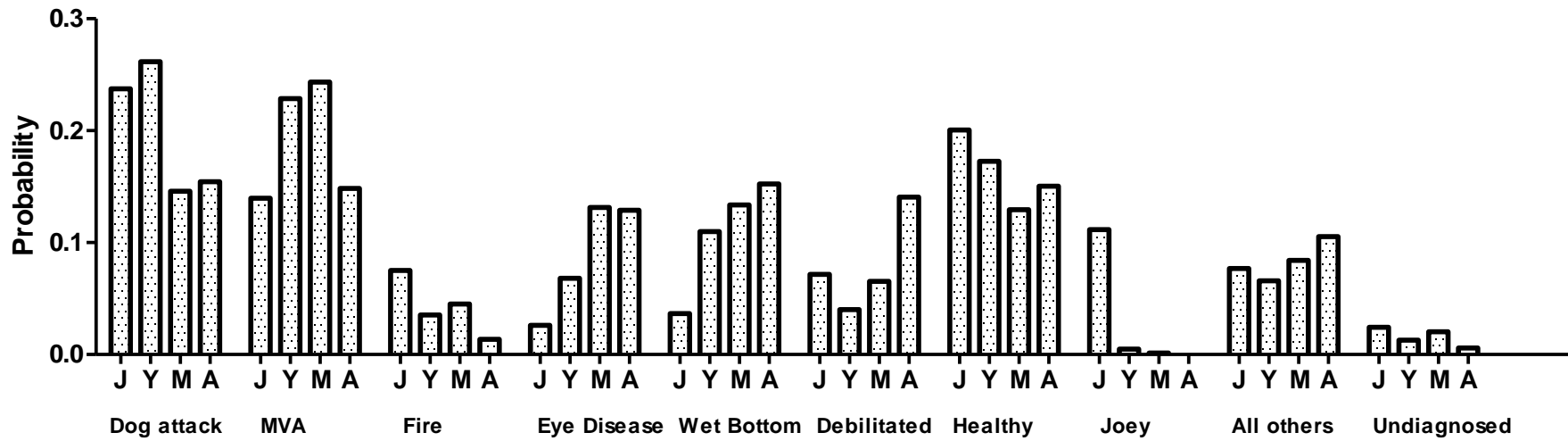


Figure 2-4. Relative probabilities of admission outcomes where age class was a significant risk factor.

Calculated by multivariable multinomial logistic regression. Age estimated according to tooth wear class (TWC) (Martin, 1981): Juvenile: TWC I – II; Young: TWC III; Mature: TWC IV. MVA: motor vehicle accident. Reference groups: Aged (TWC V- VII) and eye disease.

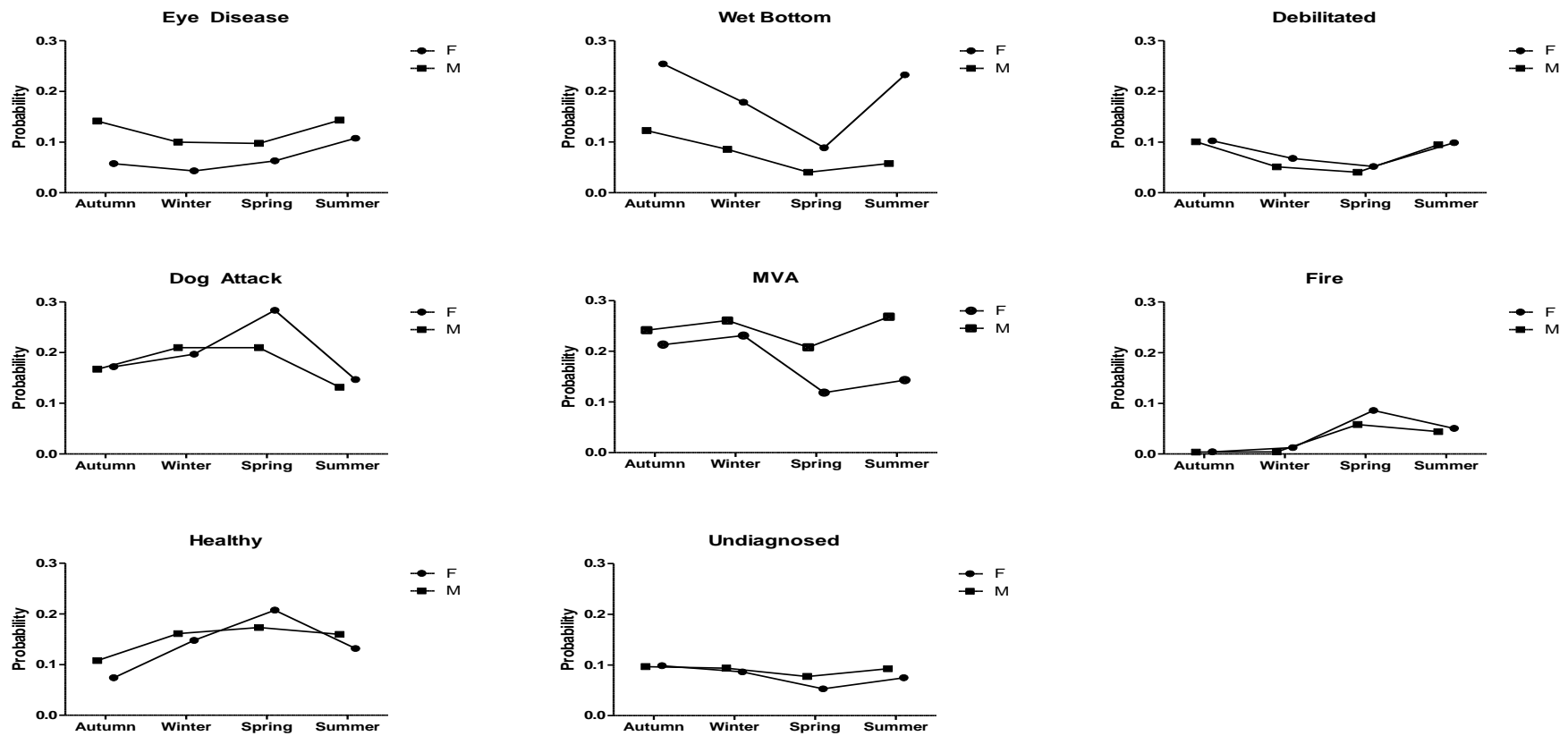


Figure 2-5. Relative probabilities of admission outcomes where sex or season was a significant risk factor.

Calculated by multivariable multinomial logistic regression. M: male; F: female; MVA: Motor vehicle accident. The only significant interaction ($p < 0.05$) between sex and season was for MVA. Reference groups: autumn, female and eye disease.

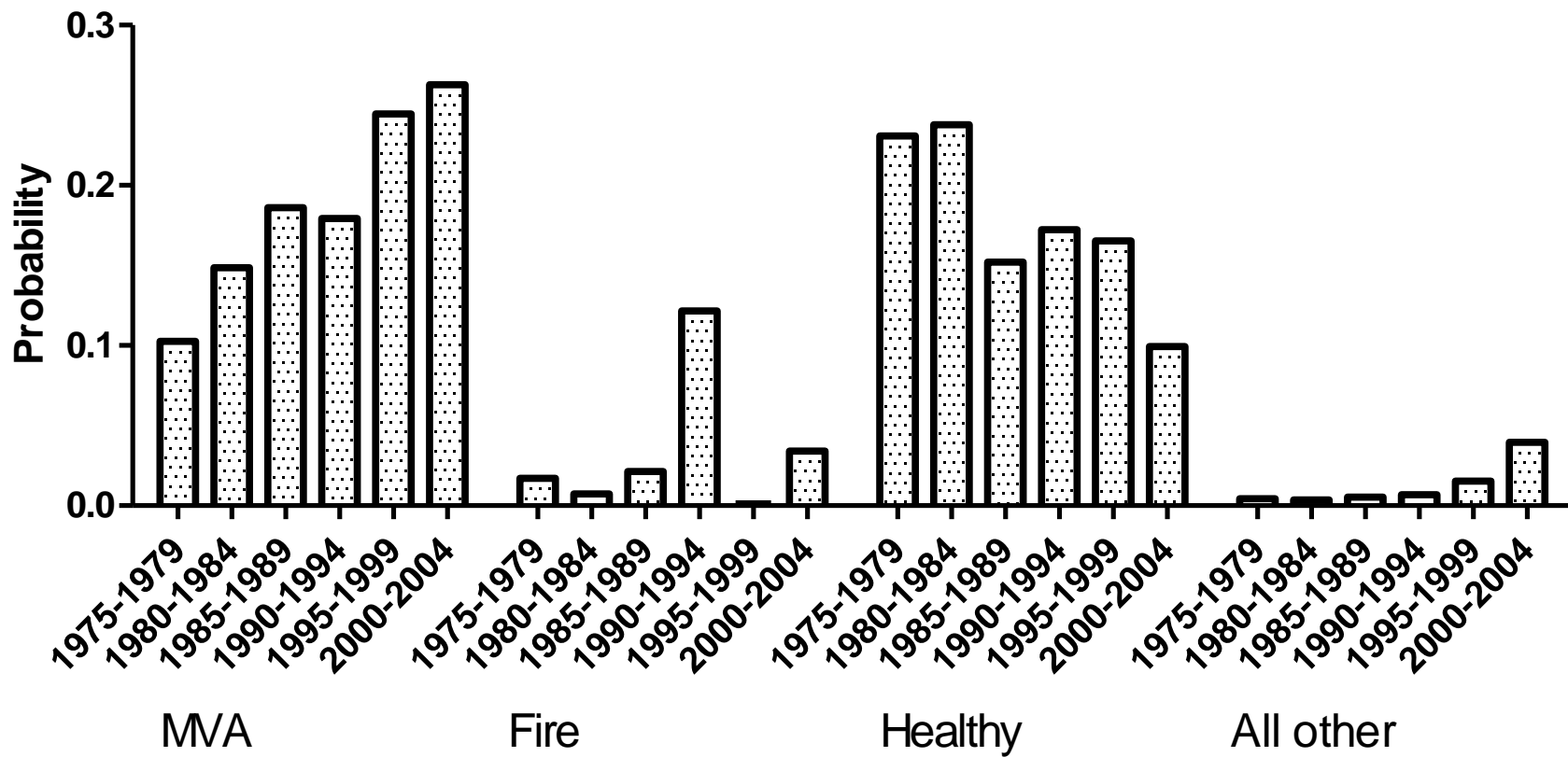


Figure 2-6. Relative probabilities of admission outcomes where five-year interval was a significant risk factor.

Calculated by multivariable multinomial logistic regression. Reference groups: eye disease and 1975 - 1979. MVA: Motor vehicle accident.

Findings of these analyses were broadly in agreement with that of the previous analysis considering hospital and wild populations (see 2.4.2). There was an increased risk of younger animals presenting for traumatic reasons (dog attacks, fire) or healthy; and older animals presenting as admissions associated with disease (eye disease, wet bottom, debilitated or undiagnosed) (Figure 2-4). Females were more likely to present with wet bottom, debilitated or undiagnosed; and males with eye disease or as motor vehicle accidents (Figure 2-5). Warmer months increased risk of presentation as a result of fire (Figure 2-5). The only significant interaction between explanatory variables in the multivariable multinomial model was between sex and season. Males were at increased risk of motor vehicle accidents compared to references in spring (OR: 2.4; CI: 1.04 - 5.66; $p = 0.039$) and summer (OR: 2.8; CI 1.8 - 6.9; $p = 0.019$) but females were at decreased risk in these seasons (Figure 2-5). Diseased animals (wet bottom, debilitated or undiagnosed) were more likely to present in autumn (Figure 2-5). Risk of motor vehicle accidents increased after 1985 in comparison with the reference group (Figure 2-6). Admission as a result of fires was more likely from 1990 - 1994 and decreased immediately following 1995 (Figure 2-6). Admission in the category “all other” significantly increased between 2000 and 2004 (Figure 2-6).

2.5 DISCUSSION

This study examined a large data-set comprising of records kept by the Koala Hospital over a 30-year period. Analysis revealed important differences in the type of animals presenting as trauma cases (younger age groups, male), or because of disease (older age groups). This information may be useful in predicting the likely effect of these threats on the local koala populations. Seasonal differences were evident for many admission categories and can be used to target public educational campaigns, efficiently deploy

resources within the Koala Hospital and may be useful in planning future scientific field-studies. Finally, changes over time revealed an increasing trend for animals presenting as motor vehicle accidents, which may be a result of fragmentation of habitat. Such evidence suggests a renewed effort should be made in preventing koala - motor vehicle accidents.

Studies of coded medical records are common, as medical data are frequently organised in this manner and are simpler to analyse than original clinical data. In this study, the large size of the database allowed statistical analyses with high power. Further, data collection began in the 1970s, meaning temporal analysis was possible. Such studies may be difficult in wildlife research where baseline data do not often exist. Finally, the dataset was made freely available to the author and, as it was already computerised, analysis was simpler and less labour intensive than using paper medical records. Such inherent advantages make the study of medical records a frequent and attractive area of research in human and veterinary literature and their use in wildlife research is increasingly being recognised (Nattrass, 1992; Shine & Koenig, 2001; Koenig, Shine *et al.*, 2002; Dique, Preece *et al.*, 2003; Dique, Thompson *et al.*, 2003; Ramp, Caldwell *et al.*, 2005; Pettett, Bird *et al.*, 2006; Trocini, Pacioni *et al.*, 2008).

The limitations of such studies are commonly recognised as data entry error, inadequate and/or inflexible coding rules, coders' interpretation of medical records leading to observer variation, and under-reporting of co-morbidities (Safran, 1991; Barrie & Marsh, 1992; Pollari, Bonnett *et al.*, 1996). In this work, the two major areas of potential inaccuracy were attribution of coding and errors relating to data entry. Code attribution by the Koala Hospital staff remains broadly incident focussed rather than diagnostically oriented and is

based on historical classification codes (C. Flanagan, pers. comm.)¹. Co-morbidities are not recognised in admission codes, despite their acknowledged role in reasons animals present to wildlife care institutes (Canfield, 1987; Trocini, Pacioni *et al.*, 2008). Although definitions of codes may seem obvious to rehabilitators, the implementation of objective case descriptions would improve rigour of data by reducing subjectivity and intra- and inter-observer variation. The addition of co-morbidities would allow more in-depth analysis of incident related admissions. In this study, rare admission codes were grouped as “all other”. The relative risk of animals presenting in this category increased between 2000 and 2004, and that of “healthy” decreased (Figure 2-6), possibly indicating a change in the way animals have been coded rather than a real change in admissions. The current supervisor started working at the Koala Hospital in 2000 and may have influenced such coding decisions. Provision of case descriptions and associated pull-down menu options would improve objectivity and remove the temptation to invent new codes. A provision for notes in each medical record could record unusual events so that information is not lost. The establishment of purposeful scientifically rigorous data collection methods, including these suggestions, would improve the validity of future studies. Despite these limitations, there is still much to be gained by analysing such data, as long as conclusions are limited to causes of admission, and not extrapolated to presume definitive diagnosis.

The second potential limitation - accuracy of translation of paper medical records to computer database - was examined by studying ages coded for joeys and, in Chapter 3 of this thesis, by examining cohorts of paper medical records relating to wet bottom or eye disease. Error rates were found to be low (11% and < 2%, respectively) in comparison to studies of medical records in other institutes (error rates up to 44%) (Safran, 1991). With

¹ Personal communication: C. Flanagan, Supervisor, Koala Hospital, Port Macquarie, NSW, March, 2010.

the exception of admission codes, this study explored objective data only (signalment and date), thus subjective interpretation by coders and inter-observer variation, acknowledged as weaknesses in other studies (Safran, 1991) were likely to have had minimal influence. Greater exploration of other aspects of the computer database may demonstrate greater inaccuracies, in particular where coders have been required to interpret medical records, an area they occasionally find challenging (E. Gabriel, pers. comm.)².

Therefore, although this dataset is limited by the incident-based subjective coding of animals and the lack of information regarding co-morbidity, the size of the database, the access to rare longitudinal information, the high accuracy of data input from paper records, and the objectivity of much of the data examined suggest useful conclusions can be made using this dataset.

The strength of conclusions based on comparison of the population dynamics of the hospital and wild populations are dependent on the accuracy of assumptions of the wild population structure. The reference wild population used in this study was drawn from bush habitat (the Innes Peninsula and Thrumpster) (Biolink, 2005; Biolink, 2008) within the catchment area serviced by the Koala Hospital. Surveys of koala population demographics are unusual outside of Victoria where koalas are intensively managed due to over-abundance (McLean, 2003). The interplay between selection pressures and population demographics may be complex and each play a role in determining final population structure (Byholm, Ranta *et al.*, 2002; Cockburn, Osmond *et al.*, 2008). Selection pressures in Victoria, where koalas are over-abundant (McLean, 2003), may be different to those in south-east Queensland, where koalas are declining (Department of Environment and

² Personal communication: E. Gabriel, Secretary, Koala Preservation Society of NSW, Port Macquarie, NSW, January 2010.

Resource Management, 2009), thus Victorian populations alone may not be a useful comparison population to that of Port Macquarie. Consequently, the demographics chosen were based on populations from within the catchment of the Koala Hospital. Although a larger sample size of wild koalas from Port Macquarie would be useful to confirm the validity of this assumption, other surveys of urban koala populations in south-east Queensland, likely to be under similar selection pressures to those of Port Macquarie due to climatic and geographic similarity, have found similar population demographics (Dique, Preece *et al.*, 2003; Biolink, 2007).

Interpretation of the odds ratios from the multivariable multinomial model must be done with care, as results are always relative to potentially biased reference groups. For example, the arbitrary reference admission reason (eye disease) in the multivariable multinomial model has a male sex bias, meaning that the odds ratio of a similarly biased group (MVA) may not be significantly different. To avoid misinterpretation, results were presented as probabilities, which are intuitively easier to understand and take into account bias in the reference group.

Results of analyses comparing the hospital population to that of the wild are more likely to identify threats to the survival of wild koalas in Port Macquarie and these data are discussed first. Although results from the statistical models often identified similar trends, these data are biased to that of the hospital population and are thus most useful for planning hospital logistics. In discussion of potential threats to the local koala population, where specific components of analyses do not agree, more weight is placed on the analysis comparing hospital demographics to that of the wild population than that utilising hospital data alone.

The over-representation of juveniles, aged adults and males among koalas admitted to the Koala Hospital might be expected if juvenile animals, in particular males, are more prone to misadventure, trauma or predation (Martin & Handasyde, 1999), and aged animals to debilitation and disease (McLean, 2003). In fact, the most frequent reasons for which koalas presented to the Koala Hospital were trauma (motor vehicle accident, or dog attack) or signs consistent with chlamydial disease (eye disease, wet bottom), and the patterns of age and sex did fit this hypothesis. These findings are in agreement with past studies identifying significant threats to survival of koala populations (Obendorf, 1983; Canfield, 1987; Weigler, Booth *et al.*, 1987; Lunney, O'Neill *et al.*, 2002; Department of Environment and Climate Change - NSW, 2008; Department of Environment and Resource Management, 2009; Department of the Environment, Water, Heritage and the Arts, 2009) and trauma is a frequent reason other species of Australian wildlife enter into rehabilitation organisations (Tribe & Brown, 2000; Trocini, Pacioni *et al.*, 2008).

Over-representation of males among motor vehicle accidents suggests that these are a potentially important factor limiting gene flow, and the loss of juveniles in this manner could contribute to decline of local populations. Male dominance in motor vehicle accidents has been observed before in koalas (Obendorf, 1983; Weigler, Booth *et al.*, 1987; Canfield, 1991; Lunney, O'Neill *et al.*, 2002; Dique, Preece *et al.*, 2003; Dique, Thompson *et al.*, 2003; Stalder, 2003; Department of the Environment, Water, Heritage and the Arts, 2009) and other species, such as macropods and bandicoots, and has been attributed to differences in the behaviour of males in comparison with females (Dufty, 1994; Coulson, 1997). Prior to the establishment of a home range, young koalas, particularly males, disperse and spend more time travelling than older animals (Mitchell, 1990; Logan & Sanson, 2002b) and may thus be at higher risk of motor vehicle collision (Canfield, 1991). Home ranges are established by about three years of age (Mitchell, 1990; Martin &

Handasyde, 1999), presumably reducing risks of motor vehicle accidents. This hypothesis is supported by the results of the study comparing hospital demographics with the wild population, in which young adults (approximately 3 - 5 years old) did have decreased risk of presenting as motor vehicle accidents. Transient males can contribute approximately equal numbers of offspring as resident males (Ellis, Hale *et al.*, 2002) so loss of significant numbers of these animals may have implications for inbreeding of local populations.

Young animals have higher fecundity than older animals in chlamydial affected populations (McLean, 2003) and loss of significant numbers of breeding animals is of obvious potential significance to population viability. Motor vehicle collisions have been identified as a serious threat to the survival of some koala populations (Martin & Handasyde, 1999; Caneris & Jones, 2004), and other species of Australian native animals (Dufty, 1994; Jones, 2000; Ramp & Ben-Ami, 2006; Roger, Laffan *et al.*, 2007). The current impact of motor vehicle accidents on the Port Macquarie koala population is unknown but is likely to be important given the types of animals affected, and that animals hit by vehicles in Port Macquarie are usually otherwise healthy (Canfield, 1987; Canfield, 1991).

Behavioural reasons, likely to be responsible for the sex and age bias of motor vehicle accident admissions, did not contribute in the same way to the risk of presentation as a result of dog attack, as both juveniles and mature adults were over-represented as a dog attack victims in comparison with wild populations and there was no sex bias. This finding confirms observations made by other authors (Dique, Preece *et al.*, 2003). The smaller size of juveniles might predispose them to dog attack; however, this does not explain why mature adults were over-represented. Older animals may be more prone to predation due to underlying disease (Canfield, 1987), but if this was the only explanation, one would expect aged adults to be similarly over-represented. There is little information in the literature

concerning the age structure of dog attack victims in other areas (Lunney, Gresser *et al.*, 2004) and only nine animals have been examined after death to ascertain rate of co-morbidities (Canfield, 1987). Examination of other care groups' records would verify whether this observation exists outside of Port Macquarie. If confirmed, larger post mortem surveys and/or behavioural studies might help explain the underlying causes.

In line with other traumatic reasons for admission, animals admitted as a consequence of fire were more likely to be juveniles and young adults than expected by chance from the wild. Dead animals are left in the field after fires to provide sustenance to scavengers such as monitors and birds of prey (C. Flanagan, pers. comm.)³, thus animals in the fire admission group reflect survivors requiring medical aid or hand-rearing, rather than the whole population affected. There is little information regarding the immediate effect of bushfires on larger marsupials, with most studies in small marsupials or native rodents (Russell, Smith *et al.*, 2003; Ecological Associates Ltd, 2006; Claridge, Seebeck *et al.*, 2007; Lunney, Lunney *et al.*, 2008). Juvenile koalas might have improved survival if, as back young, they are shielded from fire by being clasped to their mother's bellies. In addition, differences in tree use by younger, smaller animals, or anatomical factors such as body surface area may be responsible. It is not known how many of this group would survive in the wild without medical attention; however, given the extent of the injuries inflicted and lack of feed immediately post-fire, survival is likely to be low (Lunney, Gresser *et al.*, 2004). Analysis of koala behaviour and population structures post-fire would provide useful information in assessing whether wild populations can recover without intervention.

³ Personal communication: C. Flanagan, Supervisor, Koala Hospital, Port Macquarie, NSW, March, 2010.

An over-representation of young animals in the “all other” and “healthy” categories are most likely reflective of the same behavioural factor (dispersal) that makes this group prone to motor vehicle accidents and dog predation. Descriptors making up the “all other” category commonly concern misadventure (e.g. fall, drowned) or predation by animals other than dogs (e.g. cat attack, crow attack) (Appendix I). “Healthy” is mostly made up of animals in the vicinity of danger as perceived by people (e.g. dangerous area, relocation, koala seeking territory, habitat loss) (Appendix I). Although the underlying behavioural reasons that koalas present in these categories may not be different to that of trauma, detailed and non-prompted information as to why animals have been thus coded should be easily accessible in the database in future (e.g. in a notes section) to identify emerging threats, describe new syndromes and identify areas for habitat rehabilitation.

From these data it is obvious that the greatest threats to younger animals are admission categories associated with trauma or misadventure, probably as a result of behaviour unique to this group. Young animals are potentially more fecund than older age groups (McLean, 2003) and therefore adequate survival of this demographic is likely to be crucial to maintenance of viable populations. Detailed surveys including population size and demographics, and estimates of fertility are urgently needed to allow accurate modelling estimating the long-term viability of koalas in Port Macquarie, given the rate of apparent higher probability of attrition of young animals because of trauma.

Due to the high frequency of trauma admissions, the ability of all rehabilitation staff involved in the first aid of koalas to effectively and rapidly triage trauma cases, and apply or seek appropriate treatment as soon as possible if required, is crucial for improved survival of these animals, and on grounds of welfare. At the Koala Hospital, koala

retrievals, particularly for trauma, are often conducted outside of normal working hours (C. Flanagan, pers. comm.)⁴. Such animals may not have immediate access to veterinary attention due to the inconvenience and expense of out-of-hours veterinary attention. There is a high frequency of head, thoracic and jaw injuries in cases of koala motor vehicle collision (Canfield, 1991), resulting ultimately in death in the majority of cases (Dique, Thompson *et al.*, 2003) and severe soft tissue injuries may be inflicted by dogs (Flanagan, 2009). Intensive treatments, opioid analgesia or euthanasia are likely to be required in many cases, and effective triage to determine when such veterinary attention is required, particularly at night when veterinarians may be less accessible, is important. Conversely, other diseases encountered are typically chronic in nature and so are less likely to require emergency treatment. Delays of a few hours until more experienced staff or veterinarians are available are unlikely to affect outcomes in these cases.

Older animals were more likely to present with signs of disease or debilitation and loss of animals from these age groups are likely to have less impact on long-term population viability. Clinical chlamydiosis might be expected to be more prevalent in older, sexually active animals, as the main route of transmission is sexual (White & Timms, 1994; Martin & Handasyde, 1999). In addition, repeated infections may result in more overt clinical signs, as occurs in people (Grayston, Wang *et al.*, 1985), and nutritional stress as a result of worn teeth (Lanyon & Sanson, 1986) may result in clinical expression of previously subclinical infections. The demise of such animals is unlikely to have significant effects on population viability: aged females in Chlamydiaceae positive populations generally have a fecundity of < 20% (McLean, 2003) and older males have behavioural changes that could reduce breeding success (Logan & Sanson, 2002a; Logan & Sanson, 2002b). Removal of

⁴ Personal communication: C. Flanagan, Supervisor, Koala Hospital, Port Macquarie, NSW, March, 2010.

older animals may even be advantageous by freeing up home ranges for other animals (Mitchell, 1990). Population modelling has indicated that the demise of aged animals had little impact on the survival of one population of koalas (Caneris & Jones, 2004), and it is possible that the same occurs in Port Macquarie. Where resources are limited, these animals should be given a lower priority than younger animals. A prospective study examining survival post-release using radiotelemetry or a retrospective case controlled study of medical records held at the Koala Hospital examining frequency of representation of this age group would better characterise rehabilitation and breeding success in this group post-release.

Significant differences exist between the sexes that suggest both anatomical and behavioural factors are important in expression of clinical signs associated with chlamydiosis. In line with the findings of other researchers (Obendorf, 1983; White & Timms, 1994; McLean, 2003; Stalder, 2003), this study found females were over-represented as wet bottom admissions, and males as eye disease admissions. Although the female predisposition to clinical wet bottom can be explained by anatomical differences (Obendorf, 1981), the over-representation of males as presenting with clinical eye disease is more difficult to understand. Traumatic injuries sustained whilst fighting might contribute to expression of ocular chlamydiosis and in addition to presentation as direct result of traumatic eye disease in male koalas (McLean, 2003). Dust, a contributory factor to trachoma in people (Alene & Abebe, 2000), may also contribute to clinical conjunctivitis in male koalas, if they are exposed to more dust than females: for example as a result of the increased travelling by males observed during breeding season (Mitchell, 1990). The exact cause of this phenomenon is yet to be elucidated.

A study examining animals classified as “undiagnosed” or “debilitated” in more detail would be useful. Debilitated and undiagnosed are vague descriptions and these categories

would benefit from a tighter clinical description, as it is possible that there is significant cross-over within these groups. Further, emerging diseases or threats might initially be classified within this group, thus analysis of these animals might warrant specific attention.

The busiest season for admissions to the Koala Hospital was spring and total admissions have increased substantially since the 1970s. The onset of the breeding season of koalas coincides with early spring (Martin & Handasyde, 1999) and male koalas older than four years have increased frequency of movement during this time (Mitchell, 1990). The dispersal of juveniles may also contribute to the increase in admissions. Staff deployment should be maximal during this period.

Presentations in autumn were more frequently associated with disease (eye disease, wet bottom, debilitated and undiagnosed) than in other seasons. During the breeding season animals may lose condition as a result of breeding efforts, be displaced from territory or be subject to sexually transmitted diseases (e.g. chlamydiosis). As the ambient temperature declines in autumn such stressors may present as clinical disease. This influx may reflect natural attrition of non-viable animals during this season.

Although temporal population data of wild populations are unavailable, analysis of the hospital data reveals interesting trends. Substantial fires occurred in the Port Macquarie area in 1994 and 2002, causing anomalous increases in koala admissions during these years. When admissions as a result of fire are excluded from the data set, mean \pm SD annual admissions have remained stable between 1990 and 2009 (Figure 2-3; ANOVA, $p = 0.47$). Without knowledge of the temporal dynamics of the local koala population, it is difficult to draw conclusions as to why koala admissions change. The human population of the Port Macquarie - Hastings local government area has approximately tripled over the period examined, from 25,323 (Australian Bureau of Statistics, 1976) to 68,430 (Australian

Bureau of Statistics, 2006). Although human population increase and associated development may increase risks of anthropogenic admissions to the hospital, it may also increase risks of koalas being admitted simply because more people are looking for sick or “endangered” animals. Alternatively, if the risk of koalas presenting to the hospital has remained stable, the population of koalas themselves may have increased during the 1980s and plateaued over the past 20 years. For meaningful extrapolation of these data to the health of the wild population, regular accurate local wild koala population surveys are vital.

Despite these limitations, changes in relative risk of animals presenting within each five-year period may indicate changing risk factors for koalas, and suggest developing threats. The significantly increased relative risk of koalas presenting as motor vehicle accident since 1985 is alarming (Figure 2-6). Presumably, the increase in the human population of Port Macquarie has also increased the number of motor vehicles, dogs and the fragmentation of habitat. At first glance it would seem that increased motor vehicles in the area probably account for the increase in koalas presenting as result of motor vehicle accidents, as has been found in studies in south-east Queensland (Dique, Thompson *et al.*, 2003). However, analysis of the seasonal data does not support this simple hypothesis. Tourist numbers (and presumably associated cars and traffic), approximately double the population of Port Macquarie during summer (W. Beverley, pers. comm.)⁵, yet only males are at increased risk of presenting as a result of motor vehicle accident in summer in comparison with cooler seasons, and in summer and spring in comparison with females (Figure 2-5). The combination of seasonal male behavioural factors and seasonal increased numbers of vehicles most likely explains patterns of motor vehicle accidents. Vehicle

⁵ Personal communication: W. Beverley, Project support officer, Corporate & Business Services, Port Macquarie-Hastings Council, Port Macquarie, NSW, December, 2008.

collisions in other species (deer, macropods and bandicoots) have also been associated with animal behavioural factors (Dufty, 1994; Coulson, 1997; Sudharsan, Riley *et al.*, 2006). If the risk of motor vehicle accident is related to koalas spending more time travelling on the ground, as well as absolute vehicle numbers, risk factors related to increased travelling, such as time of year (breeding in spring and summer), age (juvenile) and sex (male), and interactions (male and summer, male and spring) might be expected to be connected with increased risk of motor vehicle accident in these groups.

The most logical reason that koalas might be spending more time on the ground in recent years is fragmentation of bush habitat and reduction of the number of suitable trees within urban habitat as a result of development. Radio-tracking of koalas in urban areas has demonstrated that provision of habitat corridors and patches are less important than total area and connectivity of habitat, and koalas may make frequent, quite long range movements (> 2 km) between patches of suitable habitat (White, 1999). Thus, efforts to mitigate koala motor vehicle collisions around Port Macquarie need to concentrate on providing a mosaic of more viable habitat, including trees within urban areas to reduce koala movement, rather than relying on funnelling koalas into habitat corridors or on traffic calming efforts alone.

Mitigation efforts aimed at reducing motor vehicle/wild animal collisions broadly concentrate on deterring animals from road sides and/or reducing speed of vehicles (Magnus, Kriwoken *et al.*, 2004) but to be effective, some authors propose they must take into account habitat use by the target species (Roger & Ramp, 2009), a suggestion supported by the findings of this work. Trials using differential speed zones at night did not reduce vehicle speed or reduce the number of koala-vehicle collisions (Dique, Thompson *et al.*, 2003). Studies overseas using temporary signage incorporating flashing lights have been effective in reducing deer collisions with vehicles (Sullivan, Williams *et al.*, 2004),

although this area requires further study. Night travel by vehicles has been identified as a risk factor for wildlife-vehicle collisions (Rowden, Steinhardt *et al.*, 2008) and should be discouraged using educational campaigns. Practical and cost-effective mitigation regimes that the Koala Hospital might implement could include mosaic habitat maintenance and expansion, and identification of koala-motor vehicle collision hotspots (Preece, 2007). Educational campaigns targeting driving behaviour should be timed to be maximal in anticipation of the spring breeding season, highlight collision hotspots, recommend daytime travel and reduced speed, and importantly, must also educate non-local tourists in summer. More broadly, new urban development planning should consider habitat maintenance in new suburbs, and koala fencing and underpasses for new roads. Fortunately, recent development in an area of koala habitat in the vicinity of Port Macquarie (Thrupster) has led the Port Macquarie-Hastings council to adopt a koala plan of management, incorporating many of these measures (Biolink, 2008).

Interestingly, the relative risk of presenting as a dog attack has not significantly changed since the 1970s. The change in numbers of dogs in Port Macquarie is not known, as many animals are not routinely registered, but presumably has increased along with the human population. It is possible that the increasing practice of responsible pet ownership and the legal requirements to do so (The New South Wales Companion Animals Act of 1998) may have contributed to this situation. Continued vigilance in this area is important, as dogs may be a significant threat to koala populations (Lunney, Gresser *et al.*, 2007; Department of the Environment, Water, Heritage and the Arts, 2009).

Risk of admissions as a result of eye disease or wet bottom have not changed. Such data suggest a stable host-parasite relationship, however, subclinical chlamydiosis is a frequent occurrence in koalas (Jackson, White *et al.*, 1999; McLean, 2003) and analysis of these data based on external clinical signs runs the risk of under-estimating prevalence. A study

examining chlamydial prevalence in the wild population over time would be extremely useful in studying the evolving host-pathogen-environment relationship.

Outcomes of this study that predict when animals present may be useful in making management decisions regarding resource allocation at the Koala Hospital for each season. Predictably, animals were more likely to present because of fire in spring and summer, and training of staff in anticipation of fires must be taken in advance of these seasons. Seasonal risk factors for animals presenting in different admission categories may provide targets for educational campaigns or research efforts. For example, probability of presentation as a result of dog attack was increased in autumn and winter, thus educational campaigns and dog “curfews” could target this season. Scientists wishing to investigate potentially emerging diseases might target autumn, when animals are at increased risk of presenting as diseased, debilitated or undiagnosed.

This study is the first of its kind in a koala hospital. It has the advantages of a large data set, both in terms of total numbers and in the time span covered. This study highlights the potential of research using wildlife rehabilitation databases to identify risks to wild populations, and provides information that may be useful for rehabilitation centres to plan logistics and allocate resources. In most cases, differences in the likely behaviour between the cohorts examined was considered the most important contributor to risk in each admission category and effective mitigation of threat must take this into account. A significant and increasing threat to survival of the Port Macquarie koala population was identified as an increasing risk of motor vehicle accidents since 1985. A regular scientifically rigorous ecological survey of the koala population in Port Macquarie is vital to assess whether the significant efforts of rehabilitators, the local council, and Port Macquarie residents aimed at preserving the wild koala population are effective.

CHAPTER 3 - ANALYSIS OF DIAGNOSIS AND TREATMENT REGIMES FOR CHLAMYDIOSIS AND THEIR IMPACT ON THE RECOVERY AND RELEASE OF KOALAS HELD AT THE KOALA HOSPITAL BETWEEN 1995 AND 2005

3.1 INTRODUCTION

Rehabilitation of wildlife in Australia is a high profile activity involving thousands of people and tens of thousands of wildlife cases annually (Tribe & Brown, 2000; Shine & Koenig, 2001). Despite its popularity, few studies exist concerning the success of rehabilitation and hand-rearing of wildlife in Australia. These are limited to findings of rapid and high mortality of ringtail and brushtail possums (Augee, Smith *et al.*, 1996; Tribe, Hanger *et al.*, 2005) and high mortality and poor breeding success of penguins rehabilitated following oil immersion (Giese, Goldsworthy *et al.*, 2000; Goldsworthy, Giese *et al.*, 2000). Translocated animals, which might be expected to have lower mortality than rehabilitated animals, also have poor survival. For example, 70% of healthy wild brushtail possums died within a week of translocation (Pietsch, 1994) and relocated ringtail possums survived approximately 55% the length of time of wild controls (Augee, Smith *et al.*, 1996). Studies from overseas have found similarly high mortality rates of rehabilitated and hand-reared wildlife shortly after release (Robertson & Harris, 1995; Anderson, Gress *et al.*, 1996; Fajardo, Babiloni *et al.*, 2000; Ben-David, Blundell *et al.*, 2002; Beringer, Mabry *et al.*, 2004) and extremely low three-month survival rates of many translocated species (McCullough, Jennings *et al.*, 1997; Adams, Hadidian *et al.*, 2004; Calvete, Angulo *et al.*, 2005). It seems paradoxical that although large investments of time, effort and money are made in the rehabilitation of wildlife, there are very few studies examining

aspects of the disease, host or rehabilitation efforts associated with successful release (Gage, Gerber *et al.*, 1993; Mignucci-Giannoni, 1999; Mowbray, 2009) and post-release survival (Fajardo, Babiloni *et al.*, 2000; Goldsworthy, Giese *et al.*, 2000; Molony, Dowding *et al.*, 2006).

This information hiatus probably reflects a difference in conservation philosophy between wildlife rehabilitators who usually focus on individuals, and scientists who focus on populations (Shine & Koenig, 2001). Wildlife rehabilitation undoubtedly provides benefit to individual animals, may replenish local populations, allows development of rehabilitation and husbandry techniques, provides the opportunity to study wild animals, and raises public awareness of conservation issues (Siemer, Brown *et al.*, 1991; Tribe & Brown, 2000; Turnbull, 2005). Undeniably, rehabilitation of wildlife will persist whilst debate continues as to its ultimate worth (Estes, 1991; Estes, 1998; Tribe & Brown, 2000), thus it is important that methods of rehabilitation and treatments for specific disease are studied to minimise wastage, improve animal welfare and enable the best possible outcome for rehabilitated wild animals and the populations to which they return.

Interestingly, rehabilitation of koalas for a number of different conditions appears to be relatively successful (Ellis, White *et al.*, 1990; Starr, 1990; Lunney, Gresser *et al.*, 2004; Tribe, Hanger *et al.*, 2005; Jones, 2008). Furthermore, successful breeding post-release, perhaps the most important marker of success, has also been demonstrated: in one study breeding rates in nine koalas rehabilitated after bushfire matched that of the control group (80% and 73%, respectively) (Lunney, Gresser *et al.*, 2004). Chlamydiosis, a common infectious disease in koalas, appears to be a particularly important factor determining successful breeding after translocation or rehabilitation of koalas. In Victoria the fertility of *Chlamydia* (now known as *Chlamydophila*) (Everett, Bush *et al.*, 1999) naïve animals declined after translocation into a *Chlamydia* positive population (Handasyde, 1986).

Koalas admitted with clinical signs consistent with chlamydial disease, such as conjunctivitis (Cockram & Jackson, 1976) or reproductive tract disease (Obendorf, 1981) (colloquially termed “wet bottom”) (Blanshard, 1994), account for approximately 20% of all admissions to the Koala Preservation Society of NSW’s Koala Hospital in Port Macquarie, NSW, making admissions as a result of clinical chlamydiosis second only to trauma (Chapter 2). Despite the acknowledged importance of this disease and the necessity of developing a reliable treatment, regimes differ between the three main koala rehabilitation centres in Australia (Blanshard & Bodley, 2008). Few studies exist regarding their effectiveness (Cockram & Jackson, 1976; Markey, Wan *et al.*, 2007; Blanshard & Bodley, 2008) and little is known about the prognosis, particularly for breeding success of such animals after release.

Medical records have been collected at the Koala Hospital since the 1970s and comprise a significant data set (Chapter 2). Although limited in some respects, analysis of these types of data may identify prognostic indicators and treatment regimes associated with successful rehabilitation and may be used to generate hypotheses for further study (Cockcroft & Holmes, 2003). It is imperative that treatments administered at the Koala Hospital have a sound evidence base, as wildlife rehabilitation groups and veterinarians frequently seek advice from staff of the Koala Hospital.

Given the longitudinal nature of these data, it might be possible to use the medical records as an indicator of survival and breeding success of treated animals, similar to passive resight methods used with owls (rings) (Fajardo, Babiloni *et al.*, 2000) and hump backed whales (photographs of tail flukes) (Mizroch, Herman *et al.*, 2004). Although this method is limited by the probability of animals re-presenting, other advantages are apparent.

Prospective survival trials undertaken by tracking radio-collared animals are usually limited to small numbers and relatively short monitoring times, as they are labour intensive

and costly (Lunney, Gresser *et al.*, 2004; Radford, McKee *et al.*, 2006). In addition, animals may be affected by the collars both in terms of behaviour (Cypher, 1997; Tuytens, Macdonald *et al.*, 2002) and as a result of collar injuries (Krausman, Bleich *et al.*, 2004), although these problems appear uncommon in koalas (Radford, McKee *et al.*, 2006). Use of records of a long-established wildlife rehabilitation centre allows for re-presentations of permanently identified animals over many years, a period of time rare in other studies. As koalas are relatively long lived (Martin & Handasyde, 1999), long-term passive monitoring of permanently identified animals is a relatively cheap and potentially useful way of assessing their survival. Further, it is possible to assess the breeding success of females through examination of records of evidence of breeding (presence of young, elongated nipples).

3.2 AIMS

This study examined a cohort of animals with external clinical signs consistent with chlamydial disease admitted to the Koala Preservation Society of NSW's Koala Hospital between 1995 and 2005, with the aim of describing the most recent methods of diagnosis and treatment at the Koala Hospital and assessing their impact on animal recovery and post-release survival. It was anticipated that the analysis would assist in providing invaluable background information for establishing treatment protocols for further investigation.

The specific aims of the study were to describe:

- 1/ how the diagnosis of chlamydial disease was made,
- 2/ which treatments were used, the route of administration and length of medication courses,

3/ what the outcomes were, and whether a treatment regime associated with successful release and post-release survival could be identified.

3.3 METHODS

3.3.1 Data collection and storage

Archived data available for this study incorporated information on koalas admitted to the Koala Hospital between 1st March 1995 and 28th February 2005. Paper medical records, amended only by veterinarians or trained para-veterinary supervisors (C. Flanagan, pers. comm.)¹, included an admission reason based on methods of examination and classification outlined previously (see Chapter 2); diagnostic tests performed in-house or at a veterinary diagnostic laboratory (Symbion Vetnostics, North Ryde, NSW, Australia); and treatments prescribed by visiting veterinarians, or supervisors according to treatment protocols. Before commencement of this study, admissions data had been entered into a database (Microsoft Access® 2003, Microsoft® Corporation, Redmond, Washington, USA) using defined codes (Appendix I).

3.3.2 Data retrieval

Database admission codes relating to chlamydial disease were “wet”, “eye” and “wee”, described in Appendix I. The Koala Hospital computer database was used to identify all records of animals admitted under these codes during the months of March, June, September and December, between 1st March 1995 and 28th February 2005. Paper medical records corresponding to these admissions were retrieved from storage and formed the basis of the study. Exclusion criteria included: animals whose records did not mention one of the words “conjunctivitis”, “diseased” or “discharge” for eye admissions, or “wet

¹ Personal communication: C. Flanagan, Supervisor, Koala Hospital, Port Macquarie, NSW, March, 2010.

bottom” or “Chlamydia” for “wet” admissions (n = 8); animals from Taree, NSW, whose records were unavailable for study (n = 21); animals presenting in the “wee” category (n = 2) as this category contained too few animals for meaningful analysis; and animals whose paper medical records could not be located (n = 8).

3.3.3 Statistical analysis

Data, collected from paper medical records under the categories outlined in Table 3-1 were entered into a spreadsheet using Microsoft® Office Excel® 2003 (Microsoft® Corporation, Redmond, Washington, USA). Histograms were prepared for each categorical explanatory variable and box and whisker plots of the continuous data.

The independence of categorical data was assessed using chi-square analysis in Minitab® Statistical Software (Minitab Pty Ltd, Sydney, NSW, Australia). Continuous variables (Table 3-1) were compared between the groups released/not released using a Mann Whitney test. Calculation of the odds ratios for explanatory variables, and their 95% confidence intervals, was conducted using binomial logistic regression (Hosmer & Lemeshow, 2000) in GenStat for Windows (2008, 11th ed., VSN International Ltd., Hemel Hempstead, Hertfordshire, UK). Explanatory variables are outlined in Table 3-1 and outcome variables were released, or not released (i.e. euthanased, transferred or died). Models were constructed by a manual forward stepwise approach. Statistical significance was concluded at $p \leq 0.05$.

Table 3-1. Variables used in statistical analyses of treatment data.

Variable (units/ categories)	Comments
Admission code* (Wet /Eye)	
Koala name	
Sex* (M/F)	
Age class* (juvenile/young/mature/aged)	Corresponding to tooth wear class I - II/III/ IV/ V - VII (Martin, 1981)
Crown rump length (cm) [‡]	Nose to end of tail
Head length (cm) [‡]	Nose to occiput
Month of presentation for this admission (March, June, September, December)	
Year of presentation for this admission (year)	1995 - 2005
Weight at presentation (kg) [‡]	
Crown rump length (cm)/ weight at presentation (kg) [‡]	
Head length (cm)/ weight at presentation (kg) [‡]	
Eye swabs* (Y/N)	Clearview Chlamydia MF enzyme-linked immunosorbent assay (ELISA), Inverness Medical Australia, Sinnamon Park, Qld, Australia
Date of eye swab test	
Result of eye swabs* (positive/negative/not recorded)	
Urogenital tract swabs* (Y/N)	Clearview Chlamydia MF enzyme-linked immunosorbent assay (ELISA), Inverness Medical Australia, Sinnamon Park, Qld, Australia
Date of urogenital swab test	
Result of urogenital tract swabs* (positive/negative/not recorded)	
Blood tests* (Y/N)	Routine haematological & serum biochemical analysis. Symbion Vetnostics, North Ryde, NSW, Australia
Ultrasound* (Y/N)	
Oxytetracycline/ polymyxin B eye ointment* (Y/N)	Terramycin® Ophthalmic Ointment, Pfizer, New York, USA
Other eye ointment* (Y/N)	
Which other eye ointment?	Table 3-2

Table 3-1 cont.

Variable (units/ categories)	Comments
Eye surgery* (Y/N)	
Enrofloxacin* (Y/N)	Baytril® oral suspension 25 mg/mL, or injectable solution 50 mg/mL, Bayer Animal Health, Pymble, NSW, Australia
Days on enrofloxacin‡	
Dose of enrofloxacin (mg/kg)	
Route of enrofloxacin* (p.o./s.c./n.r.)	
Other systemic antibiotic* (Y/N)	
Which other systemic antibiotic?	
Days on other systemic antibiotic‡	
Meloxicam* (Y/N)	Metacam® oral suspension 1.5 mg/mL or injectable 5 mg/mL, Boehringer Ingelheim, North Ryde, Australia
Route of meloxicam (p.o./s.c./n.r.)	
Days on meloxicam‡	
Ancillary treatment* (Y/N)	
Which ancillary treatment?	Table 3-2 Table 3-3
Total days in care‡	
Final result† (released/not released)	
Days until next admission‡	Released animals only
Breeding status at next admission (joey/no joey)	Released females only Presence of young noted in medical records

Categorical explanatory variables (*) and outcome variable (†): univariate binary logistic regression model; and continuous variable: Mann Whitney test (‡).

p.o: per os; s.c: subcutaneous; n.r: not recorded; Y/N: yes/no; M/F: male/female.

3.4 RESULTS

3.4.1 Animals included in the study

The method of case retrieval identified 88 records for inclusion in the study. Of these, 54 (61.4%) of animals were classified as “wet” and 34 (38.6%) as “eye” and there was bias in the type of presentation (chi-sq = 2.3, d.f. = 1, p = 0.13). Fifty-one animals (58%) were female and 37 (42%) were male and there was no sex bias (chi-sq = 1.12, d.f. = 1, p = 0.29). Juveniles (2 animals: 2.3%) were less frequently admitted than other age groups (aged adults: 15 animals (17%); young adults: 35 animals (39.8%); mature adults: 35 animals (39.8%); undetermined: 1 animal (1.13%); chi-sq = 28, d.f. = 3, p < 0.0001). There was no seasonal bias in included cases (December: 29 cases (33%); March: 16 cases (18%); June: 22 cases (25%); and September: 22 cases (25%); chi-sq = 1.9, d.f. = 3, p = 0.593).

3.4.2 Common diagnostic tests performed

Data indicated that swab-based chlamydial antigen ELISA (Clearview Chlamydia MF ELISA, Inverness Medical Australia, Sinnamon Park, Qld, Australia) and ultrasonography of the urogenital tract were selectively used on anatomical sites displaying clinical signs (Figure 3-1). Animals in the “eye” category were more likely to have eye swabs (chi-sq = 21.863, d.f. = 1, p < 0.0001), and animals in the “wet” category were more likely to have urogenital swabs (chi-sq = 18.312, d.f. = 1, p < 0.0001) and ultrasound imaging (chi-sq = 17.18, d.f. = 1, p < 0.0001). Ninety per cent of swabs (40 swabs) were taken within one week of admission; only 10 animals (20% of animals swabbed) were swabbed more than once. There was no difference in the proportion of animals having blood tests (haematological parameters and serum biochemistry) between the “eye” and “wet” groups (chi-sq = 0.424, d.f. = 1, p = 0.515).

Treatment appeared to be based more on clinical presentation than on test results. Animals with negative swab results had similar treatments to animals with positive swabs. All four animals that had negative results on eye swabs were treated with oxytetracycline polymyxin B sulphate eye ointment for at least seven days (median 7.5, range 7 - 12 days). Of the eight animals with negative urogenital swabs, five were treated with systemic enrofloxacin for at least eight days (median 12, range 8 - 31). Three swabs had no results recorded.

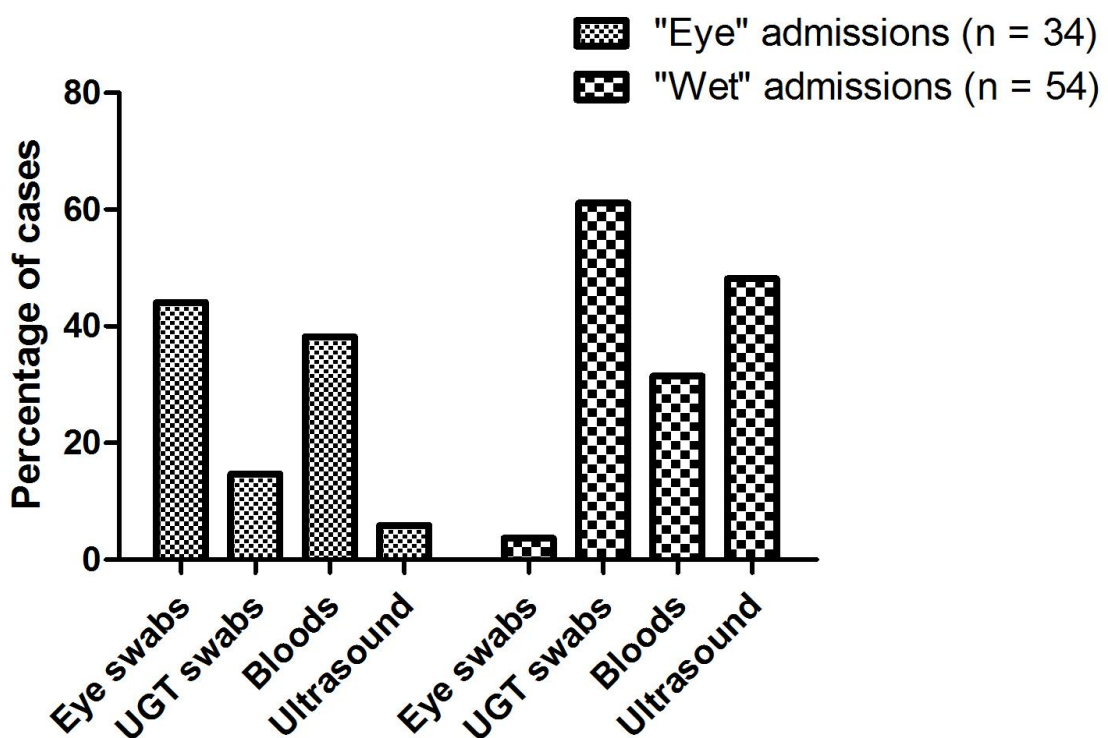


Figure 3-1. Percentage of “eye” and “wet” admissions subjected to diagnostic testing, illustrating selective use of swab and ultrasound-based diagnostics in clinically affected sites.

Cases may have received more than one diagnostic test. UGT: urogenital tract.

3.4.3 Common treatments used

Similar to diagnostics, treatment was targeted to affected sites. Animals in the “eye” category were more likely to be treated with oxytetracycline/ polymyxin B sulphate eye ointment twice daily (chi-sq = 52.62, d.f. = 1, $p < 0.0001$), other eye ointments (chi-sq = 14.72, d.f. = 1, $p < 0.0001$) and eye surgery (chi-sq = 19.97, d.f. = 1, $p < 0.0001$) than animals admitted as “wet” (Figure 3-2). Other eye ointments were most commonly chloramphenicol based and frequently contained hydrocortisone acetate (Table 3-2). Eye surgery consisted of ablation of proliferative conjunctiva and/or removal of the third eye lid (Blanshard, 1994; Blanshard & Bodley, 2008) and was performed in 30% of animals in the “eye” group. Eleven animals (12.5%) received no treatments.

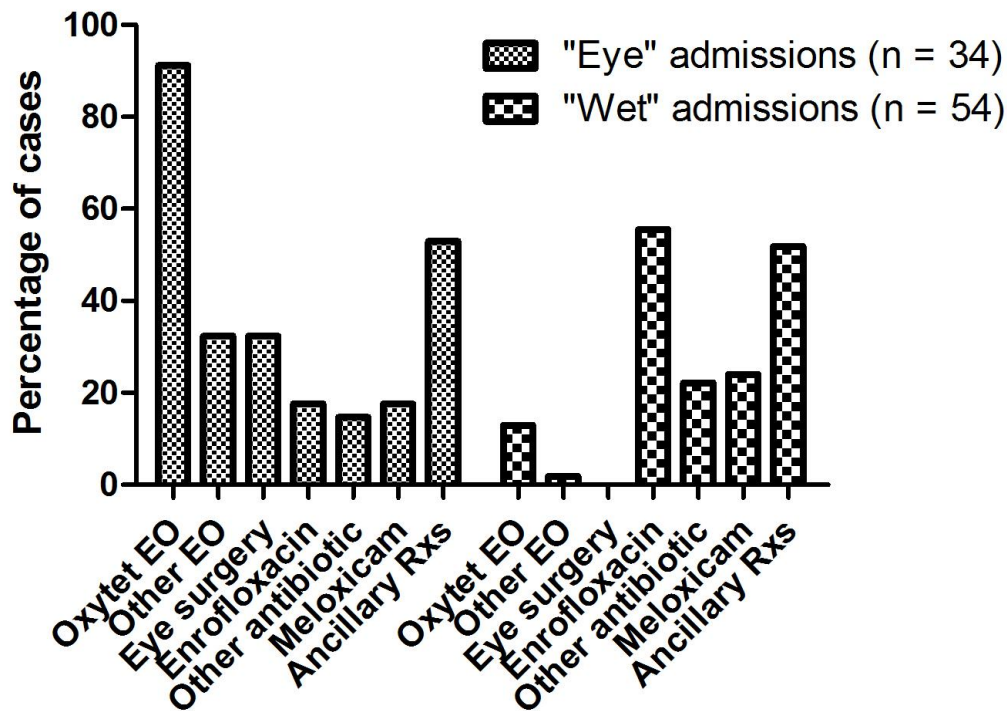


Figure 3-2. Percentage of “eye” and “wet” admissions administered different treatments.

Cases may have received more than one treatment. Eleven animals (12.5%) received no treatments. EO: eye ointment; oxytet: oxytetracycline/ polymyxin B; Rx: treatments.

Table 3-2. Detail of eye ointments other than oxytetracycline/ polymyxin B, and ancillary treatments* given to 34 “eye” cases.

Treatment	Active ingredients	Reason ¹	Route	Brand and Manufacturer	n
Other eye ointment	Chloramphenicol	Chlamydial conjunctivitis	Topical	Chlorsig®, Sigma Pharmaceuticals Ltd, South Croydon, Australia; Opticin®, Troy Laboratories, Glendenning, NSW, Australia	2
	Chloramphenicol & hydrocortisone acetate	Chlamydial conjunctivitis	Topical	Chloroint®; Troy Laboratories, Glendenning, NSW, Australia; Chlorosone®, Jurox, Rutherford, NSW, Australia	6
	Ofloxacin	Chlamydial conjunctivitis	Topical	Ocuflox®, Allergan Inc, Irvine, California, USA	1
	Benzathine cloxacillin	Chlamydial conjunctivitis	Topical	Orbenin® eye ointment, Pfizer, West Ryde, NSW, Australia	1
	Atropine sulphate	Uveitis		Ilium Atropine eye ointment®, Troy Laboratories, Glendenning, NSW, Australia	1
Ancillary treatment	Saline bathing of eyes	Remove ocular discharge	Topical		
	Vitamin B ₁₂	Nutritional support	s.c. injection	Troy Vitamin B ₁₂ ®, Troy Laboratories, Glendenning, NSW, Australia	2
	Vitamin B complex	Nutritional support	s.c. injection	Multibex Sterile injection®, Heriot Agencies, Boronia, Vic, Australia or Jurox, Rutherford, NSW, Australia	2
	Iron	Nutritional support	s.c. injection	Anaemex™, Ausrichter Animal Health, Annandale, NSW, Australia	
	Yoghurt	Probiotic	Oral		1
	Paraffin oil	Laxative	Oral		1
	Neomycin thioestrepton, nystatin, triamcinolone	Wound treatment unrelated to chlamydial disease	Topical	Panolog®, Fort Dodge Australia, Baulkham Hills, Australia	1
	Metacresolsulfonic acid and formaldehyde	Wound treatment related to urine scald	Topical	Lotagen®, Schering-Plough, North Ryde, Australia	1

* other than oxytetracycline/ polymyxin B eye ointment, systemic antibiotics and meloxicam.

s.c: subcutaneous; n: number of koalas treated.

¹ Personal communication: C. Flanagan, Supervisor, Koala Hospital, Port Macquarie, NSW, January, 2010.

Animals admitted as “wet” were significantly more likely to be treated with the systemic antibiotic, enrofloxacin, than animals admitted as “eye” (chi-sq = 14.17, d.f. = 1, $p < 0.0001$). Approximately half of animals ($n = 30$; 55%) admitted as “wet” were treated with enrofloxacin (Figure 3-2). Only a minority of animals in “wet” and “eye” groups were treated with other systemic antibiotics or the non-steroidal anti-inflammatory drug, meloxicam (Figure 3-2), which was administered for anti-inflammatory and analgesic purposes (C. Flanagan, pers. comm.)². There was no significant difference between these groups in the proportion of animals treated with these modalities. Other than enrofloxacin, the systemic antibiotic used most frequently was chloramphenicol, used in 11 cases, with one additional case each treated with sulphatroxazole/ trimethoprim, nystatin, amoxicillin/clavulanic acid, or a combination product containing sulphadiazine, streptomycin sulphate and neomycin sulphate.

3.4.4 Ancillary treatments used

Ancillary treatments were frequently used in the “eye” and “wet” groups (Figure 3-2), the most frequent being nutritional supplements, probiotics, and bathing of eyes with saline (Table 3-3). Animals in the “wet” category were frequently treated with one or more non-prescription remedies used for treatment of cystitis (methenamine hippurate, Hiprex®, Sanofi-aventis, Bridgewater, New Jersey, U.S.A; sodium citrotartrate, Ural®, Sigma Pharmaceuticals, South Croydon, Vic, Australia) and vaginal candidiasis in people (topical yoghurt (4 cases; 7.4%); nystatin, Nilstat® topical cream, Sigma Pharmaceuticals, South Croydon, Vic, Australia (1 case; 1.85%); clotrimazole, Clonea® Antifungal skin cream, Alphapharm, Glebe, NSW, Australia (1 case; 1.85%); topical yoghurt in combination with nystatin (5 cases; 9.25%) or in combination with clotrimazole (1 case; 1.85%)). Topical

² Personal communication: C. Flanagan, Supervisor, Koala Hospital, Port Macquarie, NSW, January, 2010.

antiseptics (chlorhexidine gluconate) and barrier cream (sorbolene) were also occasionally directed at preventing or treating urine scald in “wet” animals (6 animals, 11.1%). Other ancillary treatments were usually administered to one or two animals only (pentosan polysulphate, intracystic povidone iodine) or were unrelated to treatment for chlamydiosis, cystitis or incontinence. Use of multiple ancillary treatments in the same animal was more common in “wet” cases (21 animals, 38.9% of “wet” cases) than “eye” cases (4 animals, 11.7% of “eye” cases) (chi-sq = 7.62, d.f. = 1, p = 0.006). Single agent ancillary therapy was most frequently saline bathing of eyes (9 animals, 26.4% of “eye” cases), or use of methenamine hippurate (Hiprex®, Sanofi-aventis, Bridgewater, New Jersey, USA) in four “wet” cases (7.4% of “wet” cases). One case each was treated with single agent ancillary therapy of cloacal yoghurt, oral sodium citrotartrate, oral paraffin oil or a multivitamin B injection. All other cases given ancillary treatment (29 cases, 32.9% of all cases) received more than one ancillary therapy.

Table 3-3. Detail of ancillary treatments* given to 54 “wet” cases.

Treatment	Reason ³	Route	Brand and Manufacturer	No. treated†
Vitamin B ₁₂	Nutritional support	Subcutaneous injection	Troy Vitamin B ₁₂ ®, Troy Laboratories, Glendenning, NSW, Australia	1
Vitamin B complex	Nutritional support	Subcutaneous injection	Multibex® Sterile Injection, Heriot Agencies, Boronia, Vic, Australia or Jurox, Rutherford, NSW, Australia	7
Saline bathing	Removal of ocular discharge	Topical around eyes		2
Probiotics	Refaunation	Oral	Innerhealth®, Health World Limited, Virginia BC, Qld, Australia	5
			Protexin® powder, International Animal Health Products, Huntingwood, NSW, Australia	2
			Koala faeces	1
Chlorhexidine gluconate	Prevention/treatment of urine scald	Topical around rump	Hexawash® skin cleaner, Apex Laboratories, Somersby, NSW, Australia	4
Sorbolene	Barrier cream/moisturising for urine scald	Topical		2
Yoghurt	Natural remedy for vaginal candidiasis in women	Intracloacal		8
Methenamine hippurate	Acidification and sterilisation of urine	Oral	Hiprex®, Sanofi-aventis, Bridgewater, New Jersey, U.S.A	8
Sodium citrotartrate	Alkalanisation of urine	Oral	Ural®, Sigma Pharmaceuticals, South Croydon, Vic, Australia	3
Antifungals (Nystatin, Clotrimazole)	Remedy for candidiasis in women	Topical around cloaca	Nilstat® topical cream, Sigma Pharmaceuticals, South Croydon, Vic, Australia	4
			Clonea® Antifungal cream, Alphapharm, Glebe, NSW, Australia	1

Table 3-3 cont.

Treatment	Reason ³	Route	Brand and Manufacturer	No. treated†
Povidone-iodine	Topical antiseptis of the bladder	Intracystic	Betadine® antiseptic liquid, Symbion Consumer Products, Rydalmere, NSW, Australia	1
Pentosan polysulphate	Repair of glyucose- amino-glycans layer of the bladder	Injectable	Cartrophen Vet®, Biopharm, Bondi Junction, NSW, Australia	2
Bromazepam	Sedation	Oral	Lexotan®, Roche Products, Dee Why, NSW, Australia	2
Antiseptic	Topical antiseptic	Topical skin	Teatree ointment	1
			“Antiseptic” cream, brand and manufacturer unknown	1
Activated charcoal	Natural therapy for flatulence, gastrointestinal pain	Oral	Charcotabs® Activated Charcoal, Key Pharmaceuticals, North Ryde, NSW, Australia	1
Peppermint oil	Natural therapy for flatulence, gastrointestinal pain	Oral	Peppermint water, Orion Laboratories, Pty. Ltd., Balcatta, WA, Australia	1
Kaolin/Pectin	Treatment of diarrhoea	Oral	Kaomagma and pectin solution, Wyeth Australia, Baulkham Hills, NSW, Australia	1
Praziquantel (± pyrantel pamoate and febantel)	Tapeworm treatment	Oral	Droncit® or Drontal®; Bayer Animal Health, Australia, Pymble, NSW, Australia	1 1
Nandrolone laurate	Anabolism	Injectable	Laurabolin®, Intervet/Schering Plough Animal Health, Bendigo East, Vic, Australia	1
Cisapride	Gastrointestinal motility	Oral	Prepulsid®, Janssen Cilag Pty Ltd, Sydney, NSW, Australia	1

* other than oxytetracycline/ polymyxin B eye ointment, systemic antibiotics and meloxicam.

† Cases may have received more than one treatment.

³ Personal communication: C. Flanagan, Supervisor, Koala Hospital, Port Macquarie, NSW, January, 2010.

3.4.5 Duration of treatment

Antibiotic courses of systemic enrofloxacin and oxytetracycline/ polymyxin B eye ointment were administered for a median of 15 days, although the range of treatment duration was wide (Figure 3-3). Median duration of treatment with chloramphenicol was longer (25 days) for animals in the “eye” group than the “wet” group, although the former group consisted of only three animals. Non-steroidal anti-inflammatory treatment (meloxicam) was typically given for shorter periods of time to “eye” cases (median 5.5 days) than “wet” cases (median 26 days). The route of systemic medication was most frequently oral for enrofloxacin and meloxicam, and parenteral for chloramphenicol, although route of enrofloxacin administration was not recorded in a high percentage of records (Table 3-4). The method of injection (subcutaneous or intramuscular) was recorded for only five animals receiving parenteral medication. Meloxicam route could only be inferred by assuming the word “drops” was related to oral administration. Median dose rates were consistent with those used in small animal practice for enrofloxacin (5 - 10 mg/kg once daily) and meloxicam (0.1 - 0.2 mg/kg once daily) and consistent with equine doses of chloramphenicol (10 - 50 mg/kg twice daily) (Figure 3-4) (Plumb, 2005).

Table 3-4. Route, frequency, and number of animals subjected to systemic drug administration.

Drug	Frequency	Oral	Injectable	Both	Not recorded	Total
Enrofloxacin	Once daily	18	2 (subcutaneous)	2	14	36
Meloxicam	Once daily	17		2		19
Chloramphenicol	Twice daily	1	9 (intramuscular)	1		11

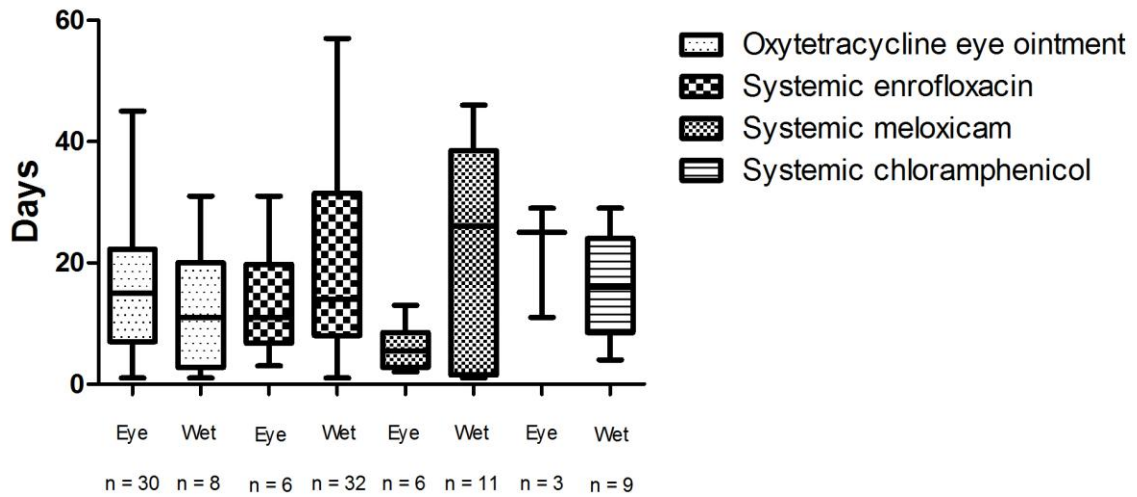


Figure 3-3. Median (range) duration of treatments administered to 77 koalas admitted for treatment of suspected chlamydia.

Included cases may have received more than one treatment. Eye: “eye” admissions; Wet: “wet” admissions.

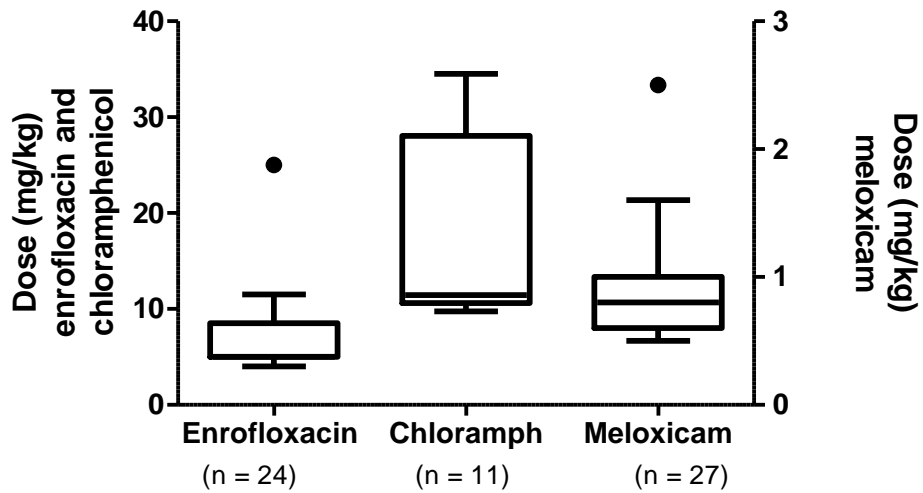


Figure 3-4. Dose ranges of systemic drugs administered in this study.

Chloramph: chloramphenicol.

3.4.6 Recovery and release analysis

The majority of animals ($n = 21$; 62%) in the “eye” admission group and approximately half of the animals (25 cases; 46%) in the “wet” group were released following treatment. The non-released group mainly consisted of animals that were euthanased (27 animals, 50% “wet” admissions; 8 animals, 23.5% “eye” admissions). Deaths were infrequent (2 cases, 6% “eye” admissions; 1 animal, 2% “wet” admissions). Two animals (5.8% of “eye” cases) were “transferred” and therefore lost to further study. The outcome for one case from each group (3% “eye” admissions, 2% “wet” admissions) could not be determined from the medical records.

Using logistic regression modeling, few factors were identified that were predictive of release. Animals that received ancillary treatments were more likely to be released than those that did not, although 95% confidence intervals were wide (Table 3-5). Young adults and mature adults were more likely to be released than aged animals ($p = 0.003$), although 95% confidence intervals were also very wide (Table 3-5). Animals that not released (i.e. euthanased or died) had significantly fewer days in care (median 11, range 1 - 236 days; Mann Whitney test, $p = 0.02$) than animals that were released (median 19, range 1 - 393 days).

Table 3-5. Summary of significant odds ratio (OR) and 95% confidence intervals (CI) for explanatory variables calculated using univariable binomial logistic regression predicting risk of whether animals were released or not released.

Explanatory variable		Odds ratio (95% CI)	p
Age class	Juvenile	7.6 (0.29 - 200.9)	0.003
	Young	14.62 (2.68 - 79.7)	
	Mature	10.59 (1.96 - 57.3)	
	Aged	1	
Ancillary treatments	No	1	0.014
	Yes	3.2 (1.23 - 8.3)	

Reference groups were “aged” and “no ancillary treatment”. Age classified according to tooth wear class (TWC) (Martin, 1981) where Juvenile: TWC I - II; Young: TWC III; Mature: TWC IV; Aged: TWC V - VII.

Among animals that were released, 43% of animals that had been admitted as “eye” and 58% of animals admitted as “wet” re-presented at least once before March 1st 2007. The median (range) number of days between release and re-presentation were not significantly different between “eye” and “wet” groups (656.5 (225 - 1939) days; 241 (16 - 1536) days, respectively; Mann Whitney test, $p = 0.15$). None of the animals originally admitted in the “eye” category re-presented with eye disease, but one re-presented with wet bottom (Figure 3-5), and one had ovarian bursal cysts diagnosed at post mortem examination after re-presenting as debilitated. Of the “wet” animals released, the majority (9 animals; 64%) re-presented with clinical signs of “wet bottom” (7 animals; 50%) or eye disease (2 animals; 14%) and one, which re-presented as a trauma case, had ovarian bursal cysts at post mortem examination (Figure 3-5). In the 14 re-presenting females (4 originally “eye” cases; 10 originally “wet” cases) evidence of breeding was uncommon: one animal originally presenting as “wet” re-presented with pouch young 23 days after release, and one originally treated for eye disease re-presented with pouch young 393 days after release.

Both those with joeys presented in winter. Other females re-presented in summer (5 animals, 36%), autumn (2 animals, 14%), winter (3 animals, 21%), or spring (2 animals, 14%).

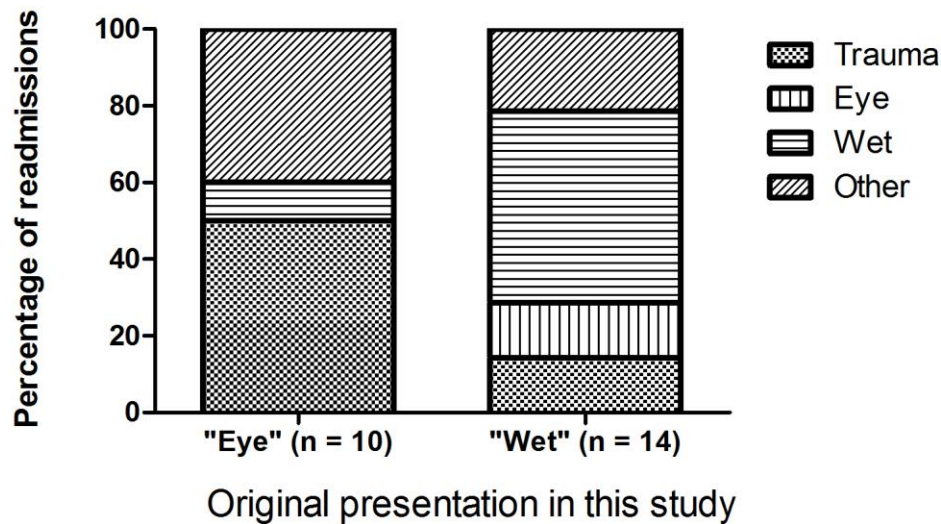


Figure 3-5. Reasons animals were readmitted post-release.

Eye: clinical signs of eye disease; wet: clinical signs associated with urogenital chlamydiosis; trauma: motor vehicle accident, dog attack; other: includes dangerous area (n = 2), debilitated (n = 2), lymphosarcoma (n = 1), unknown (n = 2).

3.5 DISCUSSION

This study found diagnosis of chlamydiosis and treatment decisions at the Koala Hospital depended mostly on clinical signs rather than diagnostic tests. Most animals were treated from a small number of drug choices for a median period of two weeks, and systemic drugs were usually administered orally. Ancillary supportive treatments were common. Few prognostic indicators for successful release were identified. Released animals frequently re-presented after extended time in the wild, indicating successful survival of

many animals; however, the majority of the “wet” animals released re-presented with similar clinical signs, suggesting either re-infection or continuation of infection. In contrast, all those that presented initially with eye infection did not re-present with clinical signs of eye disease, suggesting that treatment had been effective.

From this study it is evident that many clinical decisions at the Koala Hospital are based on clinical signs alone. Diagnostic testing and treatment were aimed at the anatomical site with clinical signs: animals with clinical eye disease had eye swabs tested and topical eye treatments; animals with clinical urogenital tract disease had urogenital tract swabs tested, ultrasonography and systemic enrofloxacin treatment. In particular, little confidence was evident in sensitivity of swabbing in detecting chlamydiosis: many animals were not tested using the ELISA; types of treatment given to animals with a negative swab result mostly did not differ from other groups, and repeat swabbing to monitor clinical response was rarely performed. This emphasis on clinical signs dictating diagnostics and mode of treatment does not take into account the possibility of subclinical disease in koalas or the possibility of non-chlamydial disease with similar clinical signs. Multiple studies have confirmed the high prevalence of subclinical disease in different koala populations (Brown, Carrick *et al.*, 1984; Mitchell, Bilney *et al.*, 1988; Weigler, Girjes *et al.*, 1988; White & Timms, 1994; Jackson, White *et al.*, 1999; McLean, 2003), although this is yet to be quantified in the clinical setting. Reliance on diagnostic tests aimed only at external clinical signs will miss both subclinical infections and infertile animals with structural, inactive disease and will misdiagnose unrelated conditions with similar clinical signs. This approach to diagnosis and treatment reflects the lack of sensitive, simple and reasonably priced diagnostic tools, a lack of evidence base for treatments and lack of information regarding the prevalence of systemic rather than local infections in koalas. There may be a tendency for lay carers to treat obvious clinical problems, rather than undertaking or

requesting more involved diagnostic tests or treatments, particularly when such tests may be perceived as poorly sensitive and specific, finances are limiting, results of diagnostic tests may preclude release, or use of other treatments may extend time in captivity. The development of a cheap, practical, point-of-care test is urgently required for diagnosis and monitoring of treatment of chlamydiosis in koalas, a similar situation to that of diagnostics for trachoma in people in the developing world (Mahilum-Tapay, Laitila *et al.*, 2007). Such tests are not widely available or have not been widely evaluated for diagnosis of chlamydiosis in koalas. This finding prompted the formulation of a study examining the sensitivity and specificity of clinical signs as a diagnostic test in detecting chlamydiosis in koalas presenting to the Koala Hospital (Chapter 8). The results of this latter study would be useful to better inform carers making decisions regarding likely diagnosis; and better inform animal management, including decisions regarding isolation of animals, replacement of cage furniture, choice of treatment modalities and decisions regarding end point of treatment.

The most frequent recorded route of systemic medication was the oral route. This route is commonly used in koalas given their relatively placid nature (Blanshard, 1994).

Pharmacokinetics of orally administered medications in koalas might be expected to be unusual, as hepatic metabolism of bromosulphalein is increased in comparison to other species, such as sheep and macropods (Pass & Brown, 1990), and small intestinal transit time is short (particulate matter: 6 minutes; soluble material: 60 minutes) (Cork & Warner, 1983). Investigations of pharmacokinetics of antibiotics in marsupial species are limited (Clark, Milton *et al.*, 1982; Kirkwood, Gulland *et al.*, 1988; McLelland, Rich *et al.*, 2009), and study of this area in koalas is warranted as the barriers to gastrointestinal absorption in this species may be profound. This background motivated the formulation of a study examining fluoroquinolones given by parenteral and oral routes (Chapter 7).

In the current study, the mainstay of treatment for conjunctivitis was oxytetracycline/ polymyxin B eye ointment, usually for two weeks. There is little data regarding the success of topical therapy in koalas (Cockram & Jackson, 1976; Markey, Wan *et al.*, 2007) despite its wide use (Blanshard, 1994; Connolly, 1999; Blanshard & Bodley, 2008). Typical courses of topical oxytetracycline/ polymyxin B in this study were not consistent with the length of courses of topical medications recommended in other species to treat conjunctival chlamydiosis (6 weeks in people, 1 - 2 weeks past clinical cure in cats) (Francis & Turner, 1993; Glaze & Gellat, 1999) and such short courses might be expected to lead to treatment failure. Similarly, the use of enrofloxacin for two weeks to treat urogenital chlamydiosis (“wet bottom”) might be expected to be unsuccessful. Third generation fluoroquinolones have been used to treat chlamydial disease in people (Ridgway, 1997), although studies using veterinary fluoroquinolones to treat chlamydiosis are few, and have conflicting reports of success (Lindenstruth & Frost, 1993; Bodetti, Johnston *et al.*, 2002; Penguin Taxon Advisory Group, 2005; Gerhardt, Schulz *et al.*, 2006; Hartmann, Helps *et al.*, 2008). One study found that koalas treated with enrofloxacin for up to 6 months were still positive for chlamydial organisms by PCR, suggesting treatment failure (Bodetti, Johnston *et al.*, 2002). Despite these treatments being theoretically unsatisfactory, the majority of koalas treated for eye disease and approximately half of koalas treated for wet bottom were released. Further animals with eye disease did not tend to re-present with eye disease, suggesting successful resolution. The apparent success of fluoroquinolone treatment of chlamydiosis in koalas evident in the current study conflicts with the equivocal results or treatment failure in other species (Lindenstruth & Frost, 1993; Ridgway, 1997; Bodetti, Johnston *et al.*, 2002; Penguin Taxon Advisory Group, 2005; Gerhardt, Schulz *et al.*, 2006; Hartmann, Helps *et al.*, 2008) and this apparent contradiction provided the background for

further work in this dissertation: a study examining the response of animals presenting with chlamydiosis to treatment with fluoroquinolones (Chapter 7 and Chapter 8).

At least 50% of released animals survived for quite extended periods in the wild, a finding in agreement with other studies in koalas (Starr, 1990; Carrick, Beutal *et al.*, 1996; Lunney, Gresser *et al.*, 2004) but in contrast to the poor success of rehabilitation efforts in many other species (Craven, 1982; Robertson & Harris, 1995; Anderson, Gress *et al.*, 1996; Fajardo, Babiloni *et al.*, 2000; Goldsworthy, Giese *et al.*, 2000; Ben-David, Blundell *et al.*, 2002; Beringer, Mabry *et al.*, 2004). It appears koalas may be particularly suitable for rehabilitation and thus effort in improving techniques is warranted.

The use of medical records proved useful in confirming the survival of at least half of animals released. The conclusions drawn from this type of study are heavily dependent on whether animals are likely to re-present. The high frequency of re-presentations in this study, possibly due to a high likelihood of reporting of diseased or displaced koalas by the public, suggest further investigation of the success rates of rehabilitation in koalas using this technique is justified within the Port Macquarie population. It would be particularly interesting to use the records to assess the survival and evidence of breeding for the different admission cohorts (Chapter 2) of animals admitted to the Koala Hospital.

Statistical modelling using these data might be used as a passive “mark-release-recapture” method to determine the relative survival of rehabilitated animals originally presenting for different reasons, providing the underlying assumptions of equal capture probability, independent capture and retention of identification marks discussed by Borchers *et al.* (2002) can be satisfied. This type of study suggests that permanently identifying released animals and accurate record keeping can be valuable tools for assessing success rates of rehabilitation.

An interesting observation was that animals originally treated for eye disease did not commonly re-present with chlamydiosis, in contrast to those originally treated for wet bottom. It is impossible to determine from the current study whether chlamydial infection at re-presentation was due to recurrence of the original infection due to treatment failure, re-infection, or a damaging and self-perpetuating host immune response leading to overt clinical signs. Ocular chlamydiosis may be self-limiting in koalas (Kempster, Hall *et al.*, 1996) and self-resolution may explain the apparent successful treatment of these animals with theoretically inadequate treatments. Animals with urogenital tract infections frequently have concurrent urogenital structural disease (McLean, 2003; Higgins, Hemsley *et al.*, 2005b). Such animals may appear to improve initially, but relapse later due to damage sustained in initial episodes of disease predisposing animals to subsequent infections (chlamydial or secondary bacterial infections), resulting in recurrence of clinical signs. This hypothesis might explain the suspected poor breeding success of females and recurrence of urogenital tract disease in “wet” cases described in this study. It appears that the prognosis for animals treated for eye disease is better than that for animals treated for urogenital disease, although both groups may survive for quite extended periods post-treatment.

From a population perspective, perhaps the most important determinant of rehabilitation success is whether animals retain their fertility (Sigg, Goldizen *et al.*, 2005). Chlamydiosis in koalas can cause occlusive fibrosis of the upper reproductive tract, which may be difficult to diagnose ante-mortem (Higgins, Hemsley *et al.*, 2005a) and associated structural changes are highly correlated with poor fertility (Brown, Carrick *et al.*, 1984; McLean, 2003). The potential fertility of animals at the point of release in this study was unknown. The fertility rate of re-admitted females was low (14.2%), although sample size was small (n = 14). Females that re-presented did so throughout the year, encompassing the

seasons in which accompanying young might be expected in animals of normal fertility (pouch young in summer and autumn, back young in winter and spring) (Martin & Handasyde, 1999). Studies of a small number of wild caught female koalas (n = 14) from bush habitat adjacent to Port Macquarie suburbs estimate fertility of approximately 43% in Port Macquarie koalas (Biolink, 2005; Biolink, 2008), which is similar to populations at Oakey and Mutdapilly in south-east Queensland (Gordon, McGreevy *et al.*, 1990; White & Timms, 1994). Unfortunately, these small sample sizes limit certainty of any conclusions as to the fertility of released females treated for chlamydiosis in comparison with the wild population. Should released females have extremely poor fertility, they may contribute little to the wild population. In addition, if these animals remain persistently infected with Chlamydiaceae they may undergo multiple infertile matings per season, increasing potential for transmission. Such animals may be detrimental to the local population, although their impact would depend on prevalence rates of chlamydiosis in the wild. Further studies, with a focus on whether these animals have successfully cleared chlamydial infections or are released still infected, and their impact on wild populations are vital to assess whether treatment of these animals is worthwhile. Prospective treatment trials examining systemic fluoroquinolone therapies (Chapter 7 and Chapter 8), a larger case controlled retrospective study using the records of the Koala Hospital, or a prospective radio-tracking study would be extremely useful in determining the prognosis and fertility of treated animals. An assessment of wild populations by a capture, test and release study as a comparison would be extremely useful.

Nutritional supplementation, probiotics and nursing care were commonly employed to treat chlamydial disease between 1995 and 2005 at the Koala Hospital. That the use of ancillary treatments was associated with increased probability of release is worthy of further investigation, although 95% confidence intervals were very wide. Most ancillary

treatments were generally supportive, rather than those associated with specific efficacy against chlamydiosis in other species. It is possible that some factors within the ancillary treatments are associated with treatment success, or that those animals more likely to be released for other reasons were more likely to receive such treatments (for example, longer times spent in care might increase probability of receiving ancillary treatments). A prospective study or a larger retrospective study examining this association would be useful in clarifying the role of these treatments.

There was a tendency for animals in the “wet” group to receive non-prescription remedies, normally associated with cystitis or vaginal candidiasis treatment in women. Such treatments have no known role in treating chlamydiosis in people and this highlights the need for evidence-based veterinary input to treatment regimes. Although supportive care may be an important part of treatment of a wild animal, such treatments should only be administered where benefit is likely. All ancillary treatments have the potential to do harm as administration will usually require extra handling of a wild animal and little data exist as to whether such treatments can be safely used. In such cases the employment of the maxim “first, do no harm” should be foremost. A close and trusting relationship between veterinarian and carer is important, so that open discussion as to the use and potential benefit or harm of such treatments is possible. The more unconventional ancillary treatments (probiotics, home remedies for vaginal candidiasis and cystitis in women, sedatives) have been phased out since 2000, when the current supervisor began working at the Koala Hospital (C. Flanagan, pers. comm.)⁴.

This study found few factors predicting whether an animal was likely to be released or not. The finding that aged unwell animals are less likely to be released from the Koala Hospital

⁴ Personal communication: C. Flanagan, Supervisor, Koala Hospital, Port Macquarie, NSW, January, 2010.

than younger animals fits with the Koala Hospital policy where aged animals are regarded as having a poorer prognosis for survival after release (C. Flanagan, pers. comm.)⁵. As 95% confidence intervals were wide, a larger sample size is required to confirm this finding.

Interestingly, morphometrics, in particular ratios between weight and crown rump length, or head length (size mass indices) (McLean, 2003), were not predictive of prognosis for release. Thus, other than age, a prognostic indicator based on physical condition of koalas at presentation was not identified. Such factors would be extremely useful in allowing efficient use of resources. A larger sample size may be required to identify such factors. Body condition score using scapular muscle mass (Connolly, 1999) was not analysed in this study, but may be a better prognostic indicator for release than those examined here.

In conclusion, this study identified common diagnostic methods and treatments employed at a major koala hospital. Decisions were often based on clinical signs alone and many medical treatments were given orally. Apparent treatment success was evident with approximately 50% of animals being released and many survived in the wild for extended periods. In particular, animals treated for eye disease did not often re-present with this problem, suggesting animals were cured of, or fully recovered from, clinical ocular chlamydiosis. In contrast, animals treated for wet bottom had high re-presentation rates with the same clinical signs, suggesting treatment failure or susceptibility to re-infection. This work provided the basis for a prospective clinical trial examining the validity of using clinical signs to diagnose and assess response to treatment, the pharmacokinetics of fluoroquinolones administered by different routes, and the response of animals to treatment using these drugs (Chapter 8).

⁵ Personal communication: C. Flanagan, Supervisor, Koala Hospital, Port Macquarie, NSW, January, 2010.

CHAPTER 4 - GENERAL METHODS FOR CLINICAL STUDIES

4.1 INTRODUCTION

This chapter describes the animal husbandry and diagnostic methods common to the clinical studies described in Chapter 5 to Chapter 8, and their associated challenges and limitations. The development of these methods was a learning process, as there are few large scale published studies of the treatment of disease in Australian wildlife. Such trials are difficult to undertake due to expense, access to animals and the public perception of research involving iconic species. Recruitment of animals often relies on opportunistic treatment of naturally occurring disease, which limits numbers. Methods of medication and sampling usually need to be adapted to the species under investigation. Specialised facilities to house wild animals may only be available in wildlife hospitals, allowing study of animals within the facilities and circumstances in which future treatment is likely to be undertaken, but necessitating adaption of scientific work within the framework and infrastructure of these institutions. Further, working within a volunteer based organisation offers unique challenges in communicating the scientific method to lay people.

4.2 RECRUITMENT TO THE STUDY

Koalas were recruited to this study opportunistically. As per normal operating procedures, members of the public reported sick, injured or displaced koalas located in dangerous areas, to the Koala Hospital, Port Macquarie, NSW for assessment in situ by volunteers from the Koala Preservation Society of NSW. If deemed necessary, for example due to clinical signs of disease, proximity to hazards such as dogs or main roads, or abnormal behaviour, volunteers retrieved koalas. Flags or plastic bags attached to extendable (up to 4 m long) poles with slightly hooked ends were placed above the koalas' heads and agitated

to encourage koalas to descend the tree to a point where they were retrieved into large canvas holding bags (Blanshard, 1994).

On presentation, the animal was assessed by the supervisor of the Koala Hospital. In cases where there was external evidence suggestive of chlamydial disease (conjunctivitis, keratitis, ocular discharge; and/or malodour, dampness and staining of the rump), animals were forwarded to investigators for assessment for entry into the clinical trial. From early 2006 (approximately half way through the first of three field seasons), healthy animals without evidence of clinical chlamydiosis were screened for chlamydial DNA collected from ocular and/or urogenital swabs by PCR (see 4.11). Animals were assessed within 12 - 24 hours of being brought into the hospital.

4.2.1 Anaesthesia

To facilitate thorough assessment, koalas were anaesthetised. Following pre-oxygenation, general anaesthesia was induced by mask and a Bain paediatric circuit using a C.I.G Midget 3 anaesthetic machine (VetQuip, Castle Hill, NSW, Australia) by gradually increasing the percentage of isoflurane in oxygen from 0 to 3.5% over 5 min. Anaesthesia was maintained with 1.5 - 2% isoflurane and 1 - 2 litres per minute 100% oxygen delivered via mask. Animals were recovered on mask oxygen and when righting reflex was re-established, they were transferred to recovery cages.

During anaesthesia, apnoea was encountered occasionally and resolved by administration of positive pressure ventilation by mouth using the reservoir bag, stimulation of breathing by toe pinch or extending both forearms laterally. Cardiac arrhythmias were encountered infrequently and assessed by auscultation only. Where arrhythmias were encountered, isoflurane concentration was decreased, the procedure shortened and subsequent anaesthetic procedures performed only when deemed essential.

4.2.2 Clinical examination

Animals underwent a full clinical examination (Blanshard, 1994) whilst anaesthetised at presentation to assess animals for inclusion in the study. Briefly, this examination included aging by tooth wear class (Martin, 1981); weighing on scales (TI 1583, Wedderburn, Summer Hill, NSW, Australia or 235-6S, Salter Brecknel, Fairmont, Minnesota, USA); thoracic auscultation; palpation of lymph nodes; examination of the oral cavity; body condition scoring by palpation of infraspinatus, supraspinatus and frontalis muscles (Connolly, 1999); examination of the pouch for reproductive status and presence/ absence of young; abdominal palpation including assessment of gut fill, hydration status (Blanshard, 1994); assessment and subjective grading of ocular abnormalities (discharge, conjunctival proliferation, chemosis); scoring of wet bottom (see 4.7 and Table 4-1); ultrasound examination (see 4.8); collection of blood for full biochemistry and haematology profiles (see 4.10) and collection of swabs from the conjunctivae and urogenital sinus (females) or penile urethra (males) for chlamydial DNA PCR (see 4.11.5).

4.2.3 Exclusion of animals

Past studies of chlamydiosis in koalas have suggested that overt expression of chlamydiosis may be more common in koalas debilitated through concomitant disease (Canfield, Love *et al.*, 1991a) or subjected to environmental stressors (Weigler, Girjes *et al.*, 1988). Animals were carefully examined for simultaneous disease and excluded if it was detected under the supposition that intercurrent disease could interfere with the outcomes of the investigation, or in the case of hepatic or renal disease, interfere with drug metabolism, and for welfare reasons.

Koalas were excluded under any of the following criteria: wet bottom score of four or more (Flanagan, 2009), healthy animals in which chlamydial DNA (by PCR see 4.11) or

antigen (by Clearview Chlamydia MF ELISA (Inverness Medical Australia, Sinnamon Park, Qld, Australia)) was not detected, presence of other disease, structural renal pathology detected by ultrasound examination, condition score of two or less, or where weight loss over a three day premedication observation period exceeded 250 grams for females and 300 grams for males. Mothers with joeys were excluded to minimise the stress on joeys.

4.3 ALLOCATION TO TREATMENT GROUPS

Following recruitment, koalas were allowed to acclimatise for 3 - 7 days, during which they were assessed 1 - 2 times daily to determine whether oral medication could be used, by attempted administration of 50 mL oral supplement twice daily (Di-vetelact® Low Lactose Milk Powder, Sharpe Laboratories, Ermington, NSW, Australia, 9 g; or Karicare® Soya All Aged Formula, Nutricia, North Ryde, NSW, Australia, 7.1 g; or Infasoy® Progress Step 2, Wyeth Australia, Baulkham Hills, NSW, Australia, 8.9 g; each in 50 mL water).

If animals were amenable to oral medications they were randomly assigned to one of several oral medication groups and supplementation continued twice daily. Animals strongly resistant to oral supplementation (exhibiting flinching, ear flicking, vocalisation, aggression and/or retreating) were randomly assigned to one of several subcutaneous or intramuscular injectable groups and supplement ceased or was substituted with 1.5 cm Nutrigel® (Troy Laboratories, Glendenning, NSW, Australia) administered orally twice daily. Nutrigel® is a sticky paste and easier to administer to resistant animals; it can be quickly placed in the mouth leaving the animal to masticate it without further intervention.

4.4 MEDICATION

When medications were administered subcutaneously, koalas were injected over the dorsal thorax (the “scruff”), a standard area for injecting domestic species; or laterally, dorsal to the axilla, as skin is loose in this area. Intramuscular injections were into the dorsal quadriceps under general anaesthesia. Where possible, injection sites were alternated between left and right sides. Oral suspensions were administered by syringe. Tablets were crushed using a pill crusher and suspended in soya or low lactose formula. Eye ointments were administered using standard technique, (i.e. a small amount of ointment was applied to the conjunctiva and massaged using the palpebrae to aid spread throughout the conjunctival sac).

4.5 HUSBANDRY

Koalas were kept according to standard methods (Blanshard, 1994). Briefly, koalas were housed individually for the first three weeks in solid-walled indoor enclosures approximately 2.0 x 2.5 x 2.5 m with a wire-netted observation and ventilation window and perches of *Eucalyptus microcorys* (Figure 4-1, Figure 4-2). Branches of at least three different species of forage trees (*Eucalyptus robusta*, *E. microcorys*, *E. tereticornis*, *E. nicholii*, *E. macrorhyncha*, *Melaleuca quinquenervia* or *Corymbia maculata*), collected by professional leaf cutters from the streets and surrounds of Port Macquarie, a small tray of soil and a trough of water were offered fresh daily. Newspaper lining the floor of the indoor units was changed once daily and the floors mopped with dilute bleach. Between cases, perches were changed completely, walls and cage furniture disinfected with dilute bleach and the enclosure rested. Cage furniture and cleaning apparatus were dedicated to each unit. After three weeks, animals were usually transferred to individual outdoor pens

of 3.0 x 4.0 m fenced to a height of 1.5 m with similar perches (Figure 4-3). Outdoor pens were raked daily and faeces removed, and leaf and water refreshed as above.

4.6 OBSERVATIONS

In the first field season, appetite was monitored subjectively. In subsequent field seasons, appetite was scored daily: the amount of leaf eaten was scored from 0 (no evidence of provided leaf eaten) to 3 (most provided leaf eaten) (Figure 4-1). The species of leaf preferred was recorded. The animal's demeanour was assessed as dull, depressed, quiet, or bright, alert and responsive. The rump of the animal was inspected daily. The presence and character of urine and urination were recorded. Faeces were observed daily and the character recorded. In the first field season, all faeces over a 24 hour period were collected and weighed daily for the first two weeks of treatment. In subsequent field seasons, total volume of faeces was recorded on a scale from 0 (no faeces observed) to 3 (many faecal pellets observed) (Figure 4-2) and, as it appeared that pellet size was an important variable, 10 random faeces were collected, placed side-by-side along the plane of their longest and shortest axes and the total length and width of 10 faecal pellets recorded. Twice weekly, animals were weighed and the condition score was monitored. Animals were examined under anaesthesia as per 4.2.2 on the day before treatment commenced (day 0), weekly during treatment and every two weeks after treatment had finished.



a)



b)



c)



d)

Figure 4-1. Grading of the amount of leaf eaten by koalas.

- a) Grade 0: No evidence of leaf eaten
- b) Grade 1: Evidence of small amount of leaf eaten; few stalks
- c) Grade 2: Moderate amounts of leaf eaten; many stalks present
- d) Grade 3: Evidence of much leaf eaten; many to most stalks denuded of leaf

Photographs illustrate indoor enclosures.

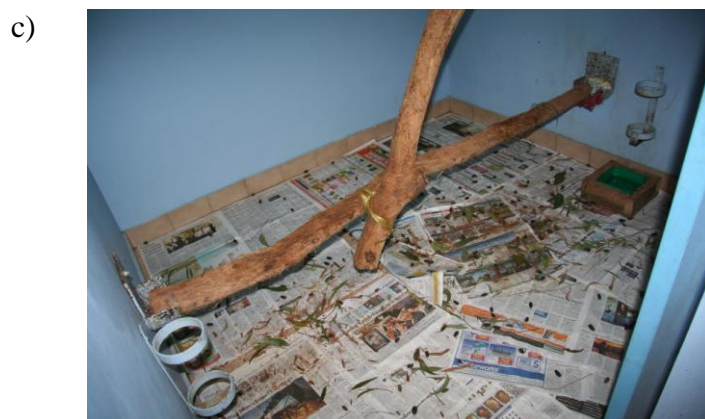


Figure 4-2. Grading of the koala faecal output in indoor enclosures.

Grade 0: No faeces present (not shown)

a) Grade 1: Few faecal pellets present; approximately 10 - 20 pellets total per enclosure

b) Grade 2: Moderate faecal pellets present; approximately 20 - 80 pellets total per enclosure

c) Grade 3: Many faecal pellets present; 80+ pellets total per enclosure

Photographs illustrate indoor enclosures.



Figure 4-3. Outdoor yards where koalas were held after the first two weeks of treatment.

4.7 WET BOTTOM SCORE

Koalas were graded at admission, then weekly throughout treatment according to a wet bottom score developed by staff of the Koala Hospital (Flanagan, 2009) and modified to include the category 0 and 0.5 (Table 4-1).

Table 4-1. Koala wet bottom score modified from Flanagan (2009).

Grade	Description
0	Normal fur, normal cloaca
0.5	Discolouration of fur around cloaca
1	Slight discolouration of fur around cloaca Evidence of mild fresh urine leakage Slight “wet bottom” smell
2	Slight discolouration of fur around cloaca/tail area Occasional urine dribbles Mild yet discernible “wet bottom” smell
3	Discolouration of tail area fur more evident Stronger “wet bottom” smell Urine discharge, greasy texture evident around cloaca/tail area
4	Fur stained, greasy, darkened Strong pungent “wet bottom” smell Inflammation of the cloacal margins, clitoris, vestibule Discharge containing urinary calculus/debris
5	Stained greasy fur covering a large area Very strong pungent acidic smell Blood in urine, crying and straining when urinating Clots, blood in both male and female urine and urogenital tracks Coat brown dry and lustreless Cloaca and tail area swollen (oedema) Grinding teeth
6	Stained, greasy, wet matted fur around rump/cloaca area Blood in urine, clots, and constant purulent discharge Crying, straining, grinding teeth, distressed, flat, ear flicking Ulcerated, oedematous cloaca/tail area
7 - 10	Progressive decline Often becomes maggot infested Death ultimately results if intervention/removal of suffering does not occur

4.8 ULTRASOUND EXAMINATION

Anaesthetised animals were placed in dorsal recumbency. A small amount of fur (approximately 2 cm x 5 cm) was clipped over the left and right cranial abdomen, in areas corresponding with the kidneys, and, in males, a similar amount of fur removed from the caudal abdomen in the area over the bladder. In females, the unhaired region of the pouch was used to visualise this area. The skin was cleansed with 70% ethanol and ultrasound coupling gel was applied.

B-mode grey-scale ultrasonography was performed using an ultrasound machine (Toshiba ECCOCEE SSA-340A, Toshiba Medical, North Ryde, NSW, Australia) with a convex 3.75 MHz, 76 mm convex probe (PVF-375MT) for the kidneys and 6.5 MHz, 11 mm endo-vaginal probe (PVF-621VT) for the bladder and reproductive organs. Images of each organ were captured using a digital capture device (ADS Video Xpress, ADS tech, Walnut, California, USA) and Ulead Movie Wizard software (Ulead Systems, Torrance, California, USA).

Ultrasound examination followed previously reported methods in the koala (Mathews, Wolff *et al.*, 1995; Stalder, 2003). Briefly, the endo-vaginal probe was placed in the midline between epipubic bones (within the pouch in females) and the bladder located on the midline, recognisable by its distinct round to triangular to ovoid shape, thin walls and anechoic lumen. Bladder wall and lumen measurements were taken from the vertical, horizontal and two diagonal axes of a captured image (Figure 4-4). The shape of the bladder and abnormalities of the bladder wall or echolucent material present in the lumen of the bladder were described.

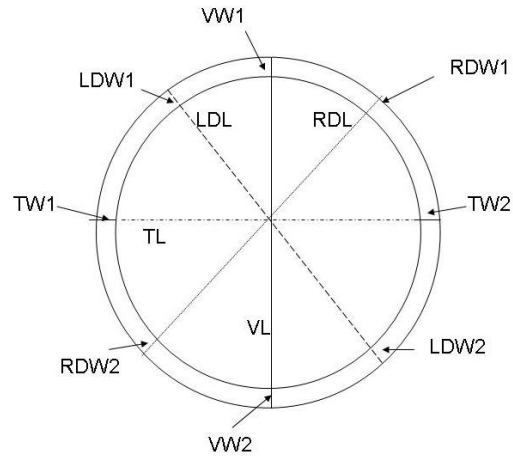
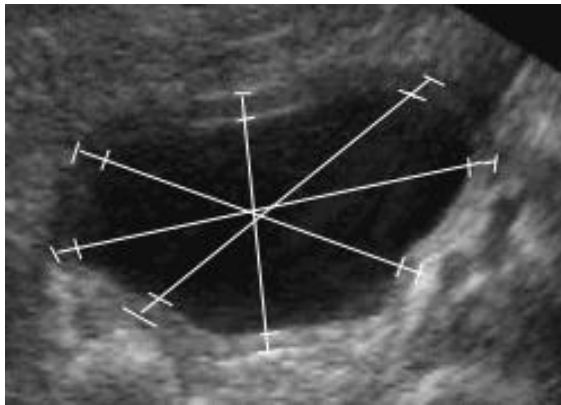


Figure 4-4. Example of a digitally captured sonogram of the bladder.

Showing measurements of the bladder wall and lumen (left). Stylised version demonstrating measurements (right). VW: vertical width (1 & 2); TW: transverse width (1 & 2); LDW: left diagonal width (1 & 2); RDW: right diagonal width (1 & 2); VL: vertical lumen; TL: transverse lumen; LDL: left diagonal lumen; RDL: right diagonal lumen.

In females, left and right uteri were located on the midline and described in terms of their size, shape and position where possible. The wall thickness and lumen was measured. The transducer was then placed laterodorsally to the epipubic bones and moved cranially on left and right sides, respectively. Abnormalities of the reproductive tract, such as echolucent masses, were described in terms of size and the presence of multiple chambers. Ovaries were not detected in this study, a similar finding to that of past researchers (Stalder, 2003). As only a limited number of ultrasound probes were available for use during this work, it is possible that such structures might be detected with alternative probes.

Left and right kidneys were imaged using the convex probe placed on the ventral body wall just caudal to the last rib and 2 - 3 cm lateral from the midline. The sagittal and transverse planes were examined and described for size, shape and relative echogenicities within the kidney. Measurements were obtained from the cranial to caudal pole of the

sagittal plane when it was maximal in size (sagittal length) ("a" in Figure 4-5). The transducer was rotated 90 degrees and maximum measurements were obtained from the hilus to the most lateral aspect (horizontal width) and from the dorsal to ventral surface of the kidney (D-V width) at the hilus ("b" in Figure 4-5).

a)



b)

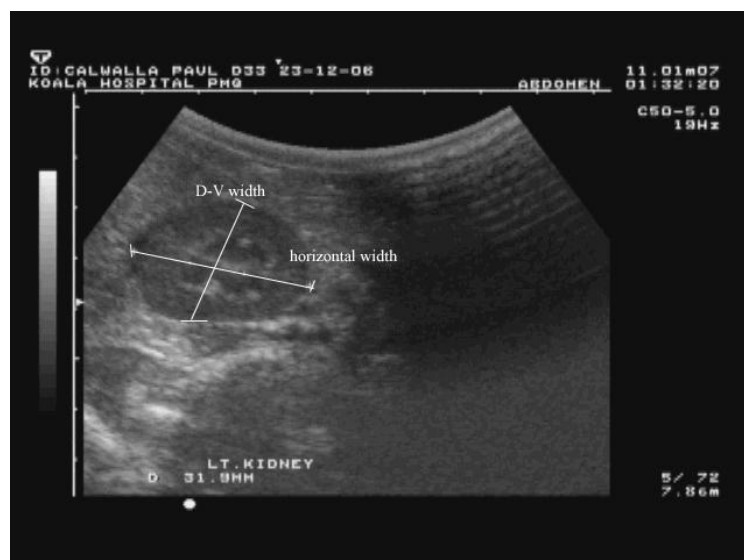


Figure 4-5. Examples of digitally captured sonograms of the left kidney.

Showing measurements a) sagittal length; b) D-V width, horizontal width.

4.9 GRADING OF THE EYES

Animals (n = 22) were anaesthetised weekly as per 4.2.1. The eyes were examined without opening of the eyelids, and then again with gentle traction of the palpebrae dorsally and ventrally by the investigator's fingers to visualise ocular pathology. Photographs of the right and left eyes were taken before and during palpebral traction. Each eye was graded as 0 (normal), 1 (mild), 2 (moderate) or 3 (severe) for chemosis, proliferation of the conjunctiva, and amount of discharge. Scores were summed to give a total eye score. Corneal opacity, lens opacity, pallor of conjunctiva, nature of discharge, blepharospasm and corneal vascularity were described.

4.10 CLINICAL PATHOLOGY

Haematological and biochemical analyses of blood and urinalysis were performed prior to commencing treatment, weekly during treatment and at least fortnightly post-treatment.

Blood (0.5 mL), collected into potassium EDTA from the cephalic vein using a 22 g butterfly catheter and 3 mL syringe, was subjected to haematological analysis for: haematocrit, haemoglobin concentration, erythrocyte count, nucleated erythrocyte count, erythrocyte indices (mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration), total leukocyte count, leukocyte differential counts (neutrophils, lymphocytes, monocytes, eosinophils and basophils) and platelet count using standard methods (Symbion Vetnostics, North Ryde, NSW, Australia). Blood (1.5 mL) was collected similarly into plain tubes, centrifuged and serum assayed for concentrations of sodium, potassium, chloride, urea, creatinine, glucose, total bilirubin, albumin, globulin, calcium, phosphate, cholesterol, triglycerides and the activity of: aspartate aminotransferase, alanine aminotransferase, gamma-glutamyl transferase, alkaline phosphatase and creatinine kinase, with a Roche Modular Chemistry Analyser

using Roche reagents (Roche, Basel, Switzerland) and standard methods by the same laboratory.

Urine was expressed manually from the bladder, and urinalysis performed (Bayer-Multistix®, Bayer Australia, Pymble, NSW, Australia). Urine specific gravity was determined using a refractometer and methylene blue stained sediment was examined under light microscopy.

4.11 FIELD PCR

Initially, diagnosis of chlamydiosis was made using the Clearview Chlamydia MF enzyme-linked immunosorbent assay (ELISA) (Inverness Medical Australia, Sinnamon Park, Qld, Australia). Poor sensitivity of this assay (described in 5.3.1) prompted the author to investigate the use of nucleic acid amplification tests such as PCR, which is regarded as more sensitive than other diagnostic tests for chlamydiosis in other species (Ostergaard, Birkelund *et al.*, 1990; Sykes, Studdert *et al.*, 1999; Amin, 2003) and is the diagnostic test of choice for chlamydial infections in people (Harindra, Underhill *et al.*, 2003). With the advent of non-toxic nucleic acid dyes for visualisation of DNA in agarose gels after electrophoresis (Williams, 2001; Invitrogen, 2007), a field site laboratory incorporating PCR became feasible. The PCR method utilised Chlamydiaceae specific primers (5' -ATGCAAAATTACTGTATGGGTA- 3' and 5' -CTGTAGAGTTTCTGTTACCTTGAAAG- 3') designed to amplify a 1030 bp fragment of the outer membrane protein B gene (Demkin & Zimin, 2005). Demkin and Zimin (2005) reported this gene as highly specific for Chlamydiaceae. Thus the primers used amplified both *C. pecorum*, *C. pneumoniae*, and any other novel pathogenic Chlamydiaceae, but not phylogenetic neighbours of Chlamydiaceae such as *Simkania negevensis*. This section describes optimisation of the field PCR and the final method used.

4.11.1 Reduction of contamination risk

To reduce contamination, the following procedures were adhered to: certified DNA-ase and RNA-ase free, filtered pipette tips, mixing vials and PCR tubes were autoclaved (120 °C for 20 minutes) before use, and bench tops and pipettes were wiped with 70% ethanol before and after use. DNA extraction, reaction set-up and post-PCR procedures were each performed in separate rooms in a facility separate from the Koala Hospital using dedicated equipment. Post-PCR product never entered the clean room. After processing, PCR product was discarded and waste products and waste consumables in contact with PCR product were discarded.

4.11.2 Optimising the PCR

The PCR reaction was optimised by increasing the time from the published method allowed for each reaction step, applying a gradient for the annealing step with temperatures of 45 - 55 °C and performing a magnesium titration from 1.5 - 5 mM MgCl₂. The final reaction conditions chosen were those that delivered the brightest band of the appropriate length without non-specific bands.

4.11.3 Sensitivity of the PCR

An experiment to detect the limit of the reaction was performed using *C. pneumoniae* DNA template from culture, diluted 1:100, 1:10,000, 1:20,000, 1:40,000 1:80,000, 1:160,000. The amount of DNA in the sample was unknown, meaning that this experiment was a subjective measure of the sensitivity of the assay. Strong bands were visible at each dilution until 1:40,000 and just visualised at 1:80,000. A clinical animal was included in this experiment and yielded a strong positive band equivalent in brightness to the *C. pneumoniae* culture dilutions of between 1:100 and 1:10,000.

4.11.4 Specificity of the PCR

Primers were tested against DNA extracted using a commercial kit (QIAamp® DNA Mini Kit, QIAGEN Pty Ltd, Doncaster, Vic, Australia) according to manufacturer's instructions from seven species of bacteria (*Staphylococcus aureus* (Veterinary Pathology and Bacteriology Culture Collection (VPB) 236), *Staphylococcus epidermidis* (VPB 238), *Proteus mirabilis* (VPB 261), *Serratia marcescens* (VPB 255), *Enterococcus faecalis* (VPB 185), *Pseudomonas aeruginosa* (VPB 591) and *Escherichia coli* (VPB 2716)) commonly found on mucous membranes of the anogenital area. There was no cross reactivity with these species. Phylogenetic neighbours of Chlamydiaceae such as *Simkania* spp, *Waddlia* spp and novel koala Chlamydiales genotypes (Devereaux, Polkinghorne *et al.*, 2003) were not available for study. In work by previous researchers reactions using these primers and *Simkania negevensis* have not produced product (Demkin & Zimin, 2005) and the basic local alignment search tool (Altschul, Gish *et al.*, 1990) did not suggest cross reactivity with these organisms. Urogenital and ocular swabs were collected opportunistically as per 4.11.5 from eight koalas bred and held in a captive facility isolated from wild animals and with no history of chlamydial disease. DNA extracted as per 4.11.6 from these animals was subjected to PCR using the protocol described in 4.11.7. All animals were negative using this method.

4.11.5 DNA collection

Plain aluminium applicator, rayon-tipped swabs (Copan, Murrieta, California, USA) were moistened with sterile saline. Gross discharge was removed from eyes with gauze swabs. Each conjunctival sac was sampled three times by vigorously rolling the tip of the swabs around the conjunctiva and external aspect of the nictitating membrane. The urogenital tract was sampled by inserting the moistened swab into the penis to the level of the prostate

(4 - 5 cm; males) or the common urogenital sinus, cranioventrally, until gentle resistance was encountered (4 - 5 cm; females). The swab was then rotated or jiggled vigorously for 5 - 10 seconds. The rectum was accidentally sampled rarely using this technique and such sampling was obvious due to faecal contamination. In these cases, a fresh swab was used and the faecally contaminated swab discarded. The second and third swab were sampled as above then labelled in the order taken and frozen at -20 °C.

4.11.6 DNA extraction

The second swab collected from each site was used preferentially. Individual swabs were placed in 250 µL of QuickExtract™ (Epicentre® Biotechnologies, Madison, Wisconsin, USA) solution in a 0.6 mL collection tube and rotated vigorously a minimum of five times. The swab was pressed against the sides of the tube to retain as much extraction solution as possible and then the remaining solution was vortexed for 10 s and incubated at 65 °C for one min. The tube was vortexed again for 15 s and incubated at 98 °C for 2 min, and was then vortexed for 15 s. This method of DNA extraction was chosen for use in the field as it did not require centrifugation (initially unavailable in the field laboratory) and was rapid. Where possible outside of the field DNA was extracted using a commercial kit (QIAamp® DNA Mini Kit, QIAGEN Pty Ltd, Doncaster, Vic, Australia) according to manufacturer's instructions to minimise the risk of PCR inhibitors that may interfere with the efficiency of the PCR ((Munoz-Cadavid, Rudd *et al.*, 2010).

4.11.7 Field PCR method

The PCR reaction mixture contained PCR grade 2mM MgCl₂ (QIAGEN, Doncaster, Vic, Australia), 200 µM of each dNTP (QIAGEN, Doncaster, Vic, Australia), 160 nM of each primer, 0.83 U Taq polymerase (HotStarTaq® DNA Polymerase, QIAGEN, Doncaster, Vic, Australia), 2.5 µL of extracted DNA template and 2.5 µL buffer (10x PCR buffer,

QIAGEN, Doncaster, Vic, Australia) made up to a final volume of 25 μ L using autoclaved purified water (Milli-Q® Ultrapure filtration system, Millipore, North Ryde, NSW, Australia). The amplification reactions were carried out in a thermal cycler (MJ Mini™ Gradient Thermal Cycler, Bio-Rad, Gladesville, NSW, Australia) using cycling conditions of: 95 °C for 15 min (Taq activation) then 40 cycles of 95 °C for 45 s (denaturation), 54 °C for 45 s (annealing), 72 °C for 60 s (extension) and then a final extension of 72 °C for 3 min. An 18 μ L aliquot of each PCR product was mixed with 2 μ L loading buffer (10% Ficoll with blue dye) and subjected to electrophoresis through a 1.5% agarose gel with SYBR® Safe 0.5X in 45 mM Tris-borate, 1 mM EDTA (TBE) buffer (Invitrogen Australia Pty Ltd, Mt Waverley, Vic, Australia) at 100 V for 30 min. Product was visualised using blue light (Dark Reader® Transilluminator, Bioscientific, Gymea, NSW, Australia) and documented using a digital camera. Samples were assayed in duplicate and compared to a 250 bp ladder (Invitrogen Australia, Pty Ltd Mt Waverley, Vic, Australia). Autoclaved purified water was used as a negative control. DNA extracted from *C. pneumoniae* culture was used as a positive control. Dilutions of 1:100 of DNA template were used routinely in PCR reactions. Clinically diseased sites negative by PCR at this dilution were re-assayed using DNA template diluted 1:10. A band at 1030 bp was considered a positive PCR result.

4.12 END POINTS

PCR was performed on swabs taken prior to beginning treatment (screening day) then weekly from week 3 for animals with subclinical disease, and weekly from week 5 for animals with clinical disease, to inform cessation of treatment.

Medication was continued for 6 weeks in animals with structural disease of the reproductive tract, as determined by ultrasound examination; until negative results were achieved on two weekly consecutive PCRs in animals without structural disease; or until

animals exhibited adverse effects such as weight loss (> 10% of starting body weight), appetite loss and depression. Animals with structural disease present at the end of treatment were euthanased. Animals without structural disease at the end of treatment were monitored for four weeks then released into suitable bush habitat near where they had been found. Animals showing severe side effects were withdrawn from the study and rescue therapy begun (Table 5-3).

4.13 POST MORTEM EXAMINATION AND COLLECTION OF SAMPLES

Animals that died or were euthanased underwent a standard midline post mortem examination (Blanshard, 1994). Where animals were euthanased, blood was collected pre-mortem for biochemistry analysis and haematological examination. Where animals could not be examined immediately post mortem, the carcass was kept at 4 °C and examined within 48 hours. Routine tissues taken for histopathology were: inguinal lymph node, axillary lymph node, liver, spleen, adrenals, heart, lungs, caecum, ileocaecal junction and ileocaecal lymphatic patch, duodenum, ileum, pancreas, thyroids, thymus (when located), palpebrae, brain, kidneys, bladder, ureters, prostate in the male, and vaginae, uteri, oviducts and ovaries in the female. Swabs were collected from the conjunctiva and urethra (males) or urogenital sinus (females) for chlamydial PCR. Bone marrow was collected in animals suspected of haematopoietic disease or lymphoproliferative disorders based on pre-mortem blood examination and/or clinical signs, by longitudinal transection of the femur using parrot-beaked by-pass pruning loppers.

All samples were placed in 10% formalin for up to 24 hours. Selection of tissues for processing followed routine procedure, apart from the urogenital tract of females, which followed standard methods for examination of koala reproductive tracts (Obendorf, 1981).

Tissues were stained with haematoxylin and eosin according to routine procedure and examined with light microscopy.

4.14 ANIMALS RECRUITED FOR TREATMENT TRIALS

In the first season (August 2005 - April 2006), 55 animals were screened. Of the 33 koalas with clinical signs of chlamydiosis, 20 were recruited and 13 animals were rejected (condition score < 2, concurrent disease). Twenty-two koalas without signs of clinical chlamydiosis were examined. Of these, 16 were not tested further and were released, four animals were rejected due to concurrent disease, one animal was tested negative with the Clearview ELISA and was released, and one animal tested positive by PCR and was recruited as a subclinical chlamydial case.

In the second season (September 2006 - April 2007), 68 animals were screened. Of these, 14 clinical and 10 subclinical animals were recruited. Thirty-six were rejected due to: negative PCR (n = 7), mother with joey (n = 3), concurrent disease (n = 8), condition score < 2 or structural reproductive tract disease (n = 18). Eight were released due to lack of accommodation.

In the third season (February - March 2008), 11 clinical animals were recruited. Treatment groups are displayed in Table 4-2.

The results of the clinical and ultrasound examination, haematological parameters, biochemistry analytes, appetite and faecal scoring, body weight and post mortem examinations are discussed in Chapter 5 for animals treated with non-fluoroquinolones and Chapter 8 for animals treated with fluoroquinolones.

Table 4-2. Number of koalas in each treatment group.

Drug	Clinical	Subclinical
Marbofloxacin 10 mg/kg p.o. SID	4	2
Marbofloxacin 1.3 - 3.3 mg/kg p.o. SID	6	0
Marbofloxacin 5 mg/kg s.c. SID	1	1
Enrofloxacin 5 mg/kg p.o. SID	1	0
Enrofloxacin 5 mg/kg s.c. SID	7‡	0
Enrofloxacin 10 mg/kg s.c. SID	12	4
Enrofloxacin 20 mg/kg p.o. SID	5	3
Doxycycline 5 mg/kg p.o. SID	1	0
Doxycycline 0.25 mg/kg p.o. SID	1	0
Doxycycline 10 mg/kg i.m. SIW	1*	0
Doxycycline 5 mg/kg i.m. SIW	1†	1
Doxycycline 2.5 mg/kg i.m. SIW	1	0
Azithromycin 10 mg/kg p.o. SID	1	0
Azithromycin 5 mg/kg p.o. SID	1	0
Azithromycin 2.5 mg/kg p.o. BIW	1	0
Chloramphenicol 50 mg/kg p.o. BID	2	0
Oxytetracycline eye ointment BID	1	0
Total	47	11

The following animals were treated with two different regimes and are thus counted twice:

*one animal re-treated after 42 days of marbofloxacin 2.5 mg/kg p.o. SID.

†one animal re-treated after 28 days of marbofloxacin 2.5 mg/kg p.o. SID.

‡ one animal re-treated after 42 days of enrofloxacin 10 mg/kg s.c. SID with enrofloxacin 5 mg/kg s.c. SID.

SID: once daily medication; BID: twice daily medication; SIW: once weekly medication; BIW: twice weekly medication; p.o: per os.

4.15 ISSUES ASSOCIATED WITH RECRUITMENT AND USE OF ANIMALS FOR TREATMENT TRIALS

Recruitment of animals relied upon naturally occurring disease in wild animals. An advantage of this strategy was that animals were drawn from, and represented, the spectrum of clinical disease currently treated in koala facilities in Australia. The major disadvantage of this strategy was that opportunistic recruitment of suitable animals suffering naturally occurring disease was slow (approx. two animals per three weeks), requiring long periods of time in the field. An experimental population of koalas, and facilities for their husbandry, or alternative hospital facilities that receive greater numbers of cases did not exist at the commencement of the study. Examination of previous admission rates to the Koala Hospital may have helped to predict the recruitment rate, however detailed information as to whether such animals would have been suitable for inclusion in a treatment trial was not always available due to differences in examination procedures between the Koala Hospital and this trial. The slow recruitment meant that planned clinical groups were reduced, and one treatment group (subcutaneous chloramphenicol) was transferred to another facility. Data from this group does not form part of this study.

Randomised controlled trials are the experimental design of choice for studies examining efficacy of treatment but may be difficult and/or expensive to implement. In this study, treatments were not blinded to investigators. Complete blinding of the trial would have been expensive as drugs would have had to be specially compounded and, as different routes of delivery were used, may not have been possible. In addition, it would have required extra sham injections and/or oral medication of wild animals, compromising welfare.

A placebo treatment group of subclinical animals was initially planned but had to be abandoned due to low numbers of subclinical cases recruited. Ethical considerations of withholding treatment precluded an untreated clinically diseased control group and thus experimental design involved comparison between treatment groups. This study was a compromise between the practicalities of working with pre-formulated commercial pharmaceuticals, cost, lay co-workers' perceptions, animal welfare and experimental design.

Medication of some animals proved to be challenging. Although koalas are generally placid, and often take oral medication well (Blanshard, 1994), some animals resisted oral supplementation vigorously. Despite three different formulas being trialled, there was no difference noted in formula acceptability or palatability and reluctance of animals to take oral medications and supplements probably related to handling rather than palatability or formulation. Pharmacological studies require that all medication be reliably consumed at one time. Animals strongly resistant to oral supplementation in a pre-treatment acclimatisation period had to be allocated to a parenteral treatment group to guarantee accurate drug administration. This meant that random allocation of koalas to treatment groups was not always possible. Animals strongly resistant to oral medication may have been more stressed in captivity. Such animals might be expected to have higher circulating stress hormones, such as cortisone, that could have influenced response to treatment. A three to seven day training period prior to beginning medication was introduced, but the length of the training period had to be balanced against extending time in captivity, the ethics of holding animals without treatment and risks to the animal of aspiration of oral medications or supplements. Generally animals amenable to oral dosing continued to accept this route throughout treatment.

The commencement of a major scientific investigation within a wildlife care hospital offered a number of major advantages and posed unique challenges. Hospital staff often had intimate knowledge of koala disease, husbandry and behaviour through long association with their care. Lay workers provided a large, enthusiastic workforce to help with many aspects of experimental work and koala husbandry. Specialised facilities and trained staff for keeping the koalas were mostly already in place at the Koala Hospital, requiring little input from researchers prior to the commencement of the study. A long association (approx. 25 years) between the Koala Hospital and the Faculty of Veterinary Science, University of Sydney, provided a solid basis for the co-operative relationship cemented by this research. However, unique challenges in working in such an environment will inevitably occur. Although some of these challenges were anticipated in advance, some, particularly the building of infrastructure, required demonstration of a real need. Others, in relation to change of protocol and demands on facilities, required careful education and explanations to lay staff.

The Koala Hospital policy - that volunteers retrieve koalas from dangerous areas (such as in the vicinity of dogs, or main roads) and immediately relocate them to suitable nearby bush habitat if deemed well on superficial inspection - had to be revised to allow screening of animals for subclinical chlamydial disease. Adoption of this policy took time, thus a number of potential recruits (n = 14) in the first few months of the 2005 - 2006 season were released without full physical examination under general anaesthesia or testing for subclinical disease, resulting in recruitment of only one subclinical case in 2005 - 2006. Detection of subclinical carriers of Chlamydiaceae by PCR and detection of clinical cases without structural disease offered hope that disease might be treated before structural damage occurred: a position enthusiastically embraced by lay workers. In the 2006 - 2007 and 2008 field seasons recruitment of such animals became a priority, whilst animals with

structural disease were euthanased soon after diagnosis to minimise the stress on lay workers that extensive time spent caring for an animal with a hopeless prognosis may bring.

The lack of holding space for animals severely limited the numbers of animals that could be enrolled in the study, particularly in the first season (2005 - 2006) and the first half of the second season (2006 - 2007). Koalas enrolled in this study were maintained for a minimum of nine weeks. Prior to the commencement of this study the Koala Hospital had a holding capacity of 18, if animals were individually housed. Three of these were already occupied by permanently disabled animals kept for educational display, and two were not available for use. As the Koala Hospital admits roughly 200 koalas a year for a variety of reasons (Chapter 2), space needed to be set aside for these animals, particularly those requiring long-term rehabilitation.

During the study, three animals escaped their enclosures, necessitating recapture, and one animal was lost to follow up by escaping from the hospital grounds completely. Escape of patients from the Koala Hospital is a relatively frequent occurrence and wild males are often found to have entered the facilities overnight (personal observation). Koalas require specialised enclosure design to prevent escape (Blanshard, 1994), which may prove expensive to build and maintain. The fencing of the Koala Hospital was due to be upgraded at the commencement of the study as part of a generalised hospital redevelopment. Fences prior to the upgrade consisted of sheet metal (1.2 m) with cyclone fencing (0.6 m) above, conforming to recommended standards (Blanshard, 1994). Although escapes from enclosures were not witnessed, they may have occurred as a result of items such as rakes/sun umbrellas, or smaller aviary-style enclosures present within the enclosures allowing koalas to acquire purchase on wire netting, or koalas acquiring purchase on the top of the sheet metal, as it did not have a rounded top edge as has been recommended

(Blanshard, 1994). Wild animals entering enclosures presumably did so by leaping from surrounding trees, which occasionally had branches overhanging enclosures. Lopping of these trees, although performed regularly by professional contractors, was not under the direct control of the Koala Preservation Society of NSW, making control of ingress challenging. As interlopers tended to end up in one large yard closest to such trees, study animals were held in yards furthest from this area to minimise risk of contact. Escapes early in the study necessitated indoor housing of the larger animals, or those that had escaped previously. As indoor spaces were few (four), this decision initially limited the ability to recruit animals into the study. The Koala Hospital staff worked with researchers to improve facilities by building an extra 10 holding yards with improved fencing and two enclosed aviaries for use by research staff, and providing exclusive use of nine previously built accommodation spaces. Cage furniture was removed or placed flush with the ground to minimise risk of escape. The development of ingress- and egress-proof yards of sufficient quantity and quality to house study koalas was a significant cost, requiring approval of the membership body of the Koala Preservation Society of NSW. The approval for and building of these yards took time but the yards were available midway through the second field season (2006 - 2007).

Performing research with wild animals within a rehabilitation setting offered many advantages but was not without difficulty. Although some challenges might be anticipated (e.g. communication of changed procedures with lay staff), others might not be predictable (e.g. difficulty in medicating animals orally, slow recruitment of suitable cases). The problems encountered in this study highlight the need for careful planning and involvement of staff of the field work site, prior to the commencement of, and during, a major scientific study. Careful planning with regard to infrastructure is ideal; however, as such changes may involve significant financial outlay, clear demonstration of its necessity may require

an initial smaller pilot study. Flexibility and the ability to adapt the study, where possible, to better suit the facilities, recruitment rate and staff expectations are ideal. A “softly, softly” approach, particularly when changes to procedures are initially implemented might be warranted. Extensive and on-going communication is required to explain the scientific process to lay staff, particularly where animal experimentation is required, as gaining the support of staff is crucial for the success of research within such facilities.

CHAPTER 5 - PILOT STUDY INTO TREATING AND MONITORING KOALAS WITH CHLAMYDIOSIS

5.1 INTRODUCTION

Chlamydophila pneumoniae and *Chlamydophila pecorum* can infect up to 85% of individuals in some populations of the koala, causing significant morbidity (Jackson, White *et al.*, 1999). Animals with clinical signs consistent with chlamydiosis account for approximately 20% of admissions to the Koala Hospital, Port Macquarie (Chapter 2). Clinical signs of chlamydiosis in koalas include conjunctivitis (Cockram & Jackson, 1976), rhinitis (Nicolson, 2002), cystitis and/or reproductive tract disease (Brown & Grice, 1986). Systemic treatment of chlamydiosis in koalas has long proved problematic due to the paucity of studies into effectiveness (Cockram & Jackson, 1976; Osawa & Carrick, 1990; Markey, Wan *et al.*, 2007), and the reported syndrome of wasting and death in koalas within two or six weeks of treatment with erythromycin (Brown, Wood *et al.*, 1984) or oxytetracycline (Handasyde, 1986; Osawa & Carrick, 1990; Osawa, Bird *et al.*, 1993), respectively. It is hypothesised that disturbance or loss of gastrointestinal microbial flora leads to this syndrome in antibiotic treated koalas (Osawa, Bird *et al.*, 1993).

Recommendations for treatment of chlamydiosis in other species (people, cats, birds) have included azithromycin (Workowski, Levine *et al.*, 2002), doxycycline (Workowski, Levine *et al.*, 2002; Dean, Harley *et al.*, 2005) and fluoroquinolones (Dorrestein, 1993; Lindenstruth & Frost, 1993; Workowski, Levine *et al.*, 2002). In people, azithromycin exhibits high efficacy, rapid and extensive tissue penetration (Alvarez-Elcoro & Enzler, 1999; Lau & Qureshi, 2002) and a long mean tissue half-life (2 - 4 days) allowing single-dose therapy (Alvarez-Elcoro & Enzler, 1999). Doxycycline remains an excellent alternative treatment choice for cats, in which azithromycin has not proved to be an

effective treatment (Owen, Sturgess *et al.*, 2003), and for people (Lau & Qureshi, 2002). Newer generation fluoroquinolones have been recommended for treatment of chlamydial infections of people (Nightingale, 2000; Workowski & Berman, 2006) and have showed some promise in treating chlamydial infections in cats (Gerhardt, Schulz *et al.*, 2006) and birds (Lindenstruth & Frost, 1993; Jung, 1994).

Revisiting the use of tetracyclines and macrolides for treatment of chlamydiosis in koalas is worthwhile as modern forms of these drugs, such as doxycycline and azithromycin, respectively, possess attributes that might decrease their side effect profile in koalas. Azithromycin has fewer gastrointestinal side effects in people than erythromycin (Hopkins, 1991); and doxycycline has better bioavailability than oxytetracycline (Plumb, 2005), allowing the use of lower doses. In addition, a slightly different spectrum of activity to the older forms of these drugs (Williams, 1991; Joshi & Miller, 1997) might cause less disturbance of gastrointestinal flora and fewer side effects.

Systemic chlamydiosis is currently treated in wildlife hospitals with chloramphenicol and/or enrofloxacin at various dose rates and routes (Table 5-1). Alternatively, animals with conjunctivitis alone are often treated with topical tetracycline based eye ointments and/or debulking surgery to remove proliferative conjunctiva (C. Flanagan, pers. comm.¹; A. MacKinnon, pers. comm.²). If azithromycin was effective in koalas, it would be extremely attractive as it could dramatically reduce time in captivity compared with current treatment regimes of up to six weeks (C. Flanagan, pers. comm.)³.

¹Personal communication: C. Flanagan, Supervisor, Koala Preservation Society of NSW, Port Macquarie, NSW, August, 2005.

² Personal communication: A. MacKinnon, Manager, Moggill Koala Hospital, Toowoomba, Qld, August, 2005.

³Personal communication: C. Flanagan, Supervisor, Koala Preservation Society of NSW, Port Macquarie, NSW, August, 2005.

This pilot study was instigated to provide preliminary information on the effectiveness and tolerability of medications: 1/ presently employed on an empirical basis to treat chlamydiosis in koalas (fluoroquinolones, chloramphenicol, tetracycline eye ointment) and 2/ currently used commonly to treat chlamydiosis in other species (doxycycline, azithromycin).

It was anticipated that the results from the pilot study would allow the refinement of diagnostic and treatment protocols, and their monitoring for efficacy and adverse effects, for future studies in this thesis.

Table 5-1. Systemic drug dosage regimes for treatment of chlamydiosis in specialised koala treatment facilities.

Drug	Dose/route/frequency	Facility	Reference
Chloramphenicol	60 mg/kg s.c. SID	Australia Zoo, Beerwah, Qld, Australia	J. Hanger, pers. comm. ⁴
Chloramphenicol	50 mg/kg p.o. BID	The Koala Hospital, Port Macquarie, NSW, Australia	C. Flanagan, pers. comm. ³
Chloramphenicol	50 mg/kg i.m. BID for 3 days then s.c.	The Koala Hospital, Port Macquarie, NSW, Australia	C. Flanagan, pers. comm. ³
Enrofloxacin	8.33 - 12.5 mg/kg p.o. SID	Moggill Koala Hospital, Toowoomba Qld, Australia	A. MacKinnon, pers. comm. ⁵
Enrofloxacin	10 mg/kg s.c. SID	The Koala Hospital, Port Macquarie, NSW, Australia	C. Flanagan, pers. comm. ³
Enrofloxacin	5 - 10 mg/kg p.o. SID	The Koala Hospital, Port Macquarie, NSW, Australia	C. Flanagan, pers. comm. ³

s.c: subcutaneous; i.m: intramuscular; p.o: per os; SID: once daily; BID: twice daily.

⁴ Personal communication: J. Hanger, Principal Veterinarian, The Australian Wildlife Hospital, Beerwah, Qld, August, 2005.

⁵ Personal communication: A. MacKinnon. Manager, Moggill Koala Hospital, Toowoomba, Qld, August, 2005.

5.2 METHODS

Animals were recruited, screened, housed, examined, allocated to treatment groups, supplemented, sampled from and observed as described in Chapter 4. Koalas were treated using dose regimes as per Table 5-2 (Results).

5.2.1 Diagnostic ELISA

Nine animals (seven females and two males) with clinical signs consistent with chlamydial disease were swabbed as per 4.11.5. The third swab from both eyes and the urogenital tract (three swabs in total) were pooled and subjected to analysis by Clearview Chlamydia MF ELISA (Inverness Medical Australia, Sinnamon Park, Qld, Australia). Due to perceived insensitivity of the diagnostic ELISA (see 5.3.1 and Discussion) and the initial lack of facilities to perform PCR in the field, nine animals with clinical signs consistent with chlamydiosis did not undergo diagnostic testing prior to treatment. Diagnosis based on clinical signs was confirmed retrospectively using qPCR.

The procedure followed was that provided by the manufacturer for chlamydial diagnosis from endocervical swabs from women. All reagents, reaction strips and collection tubes were provided by the manufacturer. Most samples were tested within 24 hours of collection although occasionally samples were refrigerated at 4 °C and tested within 48 hours, in accordance with the manufacturer's guidelines.

5.2.2 Response to treatment

Treatment was continued for up to six weeks or was discontinued when serious side effects were noted (consistent inappetence for more than three days, weight loss exceeding 10% of starting weight, depression, decreased faecal output and/or anaemia). Rescue treatments, which were administered to animals experiencing side effects, consisted of combinations of gastrointestinal motility modifiers, oral or parenteral fluids, nutritional supplements,

vitamin injections and attempted caecal refaunation (Table 5-3, Results). When inappetence, depression and faecal output continued for greater than seven days, animals were euthanased on grounds of welfare.

5.2.3 Appetite, body weight and faecal output

For the first two weeks (n = 20) or three weeks (n = 8) of treatment, all faecal pellets were collected over each 24 hour period, weighed, and the mass converted to a percentage of the faecal mass measured on day 0 or day 1 (i.e. immediately prior to treatment). Mean weekly body weights were converted to a percentage of body weight on day 0 or day 1.

5.2.4 Haematological parameters and biochemistry analytes

Detail of methods used to collect and analyse samples of urine and blood has been provided previously (4.10).

5.2.5 Statistical models

Residual plots of total eye scores and relative faecal and body weights were first examined to determine whether data approximated normality, and non-parametric data were transformed (usually with log transformation) before restricted maximum likelihood analysis (REML) using GenStat for Windows (2008, 11th ed., VSN International Ltd., Hemel Hempstead, Hertfordshire, UK). The model examined the effects of drug and time (week) and the interaction of drug and time. Statistical significance was concluded when $p \leq 0.05$.

REML analysis of biochemical and haematological analytes examined the effects and interactions of drug, route and dose; time by week and by stage of treatment (pre-treatment, in-treatment and post-treatment); and whether animals maintained, gained or lost body weight.

5.2.6 Post mortem examination

Animals euthanased during or immediately after the study had blood sampled immediately prior to death for haematological and biochemistry analysis. All animals that died or were euthanased underwent a routine post mortem and histological examination as per 4.13.

5.3 RESULTS

Twenty animals were recruited into the study with clinical signs of chlamydial disease (n = 19), and/or ultrasonographic abnormalities of the reproductive tract (ovarian bursal cysts; n = 10) and/or chlamydial DNA detected by PCR (n = 2) and/or ELISA (n = 4) (Table 5-2).

5.3.1 Diagnostics

Of the nine clinically diseased animals tested by ELISA, five animals were negative and four positive (Table 5-2). One subclinical animal (EF) tested positive for urogenital tract infection by PCR.

5.3.2 Response to treatment

Of the 20 koalas treated, 10 completed four - six weeks of treatment with marbofloxacin (n = 5), enrofloxacin (n = 5), oxytetracycline hydrochloride/polymyxin B sulphate ophthalmic ointment (n = 1), or doxycycline (n = 1). Eight animals (four treated with doxycycline, three treated with azithromycin and one treated with chloramphenicol) failed to complete treatment due to serious side effects and were euthanased (n = 7) or died (n = 1) (Table 5-2 and 5.3.3). One animal treated with chloramphenicol had treatment withdrawn after developing anaemia (see 5.3.4 and Figure 5-7).

Of the animals that completed treatment (Table 5-2): one animal developed ovarian bursal cysts (RG), ovarian bursal cysts present at the start of treatment did not resolve in six animals (TM, WR, CR, SS, KH, KS), and three koalas failed to resolve clinical signs of

cystitis (LJ, OA, AG). Two of these latter animals (OA, AG) were retreated with doxycycline and were eventually euthanased. In animals with conjunctivitis that completed four weeks of various treatments, total eye scores decreased significantly ($p = 0.004$; $n = 7$; Figure 5-1). One animal treated with oxytetracycline hydrochloride/polymyxin B sulphate ophthalmic ointment (ED; Table 5-2) escaped after 36 days of treatment and was lost to follow up.

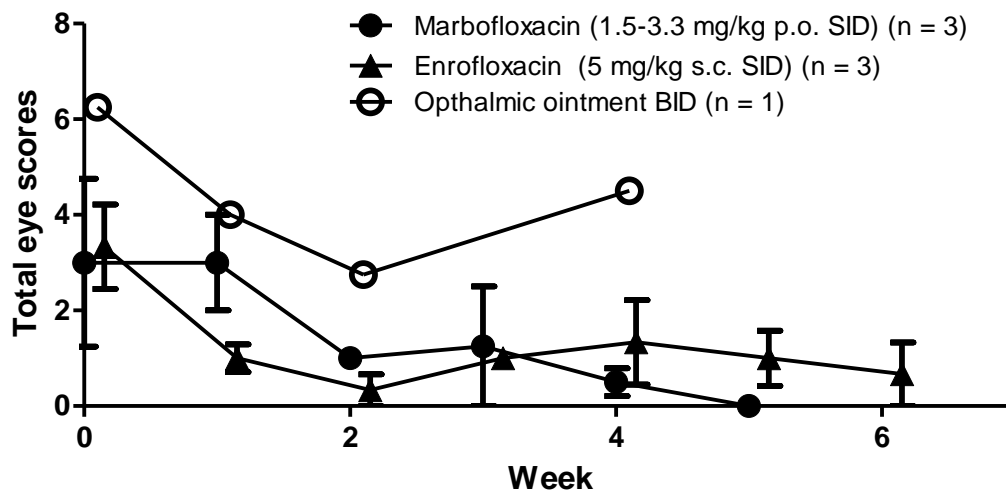


Figure 5-1. Change in mean (\pm SEM) total eye scores in seven animals treated with fluoroquinolones or oxytetracycline/ polymyxin B ointment.

s.c: subcutaneous; p.o: per os; SID: once daily.

Table 5-2. Signalment, clinical presentation, diagnostic tests, treatment regimes and incidence of side effects of animals recruited in the pilot study.

Koala	Sex	Age (years)	Clinical findings at presentation	Diagnostics (ELISA or PCR)	Drug	Serious side effects necessitating treatment withdrawal
MO	F	11.5	Rump pelage stain Bilateral conjunctivitis	ELISA negative	Chloramphenicol 50 mg/kg p.o. BID for 35 days	Anaemia
NK	M	9	Rump pelage stain Inflamed and ulcerated pericloacal skin and penile mucosa Haematuria	ELISA positive	Chloramphenicol 50 mg/kg p.o. BID for 23 days	Depression Decreased faecal output, appetite and body weight
OC	F	2	Inflamed vestibule Bilateral ovarian bursal cysts Pyuria	Not done	Doxycycline loading dose 0.5 mg/kg p.o. am, 0.25 mg/kg p.o. pm, then 0.25 mg/kg p.o. BID for two days, stopped 48 hours, then 5 mg/kg p.o. SID for four days, then 2.5 mg/kg p.o. SID for three days	Depression Decreased faecal output, appetite and body weight
TH	F	8	Rump pelage stain Unilateral conjunctivitis Unilateral ovarian bursal cyst	Not done	Doxycycline p.o. 0.25 mg/kg BID for eight days	Depression Decreased faecal output, appetite and body weight
EF	M	3.5	Subclinical	PCR positive	Doxycycline 5 mg/kg i.m. SIW for 42 days	None
CP	M	12	Rump pelage stain Incontinence and haematuria Unilateral conjunctivitis	Not done	Doxycycline 2.5 mg/kg i.m. SIW for 28 days	Depression Decreased faecal output, appetite and body weight
OA	M	7	Pelage stain Dysuria	ELISA negative	Marbofloxacin 2.5 mg/kg p.o. for 42 days, stopped 48 hours, then doxycycline 10 mg/kg i.m. SIW for 21 days	Depression Decreased faecal output, appetite and body weight after beginning doxycycline

Table 5.2 cont.

Koala	Sex	Age (years)	Clinical findings at presentation	Diagnostics (ELISA or PCR)	Drug	
AG	F	4	Conjunctivitis & dysuria	Not done	Marbofloxacin 1.3 mg/kg p.o., stopped 48 hours, then doxycycline 5 mg/kg i.m. SIW for 21 days	Depression Decreased faecal output, appetite and body weight after beginning doxycycline
MM	F	5.5	Pelage stain	PCR positive	Azithromycin 5 mg/kg p.o. SID for four days	Depression Decreased faecal output, appetite and body weight
OP	F	12	Pelage stain Bilateral ovarian bursal cysts	Not done	Azithromycin 2.5 mg/kg p.o. SID day 1, 4, 8 and 12	Depression Decreased faecal output, appetite and body weight
OK	F	12	Pelage stain Purulent vaginal discharge Bilateral ovarian bursal cysts	Not done	Azithromycin 10 mg/kg p.o. SID for seven days then once day 11 and day 14	Depression Decreased faecal output, appetite and body weight
TM	F	12	Rump pelage stain Bilateral conjunctivitis Unilateral ovarian bursal cyst Pyuria	Not done	Marbofloxacin 1.3 mg/kg p.o. SID for 42 days	None
WR	F	5	Rump pelage stain Bilateral ovarian bursal cysts Unilateral conjunctivitis	ELISA negative	Marbofloxacin 3.34 mg/kg p.o. SID for 42 days	None
RG	F	5	Pelage stain Inflamed pericloacal skin	ELISA positive	Marbofloxacin 2.3 mg/kg p.o. SID for 42 days	None

Table 5.2 cont.

Koala	Sex	Age (years)	Clinical findings at presentation	Diagnostics (ELISA or PCR)	Drug	
CR	F	10	Unilateral ovarian bursal cysts Bilateral uterine distension Rump pelage stain	ELISA positive	Enrofloxacin 5 mg/kg s.c. SID for 42 days	None
SS	F	7	Rump pelage stain Unilateral conjunctivitis Bilateral ovarian bursal cysts	ELISA negative	Enrofloxacin 5 mg/kg s.c. SID for 42 days	None
KH	F	5	Rump pelage stain Bilateral ovarian bursal cysts	ELISA negative	Enrofloxacin 5 mg/kg s.c. SID for 42 days	None
KS	F	12	Rump pelage stain Incontinence Unilateral conjunctivitis and keratitis, corneal ulceration Thickened bladder wall Bilateral ovarian bursal cysts	ELISA positive	Enrofloxacin 5 mg/kg s.c. SID for 42 days	None
LJ	F	3	Pelage stain Unilateral conjunctivitis Incontinence and pyuria	Not done	Enrofloxacin 5 mg/kg p.o. SID for 28 days	None
ED	M	4.5	Bilateral conjunctivitis	Not done	Oxytetracycline hydrochloride/polymyxin B sulphate ophthalmic ointment BID for 36 days	None

BID: twice daily; SID: once daily; SIW: once weekly; p.o: per os; s.c: subcutaneous injection; i.m. intramuscular; PCR: polymerase chain reaction; ELISA: enzyme linked immunosorbent assay. Age estimated based on tooth wear class (Martin, 1981) and past admission records.

5.3.3 Appetite, body weight and faecal output

All three animals treated with azithromycin (MM, OP, OK; Table 5-2), one animal treated with chloramphenicol (NK; Table 5-2), and five of six animals treated with doxycycline (AG, OA, OC, TH, CP; Table 5-2) experienced loss of appetite, decreased faecal output (Figure 5-2), decreased body weight (Figure 5-3, Figure 5-4 and Figure 5-5) and depression, necessitating discontinuation of treatment (Table 5-2). Two animals (AG, OA) maintained weight whilst being treated with marbofloxacin for four and six weeks, respectively (Figure 5-6) but lost weight after commencing treatment with doxycycline (Figure 5-5), used due to non-resolution of clinical signs on the initial treatment. Weight loss relative to starting weight (week 0) became significant ($p < 0.001$) by week two for azithromycin (Figure 5-4), by week three for doxycycline (Figure 5-5) and week four for chloramphenicol (Figure 5-3). In all animals that lost weight, sustained appetite loss (median 9 days, range 4 - 23 days) was first observed a few days prior to a sustained decline in weight (Figure 5-3, Figure 5-4 and Figure 5-5). Twenty-four hour faecal output appeared to decline in all animals that were inappetent and lost weight; however this was not statistically significant (Figure 5-2). No rescue therapy (Table 5-3) reversed the loss of appetite, body weight, faecal output or depression. Eight of these koalas were euthanased on grounds of welfare when inappetence, depression and weight loss persisted for at least one week and the remaining one died.

Table 5-3. Rescue treatments attempted in animals suffering side effects of azithromycin (n = 3), doxycycline (n = 5), or chloramphenicol (n = 1).

Rescue treatment	Dose and route	Frequency	Formulation	n
Vitamin B ₁₂ injection	0.05 - 0.1 mg/kg s.c.	Once	Troy Vitamin B ₁₂ ® 1 mg/mL Troy Laboratories, Glendenning, NSW, Australia	3
Fresh caecal contents	15 - 25 mL p.o.	SID - BID up to five days	Fresh caecal contents from animals that died or were euthanased for other reasons (e.g. trauma)	4
Oral rehydration salts	20 - 200 mL p.o.	Once	Vy'Trate®, Jurox, Rutherford, NSW, Australia	2
Parenteral fluids	50 - 300 mL s.c.	SID over one to four days	Compound sodium lactate (Hartmann's) intravenous solution or 0.45% sodium chloride & 2.5% glucose intravenous infusion BP both Baxter Vialflex, Toongabbie, NSW, Australia	4
	260 - 500 mL i.v.	Once		
Metoclopramide	0.5 mg/kg s.c.	Four to six doses SID - BID	Maxolon® 5 mg/ mL, GlaxoSmithKline, Boronia, Vic, Australia	3
Pumpkin and sweetcorn baby food	20 mL p.o.	SID for one - six days	Heinz® pureed pumpkin and sweetcorn, H.J. Heinz company, Australia Ltd. Southbank, Vic, Australia	3
Pulverised <i>Eucalyptus</i> leaf	30 - 50 mL p.o.	Once	Leaf tip selected from <i>Eucalyptus robusta</i> , <i>E. microcorys</i> , <i>E. tereticornis</i> , <i>E. nicholii</i> , <i>E. macrorhyncha</i> , or <i>Corymbia maculata</i>	2
Cisapride	0.2 mg/kg p.o.	BID for one day	Prepulsid® liquid 1 mg/mL, Janssen-Cilag Australia, North Ryde, NSW, Australia	1
Soya supplement	7 - 35 g in 20 - 50 mL water p.o.	SID - BID up to 44 days	Karicare® Soya All Ages Formula, Nutricia, North Ryde, NSW, Australia	7
Low lactose milk supplement	9 g in 50 mL water p.o.	BID up to 35 days	Di-vetelact® Low Lactose Milk Powder, Sharpe Laboratories, Ermington, NSW, Australia	5
High energy vitamin gel	1.5 cm p.o.	BID for 16 days	Nutrigel®, Troy Laboratories, Glendenning, NSW, Australia	1

s.c: subcutaneous; i.v: intravenous; p.o: per os.

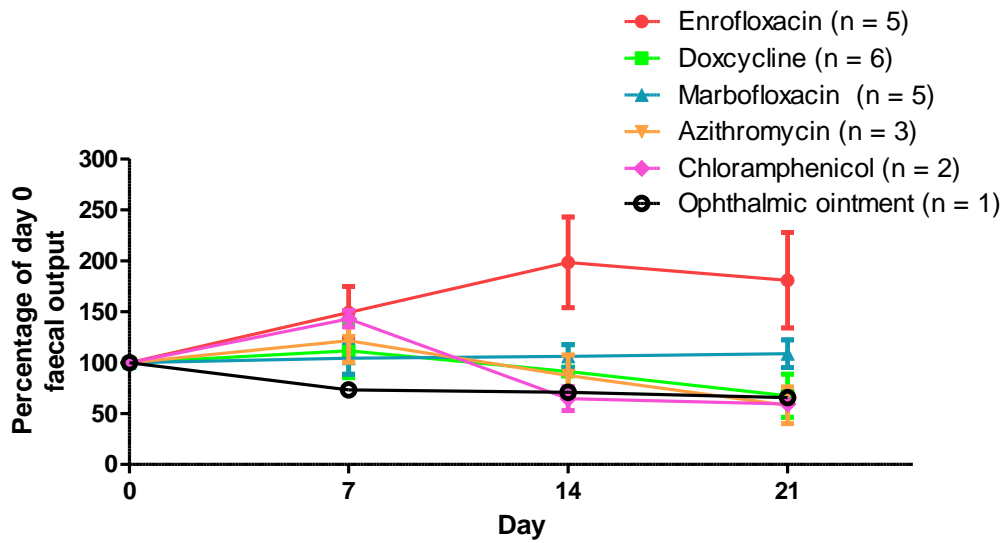


Figure 5-2. Change in mean (\pm SEM) 24 hour faecal output for each drug group for the first three weeks relative to day 0.

Drug doses and regimes are detailed in Table 5-2. Two animals are included in both doxycycline and marbofloxacin groups due to retreatment.

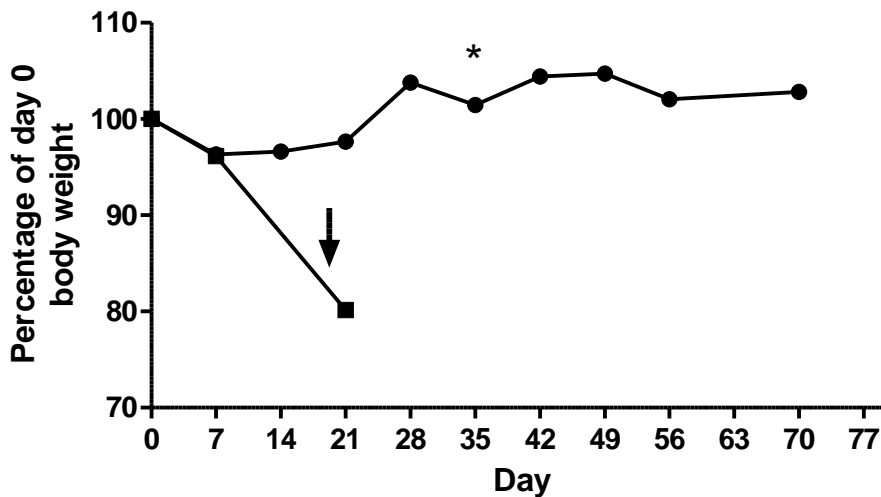


Figure 5-3. Change in body weight in two animals treated with oral chloramphenicol (50 mg/kg twice daily) relative to day 0.

Arrow indicates when appetite decline was first noted. Treatment was withdrawn in one animal on day 35 (*) due to anaemia. The other animal was euthanased on day 21.

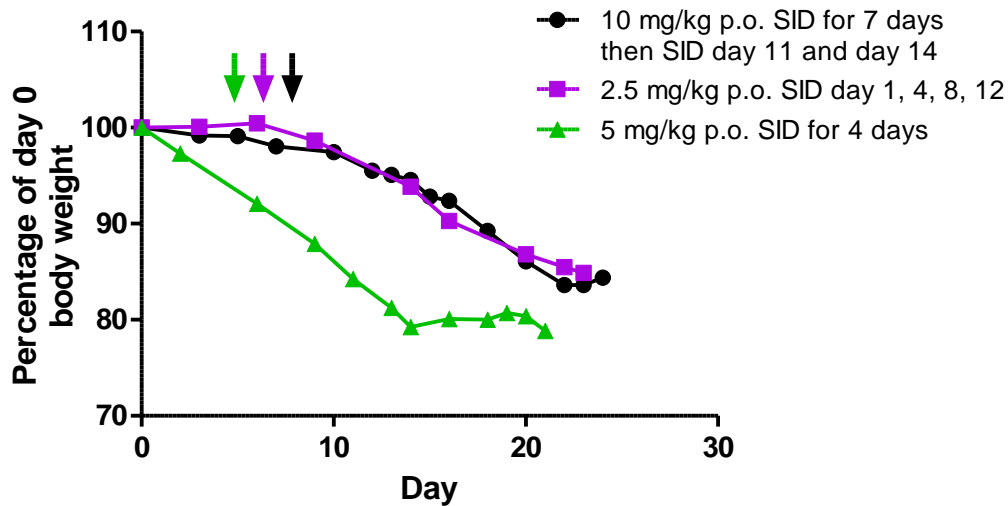


Figure 5-4. Changes in body weight in three animals treated with oral azithromycin relative to day 0.

Arrows indicate when appetite decline was first noted, p.o: per os; SID: once daily.

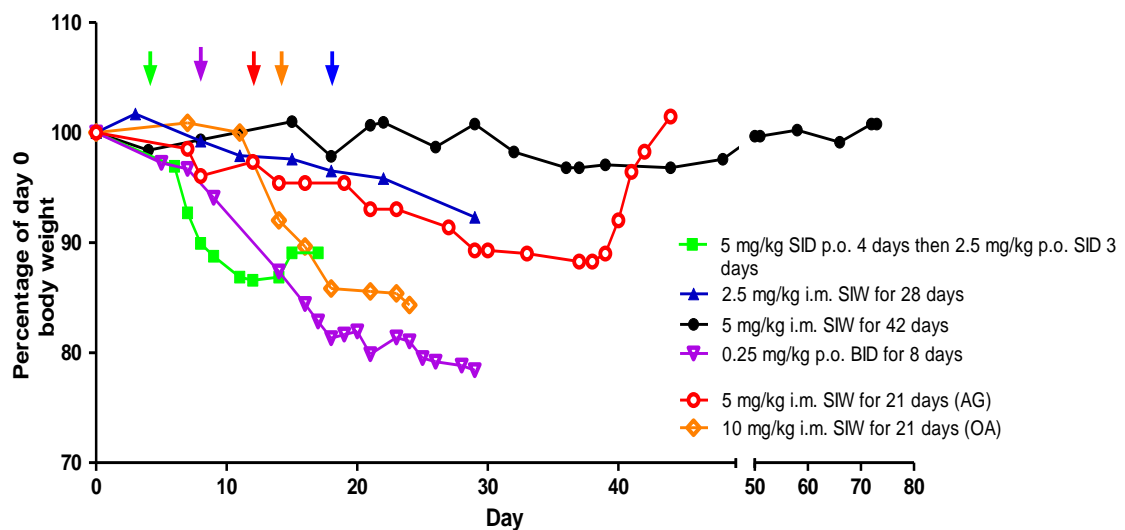


Figure 5-5. Change in body weight in six animals treated with doxycycline relative to day 0.

Arrows indicate when appetite decline was first noted, p.o: per os; i.m: intramuscular; SID: once daily; BID: twice daily; SIW: once weekly. AG & OA: animals previously treated with 28 and 42 days oral marbofloxacin 1 - 3.3 mg/kg, respectively.

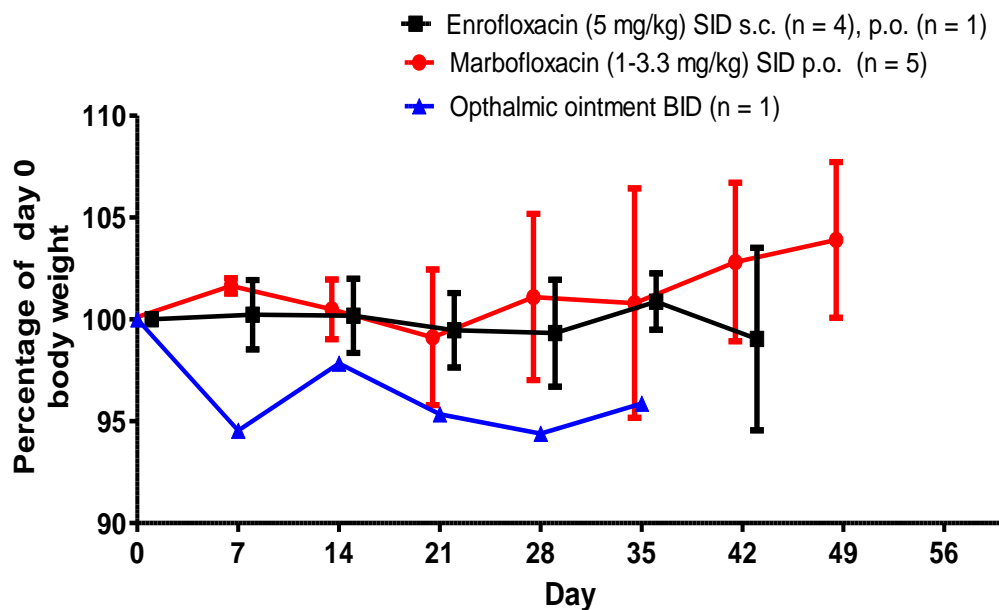


Figure 5-6. Change in body weight (mean \pm SEM) in 10 animals treated with fluoroquinolones and one animal treated with oxytetracycline/ polymyxin B ophthalmic ointment relative to day 0.

p.o.: per os; s.c.: subcutaneous; SID: once daily; BID: twice daily.

5.3.4 Haematological parameters and biochemistry analytes

Anaemia, which occurred by day 35 in one animal treated with chloramphenicol, resolved after drug withdrawal (Figure 5-7). Biochemical and haematological values of other animals remained within published reference intervals (Canfield, O'Neill *et al.*, 1989; Blanshard, 1994) with the exception of creatinine kinase activity. This was significantly greater ($p < 0.001$) in animals undergoing daily s.c. injections with enrofloxacin, when compared with other drug groups and with pre- or post-treatment groups. The last available biochemical and haematological values for inappetent animals ($n = 9$), were generally non-specific or attributable to debilitation or the stress of handling. Animals exhibited hypoalbuminaemia ($n = 5$), increase in creatinine kinase activity ($n = 7$), decrease in creatinine ($n = 3$), mildly increased cholesterol ($n = 5$) and hypoglycaemia ($n = 2$)

(Appendix III). Two animals (NK and OK) with severe acute renal oxalate nephrosis detected at post mortem had biochemical changes consistent with renal insufficiency (Appendix III), although in one of these (OK), the observed azotaemia could have been partly pre-renal and partly renal, as urine specific gravity was not available. Other changes were mild and usually confined to one animal.

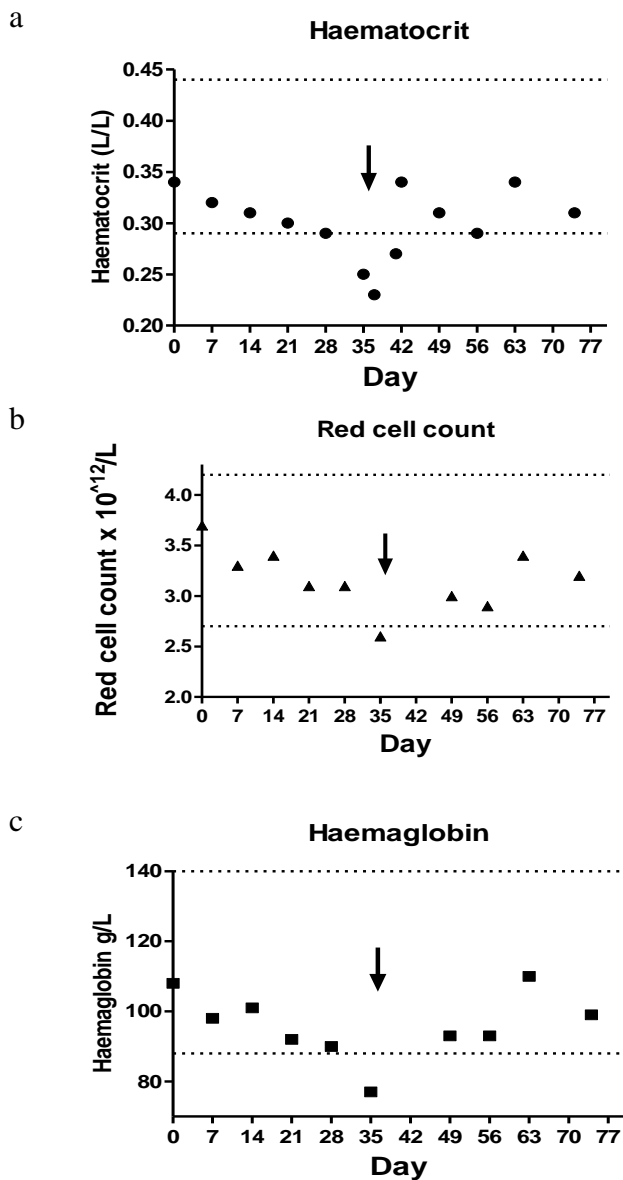


Figure 5-7. Anaemia in one animal (MO) treated with oral 50 mg/kg chloramphenicol twice daily with reduced haematocrit (a), red cell count (b) and haemoglobin (c).

Arrow represents treatment withdrawal. Dashed lines indicate reference intervals (Canfield, O'Neill et al., 1989).

5.3.5 Post mortem examination

Post mortem examination results for animals that were euthanased (n = 17) or died (n = 1) are presented in Appendix II. Most findings related to chronic inflammation and fibrosis of the urogenital tract, consistent with chlamydial disease. Of animals that declined during treatment (n = 9), all animals were thin to emaciated (condition scores 1 - 2.5) with eight of these exhibiting poor, absent or very liquid gut fill. In seven of the nine cases, post mortem examination was unremarkable, but in two declining animals, gastrointestinal tract disease and severe renal oxalate nephrosis appeared sufficiently extensive to have contributed to the significant morbidity observed. One of these animals had moderate diffuse chronic active peritonitis, associated with a small amount of plant material, without an obvious point of abdominal wall or gut penetration. Similar findings have been described in aged koalas and were attributed to perforation of the bowel by poorly masticated stems, leading to localised or subclinical peritonitis (Blanshard, 1994). The other animal had fungal gastritis, a finding not described in koalas, but associated with broad spectrum antibiotic use or generalised disease and debilitation in other species (Neitzke & Schiefer, 1974; Zwoliska-Wcislo, Budak *et al.*, 2001). Other mild, probably incidental, observations were: mild cerebral multifocal idiopathic gliosis (n = 4), evidence of previous hip trauma (n = 1), gingival recession (n = 1), mild focal aspiration pneumonia (n = 1), peritonitis (n = 1), Lafora-like cerebral bodies (n = 1) and very mild scattered tubular oxalate nephrosis (n = 1).

5.4 DISCUSSION

The results from this pilot study informed significant refinement of diagnostic, treatment and monitoring protocols for later seasons. Moreover, it clarified and led to effective monitoring of side effects for certain antibiotics, and the development of tools for objectively assessing ocular disease, ultrasonographic images, appetite and faecal output in koalas. The pilot study was a learning process, not just for the author, but also for the staff of the wildlife hospital in which the study was set. Significant challenges were encountered in the first season while adapting planned regimes to the reality of the infrastructure provided, time constraints and the experimental animals themselves.

In this study, diagnosis of clinical and subclinical chlamydial infections in koalas was required within a 24 hour period to allow swift return of non-infected animals to the wild. Although regarded as the gold standard for diagnosis of chlamydial infection in the past, cell culture has been criticised due to the difficulty of obtaining viable samples in the field, and because it is laborious and time consuming in nature, with diagnosis taking up to a week. Other screening tests such as radiology, immunofluorescence against chlamydial antigen in infected cells, complement fixation tests and antibody enzyme-linked immunosorbent assays (ELISA) have been widely criticised due to poor sensitivity and specificity and/or require specialised equipment (Brown, Carrick *et al.*, 1984; Handasyde, Martin *et al.*, 1988; Weigler, Girjes *et al.*, 1988; Carlisle, Brown *et al.*, 1989; Canfield, Love *et al.*, 1991b; Girjes, Ellis *et al.*, 1993; Emmins, 1996; Jackson, White *et al.*, 1999; Bodetti & Timms, 2000; Higgins, 2004).

Despite the Clearview Chlamydia MF ELISA having been recommended for diagnosis of chlamydiosis in koalas (Connolly, 1999), the results of this pilot study suggest poor sensitivity in the cohort of animals tested, in contrast with a previously published study

reporting a sensitivity of 91% and a specificity of 100% (Wood & Timms, 1992).

Positivity was assumed based on clinical signs alone, as other diagnostic tests were not available as point-of-care assays at this time. This assumption is flawed if clinical signs observed were the result of another organism, or if the clinical response in koalas lags behind microbial cure, as may occur in trachoma infection in people (Michel, Solomon *et al.*, 2006). However, given clinical signs were also used in the original study of 11 clinically diseased female wild koalas as a gold standard (Wood & Timms, 1992), high sensitivity of this test in this cohort was expected in the current study population. One difference between this study and that of Wood and Timms (1992) is that the previous work examined swabs only from the urogenital sinus of females, whereas the current study combined conjunctival swabs with a urogenital swab and also screened both sexes. Furthermore, the test was required to be sensitive in subclinically infected animals. Pooling of swabs from multiple sites was undertaken to increase the sensitivity of the test by providing more test material, as has been suggested by other authors (Schachter, 1985; Wood & Timms, 1992); however this technique could have introduced additional inhibitors present in conjunctival and/or penile swabs. In addition, koalas in this study were not from the same geographical population as that of the original study, and could have had different strains or species of Chlamydiaceae, affecting test results. Low sensitivity could also have been due to lower numbers of test organisms present in swabs, as might occur in animals with chronic fibrotic disease of the urogenital tract (Higgins, Hemsley *et al.*, 2005b), from the eyes (Markey, Wan *et al.*, 2007) or animals with recurrent infections, as occurs in people (Gomes, Borrego *et al.*, 2006). Further investigation of the sensitivity of this diagnostic technique in this koala population was beyond the scope of this study.

This small pilot study showed clearly that koalas tolerate poorly the medications commonly used to treat chlamydiosis in other species (doxycycline, azithromycin) but tolerate fluoroquinolones and tetracycline eye ointment well. Data on chloramphenicol was more equivocal, but presence of weight loss, inappetence and depression in one subject and reversible bone marrow suppression in the other, supports the view that use of chloramphenicol in koalas for extended periods should be closely monitored through haematological assessment (Plumb, 2005) and body weight and appetite appraisal.

In this study, systemic drugs with an anaerobic spectrum (doxycycline (Giguere, 2006), chloramphenicol (Plumb, 2005), azithromycin (Merriam, Citron *et al.*, 2006)) caused severe wasting, inappetence and depression in nine of the 11 animals treated, while no such effects were observed in the 10 animals systemically treated with drugs lacking anaerobic spectrum (fluoroquinolones) (Brown, 1996) and one animal treated with topical ophthalmic ointment. In the current study, even extremely low or infrequent doses of doxycycline and azithromycin (Table 5-2) resulted in decline, suggesting extreme sensitivity, possibly idiosyncratic, in some animals.

Observed declines in body weight, appetite and demeanour for animals treated with doxycycline or azithromycin may have been due directly to gut flora disturbances. Other factors, such as intercurrent disease or 'captive stress', appeared less likely to contribute to the declines with doxycycline or azithromycin administration because, in most cases, significant causes of morbidity were not observed at post mortem, and declines were not noted in other animals held and examined in a similar way, but treated with fluoroquinolones, or tetracycline eye ointment alone (weight change < 5% of starting weight; Figure 5-6). Anecdotally, two animals initially administered marbofloxacin without deleterious effects declined when treated with doxycycline.

In the current study, animals that declined still had a caecal bacterial layer that was indistinguishable by light microscopy from that found in untreated animals. Had animals been allowed to decline until death, this may have been lost, as found by other researchers in one koala treated with oxytetracycline for cystitis (Osawa, Bird *et al.*, 1993). The predominant organism of the bacterial matt that coats the caecum and proximal colon of koalas is a facultative anaerobic, Gram-negative pleomorphic rod that, although currently poorly characterised, is likely to fall within the family Enterobacteriaceae (Osawa, 1992). It is involved in degradation of tannin-protein complexes and is sensitive to many antibiotics including oxytetracycline, erythromycin, and chloramphenicol (Osawa, 1992; Osawa, Bird *et al.*, 1993). It has been hypothesised that disturbance or loss of this organism leads to wasting and death in antibiotic treated koalas (Osawa, Bird *et al.*, 1993). Unfortunately, no quantitative assessment of the types of microbes within the caecal bacterial matt was undertaken in the current study to provide direct evidence that disturbance of this organism was the cause of the decline. Molecular techniques such as terminal restriction fragment length polymorphism are now available to examine the changing bacterial populations in serial faecal samples collected throughout treatment or in caecal content at post mortem (Wang, Ahrne *et al.*, 2004) and might be useful to better describe these effects in such animals.

Two cases of severe acute renal oxalate nephrosis are described in this study. Oxalate nephrosis is described very rarely in koalas from Port Macquarie (Canfield & Dickens, 1982; Canfield, 1989; Stalder, 2003). In a large necropsy series (n = 1061), most of which were sourced from the Koala Hospital, only 15 koalas were reported to have oxalate nephrosis (Stalder, 2003). Oxalate nephrosis may be spontaneous or related to dehydration. Alternatively, it might be associated with a treatment-related loss of the gastrointestinal

oxalate-degrading commensals, as is postulated in people (Mittal, Kumar *et al.*, 2005).

Although the link between antibiotic use and oxalate nephrosis is not established in koalas, investigation of this area may be warranted as loss of commensals during treatment might predispose animals to later develop disease.

This study sought to find observations that might inform the earliest possible withdrawal of any drugs causing side effects, so that koalas could recover from deleterious effects.

Observation of persistent appetite loss was the earliest clinical sign associated with decline, however, as koala appetite may vary from day to day, it was really only useful in retrospect, when persistent appetite loss, together with weight loss starting a few days later was observed. Although treatment was withdrawn in all cases where side effects occurred, animals continued to decline, despite efforts to maintain nutrition and hydration, restore gastrointestinal motility and gastrointestinal refaunation. It is possible that nutritional supplementation might have avoided death had it been continued long enough, as found by previous authors (Richardson, 1984; Osawa & Carrick, 1990; Blanshard, 1994), however, as the broad aim of the research project was to investigate the treatment of chlamydiosis in koalas, therapies resulting in such severe side effects, extensive nursing and time in care, would be likely to be unacceptable to veterinarians and wildlife rehabilitators, even if koalas did eventually recover.

Past observations that inappetent animals produce fewer, smaller and drier faeces than healthy animals (Blanshard, 1994) were confirmed by this study, but total 24 hour faecal output weight was not useful in predicting appetite decline. This observation is consistent with the extended gastrointestinal transit time of koalas (99 hours for particulate matter, 213 hours for solutes) (Cork & Warner, 1983); as a result of which, changes in faecal output and character might be expected to occur several days after appetite changes.

Resolution of ocular disease, as evidenced by declining eye scores, was consistent in six animals that presented with conjunctivitis and were treated with fluoroquinolones and was suggestive of some clinical response to this drug. This grading system gave a rough estimate of clinical improvement, necessary to make clinical decisions as to the prognosis of animals for release.

Non-resolution of structural disease in all koalas in the current study is not surprising due to the severe fibrotic changes seen in this disease, associated with tubal occlusion, ovarian bursal cysts and infertility (Obendorf, 1981; Brown, Carrick *et al.*, 1984; Higgins, Hemsley *et al.*, 2005b). Such structural lesions might be expected to be resistant to antimicrobial therapy alone and return to normal function would be extremely unlikely in most cases. In further studies, described in Chapter 7 and Chapter 8, recruitment of animals without evidence of structural disease was prioritised.

5.5 IMPLICATIONS OF THE PILOT STUDY ON THE EVOLUTION OF TREATMENT PROTOCOLS FOR CHLAMYDIOSIS

At the end of the pilot study protocols for recruitment, treatment and monitoring were evaluated and refined based on the results of the pilot, the numbers of animals likely to be recruited in subsequent field seasons and the infrastructure and logistics available.

It seems likely that commensal anaerobic microflora disruption due to antibiotics played a role in the pathogenesis of the decline observed in koalas treated with azithromycin or doxycycline. The exact nature of the disruption; and why it caused such catastrophic effects in most, but not all, koalas; and whether gastrointestinal microbial disruption plays a role in the pathogenesis of oxalate nephrosis in koalas remain to be elucidated. A sensitive indicator of decline associated with treatment was not detected by this study, and

as decline was not reversed, avoidance of any decline, rather than treatment withdrawal and rescue therapy, became a key factor for studying antibiotic treatments in koalas.

Uncomplicated disease such as conjunctivitis had improved in this study whilst animals were on treatment with fluoroquinolones, suggesting that these drugs might have some benefit in treating this form of chlamydiosis in koalas; whereas complicated disease involving structural changes to the reproductive tract failed to resolve, precluding release of animals at the end of the study. As space to house animals was limited and caring for animals with poor prognosis for release for extended lengths of time was contributing to poor morale in lay staff, future recruitment concentrated on animals with a good prognosis for release after treatment (e.g. subclinical disease or uncomplicated clinical disease).

Concerns as to the possible low sensitivity of the antigen ELISA in clinical chlamydiosis; unknown sensitivity in subclinically infected koalas, male koalas, or in pooled samples; and cost of the test (approximately A\$10 or \$30 per koala if swabs were tested separately) prompted the investigation of PCR as a more sensitive point-of-care assay, as this technique is generally regarded as more sensitive than other diagnostic tests for chlamydial disease by many authors (Ostergaard, Birkelund *et al.*, 1990; Sykes, Studdert *et al.*, 1999; Amin, 2003). With the advent of non-toxic nucleic acid dyes for visualisation of DNA in agarose gels after electrophoresis (Williams, 2001; Invitrogen, 2007) and equipment becoming available towards the end of the first field season, a field site laboratory incorporating PCR became feasible (previously described in 4.11). Diagnosis of chlamydial disease and response to treatment in subsequent seasons were based on clinical signs of disease (previously described in 4.7 to 4.9) and/or PCR results.

Twenty-four hour faecal collection, found to be insensitive in predicting appetite decline, was replaced with subjective observation of faecal character and measuring of the length

and width of 10 randomly collected faeces placed length-ways and side-by-side (previously described in 4.6). Moreover, changing methods of faecal observation to a much quicker technique eased morning husbandry duties of lay staff. As appetite loss had been the earliest indicator of decline, appetite was more closely monitored with the instigation of a daily grading system out of three (previously described in 4.6) rather than subjective observations.

Grading of eyes in the field was not blinded, was undertaken by six different operators, was complicated by the allowance of a continuous scale and was not compared to reference photographs as these did not exist at the start of the study. To improve rigour, a study was designed to regrade the blinded randomised images of the eyes against rigorous criteria and reference photographs by the author (Chapter 8).

The findings of the present study generated the hypothesis that uncomplicated chlamydial disease might be treated successfully and safely with fluoroquinolones in koalas and the focus of the study was shifted to evaluating these drugs. By the beginning of the second field season refined diagnostic, recruitment, treatment and monitoring protocols were in place, allowing a more comprehensive investigation into this question.

CHAPTER 6 - PILOT STUDY USING A MODIFIED AGAR DIFFUSION METHOD TO ANALYSE THE PLASMA CONCENTRATIONS OF FLUOROQUINOLONES IN TREATED KOALAS

6.1 INTRODUCTION

The study of pharmacokinetics across species is important to determine appropriate species-specific dosage regimes, and may be useful for extrapolating efficacy and safety. Although pharmacokinetics of commonly used antibiotics in domestic species may be well studied, studies are performed infrequently in more exotic species; and rarely in marsupials (Clark, Milton *et al.*, 1982; Kirkwood, Gulland *et al.*, 1988; McLelland, Rich *et al.*, 2009). Veterinarians treating wildlife have estimated drug doses in these species through various methods of interspecies scaling, but these may not be accurate, particularly when species-specific differences in hepatic metabolism exist (Hunter & Isaza, 2008).

Koalas have been treated with various drugs since at least the 1960s, based on doses extrapolated from domestic animal species and “trial and error” (Bolliger & Finckh, 1962; Dickens, 1975; Brown, Woolcock *et al.*, 1986; Handasyde, 1986; Brown, Girjes *et al.*, 1987; Osawa & Carrick, 1990; Canfield, Love *et al.*, 1991a; Blanshard, 1992; Blanshard, 1994; Connolly, 1999; Markey, Wan *et al.*, 2007; Blanshard & Bodley, 2008). As serious side effects have been observed in koalas treated with oxytetracycline (Handasyde, 1986; Osawa & Carrick, 1990; Osawa, Bird *et al.*, 1993) and erythromycin (Brown, Wood *et al.*, 1984), enrofloxacin is now used extensively for treatment of chlamydiosis in koalas (Blanshard & Bodley, 2008) without the guidance of pharmacokinetic information. Pharmacokinetics in koalas may be unusual as koalas have increased bromosulphalein clearance compared to other species such as sheep and macropods (Pass & Brown, 1990),

and are expected to have unique metabolic pathways allowing survival on eucalypts at levels that in other species would be toxic (McLean & Foley, 1997; Stupans, Jones *et al.*, 2001). A major aim of this thesis was to investigate the pharmacokinetics of some commonly used drugs in koalas, the fluoroquinolones enrofloxacin and marbofloxacin.

Pharmacokinetic indices are calculated from sequential plasma drug concentrations taken from treated animals. One method used to determine drug concentration in plasma is an antibiotic agar diffusion assay modified by utilising paper disks (Wilson, Norris *et al.*, 2006). This method has the advantage that small volumes of plasma are required, which is particularly beneficial when dealing with wild animals. Further, the method requires little equipment, is inexpensive and has high throughput (Bennett, Brodie *et al.*, 1966). This chapter describes a pilot study evaluating this method for determining concentrations of the fluoroquinolones enrofloxacin and marbofloxacin in the plasma of treated koalas.

6.2 METHODS

Koalas (n = 6) were recruited, housed, examined, allocated to treatment groups, sampled from, observed and monitored as described in Chapter 4. All animals were medicated once daily. Two koalas were treated with 1.2 - 1.4 mg/kg oral marbofloxacin (Zeniquin® 25 mg, Pfizer, West Ryde, NSW, Australia), two with 5 mg/kg subcutaneous injections of enrofloxacin (Baytril® injectable solution 50 mg/mL, Bayer Animal Health, Pymble, NSW, Australia) and two with oral enrofloxacin (Baytril® oral suspension 25 mg/mL, Bayer Animal Health, Pymble, NSW, Australia) dosed at 5 mg/kg in one animal and 10 mg/kg in the other.

A 35.6 cm² lidded plate was constructed according to the method described by Bennett *et al.* (1966) and sterilised by autoclaving. Iso-sensitest™ agar (31.4 g/L, Oxoid Australia,

West Heidelberg, Vic, Australia) was prepared by autoclaving for 15 minutes, then poured on the plate to a depth of 2.7 mm and allowed to set for at least two hours at room temperature in a fume hood, prior to inoculation and placement of the disks and samples.

Klebsiella pneumoniae ATCC 10031 (Heinen, 2002) was obtained from the Australian Collection of Micro-organisms (Department of Microbiology, University of Queensland, Brisbane, Qld, Australia), inoculated into 10 mL Lemco yeast extract broth (Oxoid Australia, West Heidelberg, Vic, Australia), incubated overnight at 37 °C, then added to another 100 mL of Lemco yeast extract broth. This was incubated in a shaking incubator at 85 revolutions per minute at 37 °C, until the turbidity reached that of a McFarlane 0.5 barium sulphate standard. Forty evenly spaced drops of bacterial broth were placed on the prepared agar plate using a sterile glass pipette, and then spread evenly using a glass pipette spreader.

Five milligrams each of enrofloxacin, ciprofloxacin (both from Sigma-Aldrich, Castle Hill, NSW, Australia) and marbofloxacin (Pfizer Inc, Groton, Connecticut, USA) was each added to purified water to a final concentration of 2 mg/mL. To enable dissolution, 5 µL NaOH 50% (Sigma- Aldrich, Castle Hill, NSW, Australia) was added to solutions of enrofloxacin. Antibiotic solutions were vortexed for 20 s then ultrasonicated for 5 min until the drug was dissolved. Stock solutions (2 mg/mL) were stored at -20 °C, protected from light and used within three months of preparation. Aliquots of the antibiotic stock solution were added to pooled blank koala plasma at 1, 2, 4, 8, and 16 µg/mL to prepare a fresh calibration curve for each day's analysis.

Plasma from antibiotic treated koalas was stored at -20 °C and protected from light until assay. One hour prior to use, samples were defrosted at room temperature, briefly vortexed, then centrifuged for five minutes at 15,000 x g.

Immediately after inoculation of the plate with *K. pneumoniae*, blank disks (6 mm CT 998B Oxoid Australia, West Heidelberg, Vic, Australia) were placed using sterile forceps over evenly spaced predetermined locations on the agar plate. Sample (16 μ L of plasma from koalas treated with antibiotics samples at different time points) was carefully inoculated to ensure no spillage occurred onto the appropriate paper disks using a calibrated 20 μ L pipette (Gilson Pipetman®; Gilson, Middleton, Wisconsin, USA) and fresh sterile pipette tips. The negative control was pooled blank koala plasma. Each sample, standard and negative control was assayed in triplicate.

After sample placement, the plate was incubated on a flat surface at 37 °C for 18 hours. The diameter of the zones of bacterial inhibition (Figure 6-1) were measured in at least six different directions using Vernier's callipers (Mitutoyo series 505 dial Vernier's callipers, Mitutoyo, Tokyo, Japan) and the mean and standard deviation calculated for each sample. The concentrations of the unknown samples were determined by interpolation from the regression curve of the standards (Bennett, Brodie *et al.*, 1966).

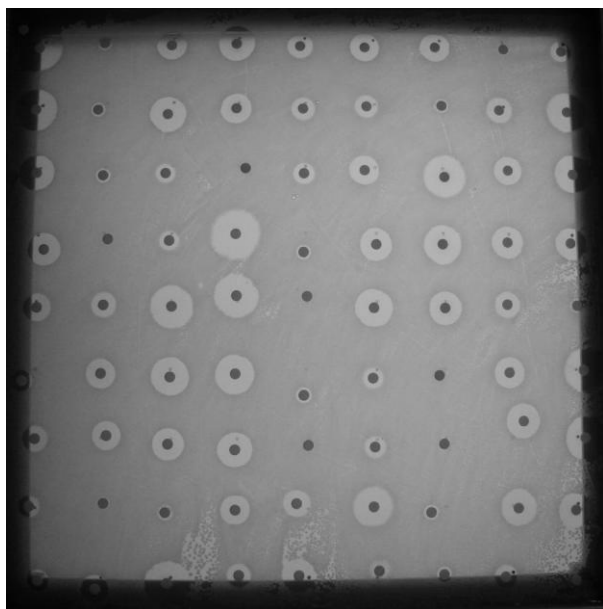


Figure 6-1. An example of a plate after incubation at 37 °C for 18 hours demonstrating the zones of inhibition in the *K. pneumoniae* lawn caused by fluoroquinolone diffusion.

6.3 RESULTS

The zones of inhibition of marbofloxacin and enrofloxacin in koalas dosed with oral 1.2 - 1.4 mg/kg for marbofloxacin and oral 5 mg/kg for enrofloxacin were barely detectable at all time points analysed (Figure 6-2 and Figure 6-3), suggesting extremely low plasma concentrations of drug in these animals. Animals subcutaneously injected with 5 mg/kg enrofloxacin had higher plasma enrofloxacin concentrations (0.6 - 1.0 µg/mL; Figure 6-3) than those treated with oral medications.

The diameter of the blank disks was 6 mm. The diameter of the zone of inhibition of the lowest antibiotic standard (1 µg/mL) was 15 mm for both enrofloxacin and marbofloxacin, which represents a 4.5 mm zone between the edge of the zone of inhibition and the edge of the disks. Inhibition zones produced by plasma samples of treated animals were even smaller, raising concerns that stochastic and measurement error would significantly affect results where the zones of inhibition could be measured, and the measurement of plasma drug concentration in animals dosed orally would not be possible.

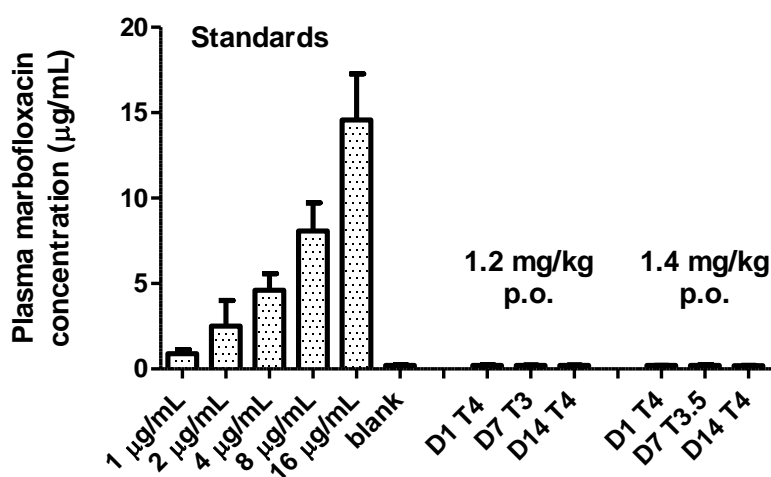


Figure 6-2. Concentration of marbofloxacin in plasma of two koalas.

Animals were dosed once daily per os (p.o.) with 1.2 mg/kg or 1.4 mg/kg marbofloxacin.

D = day of treatment; T = time since dose (h).

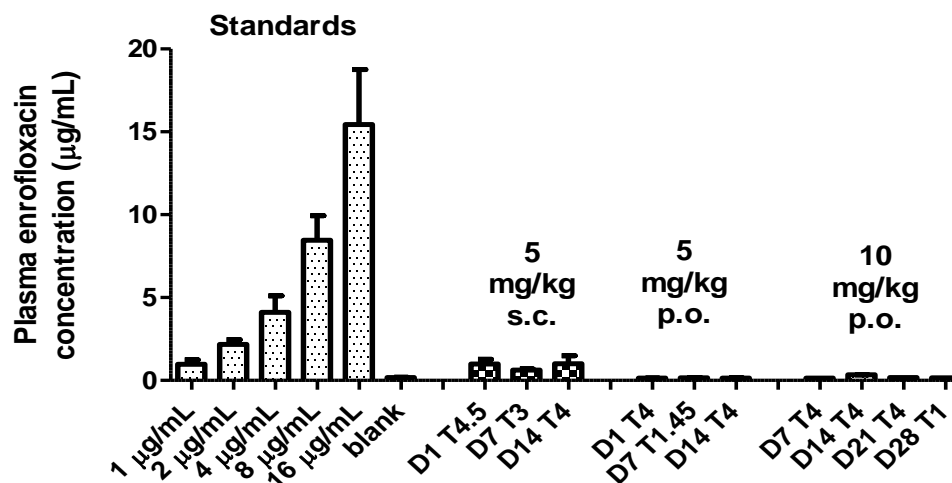


Figure 6-3. Concentration of enrofloxacin in plasma of three koalas.

Animals were dosed once daily with 5 mg/kg enrofloxacin per os (p.o.) or by subcutaneous injection (s.c.). D = day of treatment; T = time since dose (h).

6.4 DISCUSSION

The results of this pilot study indicated that at the doses administered, plasma drug concentrations for enrofloxacin and marbofloxacin in koalas were less than 1 µg/mL at the time points examined. In particular, animals dosed orally had very low or undetectable plasma concentrations of fluoroquinolones, suggesting poor bioavailability by this route. Although they have some advantages, modified agar diffusion assays are regarded as being less sensitive to low concentrations than high performance liquid chromatography (HPLC) (Klein & Edberg, 1996) and cannot distinguish antimicrobially active metabolites present in the sample from the parent compound (Heinen, 2002). Reverse phase HPLC is a method by which closely related substances dissolved in a liquid (the mobile phase) are separated by selective, polarity-based, retention onto a solid matrix (the column) (Klein & Edberg, 1996). Advantages of HPLC include the ability to separate and analyse closely related substances (e.g. drug metabolites), extreme sensitivity (usually able to quantitate between

0.5 and 1.0 µg/mL) and rapidity if automated (Klein & Edberg, 1996). The disadvantages of HPLC include a high level of technical skill required to develop methods and the initial high financial outlay for equipment (Klein & Edberg, 1996). The preliminary results of this pilot study indicated that drug concentrations in plasma of koalas dosed orally were expected to be very much less than 1 µg/mL; clearly a more sensitive method was required for quantification of plasma concentrations of fluoroquinolones in these animals. In addition, as koala metabolism is likely to be unusual, it was seen as desirable to use HPLC to detect possible metabolites. For these reasons, and because access to appropriate equipment became available from 2007, the author decided to validate a published HPLC assay (Liang, Kays *et al.*, 2002) for analysis of plasma concentrations of fluoroquinolones in treated koalas.

The final method used for HPLC analysis and key features of its validation are outlined in Chapter 7. Additional steps in the development and validation of the HPLC method, not presented in this thesis, included selection of sensitive excitation and emission frequencies for each drug of interest by “trial and error”, confirmation that a displacing agent was not required to improve recovery, verification of linearity of the standard curve and confirmation of the stability of samples in storage.

CONFIRMATION OF CO-AUTHORSHIP OF PUBLISHED WORK

I, *Joanna Griffith*, led the study design, collection and analysis of statistical data and writing up of this publication entitled “Absorption of enrofloxacin and marbofloxacin after oral and subcutaneous administration in diseased koalas (*Phascolarctos cinereus*)”.

Drs Merran Govendir, Damien Higgins and Mark Krockenberger assisted in the design of the project, and as supervisors, provided assistance in finalising the manuscript prior to publication. Dr Kong Li provided assistance in high performance liquid chromatography method development and validation and provided assistance in finalising the manuscript prior to publication.

Joanna Griffith _____ Date _____

I, as a co-author, endorse that this level of contribution by myself and the candidate indicated above is appropriate:

Damien Higgins _____ Date _____

Kong Li _____ Date _____

Mark Krockenberger _____ Date _____

Merran Govendir _____ Date _____

CHAPTER 7 - ABSORPTION OF ENROFLOXACIN AND MARBOFLOXACIN AFTER ORAL AND SUBCUTANEOUS ADMINISTRATION IN DISEASED KOALAS (*PHASCOLARCTOS CINEREUS*)

The following is a re-formatted manuscript, currently in press:

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*)” *Journal of Veterinary Pharmacology and
Therapeutics* (in press: accepted 1 Dec 2009).

The study was designed, executed and reported by the candidate under the general
supervision of the remaining authors, who were the candidate’s appointed supervisors
(principal and associate), with the exception of Dr K. M. Li, who provided assistance in
high performance liquid chromatography method development and validation.

7.1 ABSTRACT

Koalas (n = 43) were treated daily for up to eight weeks with enrofloxacin: 10 mg/kg
subcutaneously (s.c.), 5 mg/kg s.c., or 20 mg/kg per os (p.o.); or marbofloxacin: 1.0 - 3.3
mg/kg p.o., 10 mg/kg p.o. or 5 mg/kg s.c. Serial plasma drug concentrations were
determined on day one and again at approximately two weeks, by liquid chromatography.
The median (range) plasma maximum concentrations (C_{max}) for enrofloxacin 5 mg/kg s.c.
and 10 mg/kg s.c. were 0.83 (0.68 - 1.52) and 2.08 (1.34 - 2.96) $\mu\text{g/mL}$ and the median
(range) T_{max} were 1.5 h (1 - 2) and 1 h (1 - 2), respectively. Plasma concentrations of orally
dosed marbofloxacin were too low to be quantified. Oral administration of enrofloxacin
suggested absorption rate limited disposition pharmacokinetics; the median (range) C_{max}

for enrofloxacin 20 mg/kg p.o. was 0.94 (0.76 - 1.0) $\mu\text{g/mL}$ and the median (range) T_{max} was 4 h (2 - 8). Oral absorption of both drugs was poor. Plasma protein binding for enrofloxacin was $55.4 \pm 1.9\%$ and marbofloxacin $49.5 \pm 5.3\%$. Elevations in creatinine kinase activity were associated with drug injections. Enrofloxacin and marbofloxacin administered at these dose rates and routes are unlikely to inhibit the growth of chlamydial pathogens in vivo.

Key words:

koala, absorption, *Chlamydomphila*, enrofloxacin, marbofloxacin

7.2 INTRODUCTION

Chlamydomphila pneumoniae and *C. pecorum* infect up to 85% of some populations of the koala (*Phascolarctos cinereus*), a threatened, iconic Australian marsupial, causing significant morbidity (Jackson, White *et al.*, 1999). Clinical signs may include: conjunctivitis (Cockram & Jackson, 1976), rhinitis (Nicolson, 2002), cystitis and/or reproductive tract disease (Brown & Grice, 1986).

Chlamydial infection status has implications for movement of wild koalas into captive collections, export from Australia and translocation of animals between wild populations (Blanshard & Bodley, 2008). A reliable treatment for this disease is vital. Drugs such as erythromycin and oxytetracycline, used to treat chlamydiosis in other species, are poorly tolerated in koalas resulting in wasting and death (Brown, Wood *et al.*, 1984; Osawa & Carrick, 1990). Consequently, koalas with chlamydiosis are often treated with fluoroquinolones, due to their perceived safety and anecdotal effectiveness, despite a lack of information on their therapeutic efficacy or pharmacokinetics. Current treatment regimes are based on dose rates and dosage frequencies extrapolated from dogs and cats, or

by “trial and error”.

Koalas survive on a diet of eucalypt leaves, high in toxic plant secondary metabolites, and are monogastric hindgut fermentors with the largest relative caecal surface area of any animal (Snipes, Snipes *et al.*, 1993) and a short small intestinal transit time (particulate matter; 6 minutes, soluble material; 60 minutes) (Cork & Warner, 1983) which are recognised as important factors impacting on species-dependent pharmacokinetics (Lin, 1995). In addition, koalas have significantly increased bromosulphalein (BSP) clearance, compared with sheep, kangaroos and wallabies (Pass & Brown, 1990), indicating an increased rate of hepatic metabolism. Therefore, koalas may have unique pharmacokinetics due to reduced absorption, and increased metabolism and excretion rates of xenobiotics (McLean & Foley, 1997; Stupans, Jones *et al.*, 2001).

Fluoroquinolones have limited activity against Chlamydiaceae (Ridgway, 1997). In particular, sub-therapeutic plasma concentrations (e.g. 0.5 µg/mL ciprofloxacin or 1 µg/mL ofloxacin in vitro) (Dreses-Werringloer, Padubrin *et al.*, 2000) may induce a state of “persistence,” whereby the pathogens remain viable within their host cell and cannot be cultured, but result in recrudescence when antibiotics are withdrawn (Hogan, Mathews *et al.*, 2004), or may select for resistant chlamydial strains (Yokoi, Yasuda *et al.*, 2004) as well as other normal and/or opportunistic flora. Thus, knowledge of the pharmacokinetics of fluoroquinolones in koalas has important implications for the likelihood of successful treatment and resolution of chlamydiosis.

The objectives of this study were to investigate absorption of enrofloxacin and marbofloxacin in koalas following oral and subcutaneous administration and report any identifiable adverse effects.

7.3 MATERIALS AND METHODS

7.3.1 Animals

As a prospective, parallel group, clinical pharmacokinetic study, 43 wild koalas, (21 males, 22 females; age 5.98 ± 2.7 years (mean \pm SD); 1.5 - 12 years (range)) were recruited opportunistically from those admitted to the Koala Preservation Society of NSW's Koala Hospital at Port Macquarie, NSW, Australia, for treatment of chlamydial clinical signs or for relocation from dangerous areas (such as the vicinity of dogs or main roads).

Physical examination was performed under general anaesthesia, induced and maintained by mask with isoflurane in 100% oxygen. Koalas were aged according to previous hospital admission records or by tooth wear (Martin, 1981), assessed for body condition scores (Connolly, 1999) and weighed. Due to ethical considerations, only animals with clinical or subclinical chlamydiosis were recruited. Clinical chlamydiosis ($n = 33$) was defined as the presence of highly suggestive physical signs (urine stained fur and/or conjunctivitis, and/or structural changes to the reproductive tract, as determined by ultrasound (Toshiba ECCOCEE SSA - 340A)) and subclinical chlamydiosis ($n = 10$) was defined as the presence of chlamydial DNA detected by polymerase chain reaction (PCR) from urogenital or conjunctival swabs (Demkin & Zimin, 2005) in the absence of clinical signs. Koalas were excluded on the basis of the following criteria: presence of concurrent non-chlamydial disease, renal pathology as detected by ultrasound, a body condition score of two or less out of five, or when weight loss during the acclimatisation period exceeded 250 grams for females and 300 grams for males.

Koalas were acclimatised for three to seven days before commencement of the study, housed individually in indoor enclosures (approximately 2.0 x 2.5 x 2.5 m) for the first three weeks and then transferred to individual outdoor pens (3.0 x 4.0 m). Fresh branches

of at least three different species of trees selected from *Eucalyptus robusta*, *E. microcorys*, *E. tereticornis*, *E. nicholii*, *E. macrorhyncha*, *Melaleuca quinquenervia* or *Corymbia maculata*, and water were provided daily.

Initially, koalas were supplemented twice daily with a soya milk substitute (Karicare® Soya All Aged Formula, Nutricia, North Ryde, NSW, Australia, 7.1 g in 50 mL water; or Infasoy® Progress Step 2, Wyeth Australia, Baulkham Hills, NSW, Australia, 8.9 g in 50 mL water) containing 0.9 g protein, 3.3 - 3.45 g carbohydrate, 1.8 - 1.85 g fat, 30.5 - 33 mg calcium, 2.55 - 3.35 mg magnesium, 0.3 - 0.35 mg zinc, 28 - 32 µg copper and 0.6 - 0.7 µg selenium per 50 mL. Koalas strongly resistant to oral supplementation (exhibiting flinching, ear flicking, vocalisation, aggression and/or retreating) (n = 25) were randomly assigned to one of three s.c. administration treatment groups and supplementation ceased. A summary of the treatment groups is provided in Table 7-1.

Table 7-1. Koala treatment groups.

Drug	Route	Dose	Presentation	n**
Enrofloxacin	p.o.	20 mg/kg	Baytril®* oral suspension 25 mg/mL	8
Enrofloxacin	s.c.	5 mg/kg	Baytril®* injectable solution 50 mg/mL, or Enrotril®† 50mg/mL	7
Enrofloxacin	s.c.	10 mg/kg	Baytril®* injectable solution 50 mg/mL, or Enrotril®† 50 mg/mL	16
Marbofloxacin	p.o.	1 - 3.3 mg/kg	Zeniquin®‡ 25 mg and 50 mg tablets	6
Marbofloxacin	p.o.	10 mg/kg	Zeniquin®‡ 25 mg and 50 mg tablets	6
Marbofloxacin	s.c.	5 mg/kg	Marbocyl®§ injectable 5 mg/mL	2

*Bayer Animal Health, Pymble, NSW, Australia

†Troy Laboratories, Glendenning, NSW, Australia

‡Pfizer, West Ryde, NSW, Australia

§Vetoquinol, Lure cedex, France

** Two animals were subsequently treated with a different medication regime (see below) and are thus counted twice. p.o: per os; s.c: subcutaneous.

7.3.2 Drug administration and blood collection

Dose rates were based initially on those reported previously for koalas (Connolly, 1999) and dogs and cats (Pfizer Inc, 2005; Plumb, 2005). The treatment groups of enrofloxacin 20 mg/kg p.o. and marbofloxacin 10 mg/kg p.o. were formulated based on low plasma concentrations detected in koalas administered enrofloxacin 5 mg/kg p.o. (n = 1; data not shown) and marbofloxacin 1 to 3.3 mg/kg p.o. (n = 6), respectively. As a small quantity of injectable marbofloxacin was available for this study, only two koalas were treated with this preparation. These animals were transferred onto other medications (marbofloxacin 10 mg/kg p.o. or enrofloxacin 5 mg/kg s.c. respectively) after blood collection at 14 days and were thus excluded from further study.

Marbofloxacin tablets were crushed and mixed with soya formula (20 mL). Enrofloxacin oral solution was administered by syringe. Following recommended practice for supplementing koalas undergoing systemic antibiotic treatment (Blanshard, 1994; Blanshard & Bodley, 2008), both groups were then supplemented with soya supplement to a maximum of 50 mL. For all treatment groups, koalas were medicated once daily in the morning for 4 to 8 weeks. Following cessation of treatment, animals were monitored for at least 4 weeks. Two koalas were treated with a different drug or dose rate following a wash out period of at least 3 weeks (enrofloxacin 10 mg/kg s.c. followed by 5 mg/kg s.c.; or marbofloxacin 10 mg/kg p.o. followed by enrofloxacin 20 mg/kg p.o.) due to recurrence of clinical signs during a post-treatment observation period. Animals with conjunctival discharge had their eyes cleansed daily with saline.

For drug concentration determination, blood (2 mL) was collected from the cephalic vein into sodium heparin tubes before treatment (t = 0 h) and at 1, 2, 4, 8 and 24 h after medication on day 1 and 14 to 16 days later. Plasma, stored at -20 °C, was protected from

light until analysis.

7.3.3 Drug analysis

Enrofloxacin and marbofloxacin plasma concentrations were determined by a modified reversed-phase, high performance liquid chromatography (HPLC) method (Liang, Kays *et al.*, 2002). The HPLC system (Shimadzu, Rydalmere, NSW, Australia) consisted of an isocratic pump (LC20AT), autosampler (SIL 20AC) maintained at 15 °C, column oven (CTO 20AC) maintained at 25 °C, fluorescence detector (RF - 10AXL), a system controller (CBM 20A) and Shimadzu Class VP software (version 7.4). Chromatographic separation was accomplished on an Apollo C-18, 5 µm (4.6 mm x 250 mm) column (Alltech, Dandenong South, Vic, Australia) coupled with a 1 mm Optiguard® C-18 guard column (Optimize Technologies, Oregon City, Oregon, USA). The mobile phase (pH 2.0) consisted of acetonitrile: purified water (50: 50) (Ajax Finechem, Taren Point, NSW, Australia), 25 mM citric acid (Sigma-Aldrich, Castle Hill, NSW, Australia) and 10 mM sodium dodecyl sulfate (Boehringer Mannheim, Indianapolis, USA). The flow rate was maintained isocratically at 1 mL/min and sample injection volume was 5 µL. Enrofloxacin, and its major metabolite, ciprofloxacin, were detected using fluorescence at excitation and emission of 280 nm and 450 nm, respectively, and marbofloxacin at excitation and emission of 300 nm and 500 nm, respectively. Retention times for enrofloxacin, ciprofloxacin, marbofloxacin and orbifloxacin were 9.9, 8.0, 7.8 and 10.7 min, respectively. Orbifloxacin was used as the internal standard.

External calibration standards were prepared daily by adding HPLC analytical grade enrofloxacin and ciprofloxacin (Sigma-Aldrich, Castle Hill, NSW, Australia) or marbofloxacin (Pfizer Inc, Groton, Connecticut, USA) to untreated koala plasma. Prior to extraction, 1 µg/mL of internal standard (analytical grade orbifloxacin, (Schering Plough,

Kenilworth, New Jersey, USA) dissolved in 25 μ L untreated pooled koala plasma) was added to calibration standards and plasma samples.

Plasma samples collected from medicated koalas and calibration standards underwent solid-phase extraction (SPE) using Oasis® HLB 1 cm^3 30 mg column filters (Waters, Milford, Massachusetts, USA) via a vacuum manifold, according to manufacturer's instructions. Briefly, the SPE cartridge was conditioned with 1 mL methanol, followed by 1 mL deionised water. Plasma (250 μ L) was loaded, followed by 1 mL 5% methanol. Samples were eluted with 1 mL methanol. Eluates were dried at room temperature by a vacuum concentrator (SC2000, Savant Instruments, Holbrook, New York, USA) and reconstituted in 100 μ L of mobile phase. The mean % (\pm SD) recovery was determined by comparing a known concentration of enrofloxacin or marbofloxacin added to untreated koala plasma prior to, and after SPE. Enrofloxacin recovery was $93 \pm 4.87\%$ and marbofloxacin $94.6 \pm 1.94\%$.

The concentration of either enrofloxacin or marbofloxacin in plasma from treated koalas was determined by interpolation from the calibration curve with a minimum co-efficient of correlation (r^2) of 0.99. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of y-intercepts of regression line of the standards (International Conference on Harmonisation, 1996). Inter-assay variation was estimated by comparing replicates from the three standards used for calibration curves from 16 (enrofloxacin) or 7 (marbofloxacin) different assay days. Intra-assay variation (precision) was estimated from six replicates of five standard samples (0.25, 0.5, 1, 2, 4 μ g/mL). The inter-assay and intra-assay variation was $< 10\%$ for enrofloxacin for all concentrations above the LOQ and $< 16\%$ and $< 27\%$ for marbofloxacin for all concentrations above LOQ, respectively. The LOQ and LOD for enrofloxacin,

ciprofloxacin and marbofloxacin were 0.40 & 0.12 µg/mL; 0.25 & 0.08 µg/mL; 0.48 & 0.16 µg/mL, respectively.

There were no interfering peaks from endogenous compounds in pooled, drug-free koala plasma at the retention times of compounds of interest. Additional peaks at consistent retention times were considered potential metabolites if the mean area changed over time in a manner similar to that of the parent drug, i.e. peaking within 1 to 2 h of dosage and then declining between 4 and 8 h (Bremseth, Lima *et al.*, 1988).

7.3.4 Plasma protein binding

The degree of drug-binding to plasma protein was investigated using ultracentrifugation. Briefly, enrofloxacin (1 µg/mL), or marbofloxacin (2 µg/mL), in untreated thawed koala plasma was incubated at 35 °C for 30 min and then centrifuged (30 min at 3000 x g), in a fixed angle rotor centrifuge (Presvac DCS-16-RVT) through an ultrafiltration device (Centricon® YM-30, Millipore, North Ryde, NSW, Australia). The non-protein-bound ultrafiltrate was collected by pipette, adjusted to 500 µL with mobile phase and analysed by HPLC without SPE. The protein-rich fraction was collected by inverting and centrifuging the ultrafiltration device for 2 min at 3000 x g, subjected to SPE and drying as described above, reconstituted with 500 µL mobile phase and analysed by HPLC. The degree of binding of drug to the ultrafiltration device (i.e. the loss) was calculated by replicating the above steps using the same drug concentrations in phosphate buffered saline. In addition, aliquots of the same drug concentrations in koala plasma were incubated as above, subjected to SPE, dried, reconstituted in 500 µL of mobile phase and analysed to determine total (bound and unbound) drug concentration. All samples were assayed in triplicate. Percentage plasma protein binding for each drug, after correcting for losses from SPE and the ultrafiltration device, were determined by the following formula:

$$\% \text{ protein binding} = \text{bound}_{\text{drug}} / (\text{unbound}_{\text{drug}} + \text{bound}_{\text{drug}})$$

$$\text{where } \text{unbound}_{\text{drug}} + \text{bound}_{\text{drug}} = \text{total}_{\text{drug}}.$$

7.3.5 Pharmacokinetic analysis

Enrofloxacin and marbofloxacin concentration versus (vs.) time data for each animal were subjected to standard non-compartmental pharmacokinetic analysis. Only values above the LOQ were used in calculations. Maximum plasma concentration (C_{max}) and time to maximum plasma concentration (T_{max}) were determined graphically. The area under the curve (AUC) was determined by the trapezoidal method. The elimination rate constant (K_{el}) was determined by the gradient of the natural log of plasma concentration vs. time, where the relationship was linear, the gradient negative (indicating elimination), and the concentration was above the limit of quantification. The AUC extrapolated to infinity ($\text{AUC}_{0-\infty}$) was calculated as the sum of AUC up until the last measurable concentration ($\text{AUC}_{0-\text{LOQ}}$) plus C_t/K_{el} ($\text{AUC}_{\text{extrapolated}}$), where C_t was the last measurable concentration. The percentage of each contribution to the $\text{AUC}_{0-\infty}$ was determined. Elimination half life ($t_{1/2}$) was calculated using the equation $t_{1/2} = \ln 2 / K_{\text{el}}$.

7.3.6 Animal monitoring procedures

During drug treatment and post-treatment, observations of feed and water intake, urine and faecal production, and demeanour were undertaken daily and animals were weighed at least twice weekly.

Every 7 days during treatment, and 14 days post-treatment, koalas were anaesthetised, examined physically and the urogenital tract imaged by ultrasound. In addition, blood was collected into potassium EDTA for routine haematological analysis using standard methods, or plain tubes for serum biochemical analysis including creatinine kinase activity

(CK), with a Roche Modular Chemistry Analyser using Roche reagents (Roche, Basel, Switzerland). Urine was expressed manually from the bladder for urinalysis (Bayer-Multistix®, Bayer Australia, Pymble, NSW, Australia) and determination of urine specific gravity. Urine sediment was stained with methylene blue and examined under light microscopy.

Animals that died during treatment (n = 2) or were euthanased at the end of the study for other reasons (n = 16), underwent necropsy using standard techniques. Tissues were collected into 10% formalin for a minimum of 24 h, embedded in paraffin and sections subjected to standard haematoxylin and eosin staining, and examined under light microscopy. At the end of the study all other animals were released into a suitable bush habitat, close to where they had been originally found.

7.3.7 Statistical modelling

Results of serum biochemistry, haematology and weight changes over time (weeks) were analysed using residual maximum likelihood for the effect of drug, dose rate, week, route and whether the animal was within the pre-treatment, treatment or post-treatment interval. Statistical significance was concluded at $p \leq 0.05$.

7.4 RESULTS

Some pharmacokinetic parameters for enrofloxacin and marbofloxacin are presented in Table 7-2. The median (range) plasma concentration vs. time for enrofloxacin and marbofloxacin are presented in Figure 7-1 and Figure 7-2, respectively. Plasma protein binding for enrofloxacin was $55.4 \pm 1.9\%$ and marbofloxacin $49.5 \pm 5.3\%$. Plasma drug concentrations did not differ significantly between day 1 and after multiple dosing with either drug at any dose rate. The median (range) $AUC_{\text{extrapolated}}$ on day 1 were: enrofloxacin

5 mg/kg s.c. 47.8% (25.8 - 60.7%); enrofloxacin 10 mg/kg s.c. 30.2% (12.7 - 54.9%) and for marbofloxacin 5 mg/kg s.c. 23.5% (18 -28.7%). The plasma concentrations of marbofloxacin in animals dosed orally were mostly below the limit of quantification (Figure 7-2). Median (range) T_{max} for enrofloxacin dosed orally was long 4 h (2 - 8 h) and therefore calculation of K_{el} was not possible as only two points were above the LOQ during the elimination phase (Figure 7-2).

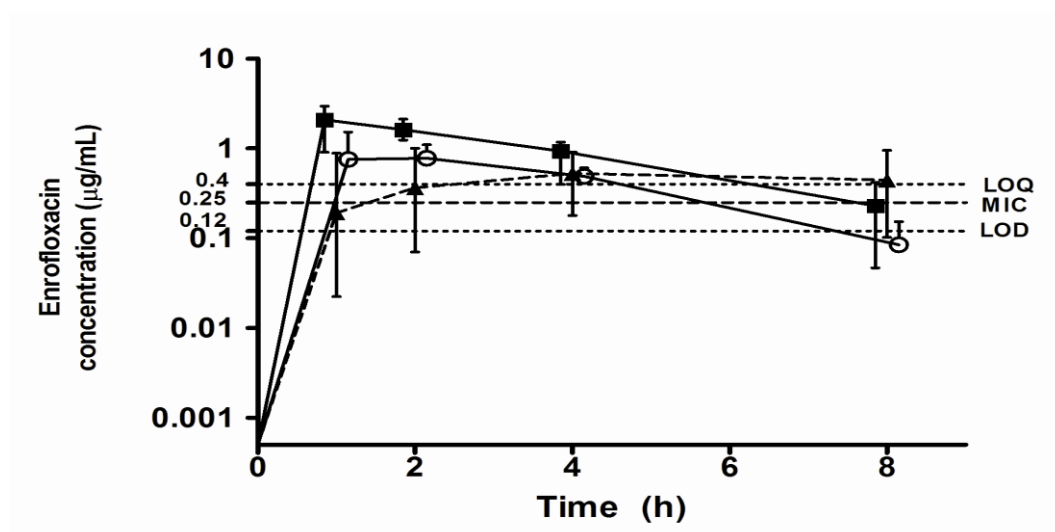


Figure 7-1. Semi-logarithmic plot of median (\pm range) plasma concentration of enrofloxacin over time in treated koalas.

Koalas were treated with 5 mg/kg subcutaneous injection (s.c.) (○) (n = 6), 10 mg/kg s.c. (■) (n = 10) and 20 mg/kg per os. (▲) (n = 6) on day 1. LOQ is the level of quantification; LOD is the level of detection; MIC is the minimum inhibitory concentration.

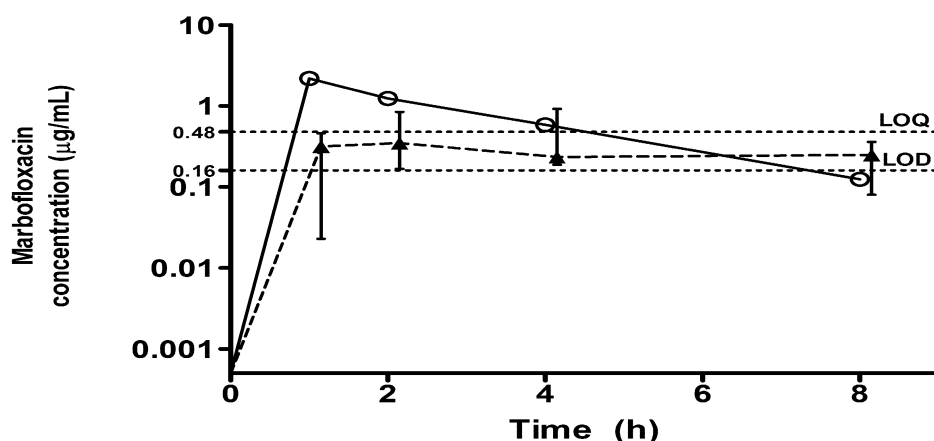


Figure 7-2. Semi-logarithmic plot of median (\pm range) plasma concentration of marbofloxacin over time in treated koalas.

Koalas were treated with 5 mg/kg subcutaneous injection (○) (n = 2), and 10 mg/kg per os (▲) (n = 6) on day 1. LOQ is the level of quantification; LOD is the level of detection.

In chromatograms of those animals dosed with enrofloxacin 10 mg/kg s.c., five suspected metabolites were detected at retention times 4.0, 7.2, 7.5, 8.0 and 9.2 min. In > 80% of plasma concentration vs. time curves, another metabolite at 6.8 minutes was detected in two thirds of plasma concentration vs. time curves (n = 12). In animals dosed with marbofloxacin 5 mg/kg s.c. (n = 2) or marbofloxacin 10 mg/kg p.o. (n = 6), one suspected metabolite (retention time 8.1 min) was noted in all plasma concentration vs. time curves.

All animals, with one exception, maintained body weight, appetite, normal demeanour and normal fecal and urine output throughout the study. Biochemical analytes and haematological indices remained within published reference intervals (Canfield, O'Neill *et al.*, 1989; Blanshard, 1994) with the exception of CK activity, which was significantly elevated in koalas during treatment with daily s.c. injections of both drugs compared to pre-treatment samples (approximately eighteen-fold and seven-fold, respectively) and post-treatment samples (approximately fourteen-fold for enrofloxacin) (Figure 7-3).

Table 7-2. Some pharmacokinetic parameters for enrofloxacin subcutaneous (s.c.) and oral (p.o.) administration and marbofloxacin s.c. administration in koalas using non-compartmental analysis.

Parameter	n	C _{max} (µg/mL)	T _{max} (h)	K _{el} (h ⁻¹)	AUC _(0-LOQ) (µg.h/mL)	AUC _(0-∞) (µg.h/mL)	t _{1/2} (h)
Enrofloxacin 5 mg/kg s.c. (d1)	6	0.83 (0.66 - 1.52)	1.5 (1 - 2)	0.28 (0.15 - 0.38)	2.40 (2.14 - 3.64)	4.9 (4.04 - 5.7)	2.43 (1.8 - 4.65)
Enrofloxacin 5 mg/kg s.c. (multiple dosing)	6	0.91 (0.58 - 1.46)	1.5 (1 - 2)	0.23 (0.14 - 0.39)	2.53 (1.63 - 2.82)	4.26 (3.18 - 7.2)	2.95 (1.74 - 4.94)
Enrofloxacin 10 mg/kg s.c. (d1)	10	2.08 (1.34 - 2.96)	1 (1 - 2)	0.32 (0.21 - 57)	5.42 (4.78 - 7.035)	8.32 (5.17 - 9.43)	2.31 (1.21 - 3.44)
Enrofloxacin 10 mg/kg s.c. (multiple dosing)	14	1.84 (0.99 - 2.74)	1 (1 - 2)	0.33 (0.17 - 0.61)	5.23 (3.08 - 11.43)	10.18 (6.3 - 15.1)	2.11 (1.13 - 3.97)
Enrofloxacin * 20 mg/kg p.o. (d1)	6	0.94 (0.76 - 1.0)	4 (2 - 8)				
Enrofloxacin * 20 mg/kg p.o. (multiple dosing)	6	0.71 (0.4 - 1.04)	4 (2 - 4)				
Marbofloxacin 5 mg/kg s.c. (d1)	2	2.18 (1.86 - 2.52)	1	0.43 (0.36 - 0.49)	4.63 (4.16 - 5.1)	6.04 (5.83 - 6.24)	1.66 (1.4 - 1.9)

Parameters are shown as median (range). C_{max}: maximum plasma concentration; T_{max}: time to maximum plasma concentration; K_{el} (h⁻¹): the excretion rate constant (first order); AUC (0-LOQ): area under the plasma concentration time curve from zero to the last point above the level of quantification; AUC_{0-∞}: area under curve between t = 0 and t = ∞; t_{1/2}: half life of terminal phase.

*calculation of K_{el}, AUC and t_{1/2} was not possible as only two points were above the LOQ during the elimination phase.

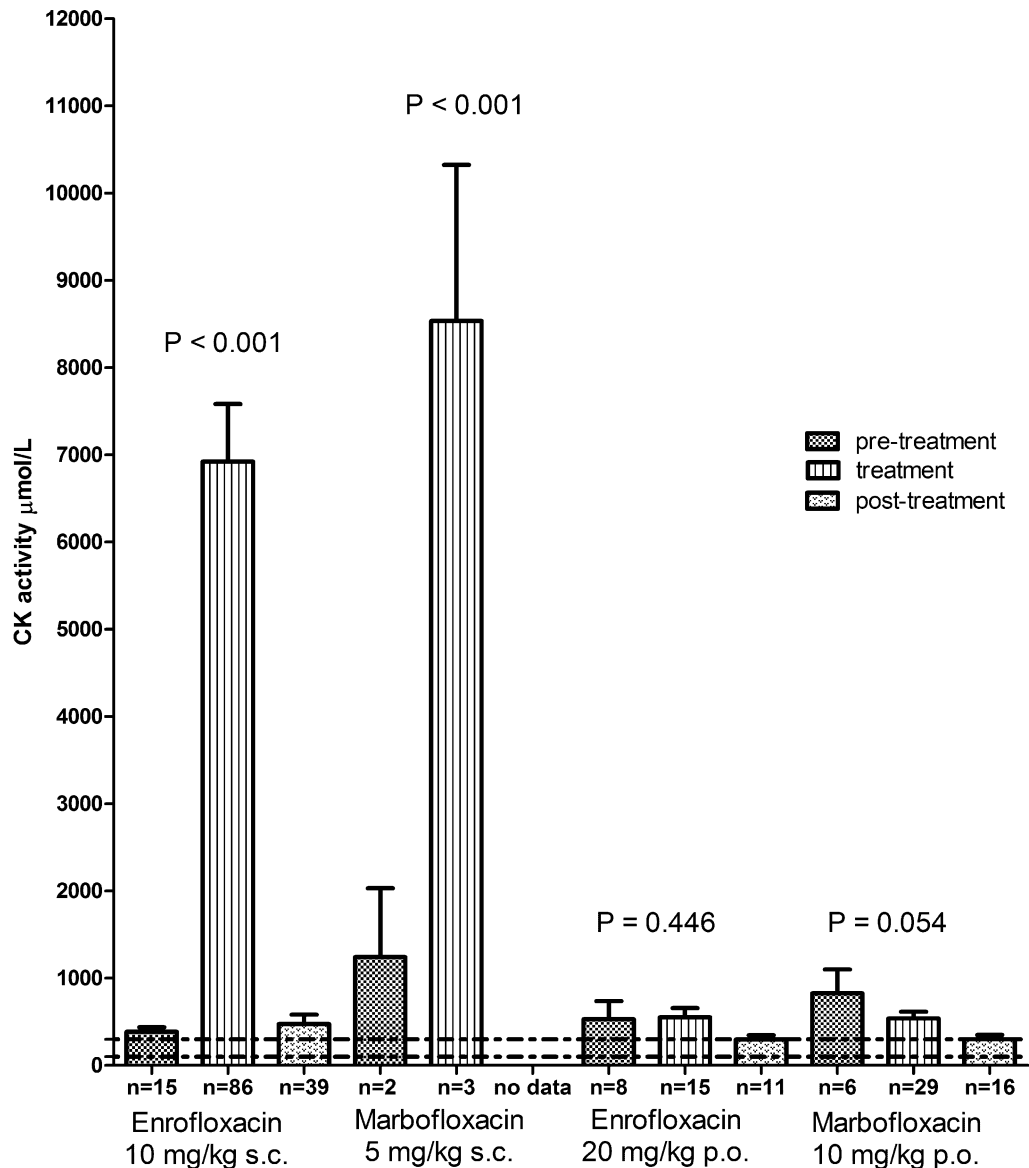


Figure 7-3. Creatinine kinase (CK) activity (mean ± SEM) in serum comparing pre-treatment, treatment and post-treatment samples in treated koalas.

Animals were treated with enrofloxacin 10 mg/kg subcutaneous injection (s.c.), 20 mg/kg per os (p.o.), and marbofloxacin 5 mg/kg s.c. and 10 mg/kg p.o. Normal range for CK activity in koalas (100 - 300 µmol/L) (Blanshard, 1994) shown with dashed lines.

Histological examination of tissues detected only two abnormalities not directly attributable to chronic chlamydial disease. One koala dosed with enrofloxacin 20 mg/kg p.o. once daily was euthanased due to persistent azotaemia with inappropriately dilute

urine, depression and inappetence three weeks after starting treatment. At post mortem examination this animal had severe, diffuse, subacute, cortical tubular oxalate nephrosis. Another koala died under anaesthesia, as a result of cardiac arrest, 13 days after commencement of enrofloxacin at 20 mg/kg p.o. This koala had an anaesthetic induced arrhythmia detected by auscultation, prior to treatment and during two subsequent general anaesthesia procedures.

7.5 DISCUSSION

This study is the first into aspects of selected drug pharmacokinetics in koalas. Our findings make a significant contribution to the understanding of drug administration in this species. There are many challenges associated with conducting a study such as this in an iconic wildlife species. This study was restricted to opportunistic recruitment of clinical cases during therapeutic treatment, which limited the size of treatment groups, particularly those amenable to oral administration. It was also restricted to conventional routes of drug administration in koalas, precluding an intravenous treatment group and six time points, to minimise the stress of handling. Despite these limitations, we can report that koalas administered oral enrofloxacin exhibit limited oral absorption with a long T_{max} compared to other species and that marbofloxacin has similarly poor relative oral absorption.

Conventional dog and cat dose rates, by both administration routes for both drugs produced relatively low C_{max} compared to other species and lack of drug accumulation. There was no clear indication of adverse effects, although CK activity increased in those animals injected subcutaneously, which has not been reported previously in this species.

Relative oral absorption of enrofloxacin compared to the s.c. route was poor, the median T_{max} was long (4 vs. 2 h) and AUC_{0-LOQ} had high individual variability. Visual inspection of the oral data presented in Figure 7-1 suggests absorption rate limited disposition. Poor

oral absorption of marbofloxacin and enrofloxacin may occur in koalas for a variety of reasons. Firstly, the koala stomach is normally full of dense, finely masticated leaf material (Blanshard & Bodley, 2008) that may entrap drugs or contain macromolecules that bind drugs, reducing their absorption, as hypothesised in sheep (González, San Andrés *et al.*, 2001). Secondly, the koala's diet of *Eucalyptus* contains metal cations such as cobalt, copper, zinc, calcium, magnesium, iron and selenium (McOrist & Thomas, 1984) that may form complexes with fluoroquinolones, reducing oral bioavailability (Turel, 2002). Soya, or low lactose milk formulas are routinely administered to sick koalas to supplement nutrition and enable administration of oral medications (Blanshard & Bodley, 2008) and supplementation was used in our study to duplicate this practice. Such supplementation could have contributed to limited absorption of drugs due to chelation with calcium ions as occurs with dairy products in people (Kivisto, Ojala-Karlsson *et al.*, 1992; Turel, 2002). The effect of chelation of enrofloxacin or marbofloxacin by the supplements in vitro or in vivo was not investigated, but should be considered for future studies. Thirdly, active transepithelial secretion of many fluoroquinolones occurs at several areas within the intestinal tract in different species, and may inhibit absorption (Ramon, Ben-Haim *et al.*, 2001). Fourthly, the mean intestinal residence time of drugs may influence the rate of absorption (Baluom, Friedman *et al.*, 1998) and the small intestinal transit time in koalas is short (particulate matter; 6 minutes, soluble material; 60 minutes) (Cork & Warner, 1983). Finally, poor oral relative bioavailability could be due to active first-pass metabolism as suggested by studies of BSP clearance in koalas indicating an increased rate of hepatic metabolism relative to other species (Pass & Brown, 1990). Our results support the hypothesis that proposed methods of interspecific scaling used to predict safe drug dose regimes in untested species may not be reliable for every drug, particularly when unique

interspecific hepatic metabolism is suspected (Hunter & Isaza, 2008).

Studies of pharmacokinetic aspects in other Australian marsupials that are related to the koala are rare (Kirkwood, Gulland *et al.*, 1988), thus aspects of drug absorption are compared here between koalas and other ‘hind-gut fermenting’ species with similar gastrointestinal tract anatomy such as rabbits and horses. Subcutaneous enrofloxacin administration (5 mg/kg after d 1) produced a C_{\max} (median 0.83; range 0.66 - 1.52 $\mu\text{g/mL}$) that was low compared to rabbits (2.23; 1.02 - 2.79 $\mu\text{g/mL}$) (Broome, Brooks *et al.*, 1991). The relatively low C_{\max} for enrofloxacin could be related to poor vascularity around the injection site (the dorsal thorax, or “scruff”) limiting absorption, or rapid clearance by efficient local and/or hepatic metabolism and/or excretion. Plasma drug accumulation was not a significant factor. The absence of difference between drug concentrations between day 1 and multiple dosing suggests enrofloxacin metabolism and excretion is complete by 24 hours in koalas. The $t_{1/2}$ for marbofloxacin administered at the same dose rate and route (1.66; 1.4 - 1.9 h) was shorter than in horses (5.47 ± 1.33 h) administered marbofloxacin 2 mg/kg intramuscularly (Carretero, Rodriguez *et al.*, 2002) and may indicate a more efficient rate of elimination in koalas.

Plasma concentrations of marbofloxacin, when administered orally, were mostly below the LOQ, indicating poor oral absorption at this dose rate. A more sensitive method would be required to detect the extremely low concentrations of marbofloxacin in koala plasma resulting from the oral dose rates used in this study, however, given the apparent poor oral absorption of this drug and that the injectable formulation of marbofloxacin is unavailable in Australia, further study of oral formulations of this drug may not have practical applications in koalas.

The plasma protein binding for enrofloxacin observed here ($55.4 \pm 1.9\%$) was similar to

that for enrofloxacin in rabbits $50 \pm 2\%$ (Bregante, Saez *et al.*, 1999), but higher than in horses (22%) (Papich & Riviere, 2001). Marbofloxacin protein binding ($49.5 \pm 5.3\%$), was higher than in calves ($29 \pm 2.2\%$) (Ismail & El-Kattan, 2007) and dogs ($21.8 \pm 6.2\%$) (Bidgood & Papich, 2005). These differences may reflect species differences in the number of plasma protein binding sites or their affinity for these drugs (Lin, 1995). Frozen plasma was used due to the practicalities of transporting samples from the remote field site. Frozen plasma may vary in pH compared with fresh plasma, affecting drug-protein binding assays (Riedel, 2006) therefore comparison between frozen and fresh plasma would be useful to confirm the values presented here.

Ciprofloxacin concentrations were below the LOD of the assay, suggesting that if present at all, low concentrations of this metabolite are produced in the koala ($< 0.08 \mu\text{g/mL}$), similar to other species (Papich & Riviere, 2001). Enrofloxacin is metabolised, via de-ethylation of the ethyl group on the piperazine ring, to ciprofloxacin. Ciprofloxacin is microbiologically active and may contribute to the overall efficacy of enrofloxacin (Papich & Riviere, 2001). The six metabolites detected in the enrofloxacin chromatogram could be ciprofloxacin, metabolites of ciprofloxacin itself or the parent compound. Marbofloxacin is metabolised into N-desmethyl-marbofloxacin in chickens (Anadon, Martinez-Larranaga *et al.*, 2002), dogs (Lefebvre, Schneider *et al.*, 1998), cattle (Schneider, Valle *et al.*, 2004), ball pythons and blue and gold macaws (Hunter, Koch *et al.*, 2007) and further metabolites have been identified in dogs (Lefebvre, Schneider *et al.*, 1998), cattle (Schneider, Valle *et al.*, 2004) and blue and gold macaws (Hunter, Koch *et al.*). The one marbofloxacin metabolite observed in this study remains to be identified.

As this study relied on opportunistic recruitment of wild animals with naturally occurring disease, animals were recruited with different states of disease (subclinical versus clinical),

varying weights, ages and sex, all factors that may influence pharmacokinetics and pharmacodynamics (Mahmood, 2006) and could explain the variability in pharmacokinetic parameters observed in this study. Small numbers of animals within each treatment group limited analysis of the influence of these factors on pharmacokinetic parameters. Our cohort represents the spectrum of expression of koala chlamydial disease for which these drugs are most commonly used. Further investigations examining pharmacokinetic parameters in healthy animals with attention to uniformity of signalment would be useful as a comparison to the results presented here.

Minimum inhibitory concentrations (MICs) of enrofloxacin or marbofloxacin have not been determined for inhibition of the growth of chlamydial species isolated from koalas. However, the MIC range of enrofloxacin for *C. pneumoniae* isolates from bandicoots has been determined (0.25 - 0.5 µg/mL) (Kumar, Kutlin *et al.*, 2007) and is similar to that of other fluoroquinolones (ofloxacin and ciprofloxacin) for *C. pecorum* isolates from cattle (0.25 - 0.5 µg/mL and 0.25 - 1.0 µg/mL, respectively) (Pudjiatmoko, Fukushi *et al.*, 1998). These organisms are most similar to koala Chlamydiaceae genetically (Everett, Bush *et al.*, 1999; Kutlin, Roblin *et al.*, 2007), and therefore a concentration of 0.25 µg/mL was chosen most likely to represent the MIC of enrofloxacin against koala Chlamydiaceae. The MIC of marbofloxacin has not been determined for chlamydial species.

A C_{\max}/MIC ratio of 8 - 10 and/or a AUC/MIC ratio of 125 - 250 is generally regarded as being 100% bactericidal with minimum risk of mutant selection for fluoroquinolones (Papich & Riviere, 2001) although these ratios may not always reflect the in vivo situation in naturally acquired disease (Aliabadi, Ali *et al.*, 2003). However, using such ratios as a guide, a theoretical in vivo bactericidal action would require a C_{\max} of at least 2.5 µg/mL for enrofloxacin. If plasma protein binding is taken into account (approximately 50%) then

a C_{\max} of 5 $\mu\text{g/mL}$ would be required for a bactericidal effect. Clearly, these concentrations were not achieved in koalas by any route and suggest a lack of efficacy. The sub-optimal regimes of fluoroquinolones suggested here may induce a state of “persistence” in infected animals leading to treatment failure, or select for resistant strains of Chlamydiaceae or other normal and/or opportunistic flora. In vivo chlamydial resistance to fluoroquinolones has been described occasionally (Yokoi, Yasuda *et al.*, 2004) and has been induced in vitro (Dessus-Babus, Bebear *et al.*, 1998). Further, resistant normal and/or opportunistic flora might pose a risk to some human care-givers through direct contact with koalas or via indirect contact with their faeces and urine (Guardabassi, Schwarz *et al.*, 2004).

Koalas in this study maintained weight during treatment in contrast to a previous study of subcutaneously administered oxytetracycline (Osawa & Carrick, 1990) and anecdotal reports of erythromycin use (Brown, Wood *et al.*, 1984), suggesting that fluoroquinolones do not cause life-threatening dysbiosis. The observed elevation of plasma activity of CK may be associated with a myopathy, although injection site biopsies would be required to determine the nature and distribution of lesions. It appears unlikely that CK activity elevations in these koalas resulted from capture myopathy (Munday, 1972) or injection trauma, as elevations were not detected in koalas handled frequently (e.g. to cleanse eyes with saline) or in records of three koalas injected with amoxicillin-clavulanate s.c. (unpublished data). Increases in CK activity following intramuscular injections of fluoroquinolones in other species have been reported (Nyska, Skolnick *et al.*, 1994; Kaartinen, Panu *et al.*, 1997).

Two koalas died during treatment with enrofloxacin (20 mg/kg p.o.); however neither can be directly attributed to enrofloxacin use. Although oxalate nephrosis in people has been associated with an antibiotic-induced reduction in gastrointestinal populations of

Oxalobacter formigenes, a commensal anaerobe important in oxalate degradation (Mittal, Kumar *et al.*, 2005), this disease is also described infrequently in untreated koalas from the study location (Canfield, 1989).

The findings of this study indicate that doses of fluoroquinolones used widely for the treatment of chlamydiosis in koalas do not reach likely therapeutic levels required. Future studies examining the clinical response to these treatments are required to minimise wastage of resources and predict treatment outcomes. In addition to their implications for drug administration, our findings of poor oral availability relative to the subcutaneous route raise interesting questions on mechanisms for absorption and metabolism of drugs and plant xenobiotics in this species.

CHAPTER 8 - THE CLINICAL AND MICROBIAL RESPONSE TO FLUOROQUINOLONE TREATMENT OF KOALAS INFECTED WITH CHLAMYDIACEAE

8.1 INTRODUCTION

Chlamydiosis, the most prevalent and important infectious disease in koalas, causes significant morbidity in many populations throughout Australia (Obendorf, 1983; Canfield, 1987; Canfield, 1989; Jackson, White *et al.*, 1999; McLean, 2003; Stalder, 2003).

Treatment of koalas with clinical signs consistent with chlamydiosis makes up a substantial part of the workload of koala rehabilitation facilities in Australia. At the Koala Preservation Society of NSW's Koala Hospital in Port Macquarie, NSW, such cases account for approximately 20% of all admissions (Chapter 2).

Despite a lack of rigorous evidence demonstrating efficacy, enrofloxacin, often given orally, is the mainstay of treatment of urogenital forms of chlamydiosis in two of the three major koala rehabilitation facilities in Australia (Blanshard & Bodley, 2008) (Chapter 3 and Table 5.1). In human medicine, older quinolone antibiotics have been unsuccessful in treating chlamydiosis (Ridgway, 1997) although, more recently, third generation fluoroquinolones have been recommended for the treatment of *Chlamydia trachomatis* (Ridgway, 1997) and *Chlamydophila pneumoniae* infections (Hammerschlag, 2003). The results of treating animal chlamydiosis with a veterinary fluoroquinolone (enrofloxacin) are equivocal. Some authors report successful treatment, possibly as a result of false negatives as relatively insensitive diagnostic techniques were used (cell culture (Lindenstruth & Frost, 1993), immunofluorescence (Gerhardt, Schulz *et al.*, 2006)); others report treatment failure (Penguin Taxon Advisory Group, 2005; Hartmann, Helps *et al.*,

2008).

Previous work in this thesis found the fluoroquinolone plasma concentrations in treated koalas to be low and thus likely to be ineffective in treating chlamydiosis (Chapter 7) (Griffith, Higgins *et al.*, 2010). This is at odds with its apparent success based on medical records (Chapter 3) and anecdotal evidence (Blanshard & Bodley, 2008). Given this evident contradiction, this author explored whether treatment with fluoroquinolones in koalas effectively reduces clinical signs of chlamydiosis and ultimately achieves microbial negativity. This information is important to determine whether it is safe to translocate treated animals into populations negative for Chlamydiaceae or with novel chlamydial strains, and is important information for future epidemiological studies of the potential impact of treated animals on the local population.

This study also provided the opportunity to study the relationship between chlamydial load and clinical signs. Diagnosis of chlamydial infection in koalas is often based on clinical signs alone (Chapter 3). The assumption that clinical signs are a sensitive diagnostic test dictates management decisions regarding which animals receive treatment, the route and end point of treatment, and husbandry decisions regarding disinfection of housing facilities and isolation of animals at the Koala Hospital. As previous studies have indicated that subclinical chlamydiosis is common in some populations of koalas in Queensland (56%) (Jackson, White *et al.*, 1999) and Victoria (39%) (McLean, 2003), and in women (Stamm, 1999), calves and cats (Shewen, 1980), and birds and sheep (Longbottom & Coulter, 2003), investigation of the validity of this assumption is warranted. Such information would better inform resource allocation and captive husbandry guidelines for koalas in rehabilitation centres and contribute to epidemiological studies of disease prevalence in wild koalas.

Given this background, the following hypotheses were generated and tested:

1/ clinical signs reflect the presence of chlamydial organisms in koalas

2/ severity of clinical signs correlate with mucosal load of *C. pecorum*

3/ treatment with fluoroquinolones at standard mammalian dose rates eliminate *C. pecorum* and *C. pneumoniae* from koalas.

8.2 METHODS

8.2.1 Design of the study

To test the hypothesis that clinical signs reflect the presence of chlamydial organisms in koalas, the presence of *C. pneumoniae* and/or *C. pecorum* was detected using real-time polymerase chain reaction (qPCR) of ocular and urogenital swabs collected immediately prior to treatment (day 0) and results compared with clinical scores of these sites.

Mucosal *C. pecorum* load was then estimated using qPCR and examined relative to severity of clinical signs and phase of antibiotic treatment. Sites positive at day 0 were subsequently tested weekly for three weeks, on the last day of treatment, two weeks after cessation of treatment, and then on the final available swab (usually four weeks after treatment end); or, where animals did not stop treatment because clinical signs did not abate, they were tested weekly for up to 10 weeks. Two animals that died during treatment were not tested. Where multiple sites were positive in one animal at the start of treatment, each site was tested individually. Clinical assessments were conducted as per Chapter 4 and data compared to chlamydial loads at associated time points.

To test the hypothesis that treatment with fluoroquinolones eliminates *C. pecorum* and *C. pneumoniae*, both eyes and urogenital tracts of all animals were examined for presence of

organisms prior to treatment and at the last time point available, using qPCR. Animals that were negative at all three sites after treatment were retested using DNA at two dilutions (1:10 and 1:100), extracted from duplicate swabs from the last available time point.

8.2.2 Animals

Koalas (16 males, 15 females) of median age 5.5 years (range 2 - 12 years) were recruited, housed, examined, allocated to treatment groups (Table 8-1), sampled from, observed and monitored as described in Chapter 4. Inclusion and rejection criteria are outlined in Chapter 4. Treatment was continued for four to 10 weeks, and was discontinued when clinical signs had resolved and animals were negative on two consecutive weekly PCRs using primers specific to all Chlamydiaceae (see 4.11). Seventeen animals (14 males, 3 females) completed treatment and were monitored for another four weeks.

Table 8-1. Fluoroquinolone treatments administered, median length of treatment and number released of 29 koalas treated for chlamydiosis.

Drug	n	Length of treatment median (range)	Released
Marbofloxacin 10 mg/kg p.o. SID	5	34 (31 - 64)	4
Marbofloxacin 1-3.3 mg/kg p.o. SID	5	44 (28 - 44)	0
Enrofloxacin 5 mg/kg p.o. SID	1	44	0
Enrofloxacin 10 mg/kg s.c. SID	11	43.5 (31 - 70)	10
Enrofloxacin 20 mg/kg p.o. SID	3	30 (30 - 38)	3
Enrofloxacin 5 mg/kg s.c. SID	4	47 (44 - 48)	0
Total	29		17

SID: once daily; p.o: per os; s.c: subcutaneous injection.

Two animals died during treatment, following administration of 20 mg/kg enrofloxacin p.o. for 20 and 14 days, respectively and were excluded from analysis. The first animal was euthanased on day 22 after a five-day history of inappetence, depression, dehydration, azotaemia and inadequately concentrated urine, and was found to have acute renal oxalate nephrosis on post mortem examination (HL in Appendix IV). The second animal died under general anaesthesia at 14 days, after experiencing cardiac arrhythmia detected by auscultation at the anaesthetic associated with death and two prior anaesthetic episodes. Post mortem examination revealed peracute myocardial hyaline necrosis (BP in Appendix IV). Neither pathological process can be directly attributed to fluoroquinolone use. Of the 12 further animals not released, 10 were euthanased due to structural disease or persistent or recurrent clinical signs, and two (OA and AG) were re-treated with intramuscular doxycycline and were subsequently euthanased (detail provided in Chapter 5 and Appendix II and III). All deceased animals underwent a standard midline post mortem examination (Blanshard, 1994) as per Chapter 4.

8.2.3 Clinical scores

Methods used clinically to describe urogenital tract disease (modified wet bottom score and ultrasonography) and ocular disease are described in Chapter 4 and Chapter 5, respectively. Any modified wet bottom score above zero was considered clinical disease. Objectivity and consistency for ocular disease scoring were improved for this study by blinded scoring of digital photographs taken at time of examination. The left and right eyes were photographed, with ocular pathology made visible by gentle manual retraction of the palpebrae. Four hundred and four images of eyes from 22 individuals were ascribed non-identifiable codes and randomised using the random number and sort functions in Microsoft® Office Excel® 2003 (Microsoft® Corporation, Redmond, Washington, USA).

The author then graded chemosis, proliferation of the conjunctiva, and amount of discharge as zero (normal), one (mild), two (moderate) or three (severe) against reference photographs and criteria (Table 8-2 to Table 8-4). The scores for each parameter for each eye were summed to derive a total eye score. Comparison was made between total eye scores determined using the above method and clinical descriptions made in the field. Total eye scores of greater than three were always described as “conjunctivitis” in corresponding field assessments, and thus a total eye score of greater than three was considered indicative of clinical disease.

Table 8-2. Grading of ocular pathology (chemosis) showing definitions used and reference photographs.





Grade	Chemosis: (Swelling of the conjunctiva)	Examples
Grade 0	None	
Grade 1	Swelling < 50% of conjunctiva and the nictitating membrane only	
Grade 2	Swelling ≥ 50% of conjunctiva and nictitating membrane total; obscuring the cornea by ≤ 50%	
Grade 3	Severe swelling of the entire conjunctiva that obscures the cornea > 50%	

Table 8-3. Grading of ocular pathology (proliferation) showing definitions used and reference photographs.









Grade	Proliferation: (Papillary conjunctival hypertrophy, i.e. proliferative conjunctival tissue exhibiting multiple small projections, or cobblestone appearance)	Examples
Grade 0	None	
Grade 1	Exuberant proliferative vegetative conjunctival hypertrophy occupying < 50% of conjunctiva and nictitating membrane total	
Grade 2	Exuberant proliferative vegetative conjunctival hypertrophy occupying ≥ 50% of conjunctiva and nictitating membrane total but extending across the cornea ≤ 50%	
Grade 3	Exuberant proliferative vegetative conjunctival hypertrophy occupying > 50% of the conjunctiva and nictitating membrane that obscures the cornea > 50%	

Table 8-4. Grading of ocular pathology (discharge) showing definitions used and reference photographs.

Grade	Discharge (Any excretion or substance emanating from the eye)	Examples
Grade 0	None	
Grade 1	Small amount of crusting, epiphora or mucopurulent discharge < 1 cm ² in total	
Grade 2	Surface area of epiphora, mucopurulent discharge or crusting 1- 2 cm ² in total	
Grade 3	Surface area of epiphora, mucopurulent discharge or crusting > 2 cm ² in surface area and/or obscuring the cornea or sticking lids together	

8.2.4 Real-time PCR

Real-time PCR has been used previously to monitor chlamydial load of diseased koalas during chloramphenicol treatment (Markey, Wan *et al.*, 2007). In this previous work, authors used primers targeting a 72 base pair region of the 16S rRNA gene, designed from a chlamydial consensus signature sequence. Initial experiments in the current study using this previously published method and computer modelling of predicted PCR product (Zuker, 2003) found significant production of heat-stable secondary structures when used with *C. pneumoniae* DNA template (Figure 8-1). Secondary structures interfere with the efficiency of the PCR reaction, and thus quantitation (Peters, Helps *et al.*, 2004). Extensive optimisation of reaction conditions are required to remove secondary structures, however this is not always possible, and redesign of primers may be required (Ashton & Headrick, 2007). A further concern regarding the use of these primers for the current study was their inability to differentiate chlamydial species in mixed infections (Markey, Wan *et al.*, 2007), which are common in koalas (Girjes, Hugall *et al.*, 1993; Devereaux, Polkinghorne *et al.*, 2003). Given this background, the author moved to designing species-specific primers and a qPCR method for quantifying chlamydial loads in koalas.

Following recommendations for general primer design for qPCR (Wang & Seed, 2006), the primer analysis programs Primer 3 (Rozen & Skaletsky, 2000) and mfold (Zuker, 2003) were used to select primers using published sequences of the *ompB* genes of koala biovars of *C. pneumoniae* and *C. pecorum* (Glassick, Giffard *et al.*, 1996), respectively. The primer pair chosen from these and trialled for *C. pneumoniae* (5'-TCCGTTCCAGAATACGCTAC-3'; 5'-CATCACTTGTAGGGGTTGTTTC-3') theoretically amplified a 154 base pair (bp) fragment, and the primer pair chosen and trialled for *C. pecorum* (5'-CCTTGTGAAGCGGAATTTGT-3'; 5'-CATCTTCGCCTTGTCTAAA-3'), a 101 bp fragment.

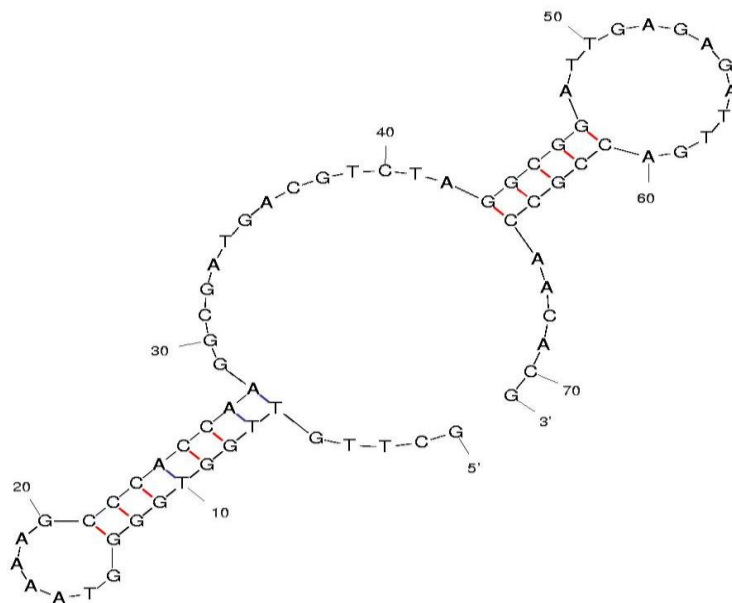


Figure 8-1. Predicted secondary structure (Zuker, 2003) of *Chlamydomonas reinhardtii* fragment stable to 71.8 °C.

Mucosal *C. pecorum* load was normalised for the number of host cells present on each swab by dividing estimated pathogen load by the estimated number of host cells present; estimated by concurrent qPCR reactions using primers based on the sequence of the koala beta-actin gene. Primers for amplification of the koala beta-actin gene were those of Markey *et al.* (2007) and in that study were shown to amplify an 82 bp fragment. DNA was extracted from swabs using a commercial extraction kit utilising silica membrane spin-columns, following manufacturer's instructions (QIAamp® DNA Mini Kit, QIAGEN Pty Ltd, Doncaster, Vic, Australia). Where DNA had been extracted in the field using a lysis and stabilising solution (Chapter 4), DNA aliquots were adjusted to a comparable concentration by dilution 1:10 with autoclaved purified water (Milli-Q® Ultrapure filtration system. Millipore, North Ryde, NSW, Australia) and then purified as above, but omitting the initial lysis and incubation steps. Without a silica based purification column reaction efficiency was affected, presumably as a result of DNA inhibitors remaining in the sample. DNA, stored at -20 °C, was subjected to at most one freeze-thaw cycle prior to

qPCR.

Triplicate qPCR reactions comprised 10 μ L of 2 x SYBR® Green PCR Master-mix (Applied Biosystems, Scoresby, Vic, Australia), 500 nM each primer, 2 μ L of DNA template, and autoclaved purified water (Milli-Q® Ultrapure filtration system, Millipore, North Ryde, NSW, Australia) in a final volume of 20 μ L. Reaction conditions were 95 °C for 10 min (activation) followed by 40 cycles of 95 °C for 15 s (denaturation), and 61 °C (60 °C for *C. pecorum* assay) for 60 s (annealing and extension). The melting profile was obtained by 90 s pre-melt conditioning at 55 °C and then heating the reactions in one-degree increments from 55 °C to 85 °C with 5 second stops.

DNA samples extracted from *C. pneumoniae* culture, *C. pecorum* culture or from the buffy coat of a koala outside of this study, using a commercial kit (QIAamp® DNA Mini Kit, QIAGEN Pty Ltd, Doncaster, Vic, Australia), were used as positive controls and autoclaved purified water (Milli-Q® Ultrapure filtration system, Millipore, North Ryde, NSW, Australia) was used as a negative control.

Efficiency of the reaction was > 95% to cycle threshold (C_T) = 30, thus this was taken as the limit of accurate quantitation and sites positive with $C_T > 30$ were recorded as low positive but not quantifiable. During method development chlamydial PCR products, based on template from pure *C. pneumoniae* or *C. pecorum* cultures, were confirmed to be of predicted size using gel electrophoresis; and purified DNA products (Ultraclean™ 15 DNA Purification Kit, MO BIO Laboratories, Carlsbad, California, USA) were sequenced by a commercial laboratory using a 3730XL DNA sequencer (Macrogen Inc, Seoul, Korea). Sequences were aligned using ClustalW (Larkin, Blackshields *et al.*, 2007) and confirmed to be those expected using basic local alignment search tool (BLAST) (Altschul, Gish *et al.*, 1990).

Both *C. pneumoniae* and *C. pecorum* primers were tested against DNA extracted using a commercial kit (QIAamp® DNA Mini Kit, QIAGEN Pty Ltd, Doncaster, Vic, Australia) according to manufacturer's instructions from seven species of bacteria (*Staphylococcus aureus* (Veterinary Pathology and Bacteriology Culture Collection (VPB) 236), *Staphylococcus epidermidis* (VPB 238), *Proteus mirabilis* (VPB 261), *Serratia marcescens* (VPB 255), *Enterococcus faecalis* (VPB 185), *Pseudomonas aeruginosa* (VPB 591) and *Escherichia coli* (VPB 2716)) commonly found on mucous membranes of the anogenital area, and against DNA extracted from the alternate chlamydial species. There was no cross reactivity with these species, or for *C. pecorum* primers and *C. pneumoniae* DNA and vice versa. Phylogenetic neighbours of Chlamydiaceae such as *Simkania* spp, *Waddlia* spp and novel koala Chlamydiales genotypes (Devereaux, Polkinghorne *et al.*, 2003) were not available for study. DNA extracted as per 4.1.1.6 from urogenital and ocular swabs taken opportunistically from eight koalas bred and held in a captive facility isolated from wild animals and with no history of chlamydial disease were subjected to qPCR using the protocol described in the previous page. All animals were negative using this method.

Samples were considered positive for the presence of *C. pneumoniae*, *C. pecorum* or beta-actin when the dissociation curve profile matched that of the positive controls. When the melt curve was equivocal, qPCR reactions were repeated using template DNA at two dilutions (1:10, 1:100).

Results were compared to standard curves based on *C. pecorum* culture or DNA extracted from a koala buffy coat, allowing relative pathogen load to be compared longitudinally and between individuals. Fresh aliquots of the standards were used for each day's analysis. The $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001), which controls for difference in starting pathogen load between anatomical sites by arbitrarily defining the starting pathogen load

as 100%, was used to examine whether changes in *C. pecorum* load over time differed between different treatment groups.

8.2.5 Sensitivity and specificity of clinical signs

Sensitivity and specificity of clinical signs as indicators of chlamydial infection at the sampled site were calculated using qPCR results as the gold standard, and the equations:

Sensitivity = true positives / (true positives + false negatives)

Specificity = true negatives / (true negatives + false positives).

Anatomical sites sampled were classified as: true positive (total eye score ≥ 3 or wet bottom score > 0 , and qPCR positive on day 0), false positive (clinical signs as above but qPCR negative at that site), true negative (total eye score < 3 or wet bottom score = 0, and qPCR negative on day 0) or false negative (no clinical signs i.e. total eye score < 3 or wet bottom score = 0, but qPCR positive at that site).

8.2.6 Statistical analysis

To assess the association between clinical scores and presence of chlamydial organisms on day 0, sites were grouped as “negative on day 0”, “positive for *C. pecorum* on day 0”, “positive for *C. pneumoniae* on day 0”, or “mixed chlamydial infections on day 0” according to whether qPCRs were positive on day 0.

To assess the response to treatment, animals were grouped according to estimated maximum plasma antimicrobial concentration (C_{\max}), based on data obtained from pharmacology assays (Chapter 7). The group defined as “high” ($n = 12$) possessed a mean C_{\max} of 2.15 ± 0.59 $\mu\text{g/mL}$ following treatment with subcutaneous (s.c.) enrofloxacin 10 mg/kg. The group defined as “medium high” ($n = 4$) had a mean C_{\max} of 0.91 ± 0.37

$\mu\text{g/mL}$ following treatment with 5 mg/kg enrofloxacin s.c. once daily. Animals defined as “medium” had a mean C_{max} of $0.46 \pm 0.35 \mu\text{g/mL}$ following treatment with enrofloxacin 20 mg/kg ($n = 3$) per os (p.o.) or marbofloxacin 10 mg/kg p.o. once daily ($n = 5$). The group defined as “low” had a mean C_{max} below the limit of detection of the assay ($< 0.12 \mu\text{g/mL}$, enrofloxacin; $< 0.16 \mu\text{g/mL}$ marbofloxacin) following treatment with enrofloxacin 5 mg/kg p.o. ($n = 1$) or marbofloxacin 1 - 3.3 mg/kg p.o. ($n = 5$).

Relative *C. pecorum* load was grouped as negative ($n = 132$; 45.1% of all assays), positive but not quantifiable ($C_T > 30$; $n = 59$; 20.1%), between 0.002 and 1 x *C. pecorum* culture standard ($n = 66$; 22.5%), and ≥ 1 x *C. pecorum* culture standard ($n = 36$; 12.3%). As animals finished treatment at different times, the stage of treatment was grouped as: pre-treatment (first screening day to day zero; $n = 29$), early treatment (day 1 - day 21; $n = 29$), late treatment (day 22 until treatment end; mean 43 ± 9.5 days; $n = 29$) and post-treatment (day 43 until day 71 ± 4.6 days; $n = 17$).

To take into account difference in sex and size, ultrasonographic measurements of urogenital tract (bladder wall thickness and lumen diameter) were converted to percentages relative to the same animal's measurements on day 0.

Clinical scores for conjunctivitis and wet bottom, and ultrasonography, appetite and faecal output, biochemical and haematological parameters, body weight and qPCR results were analysed using restricted maximum likelihood (REML) analysis using GenStat for Windows (2008, 11th ed., VSN International Ltd., Hemel Hempstead, Hertfordshire, UK) using fixed effects of presence of different chlamydial species on day 0, stage of treatment, estimated C_{max} and estimated *C. pecorum* load and random effects of koala and week. Models were built using forwards progression. Where data were non-parametric, they were normalised by log or square root transformation and then analysed. Estimated means and

standard errors predicted by the models were plotted using Graphpad Prism® 5 for Windows (Graphpad Software, La Jolla, California, USA). Statistical significance was concluded at $p \leq 0.05$.

8.3 RESULTS

8.3.1 Clinical signs as an indicator of chlamydial infection on admission

At the start of treatment (day 0), 21 of 29 chlamydia-positive animals had clinical signs consistent with chlamydial disease (total eye score ≥ 3 or wet bottom score > 0), indicating that the presence of clinical signs in a koala was a poorly sensitive indicator of presence of mucosal Chlamydiaceae in that animal (72%, Table 8-5). Specificity could not be calculated as animals negative by qPCR at all three sites (true negatives) were excluded from this study.

Table 8-5. Sensitivity and specificity of clinical signs* in predicting chlamydial infections (*Chlamydophila pneumoniae*, *C. pecorum* or both) at individual anatomical sites (eyes, urogenital tract) in 29 koalas in comparison with qPCR.

Site with clinical signs*	Chlamydial species detected by qPCR	Sensitivity of clinical signs in detecting chlamydial presence	Specificity of clinical signs in detecting chlamydial presence
Eye	<i>C. pecorum</i> only	67%	100%
	<i>C. pneumoniae</i> only	--	--
	Both	57%	100%
Urogenital	<i>C. pecorum</i> only	60%	100%
	<i>C. pneumoniae</i> only	--	--
	Both	28.5%	100%
Any site	Chlamydiaceae at any anatomical site	72%	--

*total eye score > 3 (eye), wet bottom score > 0 and/or structural change to the reproductive tract (urogenital tract); qPCR: real time polymerase chain reaction.

When sites were examined independently, the presence of clinical signs was also poorly sensitive (67% or lower), but highly specific, in determining the presence of chlamydial organisms at the individual anatomical site (Table 8-5). *Chlamydomphila pecorum* appeared to be more pathogenic: on day 0, total eye scores were greater in eyes where *C. pecorum* was detected, than in eyes that were negative or those infected by *C. pneumoniae* alone ($p = 0.004$; Figure 8-2). In fact, no site infected with *C. pneumoniae* alone was described as conjunctivitis in the field (i.e. total eye score ≥ 3). In particular, clinical signs were very poorly sensitive in predicting the presence of mixed infections at an individual site (chi-sq = 9.98, d.f. = 1. $p < 0.02$; Table 8-5). Also renal ultrasonographic measurements did not differ between these groups with respect to qPCR result or wet bottom scores (Table 8-6, Table 8-7).

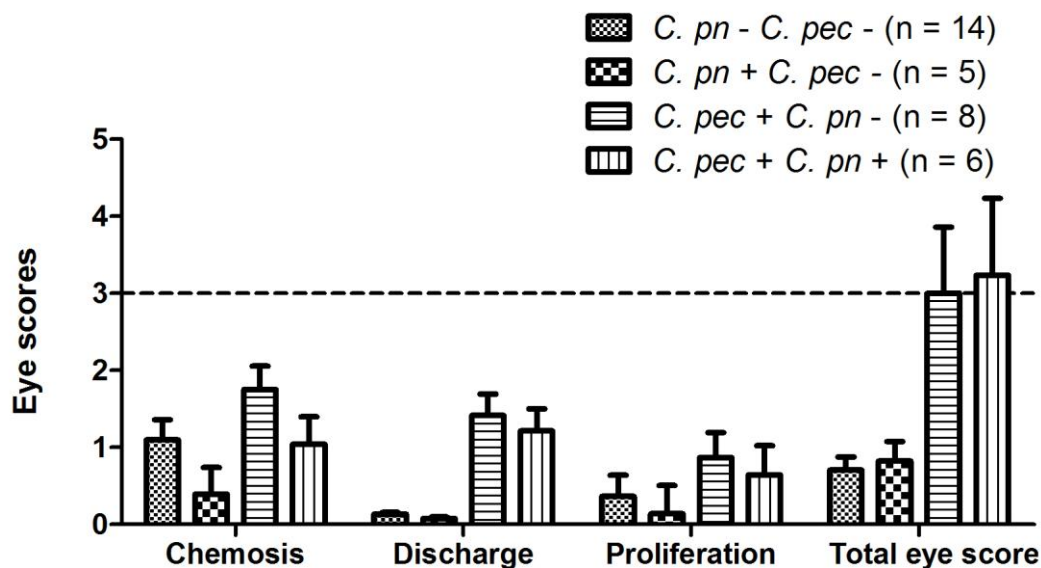


Figure 8-2. Mean (\pm SEM) eye scores prior to treatment grouped according to infection status.

Figures shown are the mean \pm SEM predicted by a restricted maximum likelihood statistical model using scores from 33 eyes from 27 koalas. *C. pn*: *Chlamydomphila pneumoniae*; *C. pec*: *C. pecorum*. Dashed line indicates clinical conjunctivitis.

Table 8-6. Measurements of the bladder and kidneys determined by ultrasonographic imaging of 23 koalas with and without clinical urogenital tract disease (wet bottom score > 0) prior to fluoroquinolone treatment.

Clinical signs	Bladder lumen diameter (mm)	p	Bladder wall thickness (mm)	p	Kidney	Sagittal length day 0	Horizontal length day 0	D-V width day 0	n
Wet bottom score > 0	12.9 ± 1.7	< 0.001	4.7 ± 0.3	< 0.001	L	45.3 ± 4.3	29.6 ± 3.4	27.9 ± 2.7	9
					R	45.5 ± 3.8	28.9 ± 3.0	29.5 ± 3.0	
Wet bottom score 0	26.9 ± 2.8		2.83 ± 0.6		L	43.6 ± 4.0	26.7 ± 3.0	27.6 ± 3.4	14
					R	42.3 ± 4.3	26.8 ± 4.6	26.9 ± 3.1	

Sagittal length (cranial to caudal pole), horizontal length (hilus to most lateral aspect), D-V width (dorsal to ventral surface). Figures shown are the mean ± SEM predicted by a restricted maximum likelihood statistical model. There was no statistically significant difference for kidney measurements between left and right kidneys, or relating to wet bottom score.

Table 8-7. Measurements of the bladder and kidneys determined by ultrasonographic imaging, and urogenital infection status of *Chlamydophila pecorum* and/or *C. pneumoniae* as detected by qPCR in 22 koalas prior to fluoroquinolone treatment.

Pathogen	Bladder wall thickness (mm)	Bladder lumen diameter (mm)	Kidney	Sagittal length (mm)	Horizontal length (mm)	D-V width (mm)	n
<i>C. pecorum</i> positive	18.11 ± 10.7	3.70 ± 1.5	L	44.7 ± 4.5	28.3 ± 3.4	27.0 ± 2.7	16
			R	44.8 ± 4.6	27.9 ± 4.3	28.3 ± 3.5	
<i>C. pneumoniae</i> negative	22.7 ± 12.0	4.5 ± 3.0	L	44.3 ± 3.5	29.5 ± 1.1	28.8 ± 1.5	4
			R	44.1 ± 4.1	28.3 ± 3.7	28.8 ± 1.8	
Positive for <i>C. pecorum</i> & <i>C. pneumoniae</i>	41.7 ± 3.25	1.95 ± 0.3	L	42.4 ± 6.9	28.9 ± 3.0	28.0 ± 5.7	2
			R	42.7 ± 8.2	29.3 ± 0.6	28.9 ± 6.9	

Bladder wall thickness and bladder lumen diameter sagittal length (cranial to caudal pole), horizontal length (hilus to most lateral aspect), D-V width (dorsal to ventral surface). Figures shown are the mean ± SEM predicted by a restricted maximum likelihood statistical model. There was no statistically significant difference for kidney measurements between left and right kidneys, or relating to wet bottom score.

Signs of clinical disease were fairly equally distributed between sites (Table 8-8). In contrast, presence of *C. pecorum* showed a significant bias (chi-sq = 23.3, d.f = 1, p < 0.0001) towards urogenital (93%) over ocular (38%) sites but *C. pneumoniae* was evenly distributed; 25% of ocular and 24% of urogenital tract swabs were positive for *C. pneumoniae* (chi-sq = 0.33, d.f = 1, p = 0.565) (Table 8-9).

Absence of signs at sites infected by *C. pneumoniae* alone and the absence of animals with single infections of *C. pneumoniae* in the urogenital tract (Table 8-9) hampered calculation of sensitivity and specificity of clinical signs in predicting *C. pneumoniae* infections.

Table 8-8. Presence of *Chlamydophila pneumoniae*, *C. pecorum* or both detected by qPCR and clinical signs* according to anatomical site (eyes, urogenital tract) in 29 koalas prior to treatment.

Infection status	Clinical signs*	Eyes	Urogenital tract
<i>C. pneumoniae</i> positive	Present	0	0
<i>C. pecorum</i> negative	Absent	7	0
<i>C. pecorum</i> positive & <i>C. pneumoniae</i> negative	Present	10	12
	Absent	5	8
Positive for both	Present	4	2
	Absent	3	5
Negative for both	Present	0	0
	Absent	28	2
Total		57	29

*total eye score > 3 (eyes), wet bottom score > 0 and/or structural change to the reproductive tract (urogenital tract).

Table 8-9. Presence of chlamydial organisms detected by qPCR and incidence of clinical signs prior to treatment in 29 koala treated with fluoroquinolones.

		Ocular [†] clinical signs	Urogenital tract clinical signs [‡]	Ocular [†] and urogenital tract clinical signs [‡]	No clinical signs	Total
qPCR positive*	Ocular only	2	0	0	0	2
	Urogenital tract only	0	5	0	3	8
	Ocular and urogenital tract	5	4	5	5	19
Total		7	9	5	8	29

*qPCR positive on day 0 for *Chlamydomydia pecorum*, *C. pneumoniae* or both

[†] total eye score > 3

[‡] wet bottom score > 0 and/or presence of ovarian bursal cysts on ultrasound.

8.3.2 Relationship between chlamydial load and clinical signs during treatment

There was no significant difference among treatment groups with different plasma antibiotic concentrations ($p < 0.845$; Figure 8-3) and therefore these data were pooled. Using the $2^{-\Delta\Delta C_T}$ method to control for differences between animals and anatomical sites, mucosal *C. pecorum* load decreased dramatically during the treatment period (Figure 8-3). Ocular clinical scores improved within two weeks of treatment regardless of treatment group ($p < 0.001$; Figure 8-4 and Figure 8-5). Throughout treatment, higher ocular *C. pecorum* loads were associated with significantly higher scores for chemosis ($p = 0.05$), proliferation ($p < 0.001$) and total eye score ($p = 0.002$; Figure 8-6). In contrast, despite reduction in urogenital chlamydial load, rump pelage stain did not change during treatment (Figure 8-7) and, although wet bottom score declined in the high dose treatment group, it

showed no clear trend in other treatment groups (Figure 8-8). The highest mean wet bottom score was associated with mid-range, rather than the highest *C. pecorum* load ($p = 0.007$; Figure 8-9). Higher urogenital *C. pecorum* loads were associated with a thicker bladder wall ($p = 0.021$) and approached significance for a smaller bladder lumen diameter ($p = 0.070$; Figure 8-10). Bladder lumen diameter increased ($p = 0.012$, Figure 8-11) and bladder wall thickness decreased over time ($p = 0.015$; Figure 8-12) in the high group; and the bladder lumen diameter also increased by late treatment in two animals in the medium high group (Figure 8-11). Renal measurements did not change in any group.

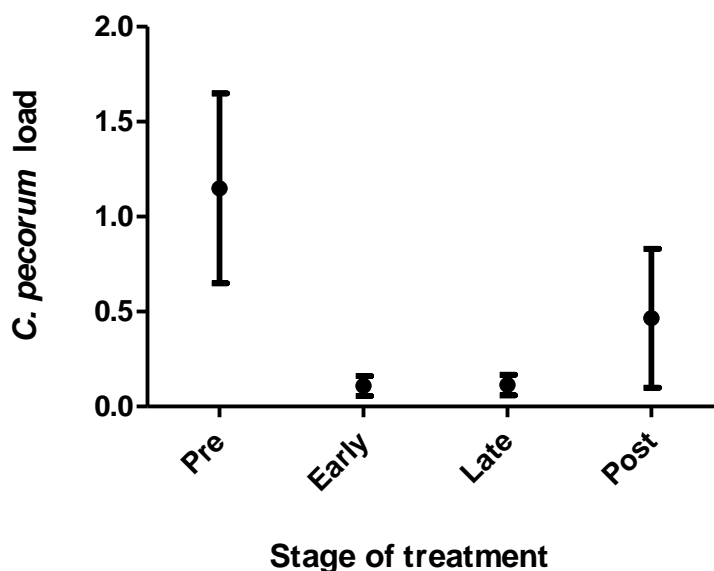


Figure 8-3. Changes in *Chlamydophila pecorum* load in 28 koalas treated with fluoroquinolones.

Figures shown are the mean \pm SEM predicted by a restricted maximum likelihood statistical model. 1 = an arbitrary standard (*C. pecorum* culture). Stage of treatment: Pre (first screening day to day 0); early (day 1 - day 21); late (day 22 until treatment end; mean day 43 \pm 9.5 SD); and post (day 43 until day 71 \pm 4.6 SD).



DAY 0

Chemosis 2, proliferation 2, discharge 3

Total eye score 7



DAY 7

Chemosis 3, proliferation 3, discharge 1

Total eye score 7



DAY 14

Chemosis 2, proliferation 2, discharge 1

Total eye score 5



DAY 29

Chemosis 0, proliferation 1, discharge 2

Total eye score 3



DAY 36

Chemosis 0, proliferation 2, discharge 0

Total eye score 2



DAY 43

Chemosis 2, proliferation 2, discharge 0

Total eye score 4

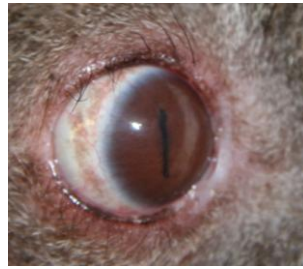
Figure 8-4. Example of eye score changes in one animal over time treated with enrofloxacin 10 mg/kg s.c. for 51 days (continues next page).



DAY 50

Chemosis 0, proliferation 1, discharge 0

Total eye score 1



DAY 57

Chemosis 0, proliferation 0, discharge 0

Total eye score 0



DAY 70

Chemosis 0, proliferation 1, discharge 0

Total eye score 1



DAY 77

Chemosis 0, proliferation 0, discharge 0

Total eye score 0

Figure 8-4 cont. Example of eye score changes in one animal over time treated with enrofloxacin 10 mg/kg by subcutaneous injection for 51 days.

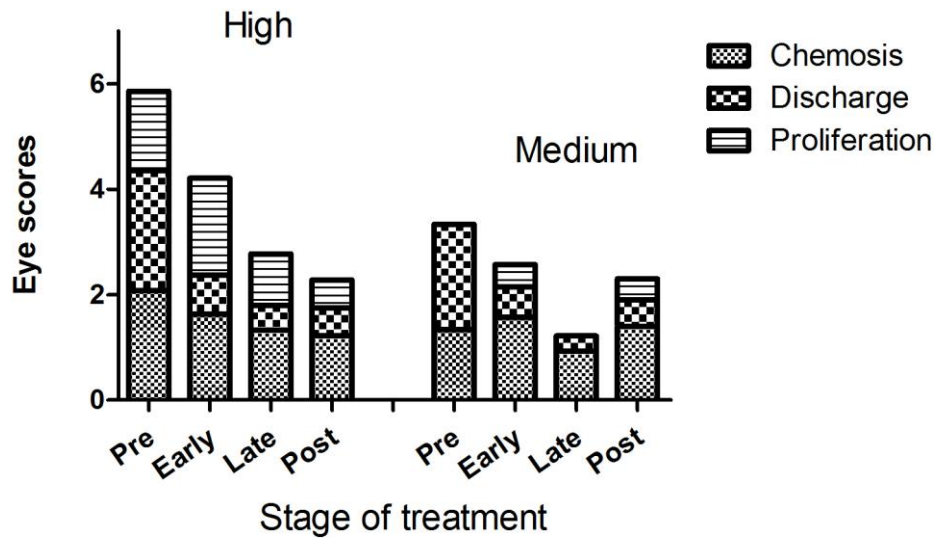


Figure 8-5. Changes in eye scores of eyes positive for *Chlamydophila pecorum* prior to fluoroquinolone treatment for two groups of animals grouped according to C_{max} .

High: mean enrofloxacin C_{max} of $2.15 \pm 0.59 \mu\text{g/mL}$; medium: mean enrofloxacin or marbofloxacin $0.46 \pm 0.35 \mu\text{g/mL}$. Total eye score is represented by total height of the bar. Figures shown are the mean \pm SEM predicted by a restricted maximum likelihood statistical model. Pre (first screening day to day 0); early (day 1 - day 21); late (day 22 until treatment end; mean day 43 ± 9.5 SD); and post-treatment (day 43 until day 71 ± 4.6 SD).

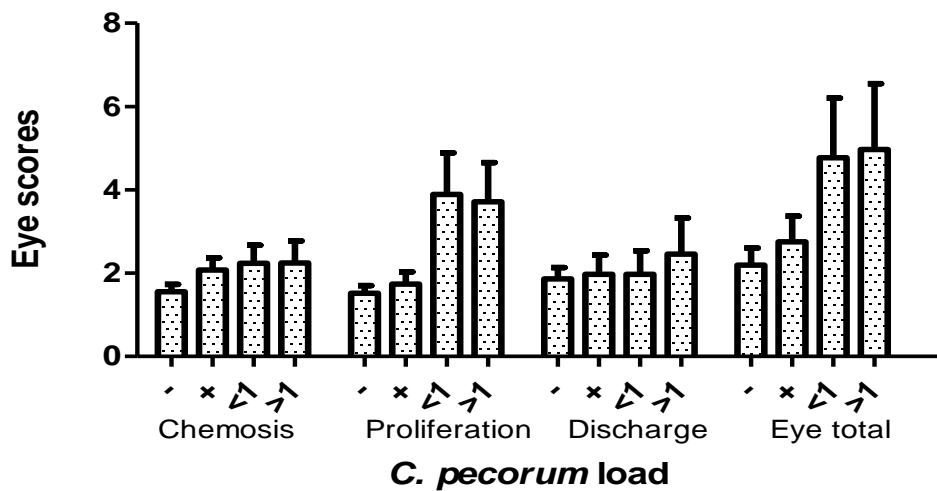


Figure 8-6. Eye scores for 10 koalas throughout fluoroquinolone treatment with respect to relative *Chlamydophila pecorum* load.

Where 1 = an arbitrary standard (*C. pecorum* culture), “-” = negative qPCR, and “+” = positive but not quantifiable. Figures shown are the mean \pm SEM predicted by a restricted maximum likelihood statistical model.



Day 0



Day 7



Day 21



Day 33



Day 42



Day 49



Day 56

Figure 8-7. Rump pelage stain in one koala treated with oral marbofloxacin 10 mg/kg once daily for 31 days then retained for post treatment monitoring.

Wet bottom score was consistently 0.5.

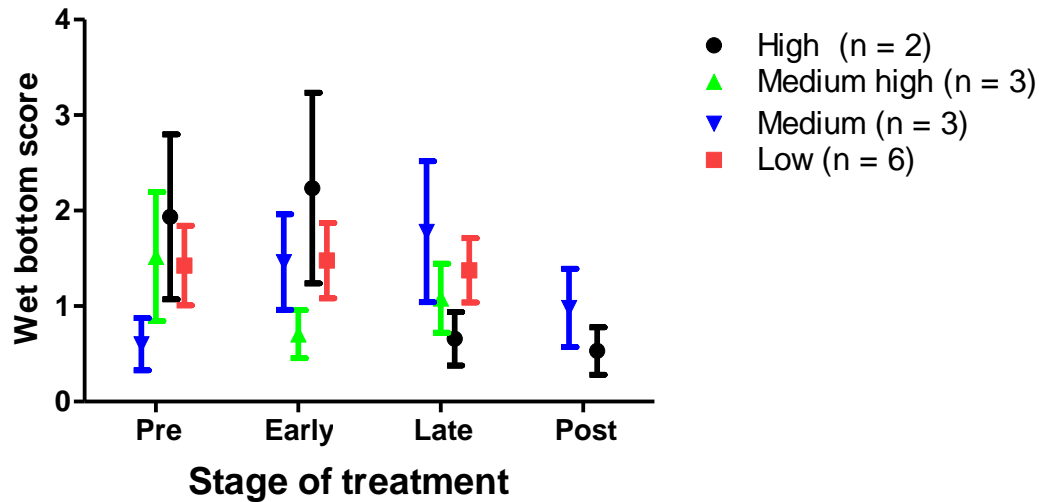


Figure 8-8. Changes in wet bottom score during treatment for groups based on maximum plasma concentration of fluoroquinolones.

High: mean C_{max} 2.15 ± 0.59 $\mu\text{g/mL}$; medium high: mean C_{max} 0.91 ± 0.37 $\mu\text{g/mL}$; medium: mean C_{max} 0.46 ± 0.35 $\mu\text{g/mL}$; low: mean $C_{max} < 0.16$ $\mu\text{g/mL}$. Figures shown are the mean \pm SEM predicted by a restricted maximum likelihood statistical model. Stage of treatment: pre (first screening day to day 0); early (day 1 - day 21); late (day 22 until treatment end; mean day 43 ± 9.5 SD); and post (day 43 until day 71 ± 4.6 SD).

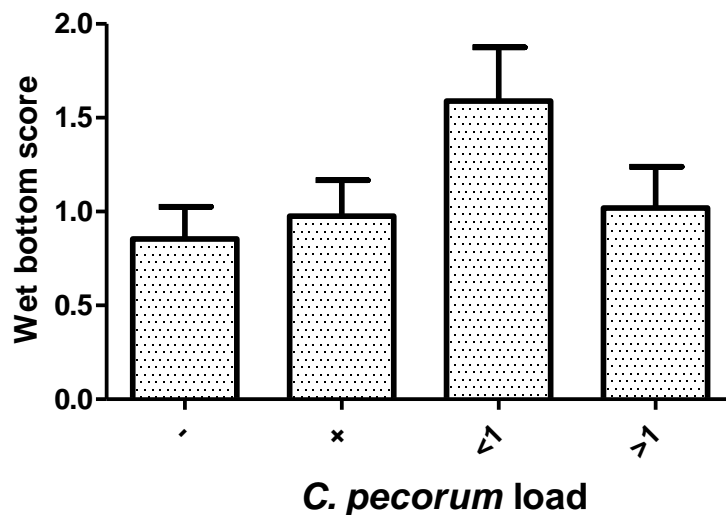


Figure 8-9. Wet bottom score for 22 koalas throughout fluoroquinolone treatment relative to *Chlamydomphila pecorum* load.

Where 1 = an arbitrary standard (*C. pecorum* culture), “-” = negative qPCR, and “+” = positive but not quantifiable. Figures shown are the mean \pm SEM predicted by a restricted maximum likelihood statistical model.

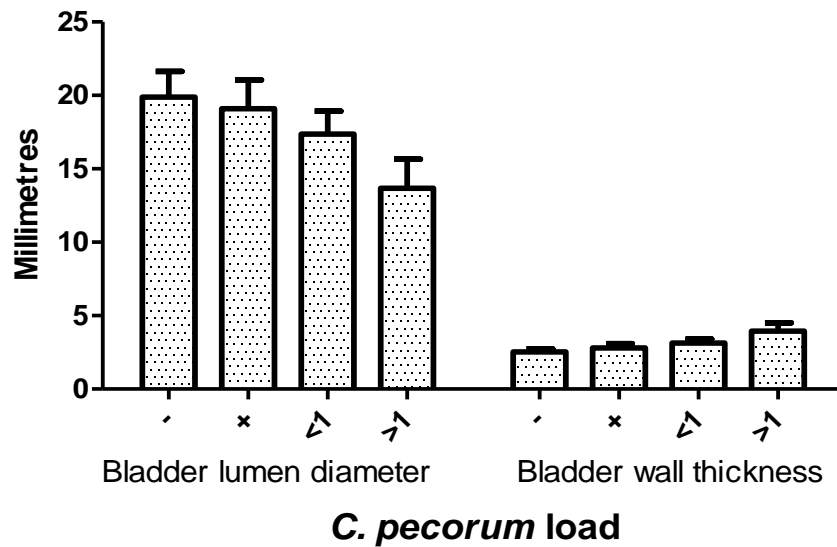


Figure 8-10. Bladder lumen diameter and wall thickness measured using ultrasonography for 23 koalas throughout fluoroquinolone treatment relative to *Chlamydomphila pecorum* load.

Where 1 = an arbitrary standard (*C. pecorum* culture), “-” = negative qPCR, and “+” = positive but not quantifiable. Figures shown are the mean ± SEM predicted by a restricted maximum likelihood statistical model.

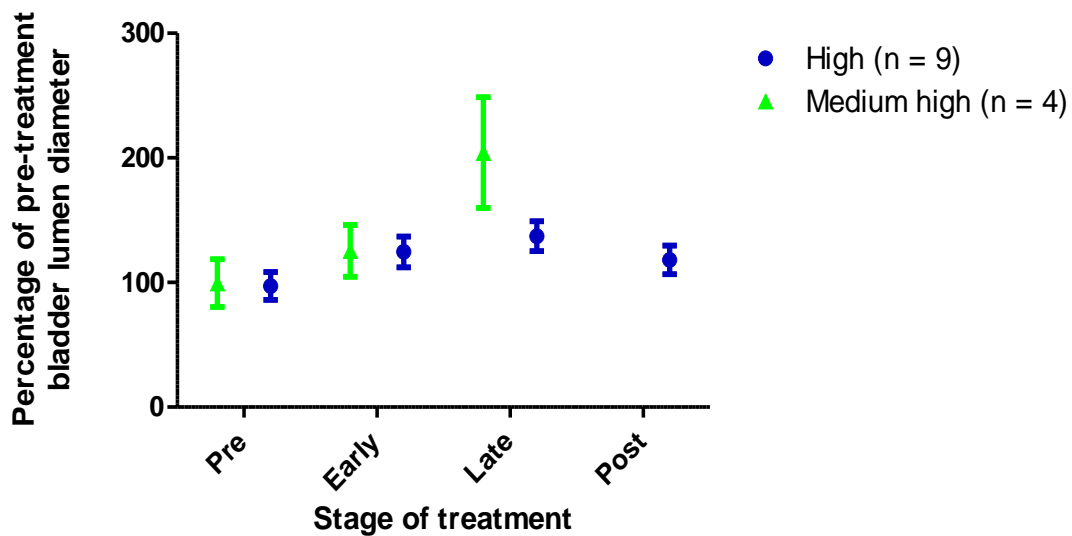


Figure 8-11. Changes in bladder lumen diameter over time compared for groups based on maximum plasma concentration of fluoroquinolones.

High: mean C_{max} $2.15 \pm 0.59 \mu\text{g/mL}$; medium high: mean C_{max} $0.91 \pm 0.37 \mu\text{g/mL}$. Figures shown are the mean ± SEM predicted by a restricted maximum likelihood statistical model. Stage of treatment: pre (first screening day to day 0), early (day 1 - day 21), late (day 22 until treatment end; mean day 43 ± 9.5 SD) and post-treatment (day 43 until day 71 ± 4.6 SD).

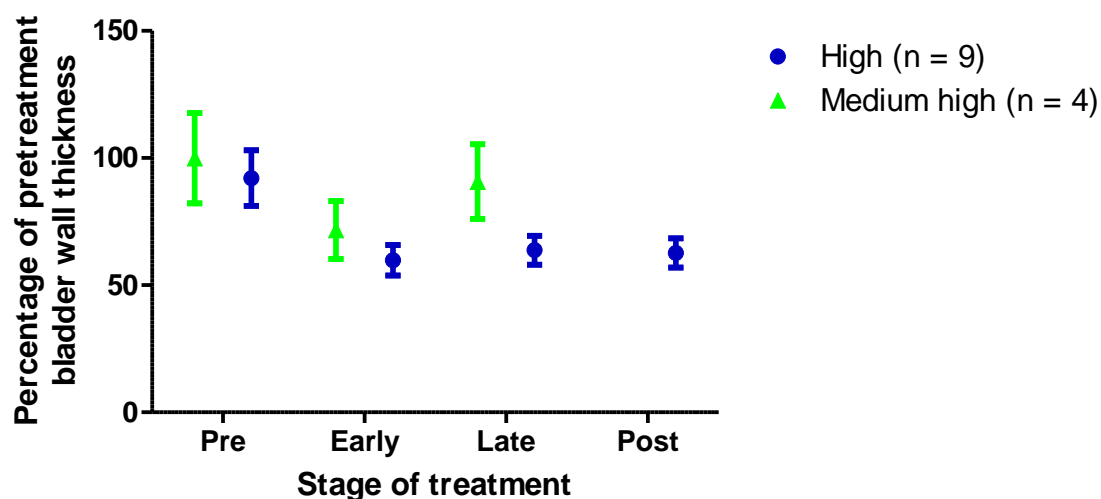


Figure 8-12. Changes in bladder wall thickness compared to pre-treatment measurements for groups based on maximum plasma concentration of fluoroquinolones.

High: mean C_{max} 2.15 ± 0.59 $\mu\text{g/mL}$; medium high: mean C_{max} 0.91 ± 0.37 $\mu\text{g/mL}$. Figures shown are the mean \pm SEM predicted by a restricted maximum likelihood statistical model. Stage of treatment: pre (first screening day to day 0); early (day 1 - day 21); late (day 22 until treatment end; mean day 43 ± 9.5 SD); and post (day 43 until day 71 ± 4.6 SD).

Ultrasonographic abnormalities of the reproductive tract, detected in six animals prior to treatment, did not change throughout treatment (described in more detail in Chapter 5).

Two additional animals, one previously described (for detail see Chapter 5) treated with once daily marbofloxacin 1-3.3 mg/kg p.o. and one treated with once daily enrofloxacin 10 mg/kg s.c., developed ovarian bursal cysts between days 7 and 14 of treatment. At post mortem examination, the latter animal (DA in Appendix IV) had bilateral ovarian bursal cysts, cystic endometrial hyperplasia and mild chronic diffuse vaginitis and ureteritis. A further animal (BB in Appendix IV) developed a 11.2 mm diameter spherical echolucent area in the renal medulla and hydroureter (8.1 mm width) of the left kidney at day 49 that resolved by day 67 (Figure 8-13). At post mortem examination, an area of focal medullary tubular nephrosis was found in the left kidney.

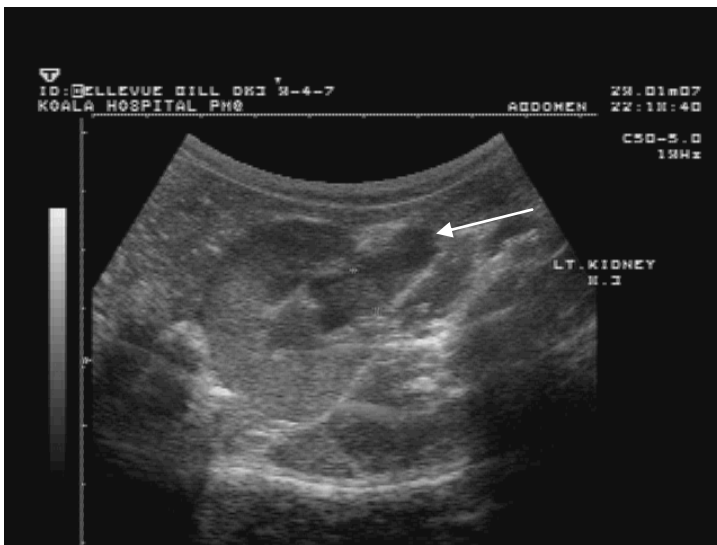
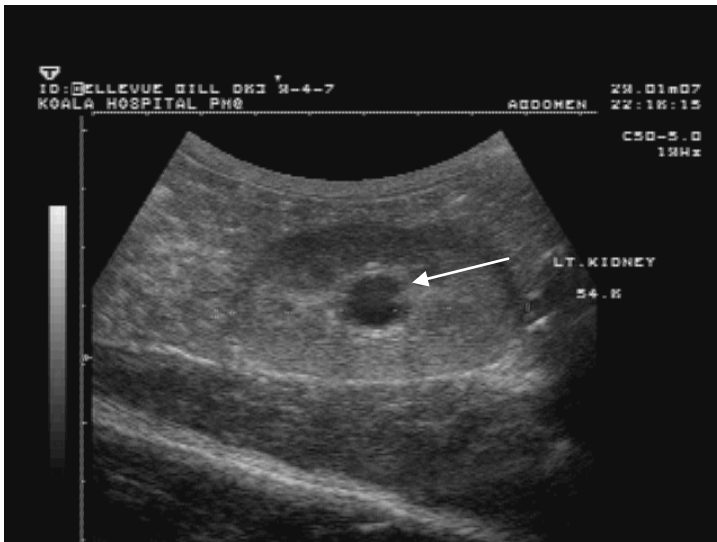


Figure 8-13. Echolucency in the renal medulla (arrow upper sonogram) and hydronephrosis (arrow lower sonogram) that developed between days 49 and 67 in one animal treated with oral marbofloxacin 10 mg/kg once daily for 64 days.

Among all biochemistry analytes and haematological parameters, only albumin related significantly to *C. pecorum* load: albumin was significantly lower in animals in early treatment with higher *C. pecorum* loads ($p < 0.001$, Figure 8-14). Others parameters did not differ with respect to pathogen load and time: most changed less than 10% of starting values and remained within the published reference intervals (Table 8-10). Trends within

these intervals reflected animals improving in health, with haematology parameters consistent with decreased inflammation (platelets, eosinophils), and decreased stress (increasing red cell count, haematocrit, decreasing glucose) and improved nutrition (increasing albumin). Minor changes in electrolytes and creatinine were consistent with improved hydration. Creatinine kinase (CK), aspartate aminotransferase (AST) and alanine transaminase (ALT) activity were elevated during treatment in animals treated by subcutaneous injection ($p < 0.001$; Figure 8-15, Figure 8-16), with creatinine kinase activity exceeding reference ranges.

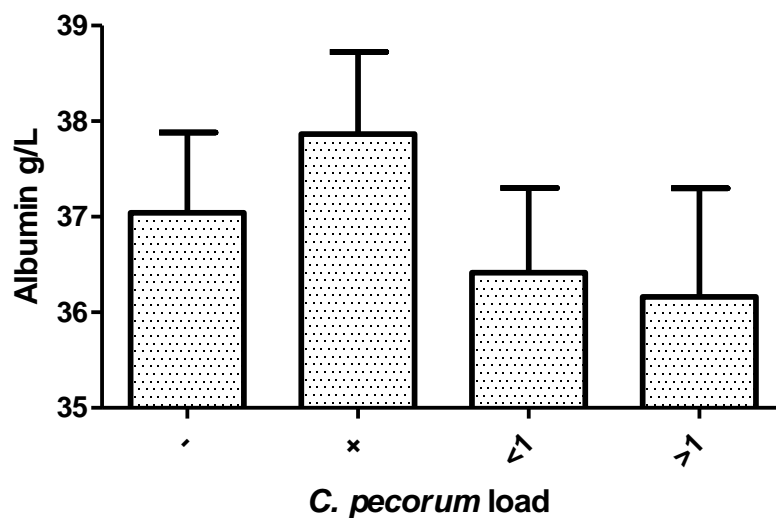


Figure 8-14. Albumin for 29 koalas throughout fluoroquinolone treatment relative to *Chlamydomphila pecorum* load.

Where 1 = an arbitrary standard (*C. pecorum* culture), “-” = negative qPCR, and “+” = positive but not quantifiable. Figures shown are the mean ± SEM predicted by a restricted maximum likelihood statistical model.

Table 8-10. Biochemical analytes and haematological parameters that changed significantly in 29 koalas throughout fluoroquinolone treatment for chlamydiosis.

	Reference intervals	Pre-treatment n = 28	Early Treatment n = 29	Late Treatment n = 29	Post-treatment n = 20	% change (pre/post)	p
Haemoglobin	88 - 140 g/L	110.1 ± 2.39	109.5 ± 2.15	112.9 ± 2.02	115.6 ± 2.2	5%	0.001
Red Cell Count	2.7 - 4.2 x 10 ¹² /L	3.48 ± 0.076	3.5 ± 0.07	3.55 ± 0.07	3.64 ± 0.07	4.5%	0.007
Haematocrit	0.29 - 0.44 L/L	0.36 ± 0.01	0.36 ± 0.01	0.37 ± 0.01	0.38 ± 0.01	5%	0.004
Neutrophils	0.5 - 6.3 x 10 ⁹ /L	3.41 ± 0.29	4.15 ± 0.32	3.36 ± 0.29	3.6 ± 0.35	N/A*	0.010
Eosinophils	< 1.1 x 10 ⁹ /L	0.52 ± 0.09	0.32 ± 0.05	0.40 ± 0.05	0.39 ± 0.06	-23.8%	0.031
Platelets	222 - 508 x 10 ⁹ /L	278.38 ± 13.80	284.29 ± 12.96	259.56 ± 11.12	263.49 ± 12.3	-5.4%	0.007
Glucose	2.7 - 7.2 mmol/L	5.60 ± 0.28	5.05 ± 0.22	4.61 ± 0.17	4.68 ± 0.21	-16.4%	< 0.001
Albumin	34 - 50 g/L	35.48 ± 0.73	36.71 ± 0.72	37.86 ± 0.71	38.94 ± 0.77	9.7%	< 0.001
Calcium	2.28 - 2.97 mmol/L	2.62 ± 0.029	2.63 ± 0.03	2.67 ± 0.02	2.68 ± 0.03	2.25%	0.042
Creatinine	80 - 150 µmol/L	97.81 ± 3.64	94.54 ± 3.33	92.11 ± 3.10	91.01 ± 3.27	-6.85%	0.018
Cholesterol	1.15 - 3.09 mmol/L	1.7 ± 0.07	1.83 ± 0.07	1.98 ± 0.06	2.05 ± 0.07	21.2%	< 0.001
Triglycerides	0.5 - 3.02 mmol/L	1.59 ± 0.13	1.67 ± 0.11	1.72 ± 0.09	2.03 ± 0.12	28.2%	0.003
Sodium	132 - 145 mmol/L	141.2 ± 0.66	139.7 ± 0.59	140.0 ± 0.51	140.8 ± 0.61	N/A*	0.049
Chloride	93 - 107 mmol/L	99.94 ± 0.69	98.13 ± 0.62	98.98 ± 0.53	100.00 ± 0.63	N/A*	0.009

Figures shown are the mean ± SEM predicted by a restricted maximum likelihood statistical model. Pre-treatment (first screening day to day 0), early treatment (day 1 until day 21), late treatment (day 22 until treatment end; mean day 43 ± 9.5 SD) and post-treatment (day 43 until day 71 ± 4.6 SD). Creatinine kinase activity is shown in Figure 8-15 and ALT and AST activity is shown in Figure 8-16.

*Statistically significant at time points other than between pre- and post-treatment.

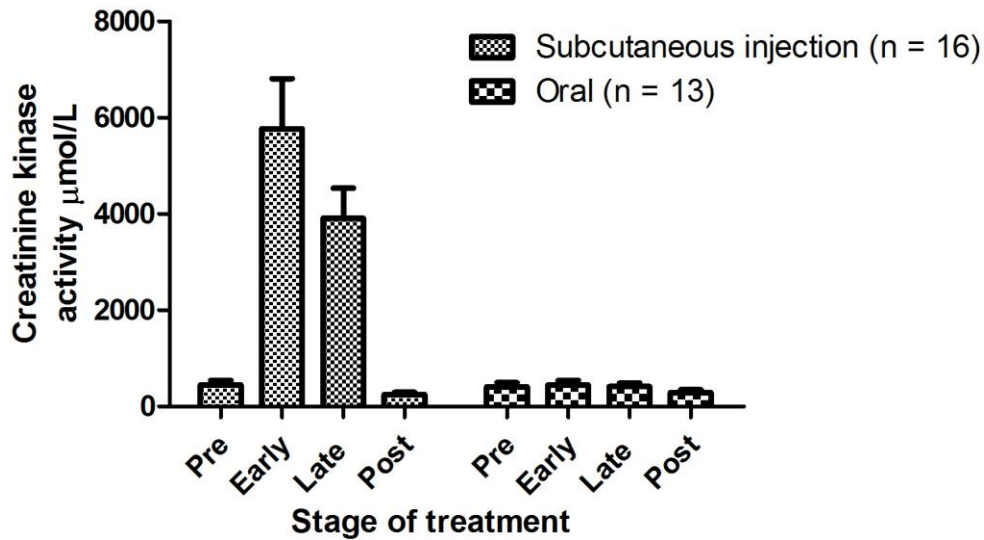


Figure 8-15. Change in creatinine kinase activity for 29 koalas throughout fluoroquinolone treatment relative to route of drug administration (subcutaneous injection, oral).

Stage of treatment: pre (first screening day to day 0); early (day 1 - day 21); late (day 22 until treatment end; mean day 43 ± 9.5 SD); and post (day 43 until day 71 ± 4.6 SD). Figures shown are the mean \pm SEM predicted by a restricted maximum likelihood statistical model.

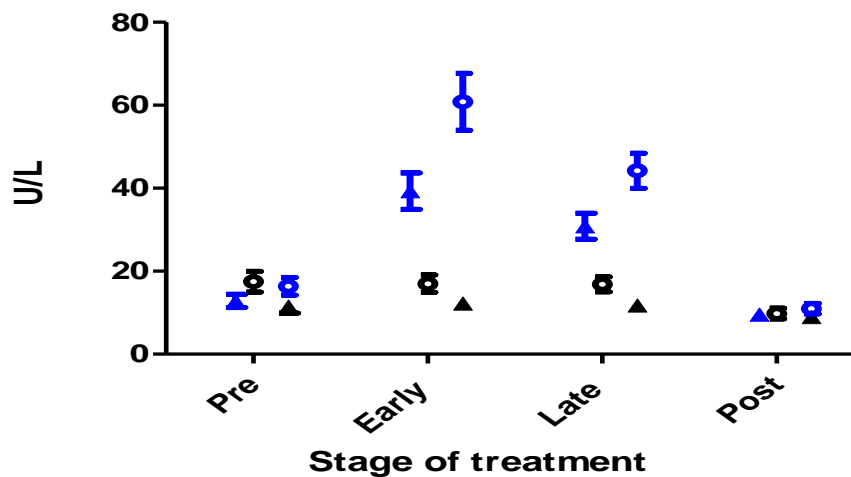


Figure 8-16. Changes in ALT and AST activity for 29 koalas throughout fluoroquinolone treatment relative to route of drug administration.

ALT (▲) and AST (○); Oral, (n = 13), shown in black; subcutaneous injection, (n = 16) shown in blue. Stage of treatment: pre (first screening day to day 0); early (day 1 - day 21); late (day 22 until treatment end; mean day 43 ± 9.5 SD); and post (day 43 until day 71 ± 4.6 SD). Figures shown are the mean \pm SEM predicted by a restricted maximum likelihood statistical model.

Appetite, weight and faecal output of koalas also indicated improving health during treatment. All koalas completing treatment maintained body mass, a robust appetite and normal faecal output throughout treatment. Appetite scores as well as faecal area and score all increased through pre-treatment, early treatment and late treatment ($p = 0.017$, appetite score; $p < 0.001$, faecal area and score; Figure 8-17, Figure 8-18).

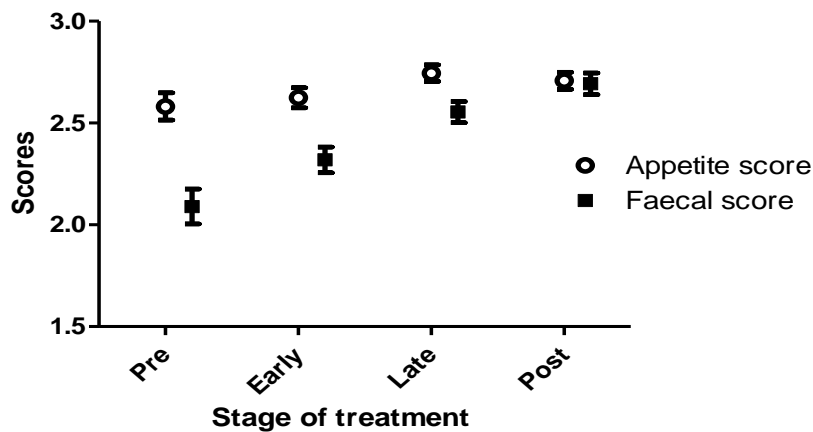


Figure 8-17. Changes in appetite and faecal scores for 29 koalas throughout fluoroquinolone treatment.

Stage of treatment: pre (first screening day to day 0); early (day 1 - day 21); late (day 22 until treatment end; mean day 43 ± 9.5 SD); and post (day 43 until day 71 ± 4.6 SD). Figures shown are the mean \pm SEM predicted by a restricted maximum likelihood statistical model.

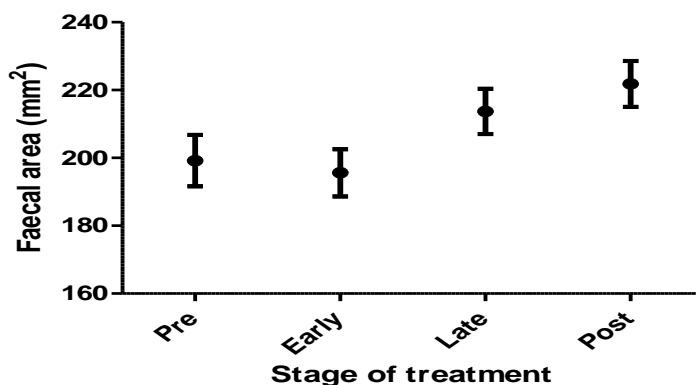


Figure 8-18. Changes in faecal area for 29 koalas throughout fluoroquinolone treatment.

Stage of treatment: pre (first screening day to day 0); early (day 1 - day 21); late (day 22 until treatment end; mean day 43 ± 9.5 SD); and post-treatment (day 43 until day 71 ± 4.6 SD). Figures shown are the mean \pm SEM predicted by a restricted maximum likelihood statistical model.

8.3.3 Presence of chlamydial organisms after treatment

After withdrawal of treatment, pathogen load increased significantly ($p < 0.001$; Figure 8-3). Twenty-four of the 29 treated koalas shed *C. pecorum* ($n = 14$) or *C. pecorum* and *C. pneumoniae* ($n = 10$) at the end of treatment or at least once during post-treatment monitoring. Of the remaining five, three were not monitored for recurrence following removal of treatment and two (6.8%) remained negative for *C. pecorum* at all three sites through post-treatment monitoring.

8.3.4 Final outcomes of animals enrolled in the study

Of 29 animals treated with fluoroquinolones, 16 were released and the remaining animals were euthanased due to non-resolution or return of dysuria and stranguria ($n = 3$), non-resolution ($n = 6$) or development of ovarian bursal cysts ($n = 2$), or subsequent treatment with doxycycline and decline in appetite, weight and demeanour ($n = 2$) (described in more detail in Chapter 5). Most post mortem findings related to chronic inflammation and fibrosis of the urogenital tract, consistent with chlamydial disease (for detail see fluoroquinolone treated animals in Appendix II and all animals in Appendix IV).

8.4 DISCUSSION

This study is the first to investigate the response of koalas with chlamydiosis to treatment with marbofloxacin and enrofloxacin and to assess the validity of commonly used methods for diagnosis of chlamydial infection in the clinical setting. Important findings were: the poor sensitivity of commonly observed clinical signs in determining presence of chlamydial organisms; correlation between ocular pathogen load and some clinical signs; improvement of ocular clinical signs during hospitalisation regardless of treatment modality; reduction of mucosal *C. pecorum* load during treatment and corresponding

changes in some urogenital clinical signs in the treatment group expected to have the highest C_{\max} ; and the rebound in mucosal *C. pecorum* load after treatment withdrawal, illustrating the failure of most animals to achieve microbial cure using these drugs.

The poor sensitivity of clinical signs as indicators of chlamydial infection (72% or less) signifies that their use as a diagnostic tool should be revised because a significant subset of positive animals are being missed using this method alone. However, specificity of clinical signs as an indicator of *C. pecorum* at an anatomical site was 100%, indicating that past assumptions leading to treatment of clinically affected animals are likely to have been correct. The lack of sensitivity of clinical signs in detecting chlamydial infection is perhaps not surprising. Previous authors have described subclinical infections in superficially healthy koalas (White & Timms, 1994; Jackson, White *et al.*, 1999; McLean, 2003), birds (Longbottom & Coulter, 2003), calves (Jaeger, Liebler-Tenorio *et al.*, 2007), people (Solomon, Peeling *et al.*, 2004) and cats (Sykes, 2005), but the current study is the first to quantify sensitivity and specificity of clinical signs in chlamydiosis in koalas within a clinical setting. It is important to note that, although sensitivity and specificity do not change, the predictive value of these signs as indicators of infection will vary with prevalence in the general hospital population (currently unknown).

The poor sensitivity of clinical signs in predicting chlamydial carriage and the current lack of sensitive, cheap, point-of-care diagnostic tests (Chapter 5) necessitates that all wild animals be assumed to be carrying chlamydial organisms regardless of clinical signs in relation to quarantine and transfer. Animals should not be transferred into known negative populations or outside of their local range, where chlamydial species, strains, and infection prevalence may be different, until proven negative with repeated negative tests using sensitive molecular techniques. As koala strains of *C. pneumoniae* may survive for up to

28 days at ambient temperatures (18 °C - 23 °C) (Rush & Timms, 1996), strict attention to disinfection of pens and change of cage furniture between all animals, regardless of clinical signs, will minimise risk of transmission on fomites. Fly control may also prevent mechanical transmission of organisms attracted to ocular discharge, as occurs in people (Emerson, Lindsay *et al.*, 1999).

The reasons why clinical signs were less sensitive in predicting mixed infections than single *C. pecorum* infections are unclear. It is possible the presence of *C. pneumoniae* may provide some level of cross-protection against clinical disease associated with *C. pecorum*; however in people, presence of multiple Chlamydiaceae species has been associated with more severe clinical signs of ocular disease and the role of mixed infections in pathogenesis is poorly understood (Dean, Kandel *et al.*, 2008). In koalas, the situation is potentially complicated further by the possible co-infection by at least nine “*Chlamydia*-like” bacteria (Devereaux, Polkinghorne *et al.*, 2003) and their role in the pathogenesis of chlamydiosis is currently unknown. *Chlamydophila pneumoniae* has been demonstrated in a variety of lesions associated with chronic diseases in people (Jackson, Campbell *et al.*, 1997; Sriram, Stratton *et al.*, 1999) but its exact role in the pathogenesis of disease can be difficult to interpret due to possible recruitment of chlamydially infected macrophages to sites of inflammation elicited by other causes (Gieffers, Pohl *et al.*, 2001; Robinson, Dobson *et al.*, 2004; Bagos, Nikolopoulos *et al.*, 2006). Clearly much work is required to understand the syndemics of these pathogens in koalas.

In this study, animals exhibiting signs of ocular chlamydiosis were also likely to have a urogenital tract infection, which was frequently subclinical (Table 8-9). Chlamydial species have spread systemically from inoculation sites in infection trials in other species (sheep and cats) (Wills, Gruffydd-Jones *et al.*, 1987; TerWee, Sabara *et al.*, 1998; Tsakos,

Siarkou *et al.*, 2001) and a proposed mechanism of systemic spread of *C. pneumoniae* in koalas and people is infection of, and circulation within, peripheral blood mononuclear cells (Bodetti & Timms, 2000). The likelihood of systemic or multi-site subclinical infection suggests that treatment of all chlamydial cases should be systemic.

The lack of site preference for *C. pneumoniae* and urogenital site preference for *C. pecorum* contrasts with findings of other authors, who have reported an ocular bias of *C. pneumoniae* infections and lack of site bias for *C. pecorum* (Girjes, Hugall *et al.*, 1993; Jackson, White *et al.*, 1999). Further, the rate of mixed infectious found here is higher than other populations (Jackson, White *et al.*, 1999; Devereaux, Polkinghorne *et al.*, 2003). These findings may be related to regional differences in pathogen strain and prevalence between this study population and others previously examined (Jackson, White *et al.*, 1999), different methodologies influencing preferential DNA amplification (nested PCR versus species-specific qPCR), the result of stochastic error as study populations were small, or could indicate a difference in the likelihood of systemic spread between the species; a possible mechanism of systemic spread in peripheral blood mononuclear cells has been demonstrated for *C. pneumoniae* (Bodetti & Timms, 2000) but is yet to be demonstrated for koala strains of *C. pecorum*.

The unsatisfactory nature of wet bottom score for monitoring response to treatment may have several explanations. Up to seven different operators scored animals throughout three field seasons, making consistent scoring difficult. The score has multiple factors within each category. This made classification of animals with clinical signs spanning several different categories challenging. The wet bottom score also places much emphasis on smell, which is highly subjective; some aspects may not be observed if intermittent (e.g. stranguria, teeth grinding); and some, such as rump pelage stain, do not change probably

until complete hair regrowth (the period of which currently unknown). Most animals in advanced stages of wet bottom were excluded from this study for other reasons (e.g. poor body condition, renal insufficiency, ovarian bursal cysts), meaning most animals with a wet bottom score of > 0 were in the lowest grades of the wet bottom score and movement within these grades was difficult to assess. This score is used most frequently at the Koala Hospital to assess animals at admission for euthanasia or treatment, rather than as a monitoring tool (C. Flanagan, pers. comm.)¹. Examination of its use as a tool for assessing response to treatment was included in this study, in part, to ease integration of researchers in an established hospital system. The difficulty encountered in its use here suggests that a useful addition to the monitoring of treatment of urogenital tract disease in koalas might be the development of a scoring system for urogenital tract clinical signs with multiple individually scored facets, such as the author developed in this work for eye disease.

In this study, clinical scores associated with higher chlamydial load (proliferation, total eye scores, bladder wall thickness) may indicate active inflammation. In contrast, others, such as the wet bottom score, may best predict chronic inactive inflammation. In people and koalas, chlamydial loads in association with inactive chronic pathology (e.g. fibrosis) tend to be lower than in patients with more active inflammation (Solomon, Peeling *et al.*, 2004; Higgins, Hemsley *et al.*, 2005a; Gomes, Borrego *et al.*, 2006). In this study, animals with clinical urogenital tract disease (wet bottom score > 0) had thicker bladder walls and smaller bladder lumens but did not have high pathogen loads. If the thicker bladder walls observed were due to inactive chronic pathology such as fibrosis, normal bladder function may have been altered in these animals contributing to incontinence and rump pelage soiling with urine and, thus, the observed elevated wet bottom scores. As the bladder wall

¹ Personal communication: C. Flanagan, Supervisor, Koala Hospital, Port Macquarie, NSW, January, 2010.

thickness did decrease during treatment in the high group, it is possible that the change in thickness of bladder wall observed in some animals was a result of resolving acute inflammation (e.g. oedema), comparable to the changes in chemosis observed during treatment in animals with conjunctivitis. As it was not possible to differentiate between oedema and fibrosis using ultrasound alone in this study, future work examining matched sonograms, bladder wall biopsy and urogenital pathogen load might be useful to better describe associations between wet bottom score, sonographic changes, pathogen load and pathological processes. A further potential limitation of ultrasonographic evaluation of the bladder wall is that the bladder wall thickness may vary depending on lumen volume (Geisse, Lowry *et al.*, 1997). It is possible that animals with higher wet bottom scores were more likely to have an empty bladder and, consequently, thicker bladder walls at imaging. Dynamic bladder studies, in which elasticity is measured through instillation of increasing volumes of saline, might help differentiate whether bladder walls are thickened as a result of pathology or simply the result of urination prior to imaging.

Absence of an association between ocular discharge and chlamydial load suggests that although discharge is a dramatic clinical sign, its presence should not necessarily be regarded as indicative of a high chlamydial load. As eyes were cleansed with saline daily on welfare grounds, it is possible that the constant removal of ocular discharge confounded this parameter, or that discharge was associated with secondary bacterial infections. A study in which discharge is not removed with saline and culture for secondary bacterial infections is performed might be useful.

Although many of the structural changes detected in this study using ultrasonography were unlikely to improve during treatment, the results presented here indicate that monitoring of the genitourinary tract in animals under treatment is warranted to detect emerging

structural changes that might alter the prognosis for release. Although weekly monitoring of the urogenital tract indicated few changes in most animals, it detected changes in the renal medulla and ureter of one animal and the development of ovarian bursal cysts in two animals during treatment (see Chapter 5 and above). Structural reproductive tract changes such as ovarian bursal cysts have been associated with infertility (Brown, Carrick *et al.*, 1984; McLean, 2003) and significant renal disease may compromise long-term survival in the wild. Assessment of these structures prior to commencing treatment, and again prior to release, is important for successful rehabilitation.

It is perhaps unsurprising that clear associations between different clinical signs and pathogen load were not always evident, as the interaction between host, environment and pathogen that manifests in clinical signs is frequently complex. Factors that have been proposed to change clinical expression of chlamydiosis in koalas and other species include the pathogenicity of the infective chlamydial strain (Morre, Rozendaal *et al.*, 2000), environmental or nutritional stressors (Weigler, Girjes *et al.*, 1988; Canfield, 1989), presence of other pathogens such as koala retrovirus (Tarlinton, Meers *et al.*, 2005) or “*Chlamydia*-like organisms” (Devereaux, Polkinghorne *et al.*, 2003), secondary infections (Canfield, 1989) and the immune response (Higgins, Hemsley *et al.*, 2005a). Investigation of these would be useful in further characterising the expression of clinical chlamydiosis in koalas.

Changes in haematological parameters and biochemistry analytes were minor and within reference ranges, which might indicate the need for better ranges, but when interpreted longitudinally they suggest a rising plane of health. Of all parameters measured, low albumin was the only parameter significantly associated with higher *C. pecorum* loads. Previous authors have also found hypoproteinaemia or hypoalbuminaemia to be a feature

of complicated cystitis (Obendorf, 1983; Canfield, O'Niell *et al.*, 1989). Together with the observed increase in appetite and faecal output, these changes suggest improving health; however it is difficult to determine whether this occurred as a result of stress, positive energy balance (food supplied, exercise restricted) or habituation to captivity. The increase in ALT, AST and CK activity noted in animals receiving injections are likely to be related to local myopathy as a result of injection (Fry, Allen *et al.*, 1994). Although ALT and AST could indicate hepatocellular damage, this is considered unlikely as other indicators of hepatopathy (bilirubin, bile acids, GGT activity) were not elevated and there was no evidence of hepatopathy in hepatic biopsies taken at post mortem. Alanine transaminase (ALT), AST and CK activity interpreted in concert may indicate myopathy more sensitively than CK activity alone (Fry, Allen *et al.*, 1994) and thus it is likely the elevations of ALT and AST also indicated injection site myopathy.

The rebound of *C. pecorum* load after removal of treatment strongly indicates a drug effect on chlamydial load, and the clinical impact of this is suggested by reduction in percentage bladder wall thickness and increase in percentage bladder lumen that occurred only in the high group. However, without a placebo group (precluded here by ethics and concern for animal welfare) it is impossible to accurately determine what degree of clinical improvement was attributable to drug treatment or supportive therapy, or if chlamydial infections resolved regardless of treatments. Clinical signs of ocular chlamydiosis in koalas may be self-limiting (Kempster, Hall *et al.*, 1996), and the acute phase of ocular chlamydial infection in koalas (Kempster, Hall *et al.*, 1996), cats (Dean, Harley *et al.*, 2005) and people (Thygeson, 1962) (six weeks), falls within the study period used in this work. Self-limiting disease could explain at least some of the improvement in clinical signs seen in many animals in this study and the reported historical success of fluoroquinolones

in treating koala chlamydiosis (Connolly, 1999; Blanshard & Bodley, 2008) (Chapter 3).

That an antibiotic effect was observed during this study, despite plasma concentrations failing to reach a minimum inhibitory concentration (MIC) considered likely to be inhibitory to koala chlamydial species (Chapter 7), could be due to a number of reasons. The MIC of koala chlamydial species might be lower than expected, or plasma concentrations of fluoroquinolones may not accurately represent those to which pathogens are exposed. The MIC for marbofloxacin has not been determined for chlamydial species. The assumed MIC required to inhibit growth of chlamydial pathogens from koalas is based on studies of enrofloxacin in *C. pneumoniae* isolates from bandicoots (Kumar, Kutlin *et al.*, 2007), which share 99.5% genetic homology with the koala biovar in a 16S rRNA gene signature segment (Kutlin, Roblin *et al.*, 2007). This assumption seems likely to be valid, as the MICs of enrofloxacin for other chlamydial species of veterinary interest are in broad agreement (0.125 - 0.25 µg/mL) (Lindenstruth & Frost, 1993; Butaye, Ducatelle *et al.*, 1997; Failing, Theis *et al.*, 2006). It appears more probable that plasma concentrations may not accurately represent those to which pathogens are exposed *in vivo*. Fluoroquinolones may accumulate at up to 63 times plasma concentrations within cells (Boeckh, Boothe *et al.*, 1999; Boothe, Jones *et al.*, 2005), tissues (Bayer Animal Health Australia, 2010; Pfizer Inc, 2010), and body fluids such as urine (Bayer Animal Health Australia, 2010). In addition, they may be transported to sites of active inflammation in white blood cells (Boothe, Boeckh *et al.*, 2009). Investigation of the MIC of fluoroquinolones required to inhibit koala species of Chlamydiaceae and assay of fluoroquinolone concentration of chlamydially infected tissues would be helpful in investigating these hypotheses.

The lack of elimination of chlamydial infections in this species following extended courses of fluoroquinolones is not completely new: treatment failure of koalas treated for up to 6

months with enrofloxacin or chloramphenicol have been described (Devereaux, Polkinghorne *et al.*, 2003), although dose and route were not reported. Chlamydial antibiotic resistance would explain the failure to achieve microbial cure, but is rare (Yokoi, Yasuda *et al.*, 2004). More likely hypotheses are the lack of effectiveness of the drug against koala chlamydial strains, and/or poor bioavailability (Chapter 7).

Another intriguing explanation for treatment failure is that the low plasma concentrations of antibiotics in this study led to survival of small numbers of chlamydial organisms in an “intermediate form” allowing persistent infections. Stressors such as cytokines, phage infections, deficiency of essential nutrients and sub-inhibitory concentrations of antibiotics, including fluoroquinolones, may induce a quiescent “intermediate” form in which chlamydial organisms exhibit abnormal inclusion morphology and are metabolically inactive. Once stressors are removed, normal chlamydial lifecycle resumes (Hogan, Mathews *et al.*, 2004; Mpiga & Ravaoarino, 2006). Although “intermediate” forms have DNA that is detectable using molecular techniques (Mpiga & Ravaoarino, 2006) during treatment cells infected with “intermediate” forms may be few in number (Drees-Werringloer, Padubrin *et al.*, 2000; Kutlin, Roblin *et al.*, 2002), or may be absent from the anatomical sites tested, leading to a false negative, as has been observed during treatment in studies of cats treated with pradofloxacin (Hartmann, Helps *et al.*, 2008) and doxycycline (Dean, Harley *et al.*, 2005). As many antibiotics are reliant on metabolic activity to be effective, such infections are difficult to resolve by antibiotics (Mpiga & Ravaoarino, 2006). The presence of this altered phase of development *in vivo* is difficult to demonstrate, but supportive evidence is provided by *in vitro* studies, and *in vivo* when other reasons for treatment failure (poor compliance, poor drug bioavailability, chlamydial resistance) are ruled out (Morrison, 2003; Mpiga & Ravaoarino, 2006).

Chlamydial load detected after treatment withdrawal remained low in comparison to starting load, and clinical signs did not return in most animals, illustrating that monitoring for the return of clinical signs for up to four weeks is not a sensitive indicator of microbial negativity. Clinical signs post-treatment might be more likely to recur in animals starting with high chlamydial load, as occurred in one case in this study.

An important outcome of this study is the opportunity to re-evaluate the goals of treating infectious disease in the rehabilitation setting. Survival rates after treatment of chlamydiosis with fluoroquinolones appear to be acceptable, although the fertility of rehabilitated animals is unknown (Chapter 3). The fact that rehabilitated animals are released carrying chlamydial organisms is an important consideration for the epidemiology of this disease in the wild population. It is likely that most koalas treated for chlamydiosis have been released without achieving microbial cure in the Port Macquarie district for the history of the Koala Hospital (35 years). The impact of these animals is unknown, but appears to have been minimal, as there is no indication that probability of admission because of chlamydiosis has increased over time (Chapter 2). However, it is possible that release of animals post-rehabilitation has been deleterious, as the chlamydial prevalence in the population prior to the establishment of the Koala Hospital is unknown. Release of animals that are microbially positive may not, by itself, be detrimental to a population in which there is a high prevalence of chlamydial carriage but, should animals be infertile and habitat scarce, their survival may be detrimental to local populations. Infertile animals will continue to occupy scarce habitat, predate trees and act as effective reservoirs of Chlamydiaceae, especially if infertility increases their promiscuity. Recapture studies of treated animals after release, examining females for presence of pouch young, or larger studies examining medical records held at the Koala Hospital could provide valuable

information regarding the successful rehabilitation and breeding success of these animals within Port Macquarie. Animals being transferred into fauna parks or from Port Macquarie should not be assumed microbially negative based on lack of clinical signs, Clearview results (Chapter 5), or after treatment with enrofloxacin or marbofloxacin for clinical chlamydiosis.

This study provides an evidence base for decisions relating to the treatment of koala chlamydiosis with fluoroquinolones. The poor sensitivity of commonly observed clinical signs for detecting chlamydial infection suggests a revision of commonly used diagnostic or case assessment practices. The improvement of clinical signs regardless of treatment modality provides strong support for the implementation of a blinded, placebo controlled study to determine the relative importance of husbandry and fluoroquinolones in changing pathogen load and resolution of clinical signs. The failure of most animals to achieve microbial cure using these drugs indicates that animals treated with doses and routes used in this study should not be regarded as microbially negative after treatment, and thus should not be transferred into microbially negative populations or those with different chlamydial strains. Assessment of the treatment goals of chlamydiosis in koalas should be re-evaluated. The prognosis and breeding success of these animals once released is a critical factor that requires examination, both for individuals and for appropriate resource allocation within the rehabilitation setting, and to allow accurate statistical modelling assessing the impact of treated animals on wild populations.

CHAPTER 9 - OVERVIEW AND GENERAL IMPLICATIONS

Despite wildlife rehabilitation in Australia involving many thousands of people and tens of thousands of animals annually, there is little formal research regarding how such rehabilitation is undertaken, whether it is successful and its impact on wild populations (Tribe & Brown, 2000). Although the direct contribution of wildlife rehabilitation to the conservation of species is debated, less quantifiable benefits, such as public education and public sympathy for the plight of wildlife, provide justification for wildlife rehabilitation as part of species conservation plans (Tribe & Brown, 2000). Studies of rehabilitation efforts are crucial to identify successful techniques on grounds of welfare and to advance knowledge of husbandry and diseases of wild animals. Research of wildlife rehabilitation techniques is further justified to minimise the risk of rehabilitated animals as a source of communicable disease to individuals (Barker, 1974; Griffin, Canfield *et al.*, 1983; Menzano, Rambozzi *et al.*, 2008) and wild populations (Jacobson, Gaskin *et al.*, 1991; Chipman, Slate *et al.*, 2008.) and of zoonotic disease to people (Allworth, Murray *et al.*, 1996).

A study examining the records of the Koala Hospital over 30 years (Chapter 2) identified the most important potential threats to the local koala population as those involving traumatic injuries, principally motor vehicle strikes. The temporal and demographic patterns identified in this work suggest that a significant behavioural component is important in predisposing different cohorts to traumatic presentations and this must be taken into account when planning for threat mitigation. Although the total extinction of koalas may not be imminent, populations in south-east Queensland have declined sharply in recent years and populations in both New South Wales and Queensland may be vulnerable to local extinction should current trends continue (Department of Environment

and Climate Change - NSW, 2008; Department of Environment and Resource Management, 2009).

Over-abundant populations of koalas in Victoria are often of poor genetic merit due to inbreeding (Houlden, England *et al.*, 1996; Houlden, Costello *et al.*, 1999), thus the preservation of populations in New South Wales and Queensland is important to maintain genetic diversity. Studies such as this one demonstrate the potential value of records kept at wildlife institutes and it is hoped this will provide impetus to improve and streamline record keeping at such organisations, which will in turn improve the rigour of future work.

The studies of medical records of the Koala Hospital in Chapter 2 identified a common reason for presentation to be clinical signs associated with chlamydiosis. Effective treatments are yet to be identified for this disease and are required for treatment of individual animals, or where translocation between populations of different infective status is required. Despite the paucity of well-defined evidence-based studies of effective treatments for this disease, treatment has been attempted for decades and is anecdotally successful. This background prompted a study of medical records of animals treated for chlamydiosis over a 10- year period at the Koala Hospital (Chapter 3) to identify commonly used medications, treatment regimes associated with successful return to the wild, and the long-term prognosis of animals returned to the wild. This work found the most commonly used treatments were not consistent with those recommended for treatment of chlamydiosis in other species, and diagnostic and treatment decisions were frequently based on clinical signs alone, despite past studies indicating these as poorly sensitive in predicting chlamydial carriage (Jackson, White *et al.*, 1999; McLean, 2003). Despite theoretically inadequate treatments and insensitive diagnostics, clinical signs improved in approximately 50% of treated cases, such that they were returned to the wild, and at least 50% of these animals survived for extended periods. The apparent contradictions between theoretically

inadequate treatment regimes and successful resolution of clinical signs and survival in the wild provided the basis for further studies examining treatments of chlamydiosis in koalas (Chapters 5-8).

Chapters 4 and 5 describe the development of field diagnostics and clinical monitoring techniques used in later studies and detail a learning experience for the author regarding the types of studies possible within a wildlife rehabilitation setting. The difficulties encountered regarding these aspects were mainly logistical: an inadequate amount of appropriate housing and insensitive diagnostic techniques initially limited the numbers recruited. During the initial stages of the project, these obstacles were overcome through development of a field site diagnostic PCR for diagnosis of chlamydiosis in koalas and input into the building of koala housing that will be useful for future research.

Chapter 5 describes a pilot study that found that drugs commonly used to treat chlamydiosis in other species were not safe to use in koalas. The cause of the severe side-effects observed in most animals treated with azithromycin or doxycycline was not definitively determined, but may have been due to gut flora disturbance, as has been described by other researchers with previous generations of these drugs (Osawa, Bird *et al.*, 1993). The current study also highlighted the difficulties of working with an iconic species within a wildlife rehabilitation setting. A somewhat unanticipated difficulty encountered was the complicated emotional involvement of lay carers with the experimental animals. The long courses of treatment administered to animals with poor prognosis for release were emotionally draining for lay workers and prompted a re-evaluation of treatment goals. The author learnt much about what types of studies are appropriate within a rehabilitation setting and about communicating the scientific process to the general public. Concern for animal and staff welfare changed the initial focus of the

scientific investigation to one examining fluoroquinolones, in which side effects had not been observed in initial studies.

Chapter 6 describes a pilot study using a modified agar diffusion method, which was found to be too insensitive to accurately detect antibiotic concentrations within plasma of koalas treated with fluoroquinolones. Working with wildlife often requires the adaption or modification of techniques used in other species and thus a more sensitive detection method (HPLC) was optimised and validated for use in Chapter 7. Difficulties were the limited time points at which blood was taken and, as animals were recruited opportunistically from diseased wild animals, lack of uniformity of experimental subjects with respect to signalment or disease status. Although the former decreased the ability to calculate some pharmacokinetic parameters, and the latter contributed to the wide inter-animal variation observed, these limitations did not alter the major conclusions regarding poor absorption of fluoroquinolones administered by the oral route in diseased koalas. The results of this study offer a significant advancement in the treatment of koalas, as the oral route is frequently used for drug administration. This study, the first of its kind in koalas, provides a sound basis and justifies future research efforts in this area, both on pharmacokinetics of other therapeutic drugs, and the mechanisms by which koalas deal with xenobiotics of therapeutic and dietary origin.

In Chapter 8, clinical signs of disease improved and *C. pecorum* loads decreased during treatment, but the pathogen load increased again after treatment withdrawal and most animals failed to achieve microbial cure. Clinical signs were poorly sensitive in predicting infection prior to and after treatment. The species-specific primers and qPCR method developed by the author in this section represent a novel method of diagnosing chlamydial infections in koalas and, as such a method was lacking prior to this work, will have application for future work examining pathogenesis and epidemiology of chlamydiosis in

koalas. The lack of inclusion of a placebo-controlled group means that the effect of treatment cannot be distinguished from other supportive measures, such as nursing care and nutritional improvements. In this respect, this study was a compromise between adhering to ideal scientific experimental design and conducting research within the wildlife rehabilitation setting. Despite being unable to distinguish which components of treatment and rehabilitation, if any, result in the resolution of clinical signs, the major finding of microbial positivity post-treatment is an important advance in the treatment of chlamydiosis in koalas and should prove to be useful information for veterinarians treating this disease, and provide rigour to decision-making regarding animal translocation.

Identification of a treatment capable of achieving microbial cure is vital for animals entering into chlamydially negative populations (e.g. zoos), but the achievement of microbial cure is perhaps not a necessary goal where treated animals are released into areas with endemic chlamydiosis. Infectious disease in wildlife cannot be controlled without huge effort and has rarely been attempted (Artois, Delahay *et al.*, 2001). Survival in the wild of treated animals appears reasonable and chlamydiosis as a reason for admission has not increased over the past 30 years at the Koala Hospital, possibly indicating a stable host-pathogen relationship despite re-introduction of carriers back to the wild population. These results suggest the impact of these animals on the wild population may not be detrimental, although further research is required to confirm these observations.

9.1 POSSIBILITIES FOR FUTURE RESEARCH

Through this work, a number of methods were developed that are applicable to future studies of wildlife rehabilitation. Appetite and faecal monitoring, conjunctivitis scoring, ultrasonographic examination and the species-specific qPCR methods developed in this study will be applicable for future studies of chlamydiosis in koalas and may be applicable to other studies where diagnosis or monitoring of the general health of koalas is required.

The establishment of the Koala Hospital as an ongoing scientific research site has done much to cement the bond between the members of the Koala Preservation Society of NSW and scientific researchers and will ease future research in this setting. Further, it is hoped that this example of research in a wildlife rehabilitation setting will encourage future researchers to consider involving these institutions in the scientific process, as there is much to be gained in encouraging dialogue and understanding between scientists and the general public.

9.1.1 Impact of rehabilitated animals on local populations

An important question in the rehabilitation of wildlife is to determine whether efforts are advantageous or deleterious to local populations and individuals. Conclusions from the studies of wildlife records in this work suggested a number of threats to local populations. With information regarding the population demography of the wild koalas of Port Macquarie, statistical models could be used to examine the effect of these threats on the long-term viability of wild koala populations. The use of the Koala Hospital records to assess long-term survival and breeding success of animals returned to the wild could be expanded to assess the relative prognosis of animals admitted for other reasons (e.g. trauma), and might improve animal welfare, refine rehabilitation methods, better allocate resources and target the efforts of the Koala Preservation Society. The results of these latter studies might also be used in statistical models to predict the impact of returned animals, which might have otherwise died, on the wild population.

9.1.2 Further examination of pharmacology in koalas

As pharmacology in koalas is very much an unexplored field, the most practical investigations are to first describe pharmacokinetics of commonly used substances in koalas to formulate accurate dosing and to establish whether the poor bioavailability

observed in this study applies to other orally administered drugs. Potential barriers to bioavailability could be examined by catheterisation of mesenteric vessels after drug administration to identify the location of the major barriers to oral absorption; investigation of activation of the cytochrome P450 enzymes by examining gene expression using real-time reverse transcriptase PCR; and demonstration of gut wall efflux pumps using inhibitors (Barthe, Bessouet *et al.*, 1998). These studies may have applications outside of pharmacology, as they could contribute to understanding of the koala's ability to deal with secondary plant metabolites and survive on a diet of eucalypt leaves. However, studies involving terminal experiments may not be suitable within the wildlife rehabilitation setting.

9.1.3 Further examination of treatments used to manage chlamydiosis in koalas

This work did not identify a treatment resulting in microbial cure of chlamydiosis in koalas. Future work could examine other promising treatments (e.g. chloramphenicol) (Markey, Wan *et al.*, 2007) to better explore whether such treatments effect a microbial cure, using methods of monitoring and pathogen detection developed in this study. Future work would also ideally include a placebo treated group to allow identification of which, if any, aspects of treatment (e.g. antibiotics, nutritional, nursing care) effect resolution of clinical signs. Such work would be best performed blinded, not only for scientific integrity, but also to mitigate carer concerns for individual animal welfare.

9.2 CONCLUSIONS

This study examined the management, diagnosis and treatment of chlamydiosis in koalas in a rehabilitation setting, an area that has received little attention in the past. As part of this study, the pharmacokinetics of fluoroquinolones were examined and significant barriers to absorption of fluoroquinolones by the oral route were identified, opening the field of pharmacokinetics and xenobiotic metabolism in this species. The results of this study have implications for treatment of koalas with other medications and may prove useful in understanding how koalas absorb and metabolise dietary toxins. Although clinical signs resolved, animals failed to achieve microbial cure when treated with fluoroquinolones, a commonly used antibiotic in this species. Such a finding will better inform treatment decisions, particularly when koalas must be translocated between populations of differing chlamydial status. The review of the historical records in this thesis suggests animals thus treated have a good prognosis for survival, despite potentially having been returned to the wild still carrying chlamydial organisms. It is possible that release of such animals is not detrimental to wild populations, but this hypothesis requires further investigation of animal prognosis and the infectious status and demographics of the local wild population. Future studies of wildlife rehabilitation in other species are required in order to improve individual animal welfare, protect and contribute to the survival of wild populations and improve the scientific understanding of wild species.

APPENDIX I

ADMISSION CODES FROM THE KOALA HOSPITAL: THEIR EXPLANATION AND RECLASSIFICATION FOR USE IN STATISTICAL ANALYSES

Koala Hospital admission codes	Explanation ¹	Reclassification	n (%)
DOG	Dog attack	Dog attack	272 (6.90)
MVA	Motor vehicle accident	Motor vehicle accident	831 (21.09)
BURN	Burns	Fire	31 (0.78)
FIR	Fire	Fire	128 (3.25)
EYE	Eye disease	Eye disease	370 (9.39)
WEE	Wet bottom and eye disease	Wet bottom	58 (1.47)
WET	Wet bottom	Wet bottom	387 (9.82)
DEB	Debilitated	Debilitated	68 (1.73)
DEH	Dehydrated	Debilitated	8 (0.20)
ULD	Underweight, lethargic, dehydrated	Debilitated	194 (4.92)
DGA	Dangerous area	Healthy	499 (12.66)
HAB	Habitat loss	Healthy	91 (2.31)
HEA	Health check	Healthy	404 (10.25)
JOE	Joey with mother	Healthy	8 (0.20)
KST	Koala seeking territory	Healthy	96 (2.44)
REL	Relocation	Healthy	34 (0.86)
ABN	Abandoned	Joey	9 (0.23)
ORP	Orphan	Joey	54 (1.37)
SEP	Separated from mother	Joey	3 (0.08)
None	None	Unidentified	1 (0.03)
UDI	Unidentified cause of injury	Unidentified	316 (8.02)

¹ E. Gabriel, Secretary, Koala Preservation Society of NSW, Port Macquarie, NSW, January 2010.

Appendix I cont.

Koala Hospital admission` codes	Explanation ²	Reclassification	n (%)
BIC	Unknown	All other	1 (0.03)
BTR	Brain Trauma	All other	1 (0.03)
CAN	Cancer	All other	1 (0.03)
CAT	Cat attack	All other	2 (0.051)
CRO	Crow attack	All other	2 (0.051)
DIA	Diarrhoea	All other	1 (0.03)
DOA	Dead on arrival	All other	5 (0.13)
DRO	Drowned	All other	13 (0.32)
ELE	Electrocuted	All other	2 (0.05)
FAL	Fall	All other	19 (0.48)
FIT	Fitting	All other	10 (0.26)
HYP	Hyperkeratosis	All other	1 (0.03)
KFI	Koala fight injury	All other	7 (0.18)
LAM	Lame	All other	6 (0.15)
MAN	Mange	All other	2 (0.05)
SCOL	Scoliosis	All other	1 (0.03)
SHO	Shot	All other	1 (0.03)
TEN	Tendon damage	All other	2 (0.05)
TIC	Ticks	All other	1 (0.03)
TUM	Tumour	All other	1 (0.03)
Total			3941 (100)

² E. Gabriel, Secretary, Koala Preservation Society of NSW, Port Macquarie, NSW, January 2010.

APPENDIX II

POST MORTEM FINDINGS OF ANIMALS THAT WERE EUTHANASED OR DIED DURING THE PILOT STUDY

Koala	Drug	Findings consistent with chlamydiosis	Findings not consistent with chlamydiosis
MO	Chloramphenicol 50 mg/kg p.o. BID	Moderate diffuse chronic active urogenital sinusitis Severe diffuse chronic fibrosing cystitis Severe diffuse uterine fibrosis Moderate chronic active conjunctivitis	None
NK	Chloramphenicol 50 mg/kg p.o. BID	Mild multifocal lymphocytic interstitial nephritis Severe diffuse acute haemorrhagic cystitis Moderate to severe diffuse chronic active prostatitis Severe diffuse chronic fibrosis of the bladder	Thin Gastrointestinal tract relatively empty Severe acute diffuse oxalate diffuse leading to acute renal failure Multifocal severe acute fungal gastric ulceration/gastritis Mild chronic multifocal gliosis
OC	Doxycycline 5 mg/kg p.o. SID	Moderate chronic active pyometra Moderate diffuse chronic vaginitis Mild chronic ureteritis Sever chronic active bursitis and salpingitis Moderate chronic active diffuse cystitis Moderate diffuse chronic urogenital sinusitis	Thin Liquid gastrointestinal contents
CP	Doxycycline 2.5 mg/kg i.m. SIW	Mild chronic diffuse ureteritis Severe diffuse chronic active cystitis Severe diffuse chronic active fibrosing prostatitis	Thin Gastrointestinal tract relatively empty

Appendix II cont.

Koala	Drug	Findings consistent with chlamydiosis	Findings not consistent with chlamydiosis
TH	Doxycycline 0.25 mg/kg p.o. SID	Left ovarian bursal cyst Diffuse moderate chronic vaginitis Diffuse moderate chronic cervicitis Moderate diffuse chronic active urogenital sinusitis Severe diffuse chronic active cystitis Mild diffuse chronic lymphocytic ureteritis Moderate chronic diffuse salpingitis (left) Moderate chronic diffuse plasmacytic lymphocytic conjunctivitis	Thin Gastrointestinal tract relatively empty Left femoral fracture (old) with secondary chronic fibrosis and arthritis
MM	Azithromycin 5 mg/kg p.o. SID	Moderate diffuse chronic plasmacytic lymphocytic urogenital sinusitis Moderate diffuse chronic active vaginitis Moderate diffuse chronic active cervicitis	Emaciated Gastrointestinal tract relatively empty Gingival recession lower incisors Left hip capital ligament is not intact
OP	Azithromycin 2.5 mg/kg p.o. SID	Chronic fibrotic ongoing urogenital tract disease	Emaciated Lafora-like bodies in cerebrum
WR	Marbofloxacin 3.34 mg/kg p.o. SID	Mild chronic focal plasmacytic pyelonephritis Chronic diffuse severe plasmacytic cystitis Moderate chronic active diffuse vaginitis Ovarian bursal cysts with adhesions Fibrous adhesions between R uterus and ovarian bursal cyst obliterating normal tissue Hydrometron	None

Appendix II cont.

Koala	Drug	Findings consistent with chlamydiosis	Findings not consistent with chlamydiosis
TM	Marbofloxacin 1.3 mg/kg p.o. SID	Moderate diffuse plasmacytic lymphocytic pyometron Moderate diffuse chronic active salpingitis Chronic diffuse moderate plasmacytic lymphocytic pyometra Moderate diffuse chronic plasmacytic urogenital sinusitis Chronic diffuse moderate plasmacytic fibrous cystitis Mild chronic diffuse plasmacytic ureteritis Moderate chronic active vaginitis Caecal adhesion to left uterus	Mild portal triad and centrilobular hepatic fibrosis Multifocal moderate pulmonary lipid granulomata
RG	Marbofloxacin 2.3 mg/kg p.o. SID	Moderate diffuse neutrophilic interstitial pneumonia. Moderate diffuse chronic plasmacytic pyelonephritis Mild diffuse chronic plasmacytic pyelonephritis Severe chronic diffuse chronic active cystitis Bilateral moderate chronic diffuse chronic vaginitis Mild diffuse chronic active ureteritis Moderate to severe diffuse chronic active urogenital sinusitis Bilateral moderate to severe diffuse chronic active pyometron Mild diffuse neutrophilic salpingitis	None
CR	Enrofloxacin 5 mg/kg s.c. SID	Bilateral severe chronic inactive uterine fibrosis Diffuse moderate chronic lymphocytic vaginitis Diffuse severe chronic active urogenital sinusitis Severe bilateral chronic-inactive cervical fibrosis Bilateral ovarian bursal cysts Diffuse moderate chronic cystitis with generalised oedema	None

Appendix II cont.

Koala	Drug	Findings consistent with chlamydiosis	Findings not consistent with chlamydiosis
SS	Enrofloxacin 5 mg/kg s.c. SID	Chronic severe inactive uterine fibrosis Mild chronic diffuse plasmacytic lymphocytic vaginitis Moderate diffuse chronic active cervicitis and urethritis Mild diffuse acute urogenital sinusitis Moderate diffuse plasmacytic pyelonephritis Bilateral ovarian bursal cysts	None
KH	Enrofloxacin 5 mg/kg s.c. SID	Mild diffuse lymphocytic plasmacytic pyelonephritis Severe diffuse chronic cystitis Moderate diffuse chronic active urogenital sinusitis Moderate diffuse chronic vaginitis Mild diffuse chronic plasmacytic lymphocytic metritis Bilateral ovarian bursal cysts	None
KS	Enrofloxacin 5 mg/kg s.c. SID	Severe diffuse chronic inactive occlusive vaginal fibrosis Mild diffuse plasmacytic chronic conjunctivitis Bilateral diffuse severe chronic inactive salpingitis Bilateral ovarian bursal cysts Severe diffuse chronic inactive cervical occlusive fibrosis Severe diffuse chronic inactive uterine fibrosis Chronic inactive multifocal renal fibrosis Severe diffuse fibrotic chronic active cystitis	Adhesion between liver and kidney
LJ	Enrofloxacin 5 mg/kg p.o. SID	Severe diffuse chronic active vaginitis & cystitis Moderate chronic active ureteritis Mild chronic diffuse pyometra Moderate diffuse chronic active pyelonephritis	None

APPENDIX III

LAST AVAILABLE BIOCHEMICAL AND HAEMATOLOGY ANALYSES IN ANIMALS FAILING TO COMPLETE TREATMENT IN THE PILOT STUDY

	Reference intervals	S.I. units	MM	OK	OP	NK	OA	AG	OC	TH	CP
Day			20	21	21	28	27	42	20	29	28
Dose		mg/kg	5	10	2.5	50	10	5	5	0.25	2.5
Route			p.o.	p.o.	p.o.	p.o.	i.m.	i.m.	p.o	p.o	i.m.
Haemoglobin	88 - 140	g/L	102	88	133	128	111	92	100	96	101
Red cell count	2.7 - 4.2	$\times 10^{12}/L$	3.6	2.8	3.8	4.2	3.8	2.8	3	3.1	3.3
Haematocrit	0.29 - 0.44	L/L	0.34	0.27	0.4	0.4	0.36	0.29	0.32	0.31	0.33
Mean corpuscular volume	94 - 117	fL	96	97	104	96	94	100	106	101	100
Mean corpuscular haemoglobin	33 - 35	pg/L	29	31	35	30	29	33	34	31	30
Mean corpuscular haemoglobin concentration	298 - 330	g/L	302	320	336	320	308	323	316	306	304
Platelets	222 - 508	$\times 10^9/L$	47	152*	208	273		388		170*	933
Nucleated red blood cells		%WBC	79	21	37	5	22	15	197	9	
White blood cells	2.8 - 11.2	$\times 10^9/L$	1.5	8.3	3.9	14.2	4.7	5.8	7.7	4.5	7.2
Neutrophils	0.5 - 6.3	$\times 10^9/L$	0.9	6.6	2.5	12.1	2.6	4.2	4.8	3	4.7
Lymphocytes	0.2 - 5.8	$\times 10^9/L$	0.5	1.1	1.1	1.7	1.6	1.6	2.7	1.4	2.3
Monocytes	< 0.6	$\times 10^9/L$	0.1	0.6	0.4	0.4	0.2	0.1	0.2	0	0.1
Eosinophils	< 1.1	$\times 10^9/L$	0	0	0	0	0.3	0	0	0	0.1
Basophils	< 0.1 [†]	$\times 10^9/L$	0	0	0	0	0	0	0	0	0
Sodium	132 - 145	mmol/L	127	143	145	145	145	139	137	142	143
Potassium	3.5 - 6.8	mmol/L	4.4	5.5	5.8	4.2	4.7	4.2	5.3	4.9	4.9
Chloride	93 - 107	mmol/L	92	111	96	106	99	93	102	107	96

Appendix III cont.

	Reference intervals	S.I. units	MM	OK	OP	NK	OA	AG	OC	TH	CP
Bicarbonate	12 - 30	mmol/L	17	16	27	15	29	23	17	18	23
Anion Gap	11.3 - 32.3	mmol/L	22	22	28	28	22	27	23	22	29
Urea	0.2 - 6.6	mmol/L	4.8	11.2	3.5	28.6	5.3	3.5	6.3	7	2.2
Creatinine	80 - 150	µmol/L	70	110	85	230	110	70	115	125	70
Glucose	2.7 - 7.2	mmol/L	3.8	1.4	4.8	3.5	1.8	5.1	3.7	2.8	6.3
Bilirubin	< 8	mmol/L	5	1	2	0.5	2	1	0.5	2	1
Aspartate transaminase	1 - 134	U/L	45	61	18	86	27	23	6	23	18
Alanine transaminase	< 236	U/L	41	56	17	30	16	12	10	19	13
Gamma glutamyl transferase	< 16	U/L	7	6	10	16	9	10	11	10	7
Alkaline phosphatase	25 - 375	U/L	69	91	67	79	60	65	170	165	119
Protein	53 - 83	g/L	53	50	63	56	69	53	70	61	61
Albumin	34 - 50	g/L	26	29	35	33	41	29	42	34	33
Globulin	13 - 39	g/L	27	21	28	23	28	24	28	27	28
Albumin/ Globulin			1	1.4	1.2	1.4	1.5	1.2	1.5	1.3	1.2
Calcium	2.28 - 2.97	mmol/L	1.92	2.16	2.63	2.82	2.6	2.26	2.65	2.45	2.67
Phosphate	0.79 - 2.44	mmol/L	0.92	1.56	1.41	3.21	1.05	1.26	1.75	1.23	1.1
Creatinine kinase	100 - 300 [†]	U/L	10483	8199	606	11959	1241	499	264	3512	290
Cholesterol	1.15 - 3.09	mmol/L	3.9	2.6	3.8	3.2	3	2.2	3.5	3.4	2.3
Triglyceride	0.5 - 3.02	mmol/L	1.4	0.2	2.1	1.8	2.8	3.7	1.8	0.9	4.6
Urine specific gravity			NA	NA	NA	1.020	1.027	NA	1.037	1.052	NA

*platelets clumped

[†] (Blanshard, 1994); all others (Canfield, O'Neill et al., 1989)

Animals treated with azithromycin (MM, OK, OP); marbofloxacin then doxycycline (OA, AG); or doxycycline (OC, TH, CP). All animals were euthanased except OK, which died. Parameters outside reference range are highlighted in bold. NA: not available.

APPENDIX IV

POST MORTEM FINDINGS OF ANIMALS THAT WERE EUTHANASED OR DIED OUTSIDE OF THE PILOT STUDY

Koala ID	Drug (all once daily)	Findings consistent with chlamydial disease	Findings inconsistent with chlamydial disease
BB [†]	Marbofloxacin	Severe focal medullary tubular nephrosis	Right capital ligament rupture
	10 mg/kg p.o.	Severe diffuse chronic bladder oedema and fibrosis	Moderate diffuse periodontal disease
OJ [‡]	Enrofloxacin	Severe chronic active diffuse urogenital sinusitis	
	10 mg/kg s.c.	Moderate diffuse chronic plasmacytic cystitis	
HL [*]	Enrofloxacin 20 mg/kg p.o.	Moderate chronic multifocal interstitial nephritis	Mild diffuse chronic active cholangitis
		Diffuse moderate chronic plasmacytic cystitis	Nodular hyperplasia of the adrenal medulla
		Mild multifocal lymphocytic chronic active urogenital sinusitis	Diffuse moderate chronic splenic lymphoid hyperplasia
		Diffuse mild chronic active vaginitis	Hyperplastic lymph node
		Diffuse severe chronic fibrosis of the urinary bladder, vaginae, cervixes, and uteri	Focal chronic type II pneumocyte hyperplasia and mild focal chronic lymphocytic pneumonia
		Diffuse severe chronic cystic salpingeal fibrosis	Diffuse moderate chronic epicardial fibrosis
			Diffuse moderate chronic myocardial fibrosis and mild scattered myocardial necrosis
			Diffuse severe subacute cortical tubular oxalate nephrosis
BP [*]	Enrofloxacin 20 mg/kg p.o.	Bilateral proliferative conjunctivitis with bilateral corneal oedema	Severe peracute diffuse myocardial hyaline necrosis
		Mild chronic interstitial nephritis, cystitis and urethritis	
DA [§]	Enrofloxacin 10 mg/kg	Mild chronic diffuse vaginitis and ureteritis	Cystic endometrial hyperplasia Bilateral ovarian bursal cysts

*died; †euthanased due to non-resolution of clinical signs; ‡euthanased due to recurrence of clinical signs; §euthanased due to development of ovarian bursal cysts

p.o: per os; s.c: subcutaneous injection; SID: once daily.

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