OXALATE NEPHROSIS IN A POPULATION OF SOUTH AUSTRALIAN KOALAS (*Phascolarctos cinereus*)

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ABSTRACT

Renal disease had been reported to occur at high prevalence in the koala population of the Mount Lofty Ranges in South Australia, but the cause was unclear. Kidney crystals consistent with calcium oxalate had been observed in several koalas, suggesting that oxalate nephrosis may occur. The aims of this study were to describe renal pathological changes and confirm oxalate deposition in these koalas and also to investigate possible causes of disease.

Oxalate nephrosis was found in 55% of 51 captive and rescued wild koalas from the Mount Lofty population. Renal histopathological changes associated with crystals included intratubular and interstitial inflammation, tubule dilation, glomerular atrophy, tubule loss and cortical fibrosis. Renal insufficiency was confirmed in affected koalas by azotaemia in association with poorly concentrated urine, and decreasing urine specific gravity was significantly associated with increasing severity of histopathological changes. The number of males and females, and captive and rescued wild koalas showing oxalate nephrosis was similar. Age was not found to be a predisposing factor, but many koalas <2 years old were affected. Urinary crystals in all koalas with oxalate nephrosis showed an atypical morphology for calcium oxalate. Hyperoxaluria was also found, suggestive of a primary cause for disease.

To investigate whether a dietary cause existed for oxalate nephrosis in koalas, oxalate concentration was measured in juvenile, semi-mature and mature leaves from manna gum (*E. viminalis*), red gum (*E. camaldulensis*), SA blue gum (*E. leucoxylon*) and messmate stringybark (*E. obliqua*) in spring. Eucalypt leaves were found to be low in oxalate overall (<1% dry weight) with occasional samples that were higher in oxalate. Mount Lofty eucalypts were found to have higher oxalate content overall than those eaten by koalas in Moggill, Queensland, where the prevalence of oxalate nephrosis is lower.

To investigate whether endogenous overproduction of oxalate could occur due to an inherited liver enzyme dysfunction, similar to primary hyperoxaluria type I in humans, the activity of alanine: glyoxylate aminotransferase (AGT) was measured in liver samples. Koalas with oxalate nephrosis showed no decrease in AGT activity compared with samples from unaffected Queensland koalas, indicating normal activity of this enzyme.

Water content of eucalypt leaves was also measured, since dehydration is a key risk factor for renal calcium oxalate deposition. Mount Lofty eucalypt leaves were found to be lower in moisture in autumn compared with those in Queensland, particularly juvenile and semi-mature leaves of *E. obliqua* and *E. leucoxylon*.

The pathological, histopathological and clinicopathological description of oxalate nephrosis in koalas provided by this study will assist veterinarians and pathologists in the diagnosis of this disease. Investigation of the pathogenesis of oxalate nephrosis in the Mount Lofty koala population found that neither high eucalypt leaf oxalate or decreased AGT activity were the primary cause. Further research is needed, but based on the low genetic diversity of the Mount Lofty koalas, an inherited pathogenesis of oxalate nephrosis remains likely. To decrease the risk of oxalate nephrosis, water supplementation should be provided for captive and wild Mount Lofty koalas during the hot, dry summer.

DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution in my name and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide.

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LIST OF ABBREVIATIONS

AGT	alanine: glyoxylate aminotransferase
ALT	alanine aminotransferase
AST	aspartate aminotransferase
DW	dry weight
EDX	energy dispersive X-ray analysis
ELISA	enzyme-linked immunosorbent assay
GGT	gamma glutamyl transferase
HCl	hydrochloric acid
HE	haematoxylin and eosin stain
HPLC	high performance liquid chromatography
IRS	infrared spectroscopy
KI	Kangaroo Island
ML	Mount Lofty, South Australia
N	number of samples
NSW	New South Wales
PCR	polymerase chain reaction
Qld	Queensland
SA	South Australia
SD	standard deviation
SE/SEM	standard error of the mean
SEM	scanning electron microscopy
TP	total protein
TWC	tooth wear class
USG	urine specific gravity
Vic	Victoria

Note: In 2009, *Chlamydophila* spp. which affect koalas (*C. pecorum* and *C. pneumoniae*) were reclassified as *Chlamydia*, hence both of these terms are used in this thesis.

CHAPTER 1 INTRODUCTION

The koala (*Phascolarctos cinereus*) is a unique Australian marsupial found in the eastern and southern mainland states of Australia. Due to population declines in the eastern populations, research has focussed on understanding key aspects of koala biology such as diet (Moore and Foley 2000), digestive physiology (Cork et al. 1983) and social structure (Ellis et al. 2002). In addition, infectious diseases which cause significant morbidity and mortality in the eastern koala populations, particularly chlamydiosis, have been well investigated (Brown and Woolcock 1988, Cockram and Jackson 1981, Hemsley and Canfield 1997, Polkinghorne et al. 2013, Timms 2005).

In contrast, few studies have focussed on South Australian koala populations, particularly that of the Mount Lofty Ranges. Little is known about the disease status of these koalas, however a previous study found a high prevalence of kidney dysfunction in the koala population in the Adelaide Hills region of the Mount Lofty Ranges, estimated at 11% in 2000 (Haynes et al. 2004). Both wild and captive koalas were found to be affected and showed varying degrees of lethargy, weight loss, polydipsia and polyuria in the absence of clinical signs of chlamydiosis (Haynes et al. 2004). In contrast, kidney disease is uncommon in koalas in the eastern states, unless associated with chlamydiosis (Canfield 1989, Connolly 1999).

A previous study investigated dietary aluminium as a possible cause of kidney disease in the Mount Lofty koalas, with aluminium evident in eucalypt leaves, kidney tubule cells and bone, but the significance of these findings was unclear (Haynes et al. 2004). However, one koala in this study showed renal crystals consistent with calcium oxalate (Haynes et al. 2004). Routine post mortem examinations had also found pale yellow deposits within kidneys of several Mount Lofty koalas, which histopathological examination indicated was suggestive of calcium oxalate (I. Hough; W. Boardman, 2008, pers. comm.).

Renal calcium oxalate deposition had also been reported in low numbers of koalas in the eastern states of Australia as a necropsy finding (Canfield 1987b, Canfield 1989, Connolly 1999), with unclear cause and significance (Blanshard 1994). In addition, calcium oxalate deposition associated with renal dysfunction, or oxalate nephrosis, had been reported in individual koalas (Canfield and Dickens 1982, Dickson 1989a, Main 1992). In mammalian species, the main causes of deposition of calcium oxalate in the kidney include high dietary oxalate intake, gastrointestinal factors affecting oxalate absorption, liver enzyme dysfunction causing overproduction of oxalate, decreased oxalate excretion by the kidney and calcium imbalances (Asplin 2002, Weiss et al. 2007). The aims of the current study were to describe the pathological features of the renal disease so as to confirm whether oxalate nephrosis was occurring in the Mount Lofty Ranges koala population, and to investigate a possible cause.

1.1 KOALA DISTRIBUTION AND HISTORY

Koala populations are found throughout the eastern states of Australia and also extend into South Australia. Three 'races' of koalas are recognised: *Phascolarctos cinereus adjustus* which occur in northeastern Australia, primarily Queensland; *P. c. cinereus* in the intermediate eastern regions, mainly New South Wales; and *P. c. victor*, in the south-eastern regions, including Victoria and South Australia (Martin and Handasyde 1999). However, despite morphological differences in body size and fur colour between northern and southern koalas (Lee and Martin 1988), genetic analyses do not support classification as separate subspecies (Houlden et al. 1999).

Following European settlement, koala population numbers drastically declined during the late 1800s (Lewis 1934, Phillips 1990), due to intensive hunting for pelts, habitat loss, fire, drought and disease (Lee and Martin 1988, Martin and Handasyde 1999, Melzer et al.

2000). Similarly in South Australia, population numbers fell and koalas were considered extinct by the early 1930s from their original range in the lower southeast region of the state (Robinson et al. 1989).

To address the declining southern koala populations, between two and five Victorian koalas were translocated to French Island in the 1890s, where the colony flourished (Jackson 2007, Lewis 1934). In the years 1923 and 1925, eighteen koalas, some with pouch young, were taken from this French Island population and introduced to Kangaroo Island, off the coast of South Australia (Lindsay 1950, Robinson 1978, Robinson et al. 1989). This colony also rapidly expanded and between 1959 and 1965, nineteen koalas were transported from Kangaroo Island to various locations in the Riverland region of South Australia (Robinson 1978, Robinson et al. 1989). In 1965, six koalas from Kangaroo Island were released into the Adelaide Hills and Ashbourne areas of the Mount Lofty Ranges, then in 1969 six were translocated to the Eyre Peninsula, and also to their original range in the lower southeast of South Australia, near Lucindale (Figure 1) (Robinson 1978, Robinson et al. 1989).

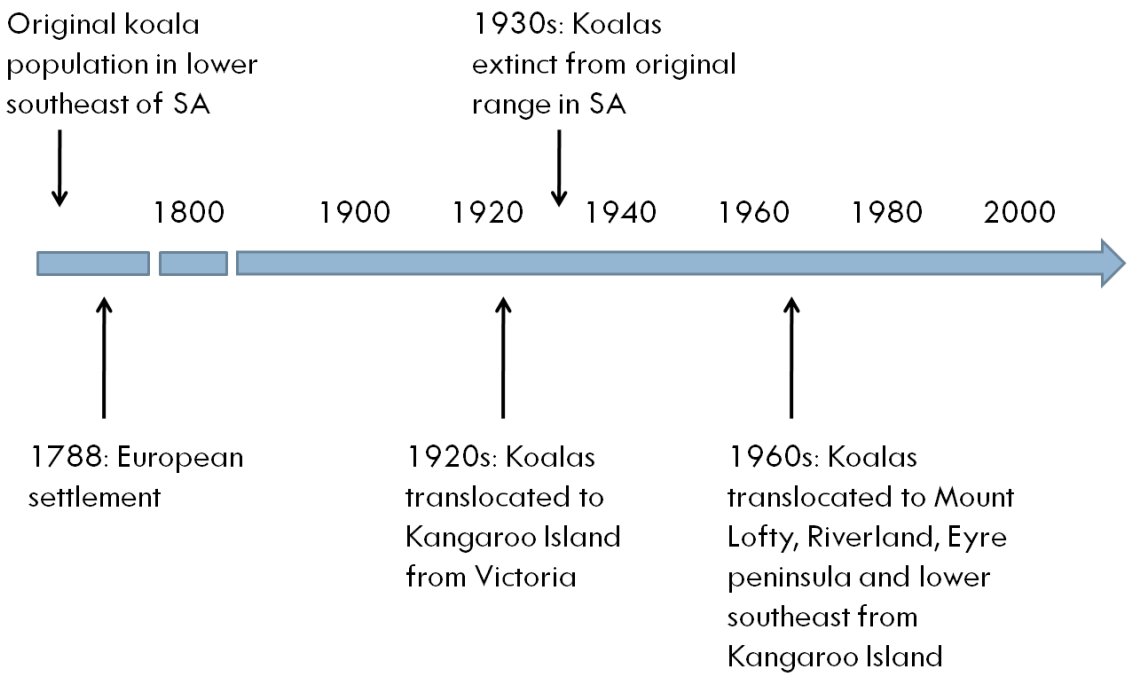

Figure 1 Timeline of koala history in South Australia (SA).

In addition to the translocated koalas from Kangaroo Island, the current population in the Adelaide Hills region of the Mount Lofty Ranges is thought to include koalas that escaped from the Belair Recreation Park and Cleland Conservation Park, which were of Kangaroo Island descent, as well as from a private colony suspected to include koalas from New South Wales (Robinson 1978) or Queensland (Houlden and St John 2000). Despite this, Seymour et al. (2001) found no evidence to support genetic contribution from New South Wales or Queensland koalas to the Mount Lofty Ranges population.

Low genetic variability occurs in South Australian koala populations, due to their history of several translocations causing genetic 'bottlenecks' (Houlden et al. 1996, Taylor et al. 1997), as well as originating from island populations, in which inbreeding is often increased (Lee et al. 2012). Evidence of reduced genetic variation has been shown by low heterozygosity and allelic diversity (mean number of alleles per locus) (Houlden and St John 2000, Montgomery 2002), in koalas from French Island, the Mount Lofty Ranges, Kangaroo Island and the Eyre Peninsula (see Table 1) (Houlden et al. 1996, Houlden and St John 2000, Montgomery 2002, Seymour et al. 2001, Taylor et al. 1997). Previous studies have also found that higher levels of testicular aplasia occur in the koala populations of French Island, Eyre Peninsula and Kangaroo Island (see Table 1) (Cristescu et al. 2009, Montgomery 2002, Seymour et al. 2001). In contrast, koala populations in the eastern states have higher genetic diversity, such as those of the Strzelecki Ranges in Victoria and Pilliga State Forest in New South Wales (see Table 1) (Montgomery 2002, Seymour et al. 2001), as well as those in Queensland, which have up to twice as much genetic variation as southern koala populations (Fowler et al. 1998, Fowler et al. 2000).

Table 1 Comparison of genetic variability between koala populations

	Allelic diversity (mean)	**Inbreeding coefficient** (mean)	**Testicular aplasia** (%)
Pilliga State Forest, NSW[1]	7.0	0.12	0
Strzelecki Ranges, Vic	4.7	0.43	-
South Gippsland, Vic	4.3[2]	0.43[1]	2.4[1]
French Island, Vic	3.0[2]	0.57	4.3
Mount Lofty Ranges, SA	2.7	0.59	-
Kangaroo Island, SA	2.0	0.63	12.9
Eyre Peninsula, SA	1.7	0.75	23.9

Data from Seymour et al. (2001)

[1] Montgomery (2002)

[2] Houlden and St John (2000); Houlden et al. (1996)

1.2 THE EUCALYPT DIET OF KOALAS

The koala has a specialised diet which consists primarily of leaves from *Eucalyptus* species (Hume 1982, Lee and Martin 1988). Eucalypt leaves are relatively poor in nutritional quality, being high in fibre and low in nitrogen (Cork et al. 1983, Hume 1982). To cope with this diet, koalas have a large caecum and proximal colon, which increases the capacity for microbial hindgut fermentation (Cork et al. 1983, Hume 1982). Despite this specialisation of the gastrointestinal tract, cell contents such as lipids and carbohydrates provide the highest metabolisable energy for the koala, and their release from the leaves is aided by efficient

mastication (Cork and Hume 1983, Cork et al. 1983, Lanyon and Sanson 1986). To further cope with a low energy diet, the koala maintains a sedentary lifestyle and has a low basal metabolic rate, approximately 70% of that expected for a similar sized marsupial (Degabriele and Dawson 1979).

Approximately 600 species of *Eucalyptus* trees occur in Australia, but relatively few of these are eaten regularly by koalas (Pahl and Hume 1990, Phillips 1990, Tyndale-Biscoe 2005). In South Australia, as well as Victoria, the preferred eucalypt species of koalas is manna gum (*Eucalyptus viminalis*) (Nicolle 1997, Phillips 1990), which is found in the Mount Lofty Ranges, Kangaroo Island, the southeast region of South Australia and across Victoria (Sutton 1934). The favoured eucalypt species of koalas in New South Wales and Queensland are more varied, but include forest red gum (*E. tereticornis*) and small fruited grey gum (*E. propinqua*) (Jackson et al. 2003, Phillips 1990).

Koalas are selective eaters and may favour particular eucalypt species, as well as individual trees within their preferred species (Hindell and Lee 1987). Young foliage is generally preferred to mature leaves, and this may be due to higher levels of moisture, higher crude protein, and lower proportions of fibre and lignin (Cork 1984, Hume and Esson 1993, Pahl and Hume 1990, Ullrey et al. 1981). Koalas may also vary their tree preferences seasonally, for instance choosing species with higher water content in summer (Ellis et al. 1995) and have been shown to decrease intake of foliage below a leaf moisture threshold, estimated as 65% moisture by Pahl and Hume (1990), 55% by Hume and Esson (1993) and between 45 and 50% for leaves of *E. punctata* (Degabriele et al. 1978).

1.3 WATER BALANCE IN KOALAS

Water intake in koalas is primarily from the moisture found within eucalypt leaves, but this may be supplemented by water droplets on the leaf surface following rainfall (Ellis

et al. 2010) or overnight dew (Degabriele et al. 1978). Also, all koalas may drink if they are ill or in drought conditions (Lee and Martin 1988, Phillips 1990) and large male koalas are more likely to drink free water than females (Tyndale-Biscoe 2005). Physiological conservation of water is achieved in the koala primarily by reducing the moisture content of faeces (Degabriele et al. 1978). One study showed that in water deprivation states, the faecal moisture decreased from 52% to 43% (Degabriele et al. 1978), which is lower than that found in the euro (*Macropus robustus erubescens*), an arid-zone macropod (Freudenberger and Hume 1993), and similar to that found in the camel (Schmidt-Nielsen 1964).

Water conservation in koalas is also aided by the kidneys, which have a unilobar structure with a single papilla (Degabriele et al. 1978, Sonntag 1921) and can produce relatively concentrated urine, with urine specific gravity (USG) up to 1.135 (Canfield et al. 1989a). However, Degabriele et al. (1978) when investigating water metabolism in the koala found that the koala kidney does not show any relative increase in medullary thickness, as occurs in desert animals with high urine concentrating ability (al-Kahtani et al. 2004, Degabriele et al. 1978), indicating adaptation to an environment with adequate water supply. Furthermore, the difference in cortical and juxtamedullary glomerular volumes in koala kidneys was found to be 60% (Degabriele et al. 1978), significantly less than that of semi-desert dwellers (Munkacsi and Palkovits 1965). This again suggests that koalas have adapted to an environment with adequate water availability, which reflects the distribution of koalas across Australia in regions with consistent annual rainfall (Degabriele et al. 1978).

1.4 CAUSES OF KOALA DISEASE AND DEATH

Morbidity and mortality studies of koalas have mainly been conducted in New South Wales and Queensland, where populations are in decline and listed as vulnerable (DEWHA 2009, Phillips 2000). A major limitation to koala distribution and abundance is habitat loss

due to encroachment of human settlement into areas of eucalypt forest (Phillips 2000). Other threats to koalas include trauma, urogenital disease, respiratory disease, gastrointestinal disease, multiorgan disease and neoplasia (Butler 1978, Canfield 1990a, Connolly 1999, Griffith et al. 2013, Weigler et al. 1987). Death may also occur due to an unidentifiable cause whereby koalas show 'no visible or significant lesions' at post mortem examination (Canfield 1990a), which may be attributed to 'koala stress syndrome' (Obendorf 1983) or 'koala wasting disease' (Degabriele 1989). The causes of death in wild and captive koalas differ significantly; Canfield (1990a) found that in New South Wales, trauma and urogenital disease accounted for more deaths in wild koalas, whilst gastrointestinal disease and death with 'no visible lesions' were more common in captive koalas.

1.4.1 Trauma

Koala death due to trauma can occur from motor vehicle accident, dog attacks, falling from trees, bushfires or drowning (Canfield 1990b). Motor vehicle accident is the most common cause of death for many wild animals, for example 47% of echidnas (*Tachyglossus aculeatus*) (McOrist and Smales 1986). In a study undertaken in New South Wales, Canfield (1990a) found trauma to be the primary necropsy finding in 38% of free-ranging koalas (N=162). Similarly, Weigler et al. (1987) found that 35% of deaths in wild Queensland koalas (N=58) were due to motor vehicle accident, whilst 19% were due to dog attacks. Trauma to the head is the most common injury in koalas from motor vehicle accident, and accounted for 71% in a study of New South Wales koalas (Canfield 1987b), with similar findings reported in other studies (Canfield 1991, Hemsley and Canfield 1993). Due to roaming, male koalas are most at risk of trauma from motor vehicles (Dique et al. 2003), with accidents more common during the breeding season in spring (Canfield 1991, Griffith et al. 2013). There appears to be no correlation between the presence of underlying disease in koalas

and death due to motor vehicle accident; Canfield (1991) found only 16% (12/75) of trauma cases with concurrent disease and similarly Weigler et al. (1987) reported only 11% (4/36). In both of these studies, the disease that was found in conjunction with motor vehicle trauma was consistent with chlamydiosis (Canfield 1991, Weigler et al. 1987).

1.4.2 Chlamydiosis

Chlamydia spp. are intracellular bacteria which infect many avian and mammalian species, including humans, and are a well documented cause of morbidity in most koala populations (Polkinghorne et al. 2013, Shewen 1980, Timms 2005). Disease consistent with chlamydial infection has been observed in koalas since the late 1800s (Backhouse and Bolliger 1961, Pratt 1937), and recent studies have shown that *Chlamydiae* may have crossed to koalas from livestock brought by the early settlers (Jackson et al. 1997, Jelocnik et al. 2013). Initially, disease in koalas was attributed to *Chlamydia psittaci* (Brown 1984, McColl et al. 1984), but improved molecular techniques led to the redesignation of koala strains to *C. pecorum* and *C. pneumoniae* (Girjes et al. 1988, Glassick et al. 1996). In 1999, koala strains were grouped into a new genus *Chlamydophila*, but following further studies in 2009, they were reclassified as *Chlamydia* (Polkinghorne et al. 2013, Stephens et al. 2009).

A primary site of *Chlamydia* infection is the reproductive tract of koalas, which causes infertility in females due to pathological changes such as cystic dilation of the ovarian bursae and oviducts, metritis, pyometra and vaginitis (Blanshard and Bodley 2008, Obendorf 1981, Obendorf and Handasyde 1990, Polkinghorne et al. 2013). A second site of infection is the urinary tract, mainly causing cystitis (‘dirty tail’ or ‘wet bottom’), but also urethritis, ureteritis, prostatitis, and nephritis (Brown et al. 1987, Canfield and Spencer 1993). *Chlamydia* also causes ocular pathology such as keratoconjunctivitis or ‘pink eye’, which can result in blindness (Cockram and Jackson 1981, Girjes et al. 1988), as well as infection of the

respiratory tract, including mild rhinitis and occasionally pneumonia (Jackson et al. 1999, Timms 2005). *C. pecorum* is responsible for most infections in koalas and is regarded as having higher pathogenicity (Polkinghorne et al. 2013).

Diagnosis of chlamydial infection in koala populations can be challenging for researchers. Over the years several methods have been used, including physical examination, radiography or ultrasonography, serology, complement-fixation test, immunofluorescence, histopathology and cell culture (Blanshard 1994, Connolly 1999). Currently, the gold standard for diagnosis of chlamydiosis is based on confirmation by polymerase chain reaction (PCR) (Polkinghorne et al. 2013). Since chlamydial infection can also be subclinical, whereby there are no signs of disease seen in koalas (Timms 2005), prevalence of infection can be underestimated (Polkinghorne et al. 2013). For example, a previous study found that only 9% of koalas in southeastern Queensland showed clinical signs of chlamydial disease, but 71% tested positively by culture (N = 65) (Weigler et al. 1988). A recent review by Polkinghorne et al. (2013) further highlights the differences between prevalence of *Chlamydia* infection detectable by PCR in comparison with clinical signs of chlamydiosis in koala populations across Australia.

In South Australia, the prevalence of chlamydiosis appears to be low when compared with koalas in the eastern states. On Kangaroo Island, koalas have shown mixed results using various *Chlamydia* tests over the years (Robinson et al. 1989, Whisson and Carlyon 2010), but recent negative PCR results and a consistent lack of clinical signs suggests that this population are *Chlamydia*- free (Polkinghorne et al. 2013, Timms 2005). French Island, from which Kangaroo Island koalas originated, is also considered *Chlamydia*- free (Lee and Martin 1988, Polkinghorne et al. 2013).

In the Mount Lofty population, *Chlamydia* has been detected in koalas, with one study over a decade ago finding that up to 39% koalas (N=23) tested positive for *Chlamydia*

using an ELISA technique in combination with PCR (Houlden and St John 2000). A recent study reported 90% prevalence in 17 Mount Lofty Ranges koalas using PCR (Polkinghorne et al. 2013), based on samples also taken around 1996-1997 (A. Polkinghorne and P. Timms, 2013, pers. comm.). In the majority of these cases, *C. pecorum* was detected (90%), but a significant number also showed *C. pneumoniae* (53%) (Polkinghorne et al. 2013). Captive koalas at Cleland Wildlife Park also showed a low level of PCR positivity (18% of 28 koalas) (Polkinghorne et al. 2013). Despite these results showing detection of *Chlamydia* in Mount Lofty koalas, clinical evidence of chlamydiosis had not been observed in captive or wild koalas until 2012, when it was confirmed in three cases of conjunctivitis (O. Funnell, L. Woolford, W. Boardman, pers. comm.). The current prevalence of *Chlamydia* in the Mount Lofty koala population is therefore unknown.

1.4.3 Cryptococcosis and koala retrovirus

Other significant infections of koalas include cryptococcosis and koala retrovirus. *Cryptococcus neoformans* is a fungus that causes respiratory tract infection in koalas (Blanshard 1994, Krockenberger et al. 2003) and *C. n.* var. *gattii* has been identified on leaves of various *Eucalyptus* spp. (Ellis and Pfeiffer 1990, Krockenberger et al. 2002b), which possibly act as a source of infection for koalas (Krockenberger et al. 2002a). In New South Wales, disease caused by *Cryptococcus* occurs in koalas at a prevalence of 2.5% (N=1061) (Stalder et al. 2003), but is also found in the nasal cavity of healthy koalas (Krockenberger et al. 2002a), isolated in 100% of captive koalas at Taronga Zoo (Barnes 1999).

Koala retrovirus (KoRV) is a recently described infection of koalas (Hanger et al. 2000) and has been shown to be associated with lymphoid neoplasia and leukaemia (Tarlinton et al. 2005), which has been observed in koalas for many years (Backhouse and Bolliger 1961, Canfield et al. 1987). The prevalence of KoRV infection is varied across Australia, with 100%

of koalas affected in Queensland and New South Wales, 73% in Victoria, but only 15% in Kangaroo Island (Simmons et al. 2012). Also, whilst northern populations have an endogenous form of infection with high proviral load, southern populations appear to have an exogenous form with low levels of provirus (Simmons et al. 2012). However, KoRV seems to currently be undergoing active endogenisation in koalas as the infection spreads southward on the Australian mainland (Simmons et al. 2012, Tarlinton et al. 2008, Tarlinton et al. 2006). Infection with koala retrovirus may induce immunosuppression, which has implications for increased susceptibility to *Chlamydia* infection, but this is yet to be demonstrated (Tarlinton et al. 2005, Young et al. 2008). The retrovirus status of the Mount Lofty koala population is currently unknown.

1.4.4 Other causes

Necropsy surveys have found that many koalas have an unidentifiable cause of death, or 'no visible lesions' at post mortem examination (Canfield 1990a). These cases may be attributed to 'koala stress syndrome' (Obendorf 1983) or 'koala wasting disease' (Degabriele 1989). 'Koala stress syndrome' was first recognised by Obendorf (1983) as a disease of inappetance and weight loss in both captive and wild koalas in Victoria. The cause was postulated as stress due to concurrent disease or trauma, or hospitalisation with frequent handling and treatment (Obendorf 1983). At post mortem only non-specific changes were found to occur which included atrophy of lymphoid tissue and adrenal cortex, and occasionally acute tubular necrosis (Butler 1978, Canfield 1990a, Obendorf 1983).

"Wasting disease" has been reported in captive koalas and is described as the death of koalas due to apparent starvation, despite having a full stomach at post mortem examination (Braysher 1978, Cork and Sanson 1990, Degabriele 1989). This disease is most common in aged koalas and has been linked to advanced tooth wear decreasing the

efficiency of mastication (Lanyon and Sanson 1986, Wood 1978). It has also been found to occur in winter or following a drought, in which only older fibrous eucalypt leaves are available due to decreased new leaf growth, and nitrogen intake is therefore limited for koalas (Braysher 1978, Degabriele 1980, Degabriele 1981, Wood 1978).

1.5 RENAL DISEASE IN KOALAS

The prevalence of renal disease in koalas is difficult to estimate, since the majority of studies on morbidity and mortality in koalas have been undertaken in New South Wales, in *Chlamydia*-infected populations. Due to the predilection of *Chlamydia* infection for the urogenital tract, the majority of kidney disease in koalas has been found to be associated with chlamydiosis. For instance, in one disease survey 55% of koalas (60/110) showed urinary tract pathology and 10% kidney pathology, but the majority were suspected to have chlamydial disease (Connolly 1999). In another necropsy survey of 127 koalas, a total of 33 showed urogenital disease (26%), ten had kidney disease associated with cystitis (8%) and only five showed kidney disease alone (4%) (Canfield 1987b).

Forms of renal disease found in conjunction with cystitis include hydronephrosis due to urinary retention, and pyelonephritis due to ascending infection (Canfield 1989, Canfield and Spencer 1993). Pyelonephritis can involve secondary opportunistic bacteria including *Staphylococcus* spp, *Streptococcus* spp, *Escherichia coli*, and *Proteus* spp (Higgins et al. 2005, Obendorf and Handasyde 1990). Acute and chronic nephritis can result from these infections, with the latter resulting in the kidney being small, hard and irregular in shape (Obendorf 1988).

Renal disease in koalas in the absence of chlamydiosis appears to be uncommon. In the only survey specifically of urinary tract disease in koalas, performed in New South Wales between 1980 and 1988, only 7 out of 235 koalas (3%) had renal disease unassociated with

cystitis (Canfield 1989). Reports of renal disease in individual koalas include acute tubular necrosis (Spencer and Canfield 1993), membranous glomerulonephritis (Canfield 1987a), and acute nephrosis and pyelonephritis, thought to be related to systemic toxicities or septicaemia (Canfield 1987b). In one case of renal failure, elevated values of urea (29.9 mmol/L; normal koala reference interval 0.2-6.6 mmol/L) and creatinine (581 µmol/L; 80-150 µmol/L) were reported in conjunction with low urine specific gravity (1.017; normal koala reference interval 1.062-1.135), indicative of poor kidney function (Canfield et al. 1989a, Canfield et al. 1989b, Spencer and Canfield 1993).

1.6 RENAL CALCIUM OXALATE DEPOSITION IN KOALAS

Renal calcium oxalate crystal deposition has been reported at low prevalence in koalas across Australia, as both a necropsy finding (Canfield 1987b, Canfield 1989, Connolly 1999) and as a disease associated with renal dysfunction (Canfield and Dickens 1982, Dickson 1989a, Main 1992). Calcium oxalate stone deposition has also been reported, with renal nephrolithiasis found in one koala, in which the calculus was composed of 70% oxalate, 30% calcium and 0.5% ammonium (Connolly 1999), and in another koala, a bladder urolith was found to consist of calcium oxalate and uric acid (Canfield 1989).

In necropsy surveys of koalas undertaken in New South Wales, renal calcium oxalate deposition has been reported at low levels, at <3% of 110 koalas (Connolly 1999) and <2% of 127 koalas (Canfield 1987b). In another study, 4 koalas out of 235 (<2%) showed renal calcium oxalate deposition overall, and in 67 koalas with urinary tract disease likely due to *Chlamydia* infection, 6% prevalence occurred (Canfield 1989). Yet in two other studies investigating renal complications of cystitis, no deposition of oxalate was reported (Canfield and Spencer 1993, Obendorf 1988). Another study of the role of *Chlamydia* in renal disease in New South Wales and Queensland koalas found only one koala out of 79 with renal

intratubular crystals of an unknown type, and this particular koala showed negative renal cultures for *Chlamydia*, bacteria and fungi (Higgins et al. 2005).

Calcium oxalate deposition associated with renal dysfunction, or oxalate nephrosis, has been reported in few koalas (Canfield and Dickens 1982, Dickson 1989a, Main 1992). Only one case, a suspected poisioning, has been described in detail; an adult male koala which had been on antibiotic treatment for 'dirty tail' or cystitis. The kidney pathology in this case was severe with enlarged, soft kidneys which on histopathological examination showed extensive necrosis of renal tubule epithelial cells, and tubular dilation with crystals and amorphous pink material (Canfield and Dickens 1982). Crystals were birefringent using polarisation microscopy, stained positively to Pizzolato's peroxide silver method for oxalate and analysis of the 'urethral-bladder plug' indicated calcium oxalate (Canfield and Dickens 1982). Other cases of oxalate nephrosis include a female koala and her ten month old joey at Perth Zoo, which both showed renal failure and Pizzolato-positive renal crystals associated with pyelonephritis and fibrosis at histopathological examination (Dickson 1989b, Dickson 1989a); and a young female koala with azoturia and weight loss, which showed renal crystals consistent with oxalate, renal fibrosis and tubule dilation (Main 1992).

In addition to koalas, renal calcium oxalate deposition has also been reported to occur sporadically in other Australian marsupials such as wombats (L. Woolford, pers. comm. 2013)(Hartley 1991), a ringtail possum (Hemsley and Canfield 1993), the endangered Gilbert's potoroo (D. Forshaw, pers. comm. 2013), a scaly-tailed possum and a swamp wallaby (Ellis et al. 1983).

1.7 CAUSES OF RENAL CALCIUM OXALATE DEPOSITION IN OTHER SPECIES

Calcium oxalate is the most common deposit found in the kidneys of humans (Chonko and Richardson 1994, Hagler and Herman 1973b) and also occurs in animals,

particularly livestock (James 1972, Maxie and Newman 2007). Renal calcium oxalate deposition can occur as part of an acute or chronic illness and may be mild, with little apparent effect on renal function, or severe, causing end-stage renal failure and death (Asplin 2002, Weiss et al. 2007). Renal dysfunction occurs due to crystal obstruction of the tubules (Osborne and Polzin 1991), direct damage from the crystals causing necrosis of the tubular epithelium (James 1972, Weiss et al. 2007) and/or toxic effects of oxalate ions to the tubular epithelium prior to crystal formation (Hackett et al. 1994, Khan and Hackett 1993, Scheid et al. 1996). Following the rupture of crystals from tubules into the interstitium, inflammation involving giant cell reaction, and fibrosis may occur (Chonko and Richardson 1994). Renal parenchymal damage leads to dysfunction characterised by poor urine concentrating ability and azotaemia, or increased levels of urea and creatinine in circulation, due to decreased excretory ability of the kidneys (Osborne and Polzin 1991).

Calcium oxalate deposition in the kidneys of humans and animals usually occurs due to increased renal excretion of either oxalate or calcium i.e. hyperoxaluria or hypercalciuria (Weiss et al. 2007). Hyperoxaluria occurs with increased levels of circulating oxalate since the kidney is the primary site of excretion for oxalate, in which it is freely filtered by the glomerulus and actively secreted in the proximal convoluted tubule (Robijn et al. 2011). Supersaturation of the urine with oxalate greatly increases the risk of formation of insoluble calcium oxalate (Asplin 2002). Hypercalciuria also increases the risk of calcium oxalate precipitation (Asplin et al. 2000), but to a lesser extent since calcium is excreted in ten-fold greater amounts than oxalate in normal human urine (Asplin 2002). Therefore, significant increases in urinary calcium are necessary for calcium oxalate formation, compared with only mild increases in urinary oxalate (Asplin 2002, Chonko and Richardson 1994, Robertson and Peacock 1980).

Hyperoxaluria can be caused by high dietary intake of oxalate, gastrointestinal factors affecting oxalate absorption, endogenous overproduction of oxalate resulting from inherited liver enzyme dysfunctions, or inadequate excretion of oxalate secondary to kidney failure (Asplin 2002, Chonko and Richardson 1994, Robijn et al. 2011). Hypercalciuria usually occurs due to disorders affecting calcium homeostasis (Weiss et al. 2007).

1.7.1 Dietary sources of oxalate

Dietary oxalate is an exogenous source of oxalate and has been found to make a significant contribution to circulating oxalate in the body, accounting for up to 53% of urinary oxalate in humans (Holmes et al. 2001). Dietary sources of oxalate are primarily plant-derived and occur in the form of soluble and insoluble oxalate salts of calcium, potassium or sodium (McBarron 1977). Many plants contain low to moderate levels of oxalate, such as spinach, rhubarb, soy and beetroot (Reyers and Naude 2012, Siener et al. 2006), but some contain toxic levels of oxalate, such as halogeton (*Halogeton glomeratus*), curly dock (*Rumex crispus*) and soursobs (*Oxalis pes-caprae*) (James and Panter 1993, McBarron 1977). Toxic plants contain >10% oxalate on a dry weight basis and particularly affect grazing livestock (James and Panter 1993, McBarron 1977, Panciera et al. 1990), resulting in death after either an acute or chronic illness (James 1972, McKenzie 2012).

Some fungi, such as *Aspergillus* and *Penicillium* spp., have been found to be able to produce oxalate (Reyers and Naude 2012, Weiss et al. 2007) and are also a potential cause of oxalate toxicity in livestock due to contamination of feed with mould (Cheeke 1995, da Costa et al. 1998). In humans, high intakes of oxalate precursors, such as ascorbic acid (Vitamin C), fructose, xyulose and the amino acid hydroxyproline, result in increased oxalate in the body, but their ability to induce oxalate nephrosis is questioned (Asplin 2002). Deficiencies of vitamins in the diet, such as pyridoxine (Vitamin B6) which has a role in

oxalate metabolism, and thiamine (Vitamin B1), are also considered to be causes of increased urinary oxalate in humans and laboratory rats (Chonko and Richardson 1994, Di Tommaso et al. 2002). In addition, renal calcium oxalate deposition can occur following ingestion of ethylene glycol (antifreeze) in both humans and animals (Hess et al. 2004, Jones and Hunt 1983). Another exogenous source of oxalate reported in humans is methoxyflurane anaesthetic gas (Asplin et al. 2000, Weiss et al. 2007).

1.7.2 Gastrointestinal factors

The gastrointestinal absorption of oxalate can be increased or decreased by a variety of factors. In humans, over-absorption of oxalate can occur in patients who have bowel disease causing steatorrhea, such as Crohn's disease or pancreatic insufficiency, or following surgery such as jejuno-ileal bypass, and can be significant enough to cause renal failure (Asplin 2002, Chonko and Richardson 1994, Weiss et al. 2007). Conversely, intestinal absorption of oxalate may be decreased if the diet contains high levels of calcium, due to the production of insoluble calcium oxalate which is excreted in the faeces (Brogren and Savage 2003, James 1972).

The absorption of oxalate will also be decreased if oxalate-degrading bacteria are well established in the gut (Hoppe et al. 2006, James 1972). These bacteria occur in the gastrointestinal tract of humans (Allison et al. 1986), as well as ruminants, horses, pigs, rats, rabbits and guinea pigs (Allison and Cook 1981, Argenzio et al. 1988), with *Oxalobacter formigenes* identified as a key species (Allison et al. 1985). In ruminants, the colonisation of the rumen by oxalate-degrading micro-organisms is thought to decrease their susceptibility to toxicity from ingestion of high oxalate plants, due to the ability of the bacteria to adapt to increasing oxalate concentrations (James and Panter 1993). Also, the higher pH of the rumen favours formation of insoluble calcium oxalate, whereas the low stomach pH in monogastric

species increases solubilisation of plant oxalates (Reyers and Naude 2012). Despite this, sheep flocks are occasionally affected by widespread acute or chronic oxalate toxicity due to grazing soursobs (*Oxalis pes-caprae*) (Bull 1929, McIntosh 1972).

1.7.3 Endogenous oxalate production

Oxalate is produced endogenously in small amounts as a by-product of the glyoxylate metabolic pathway in mammals (Raju et al. 2008). However, in humans an inherited group of diseases, called the primary hyperoxalurias, result in overproduction of oxalate by the liver due to the deficiencies of enzymes involved in this pathway (Asplin 2002, Cochat et al. 2006). The most common form, primary hyperoxaluria type I (PH I) is caused by dysfunction of the liver enzyme alanine: glyoxylate aminotransferase (AGT), which catalyses the conversion of glyoxylate to glycine (Asplin 2002, Cochat et al. 2006). In addition to oxalate, glycolate is produced in excess, and is also found in increased concentration in the urine (Asplin 2002, Cochat et al. 2006). In animals, a PH I - like deficiency of AGT has been reported in a litter of purebred Tibetan spaniels (Danpure et al. 1991) and also in Coton de Tulear dogs (Vidgren et al. 2012).

Primary hyperoxaluria type II (PH II) is a rare disease in humans (Johnson et al. 2002, Mansell 1995), also causing overproduction of oxalate by the liver (Giafi and Rumsby 1998, Mistry et al. 1988, Rumsby 2006). It occurs due to deficiencies of two liver enzymes, glyoxylate reductase (GR) and hydroxypyruvate reductase (HPR) and results in increased oxalate and glycerate in the urine (Asplin 2002). A disease similar to primary hyperoxaluria type II has been found to occur in cats (Blakemore et al. 1988, Danpure 1989, McKerrell et al. 1989). Also, primary hyperoxaluria type III has recently been identified as the dysfunction of hepatic enzyme 4-hydroxy-2-oxoglutarate aldolase and leads to accumulation of glyoxylate and hence oxalate, but its pathogenesis is still not well understood (Belostotsky et al. 2010,

Hoppe 2012). Further human cases remain unclassified, with PH I, II and III ruled out as the cause (Hoppe 2012, Milliner 2005).

In early stages, primary hyperoxaluria in humans is treated conservatively to reduce renal calcium oxalate crystal formation. High water intake has been identified as a key therapy to increase urine volume and decrease urinary oxalate saturation, whilst avoidance of high oxalate foods is recommended so as not to contribute further to the oxalate load (Asplin 2002, Cochat et al. 2012). Also, pyridoxine (vitamin B6) therapy has been found to reduce urine oxalate in up to a third of PH type I patients, as it is the main co-factor of AGT and increases any residual enzyme activity (Asplin 2002, Cochat et al. 2006, Fargue et al. 2013, Hagler and Herman 1973a). Neutral orthophosphate, magnesium and citrate may also reduce calcium oxalate formation by producing soluble oxalate complexes, inhibiting crystal formation or reducing oxalate saturation (Asplin 2002, Cochat et al. 2012, Milliner et al. 1994). However, as the disease inevitably progresses the prognosis becomes poor for those affected, with severe cases resulting in renal failure early in life (Asplin 2002), for which a combined liver-kidney transplant is the only cure (Asplin 2002, Cochat et al. 2012, Harambat et al. 2011).

1.7.4 Renal failure

In end-stage kidney failure, when <10% of kidney function remains, uraemia occurs and involves the build up of toxins in the body due to the inability of the kidney to excrete these substances (Mydlik and Derzsiova 2008, Whelton et al. 1994, Zollinger and Milhatsch 1978). One of these uraemic toxins is oxalate, since it mirrors the increase in urea and creatinine concentrations that occur with worsening renal function (Mydlik and Derzsiova 2008, Zarembski et al. 1966). Hence renal failure can result in calcium oxalate deposition in the kidney as a secondary process (Chonko and Richardson 1994, Salyer and Keren 1973), in

which crystals are seen mainly within the lumen of proximal and distal convoluted tubules and are associated with little tubule epithelial cell damage (Bosman and La Ginestra 1967, Fanger and Esparza 1964) or inflammatory reaction (Salyer and Keren 1973). Despite the increased plasma oxalate that occurs with renal failure, urinary oxalate is usually decreased due to impaired excretion (Chonko and Richardson 1994, Harambat et al. 2011, Hodgkinson 1977, Milliner 2005).

1.7.5 Disorders of hypercalciuria

Calcium oxalate deposition in the kidney may also occur due to disorders causing significant increases in urinary calcium (Asplin 2002, Robertson and Peacock 1980). In humans, most cases of hypercalciuria are due to primary hyperparathyroidism in which overproduction of parathyroid hormone increases calcium absorption at the intestine, bone and kidney (Weiss et al. 2007). Malignant neoplasms, including carcinomas and those that invade bone, such as multiple myeloma, may also cause hypercalcaemia and hypercalciuria (Weiss et al. 2007). However, many human cases of hypercalciuria are idiopathic, and may be associated with intestinal calcium overabsorption or renal calcium leakage (Arrabal-Polo et al. 2013, Asplin et al. 2000).

1.8 INVESTIGATION OF RENAL DISEASE IN THE MOUNT LOFTY KOALAS

The koala population of the Mount Lofty Ranges in South Australia was found by a previous study to have a high level of renal disease, affecting five of 45 koalas (11%) in 2000 (Haynes et al. 2004). Both wild and captive koalas were found to be affected and showed varying degrees of lethargy, weight loss, polydipsia and polyuria in the absence of clinical signs of chlamydiosis (Haynes et al. 2004). Dietary aluminium was investigated as a possible cause, with aluminium evident in eucalypt leaves, kidney tubule cells and bone, but the significance of these findings was unclear (Haynes et al. 2004). However, one koala in this

previous study showed intratubular renal crystals which stained positively with Pizzolato's silver peroxide technique, consistent with calcium oxalate (Haynes et al. 2004). Routine post mortem examinations had also found pale yellow deposits within kidneys of several Mount Lofty koalas, which histopathological examination indicated was suggestive of calcium oxalate (I. Hough; W. Boardman, 2008, pers. comm.). To investigate this disease further, renal pathological changes needed to be described and deposits analysed to confirm calcium oxalate. If oxalate nephrosis was confirmed to occur in Mount Lofty koalas, investigation of its cause needed to be based on what occurs in other mammalian species affected by this disease.

Ingestion of oxalate-containing plants is the most common cause of renal calcium oxalate deposition in herbivores (Maxie and Newman 2007). However, little was known of the oxalate content of eucalypt leaves which comprise the diet of koalas. A previous limited analysis of eucalypt leaf samples as a possible source of dietary oxalate for affected koalas at Perth Zoo found very low quantities (Dickson 1989b). In contrast, a previous study of karri (*E. diversicolor*) found moderate levels of total oxalate in leaves (3.7-4.4% on a dry weight basis) (O'Connell et al. 1983), but this eucalypt species is not known to be eaten by koalas (Jackson et al. 2003). More recently, manna gum (*E. viminalis*) was found to be the cause of oxalate nephropathy in a colony of captive marmosets (Vanselow et al. 2011). Therefore eucalypt oxalate would need to be investigated as a possible cause of oxalate nephrosis in koalas.

Koalas in the Mount Lofty Ranges population have low genetic variation (Houlden and St John 2000, Seymour et al. 2001). Hence, primary hyperoxaluria, which occurs at higher prevalence in inbred human populations (Kamoun and Lakhoua 1996), would also need to be considered as a possible cause of oxalate nephrosis in these koalas. In particular, a disease similar to primary hyperoxaluria type I, in which dysfunction of the liver enzyme AGT occurs, would be important to investigate since supplementation with pyridoxine, the

co-factor of AGT, had been observed to improve the clinical condition of captive Mount Lofty koalas with renal disease (I. Hough, A. Sulley, 2008, pers. comm.).

Other potential causes of oxalate nephrosis such as gastrointestinal disease, renal failure and calcium disorders would also need to be considered for the koalas of the Mount Lofty population. In addition, since low water intake has been identified as a key risk factor for the progression of renal calcium oxalate deposition in humans (Asplin 2002, Cochat et al. 2012), the assessment of moisture content of eucalypt leaves could indicate whether water intake was adequate in affected koalas, given that the Mount Lofty region had recently experienced a prolonged drought (CSIRO 2007).

1.9 RESEARCH AIMS

1. The initial aim of the current research study was to confirm that renal disease in the Mount Lofty koala population was associated with calcium oxalate deposition, by describing:

    a) The gross and histopathological changes that occurred in the kidneys of koalas with renal disease, including the composition of renal crystalline deposits.
    b) Plasma and urine biochemistry of affected koalas compared with that of other koala populations.
    c) Prevalence of renal disease and if any predisposing factors such as age, sex and origin of koalas as captive or wild occurred.

2. If oxalate nephrosis was confirmed, the subsequent aim was to investigate possible causes, by determining:

    a) Oxalate concentration in various species of eucalypt leaves eaten by koalas in the Mount Lofty Ranges compared with those eaten by koalas in other regions.
    b) Activity of liver enzyme alanine: glyoxylate aminotransferase (AGT) in koalas with oxalate nephrosis.
    c) Seasonal changes in moisture content that occur in leaves of various eucalypt species eaten by koalas, to assess water intake by affected koalas.

CHAPTER 2

Pathological features of oxalate nephrosis in a population of koalas (*Phascolarctos cinereus*) in South Australia

Speight KN, Boardman W, Breed WG, Taggart D, Woolford L and Haynes JI. (2013) Veterinary Pathology 50 (2) 299-307.

CONTEXTUAL STATEMENT

The koala population of the Mount Lofty Ranges in South Australia has been found to have a high level of renal disease, estimated at 11% in 2000 (Haynes et al. 2004). Dietary aluminium was previously investigated as a possible cause, but its significance remains unclear (Haynes et al. 2004). A koala in this earlier study, as well as several veterinary histopathological reports, showed renal crystals consistent with calcium oxalate in affected koalas, suggesting that oxalate nephrosis may occur. Renal calcium oxalate crystal deposition has previously been reported at low prevalence in other Australian koala populations (Canfield 1987, Canfield 1989, Connolly 1999), but only described in detail in one case report in the scientific literature (Canfield and Dickens 1982).

Chapter 2 describes the composition of crystalline deposits in the kidneys of koalas from the Mount Lofty population, the associated renal gross pathological and histopathological changes which occur, the current prevalence of disease and any predisposing factors such as age, sex and origin of koalas.

STATEMENT OF AUTHORSHIP: Chapter 2

Pathological features of oxalate nephrosis in a population of koalas (*Phascolarctos cinereus*) in South Australia. (2013) *Veterinary Pathology,* 50 (2) 299-307.

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Veterinary Pathology, v. 50(2), pp. 299-307

CHAPTER 3

Plasma biochemistry and urinalysis of koalas (*Phascolarctos cinereus*) with oxalate nephrosis

Speight KN, Boardman W , Breed WG , Taggart D, Rich B, Woolford L and Haynes JI. (in press) Veterinary Clinical Pathology. Accepted 4th February 2013.

CONTEXTUAL STATEMENT

In **Chapter 2**, oxalate nephrosis was shown to affect 55% of captive and rescued wild koalas in the Mount Lofty population in South Australia. In the eastern states of Australia, oxalate nephrosis is an uncommon disease of koalas, with < 3% prevalence reported (Canfield 1987, Canfield 1989, Connolly 1999). Biochemical abnormalities of renal disease in koalas are not well described, and those associated with oxalate nephrosis in koalas are unknown.

Chapter 3 characterises the plasma and urine biochemical abnormalities associated with oxalate nephrosis in the Mount Lofty koala population, including urinary crystal morphology and composition.

STATEMENT OF AUTHORSHIP: Chapter 3

Plasma biochemistry and urinalysis of koalas (*Phascolarctos cinereus*) with oxalate nephrosis. *Veterinary Clinical Pathology,* Accepted 4th February 2013.

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3.1 ABSTRACT

Oxalate nephrosis is a leading disease of the Mount Lofty Ranges koala population in South Australia, but associated clinicopathological findings remain undescribed. The aims of this study were to determine plasma biochemical and urinalysis parameters, particularly for renal function and urinary crystal morphology and composition, in koalas with oxalate nephrosis. Blood and urine samples from Mount Lofty Ranges koalas with oxalate nephrosis were compared with those unaffected by renal oxalate crystal deposition from Mount Lofty and Kangaroo Island, South Australia and Moggill, Queensland. Plasma and urine biochemistry was analysed using a Cobas Bio analyser and urinary oxalate by high performance liquid chromatography. Urinary crystal composition was determined by infrared spectroscopy and energy dispersive X-ray analysis. Azotaemia was found in 93% of koalas with oxalate nephrosis (n=15). Renal insufficiency was shown by poorly concentrated urine of specific gravity (USG) <1.035 in 100% azotaemic animals, and <1.030 in 83%. Koalas with oxalate nephrosis were hyperoxaluric compared with Queensland koalas ($P<0.01$). Urinary crystals from koalas with oxalate nephrosis had atypical morphology and were composed of calcium oxalate. Mount Lofty Ranges koalas unaffected by renal oxalate crystal deposition also showed renal insufficiency (43%), although only 14% had USG <1.030 (n=7). Unaffected Mount Lofty Ranges and Kangaroo Island koalas were also hyperoxaluric compared with Queensland koalas ($P<0.01$). Koalas with oxalate nephrosis from the Mount Lofty Ranges show renal insufficiency, hyperoxaluria and pathognomonic urinary crystals. The findings of this study will aid veterinary diagnosis and further investigations of this disease.

3.2 INTRODUCTION

The Mount Lofty Ranges koala population in the Adelaide Hills region of South Australia (SA) appears to have a high level of renal dysfunction (Haynes et al. 2004) compared to koalas found elsewhere in Australia (Canfield 1989), with koalas showing clinical signs typical of renal disease such as polydipsia, polyuria and weight loss (Haynes et al. 2004). A recent study has shown that oxalate nephrosis also occurs at high prevalence in the Mount Lofty population, with 55% of captive and rescued wild koalas found to be affected (Speight et al. 2013). Oxalate nephrosis is far more common in this koala population than in the eastern states of Australia, such as in New South Wales, where less than 3% of koalas are affected (Canfield 1987, Canfield 1989, Connolly 1999). Koalas with oxalate nephrosis show characteristic renal histopathological changes associated with deposition of calcium oxalate crystals (Weiss et al. 2007), such as intratubular and interstitial inflammation, nephron loss and fibrosis, glomerular atrophy and tubule dilation (Canfield and Dickens 1982, Speight et al. 2013).

Oxalate precipitation in the kidney and lower urinary tract typically occurs with increased circulating oxalic acid. Oxalic acid is excreted by the kidneys and may precipitate as insoluble calcium oxalate within the renal tissue (Asplin 2002). Hyperoxaluria (elevated levels of oxalate in the urine) may also occur, with calcium oxalate crystals forming in urine typically as either dumbbell-shaped calcium oxalate monohydrate crystals or as envelope-shaped calcium oxalate dihydrate crystals (Osborne and Stevens 1999).

Oxalate nephrosis can cause renal dysfunction by crystal obstruction of tubules (Osborne and Polzin 1991), necrosis of tubular epithelium (Weiss et al. 2007), or toxic effects of oxalate ions to tubular epithelium prior to crystal formation (Hackett et al. 1994, Khan and Hackett 1993, Scheid et al. 1996). This renal dysfunction results in decreased glomerular filtration and inadequate excretion of metabolic wastes, leading to azotaemia (elevated

plasma creatinine and urea) (Osborne and Polzin 1991). Urine specific gravity (USG) is used to show that the azotaemia is of renal origin and to further classify the dysfunction as renal insufficiency or failure (Lane et al. 1994, Osborne and Polzin 1991). Hence, knowledge of the urine concentrating capacity of different species is important for interpretation of USG and accurate classification of renal dysfunction.

In koalas, plasma creatinine and urea reference intervals and USG values have been primarily determined in animals from the eastern states of Australia (Blanshard and Bodley 2008, Canfield et al. 1989a, Canfield et al. 1989b). In one study of koalas from New South Wales, the reference interval for plasma creatinine was found to be 80 to 150 µmol/L and for urea, 0.2 to 6.6 mmol/L (Canfield et al. 1989b); whilst in another study mean USG was determined as 1.087 (interval 1.062-1.135) (Canfield et al. 1989a). A previously published case of renal failure in a Victorian koala due to acute tubular necrosis described elevated values of plasma creatinine (581 µmol/L) and urea (29.9 mmol/L) with decreased USG (1.017), consistent with azotaemia of renal origin (Spencer and Canfield 1993). In the Mount Lofty Ranges region in SA, a study of renal disease occurrence in 11 koalas between the years 1995-2000 reported mean urea as 30 ± 4 mmol/L and USG <1.040 (Haynes et al. 2004).

Although it has recently been confirmed that oxalate nephrosis is a leading disease in the koala population of the Mount Lofty Ranges (Speight et al. 2013), clinicopathological findings in affected koalas remain uncharacterised. In addition, little is known of blood and urine biochemistry and health status of SA koala populations. The current study describes plasma biochemistry and urinalysis of koalas with oxalate nephrosis in the Mount Lofty Ranges population and compares results with koalas unaffected by renal oxalate crystal deposition also from the Mount Lofty Ranges, as well as with related koalas on Kangaroo Island in SA (Robinson 1978), and with geographically distant Queensland koalas. In addition,

the morphology and composition of urinary crystals from koalas with oxalate nephrosis are described.

3.3 MATERIALS AND METHODS

3.3.1 Koalas

Blood, urine and kidney samples were collected from a total of 57 rescued wild and captive koalas that were euthanased on welfare grounds at Cleland Wildlife Park, Mount Lofty, SA and at Moggill Koala Hospital, Moggill, Queensland. Captive koalas at Cleland Wildlife Park had been group housed with access to ad lib water and eucalypt leaves and received regular veterinary care. Koalas were aged using the tooth wear classification method of Martin (1981) and Martin and Handasyde (1990) or from animal management records. Where possible, blood was collected by cardiac puncture into lithium heparin tubes (Sarstedt, Germany), refrigerated and centrifuged within 24 hours for plasma separation and stored at -70˚C until analysis.

Necropsy was performed at varying times post mortem (median 2 hours, range 0 – 48 hours) to determine the health status of koalas. Urine was collected by direct cystocentesis and urinalysis performed, including microscopic sediment examination, with aliquots stored at -70˚C. Kidney samples were processed routinely for histological examination to determine the presence or absence of renal oxalate crystals (see Speight et al. 2013), to allow classification of koalas into groups affected by oxalate nephrosis from Mount Lofty and those that were unaffected by renal oxalate crystal deposition from Mount Lofty and Queensland.

25 Mount Lofty (ML) rescued wild and captive koalas were classified as affected by oxalate nephrosis and showed renal histopathological changes associated with oxalate crystal deposition (see Speight et al. 2013). Kidney sections were scored semi-quantitatively

0 – 3 (none, mild, moderate, severe) for cortical fibrosis, tubule dilation, intratubular inflammation and interstitial inflammation. Necropsy findings, in addition to oxalate nephrosis associated with poor body condition, were concurrent gastrointestinal disease (16%), including intestinal torsion in three captive koalas, and respiratory disease (4%).

17 rescued wild and captive Mount Lofty koalas were classified as unaffected by renal oxalate crystal deposition, with the main post mortem findings including trauma likely due to motor vehicle accident (53%), poor body condition (18%), respiratory disease (18%) and gastrointestinal disease (12%). In the absence of oxalate crystals, renal histopathological changes were mild interstitial inflammation in 2/17 koalas (12%) and mild calcium phosphate deposition in 41% koalas.

Histological examination of the kidneys of Queensland (Qld) rescued wild koalas showed 15 koalas unaffected by renal oxalate crystal deposition for inclusion in the study, and two koalas with oxalate nephrosis for exclusion from the study. Of the 15 unaffected Qld koalas, renal histopathological changes included moderate interstitial inflammation (35%), variable cortical fibrosis (12%) and mild to moderate medullary calcium phosphate deposition (94%). The main post mortem findings included lesions consistent with ocular and/or urogenital chlamydiosis (67%), poor condition (20%), motor vehicle trauma (7%) and dog attack (7%).

In addition, live wild-caught koalas on Kangaroo Island (KI), SA were sampled for blood and urine only. Blood and midstream urine samples were collected from koalas whilst under isoflurane anaesthesia. Urine was examined microscopically to detect crystals similar in morphology to Mount Lofty koalas with oxalate nephrosis, so as to determine unaffected koalas for inclusion in the study (n=24) and those to be excluded (n=1). For final numbers of blood and urine samples collected from the four koala groups, see Table 1.

Tooth wear class (TWC), sex and origin of koalas as captive, rescued wild or wild caught are summarised in Table 2. Mean TWC for ML koalas with oxalate nephrosis was 2.0 ± 1.3 (mean ± SD) and for ML koalas unaffected by renal oxalate crystal deposition, 2.0 ± 1.2, showing many young animals approximately 2 - 3 years old in both ML groups (Martin and Handasyde 1990, Martin 1981). In contrast, the Qld group were mostly older animals approximately 6 - 12 years old, mean TWC 5.6 ± 1.1; as were the KI cohort of which most were approximately 4 - 6 years old, mean TWC 4.0 ± 1.1 (Martin and Handasyde 1990, Martin 1981). Whilst the sex ratio for koalas with oxalate nephrosis was approximately equal; male koalas dominated the ML group unaffected by renal oxalate crystal deposition (82%), and numbers of female koalas were higher in both the Qld (80%) and KI (63%) groups.

Voided urine samples were also collected from 6 captive resident koalas (4 female, 2 male) at Cleland Wildlife Park with clinical signs of renal dysfunction such as polydipsia, polyuria and inappetance, and heavy urinary sediment. These urine samples were examined microscopically for crystals and where crystal morphology was similar to koalas with oxalate nephrosis, these samples were included in crystal composition analyses results. All koalas were sampled with approval of the University of Adelaide Animal Ethics Committee, Department of Environment and Natural Resources (SA) and Department of Environment and Resource Management (Qld).

3.3.2 Plasma biochemistry

Plasma samples were analysed for total protein (TP), albumin, glucose, creatinine, urea, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT), urate, calcium and phosphate on a Cobas Bio analyser (Roche, Switzerland) using standard Roche kits (Roche Diagnostics, Switzerland). Roche analyser calibrator for automated systems (C.F.A.S) was run concurrently with each batch of assays as

a standard, control and recovery for each analyte. Ten assays were regularly run on randomly selected analytes to confirm Cobas Bio precision, with relative standard deviation (rsd) values less than 5%. Results were compared with reference intervals for koala biochemical analytes established by Canfield et al. (1989b). Any haemolysis of samples was recorded.

3.3.3 Urine biochemistry

Urine specific gravity was measured using a handheld refractometer and concentrated samples were diluted with equal volumes of water if required. Samples of urine were analysed for creatinine, urate, calcium and phosphate on a Cobas Bio analyser using standard Roche kits (as above). Urinary analytes were expressed per unit of creatinine to standardise results and allow comparison between groups.

For measurement of oxalate, urine was diluted 1:9 in 0.01M HCl and analysed by the reverse phase high performance liquid chromatography method of Grace-Alltech Chrom 9384, modified using a Waters 600 Pump and 600E Controller coupled to a Waters 2489 UV/Visible Detector (Waters Corporation, Milford MA). Prevail Organic Acid columns (Alltech, Deerfield, IL, USA) were used as the stationary phase with a guard column (C18, 75 x 4.6mm: Particle size 5µm) positioned ahead of the analytical column (C18, 250 x 4.6mm: Particle size 5µm).

The mobile phase consisted of 97% 0.025M potassium dihydrogen phosphate buffer pH 2.5 and 3% acetonitrile (HPLC grade, Burdick & Jackson, Muskegon, MO, USA) was applied at a flow rate of 1mL/min for 6 minutes then at 2mL/min for a further 5 minutes to clear the column of residual solutes. Column temperature was maintained at 30˚C and oxalic acid was detected at 210nm.

Chromatograph output was processed using Delta Chromatography Data Systems software. A 35µL sample was injected with the oxalate peak appearing approximately 3 minutes after injection. Oxalate concentration was calculated from a calibration curve produced for oxalate by plotting peak height in mm versus concentration in µg/mL. Least squares regression was performed to determine the slope, intercept and coefficient of determination. Oxalate concentrations of samples were calculated using the following formula:

$$\text{Oxalic acid (µg/mL)} = \frac{\text{Peak height for assay x standard concentration x sample dilution}}{\text{Peak height for standard x sample volume (mL)}}$$

The oxalate calibration curve was linear (R^2= 0.99). A typical chromatogram for urinary oxalate measurement is shown in Figure 1. The mean recovery of oxalate added to urine samples was 93.5 ± 3.1% (mean ± SD) with 7% within-sample variation.

3.3.4 Urinary crystal examination and analysis

Urinalysis included measurement of urinary pH using Combur-9 dipsticks (Roche Diagnostics, Switzerland) and microscopic examination of urinary sediment. Urine samples from koalas with heavy crystal precipitation were also filtered and dried on filter paper at 37˚C (n=3) (Thurgood and Ryall 2010). Each filter paper (Whatman International Ltd, England) was mounted on an aluminium stub, carbon coated and examined on a Philips XL30 field emission scanning electron microscope (SEM) (Philips Electronics, The Netherlands). For elemental analysis of crystal composition, energy dispersive X-ray analysis (EDX) was performed at an accelerating voltage of 10 kV on 5 different crystals, as well as at a control location for each sample, and data were analysed using EDAX Genesis software (EDAX Inc., New Jersey, USA).

Urinary crystal samples were also harvested from the dried filter paper and analysed with infrared spectroscopy (n=4), after being incorporated into a potassium bromide disc for

qualitative analysis on a Varian 800 Scimitar series spectrophotometer (Varian Inc., Palo Alto, USA) using Resolutions software (Agilent Technologies, California, USA). Transmittance spectra were obtained over a range of 2000 to 600 cm^{-1} and compared to reference spectra for calcium oxalate and other urinary stones.

3.3.5 Data analysis

Plasma and urine biochemistry data were checked for normality and homogeneity and in all cases analysed using the nonparametric Kruskal-Wallis test. Mann Whitney U tests were used for post hoc pairwise comparisons using SPSS software, with P-values adjusted by Holm's stepdown Bonferroni procedure for multiple comparisons using SAS software (Holm 1979). Outliers were detected using Dixon's test (Dixon 1953, Horn and Pesce 2003) and excluded from the data based upon statistical analysis results. Spearman's test was used to determine the correlation between severity of renal histopathological lesions and USG. Chi squared test for association and t tests were used to determine differences between koala groups for USG.

Table 1. Number of blood and urine samples collected from koala groups.

	Oxalate nephrosis SA	Mt Lofty Ranges SA	Moggill Qld	Kangaroo Is. SA
Total blood samples	15	11	15	23
Total urine samples	22	13	11	20
Concurrent blood and urine	12	7	11	19
Total N sampled	25	17	15	24

Koalas with oxalate nephrosis from Mount Lofty Ranges, SA; koalas unaffected by renal oxalate crystal deposition from Mount Lofty Ranges, SA; Moggill, Qld and Kangaroo Island, SA.

Table 2. Tooth wear classification (TWC), sex and origin of koala groups.

	Oxalate nephrosis	Mt Lofty Ranges	Moggill	Kangaroo Is.
TWC[a]	**SA**	**SA**	**Qld**	**SA**
I	12	7	-	1
II	3	3	-	-
III	4	1	-	4
IV	3	3	2	16
V	1	-	6	1
VI	-	-	3	1
VII	-	-	4	1
unknown	2	3	-	-
TOTAL	**25**	**17**	**15**	**24**
Sex	12 M, 13 F	14 M, 3 F	3 M, 12 F	9 M, 15 F
Origin	12 C, 13 R	5 C, 12 R	15 R	24 W

Koalas with oxalate nephrosis from Mount Lofty Ranges, SA; koalas unaffected by renal oxalate crystal deposition from Mount Lofty Ranges, SA; Moggill, Qld and Kangaroo Island, SA. M= male; F=female; C=captive koalas kept at Cleland Wildlife Park, Mount Lofty Ranges, SA; R=rescued wild koalas, W= wild caught koalas. [a]Tooth wear class (TWC) method of Martin (1981).

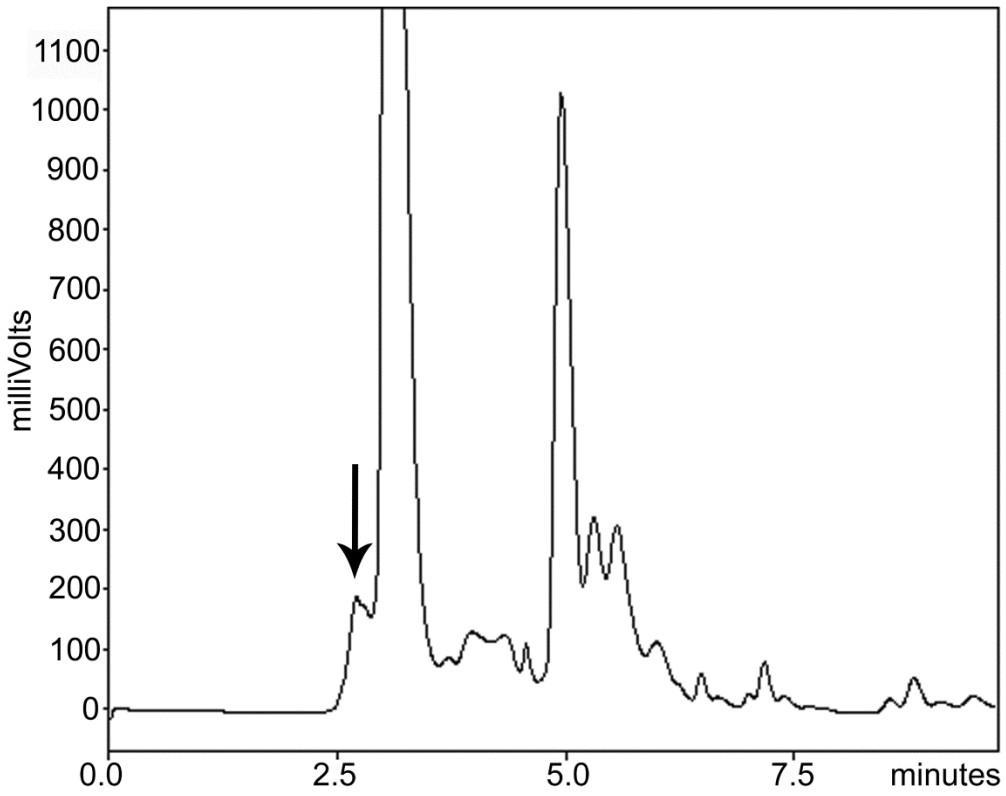

Figure 1. Urinary chromatogram showing oxalate peak (arrow) eluting at 3 minutes.

3.4 RESULTS

3.4.1 Renal insufficiency

Results of analyses of plasma biochemistry for all groups of koalas are shown in Table 3. Individual koalas were classified as azotaemic if both plasma creatinine and urea values were greater than reference intervals published by Canfield et al. (1989b). Azotaemia was evaluated in conjunction with USG to assess renal function of each koala where concurrent blood and urine samples were available. This assessment was based on the reference interval 1.062-1.135 established in New South Wales koalas in the study by Canfield et al. (1989a).

Azotaemia was present in 93% of koalas with oxalate nephrosis (n=15), with plasma creatinine and urea significantly elevated in koalas with oxalate nephrosis compared with those from Queensland and Kangaroo Island ($P<0.001$). Median urine specific gravity of koalas with oxalate nephrosis was found to be 1.020 (range 1.012 – 1.048), and was significantly lower than koalas unaffected by renal oxalate crystal deposition from ML ($P<0.01$), as well as Qld and KI koalas ($P<0.001$). Median USG of koalas with oxalate nephrosis was also well below the published reference interval.

Concurrent plasma and urine sample analysis (n=12) in koalas with oxalate nephrosis showed that 83% of koalas with azotaemia had USG <1.030, whilst 100% showed azotaemia with USG <1.035. In 25% koalas (3/12), urine was much less concentrated with USG ≤1.017. Decreasing USG was found to be significantly correlated with increasing severity of renal histopathological changes: cortical fibrosis ($R=-0.524$; $P<0.05$), tubule dilation ($R=-0.651$; $P\leq0.001$), intratubular inflammation ($R=-0.594$; $P<0.005$) and interstitial inflammation ($R=-0.526$; $P<0.05$) (Figure 2). No significant difference was found in USG between koalas with oxalate nephrosis which had been living in captivity and those rescued from the wild population. Also, no significant differences in USG were found within koala groups based on

variability of timing of urine collection occurring <2 and >2 hours post mortem.

Azotaemia was also found in 73% of koalas unaffected by renal oxalate crystal deposition from ML (n=11), but creatinine and urea values did not significantly differ from other groups, except for higher plasma urea than those from KI (P<0.001). In azotaemic koalas from which concurrent urine samples were obtained (n=7), 43% showed USG <1.035 but only 1 koala (14%) showed USG <1.030 (USG=1.015). Whilst USG <1.035 and <1.030 were found to be statistically associated with koalas with oxalate nephrosis (P<0.005), no association between USG and koalas unaffected by renal oxalate crystal deposition was evident. Overall, USG was significantly higher in unaffected ML koalas than those with oxalate nephrosis (P<0.01), and significantly lower than those from KI (P<0.001).

The median USG for Qld koalas (1.048) was also less than the reference interval established in New South Wales koalas. Although 6 of 15 (40%) Qld koalas were azotaemic, only 2/11 (18%) had a concurrent USG <1.035, with none <1.030. KI koalas showed the highest median USG (1.090) of all groups, within the reference interval; as well as the highest maximal value of 1.174, well above the reference interval. Only one KI individual showed a marginal azotaemia, but this was paired with a USG of 1.084 showing normal renal function with azotaemia of prerenal origin.

3.4.2 Plasma biochemistry

Koalas with oxalate nephrosis showed median total protein (TP) and albumin within the published reference interval. In ML koalas unaffected by renal oxalate crystal deposition, median total protein and albumin values were slightly below the published adult reference intervals, with albumin significantly lower than those from Qld (P<0.05) and TP and albumin both lower than those from KI (P<0.05). Median plasma glucose was within the reference

interval for all groups, although some low values occurred, likely due to collection of blood into lithium heparin rather than sodium fluoride anticoagulant.

Median ALT was within the reference interval for all groups, whilst median AST was markedly elevated from the reference interval in koalas with oxalate nephrosis. The ALT and AST results of KI koalas were significantly lower than all other groups of koalas ($P \leq 0.005$ and $P < 0.05$ respectively). All groups of koalas showed median GGT values within the published reference interval, with koalas from Qld showing significantly lower GGT than those with oxalate nephrosis and KI koalas ($P < 0.001$).

Median plasma urate was elevated in both groups of koalas from Mount Lofty to a similar extent and was significantly higher in both groups compared with those from Qld and KI ($P < 0.001$). No reference interval exists for this analyte in koalas, but based on control KI koalas in this study, 0 - 6.39 µmol/L (mean ± 2SD) is suggested. Both the ML koala groups showed marked elevations in median plasma urate above this suggested reference interval whilst the median value for Qld koalas was within the interval.

Median plasma calcium was marginally elevated from the reference interval in KI koalas and marginally low in unaffected Mount Lofty koalas but these changes were not statistically different from other groups. ML koalas unaffected by renal oxalate crystal deposition showed a moderate elevation of plasma phosphate above the adult reference interval, whilst plasma phosphate in KI koalas was within the reference interval but significantly lower than both ML koala groups ($P \leq 0.005$).

Although 5 of 15 blood samples from koalas with oxalate nephrosis were markedly haemolysed, no significant differences were found to occur for results of any plasma analyte (including AST) in haemolysed versus non-haemolysed samples. In the ML koala group unaffected by renal oxalate crystal deposition, a total of 2 of 11 samples were markedly

haemolysed, with one showing a high AST (1882 IU/L) but this was not statistically significant. There were no samples from Qld or KI that were notably haemolysed.

3.4.3 Urine biochemistry

Results of analyses of urine biochemistry of koalas are shown in Table 4. Except for USG (see previous section), reference intervals were unavailable for analytes as these tests have not previously been performed in koalas. The urate: creatinine ratio was significantly lower in koalas with oxalate nephrosis compared with all other koala groups ($P<0.05$). No significant differences were found between any koala groups for urinary calcium excretion. Phosphate: creatinine ratio was significantly lower in KI koalas compared with ML koalas affected by oxalate nephrosis ($P<0.001$).

Koalas with oxalate nephrosis, unaffected ML koalas and KI koalas all showed significantly higher urinary oxalate: creatinine ratios than koalas from Qld ($P<0.01$), suggestive of hyperoxaluria occurring in all South Australian koala groups. Both ML koala groups showed high maximal values, but these groups were not significantly different. The median urinary oxalate for ML koalas unaffected by renal oxalate crystal deposition was approximately 20-fold higher than koalas from Qld.

3.4.4 Urinary crystals

In koalas with histological evidence of oxalate nephrosis, a gross yellow particulate precipitate was present in the majority of urine specimens. Microscopic sediment examination showed urinary crystals with similar morphology in 12 of 16 (75%) urine samples from koalas with oxalate nephrosis. These urinary crystals consisted of pale brown spicules arranged in wheat-sheaf or bow-tie formations (Figure 3). When viewed using SEM, the crystals were shown to be narrow plates with jagged ends either arranged in bow-tie

formations or clustered into round spherule formations, with a diameter of up to approximately 40 µm (Figure 4).

In one koala, small numbers of typical calcium oxalate dihydrate crystals were observed in addition to these bow-tie crystals. In the remaining 4 urine samples examined from koalas with oxalate nephrosis, irregular plate-like crystals were seen in 2 samples and no crystals in 2 samples. Fresh voided urine samples from 6 captive koalas with clinical signs of renal dysfunction also showed yellow precipitate and similar bow-tie crystal morphology in all samples upon sediment examination. In one sample of fresh urine from a captive koala, the crystals were observed in cast formation, suggestive of a tubular origin.

No crystals of bow-tie or spherule morphology were seen in the sediment of 7 urine samples from ML koalas unaffected by renal oxalate crystal deposition, but 2 koalas showed irregular plate-like crystals. None of 3 Qld koalas for which urine sediments were examined showed any urinary crystals. 2 of 21 (10%) Kangaroo Island koalas showed typical envelope-shaped calcium oxalate dihydrate crystals, with one of these samples also showing small rectangular crystals. Another KI koala also had small rectangular crystals, possibly calcium oxalate monohydrate. There were no significant differences found in average urinary dipstick pH between the four koala groups: mean 5.7 for koalas with oxalate nephrosis, 5.4 for ML koalas unaffected by renal oxalate crystal deposition, 5.0 for Qld and 5.1 for KI koalas.

3.4.5 Urinary crystal composition

EDX analysis of the urinary crystals showed co-location of carbon, oxygen and calcium in all readings from the 3 koala samples, consistent with a composition of calcium oxalate. Infrared spectroscopy of urinary crystals showed identical spectra for all 4 samples. The absorption peaks corresponded to a composition of calcium oxalate, with some uric acid and phosphate also present.

Table 3. Comparison of plasma biochemistry results.

Plasma analyte	Oxalate nephrosis SA		(n)	Mt Lofty Ranges SA		(n)	Moggill Qld		(n)	Kangaroo Is. SA		(n)	Canfield *et al* ., (1989) interval
TP[a] (g/L)	69.4	*(16.8-89.9)*	(14)	54.8	*(15.9-75.0)*	(11)	66.0	*(40.5-85.4)*	(15)	70.5	*(58.7-83.6)*	(22)	53-78 / 58-83*
Albumin (g/L)	39.0	*(27.6-68.7)*	(13)	30.5	*(16.3-40.8)*	(11)	38.7	*(28.6-45.4)*	(15)	46.1	*(37.5-56.0)*	(21)	34-50
Glucose (mmol/L)	5.0	*(0.1-7.5)*	(10)	5.6	*(0.6-10.4)*	(11)	3.3	*(0.1-11.5)*	(15)	5.0	*(3.1-7.5)*	(21)	2.7-7.2†
Creatinine (µmol/L)	478.0	*(136.0-2098.0)*	(15)	152.4	*(120.0-3249.0)*	(11)	181.0	*(100.0-384.0)*	(15)	137.0	*(76.0-175.0)*	(23)	80-150
Urea (mmol/L)	26.8	*(8.9-56.9)*	(14)	9.6	*(3.9-32.6)*	(11)	8.5	*(1.3-22.5)*	(15)	3.0	*(0.8-7.1)*	(23)	0.2-6.6
ALT[b] (U/L)	133.0	*(2.0-1652.0)*	(13)	58.0	*(3.1-983.0)*	(11)	25.0	*(11.0-101.0)*	(15)	8.0	*(1.0-65.0)*	(21)	0-236
AST[c] (U/L)	604.0	*(39.0-2129.0)*	(13)	100.9	*(0.3-1882.0)*	(11)	88.0	*(20.0-1013.0)*	(15)	22.0	*(1.0-104.0)*	(21)	0-134
GGT[d] (U/L)	13.8	*(7.6-19.7)*	(8)	12.9	*(1.0-18.1)*	(6)	6.8	*(3.9-12.6)*	(13)	13.0	*(8.4-18.2)*	(17)	0-16
Urate (µmol/L)	35.5	*(1.0-147.2)*	(14)	34.5	*(3.0-204.0)*	(10)	1.7	*(0.0-18.0)*	(15)	3.0	*(0.0-6.0)*	(23)	-
Calcium (mmol/L)	2.83	*(1.38-3.74)*	(7)	2.20	*(1.47-3.84)*	(6)	2.69	*(1.78-4.58)*	(13)	2.99	*(2.02-5.47)*	(21)	2.28-2.97
Phosphate (mmol/L)	1.67	*(1.00-2.59)*	(10)	2.40	*(1.11-4.29)*	(9)	1.53	*(0.12-3.87)*	(14)	0.83	*(0.32-1.39)*	(17)	1.25-2.44/0.79-1.96*

Koalas with oxalate nephrosis from Mount Lofty Ranges, SA; koalas unaffected by renal oxalate crystal deposition from Mount Lofty Ranges, SA; Moggill, Qld and Kangaroo Island, SA [median (*range*)]. Shaded values fall outside the published reference interval in Canfield et al. (1989b).

[a]TP = total protein; [b]ALT = alanine aminotransferase; [c]AST = aspartate aminotransferase; [d]GGT = gamma glutamyl transferase.

* Reference interval shows juvenile / adult reference intervals published in Canfield et al. (1989b).

† Refers to reference intervals published in Blanshard and Bodley (2008).

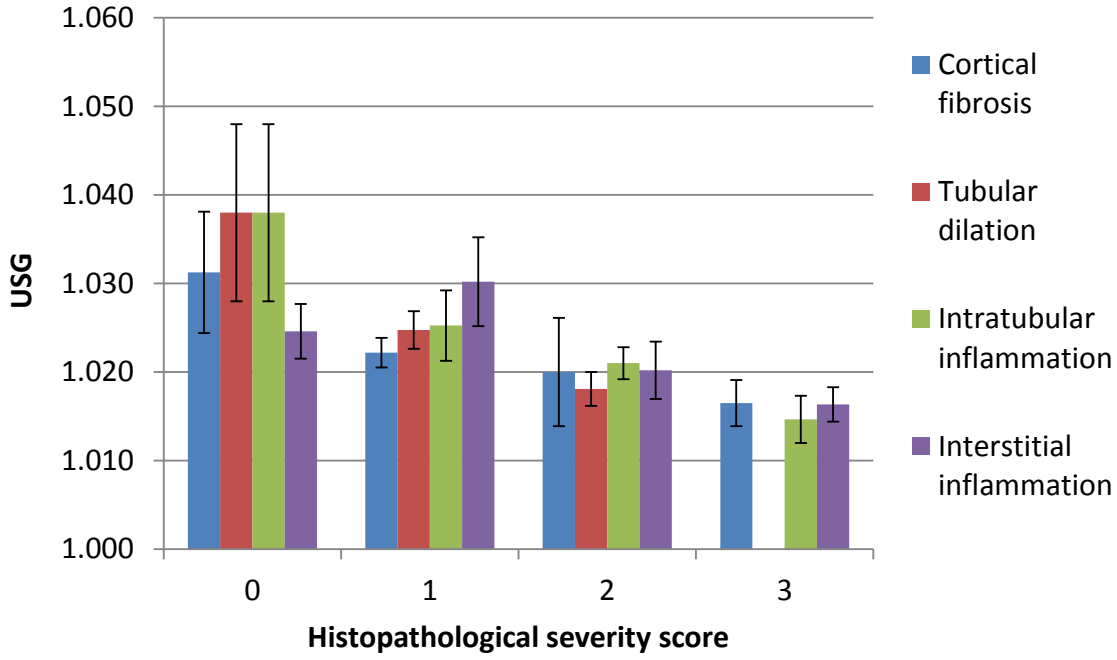

Figure 2. USG correlation with severity of renal histopathological changes. Error bars show SEM.

Table 4. Comparison of urine biochemistry results.

Urine analyte	Oxalate nephrosis		Mt Lofty Ranges		Moggill		Kangaroo Is.	
	SA	[n]	SA	[n]	Qld	[n]	SA	[n]
Urine specific gravity (USG)	1.020 *(1.012-1.048)*	[22]	1.032 *(1.015-1.084)*	[13]	1.048 *(1.032-1.144)*	[11]	1.090 *(1.016-1.174)*	[20]
Creatinine (mmol/L)	4.3 *(1.4-13.7)*	[18]	7.1 *(1.2-25.3)*	[10]	17.4 *(0.8-43.8)*	[11]	18.4 *(4.0-62.4)*	[21]
Urate:creatinine (µmol/mmol)	3.1 *(0.0-43.0)*	[18]	26.5 *(0.1-225.0)*	[8]	15.0 *(2.8-135.9)*	[11]	29.9 *(6.6-152.5)*	[21]
Calcium:creatinine ratio (mmol/mmol)	0.9 *(0.07-3.5)*	[18]	1.2 *(0.1-4.9)*	[8]	0.8 *(0.0-2.4)*	[11]	0.5 *(0.1-2.6)*	[21]
Phosphate:creatinine ratio (mmol/mmol)	2.3 *(0.1-15.9)*	[15]	0.6 *(0.3-1.5)*	[4]	1.0 *(0.0-6.7)*	[9]	0.1 *(0.0-1.4)*	[14]
Oxalate:creatinine ratio (µmol/mmol)	209.4 *(36.4-1052.5)*	[16]	625.1 *(85.4-1364.8)*	[6]	31.3 *(9.0-140.4)*	[7]	148.7 *(36.2-488.1)*	[17]

Koalas with oxalate nephrosis from Mount Lofty Ranges, SA; koalas unaffected by renal oxalate crystal deposition from Mount Lofty Ranges, SA; Moggill, Qld and Kangaroo Island, SA [median (range)]. Shaded values fall below the reference interval of USG for koalas 1.062-1.135 reported by Canfield et al. (1989a).

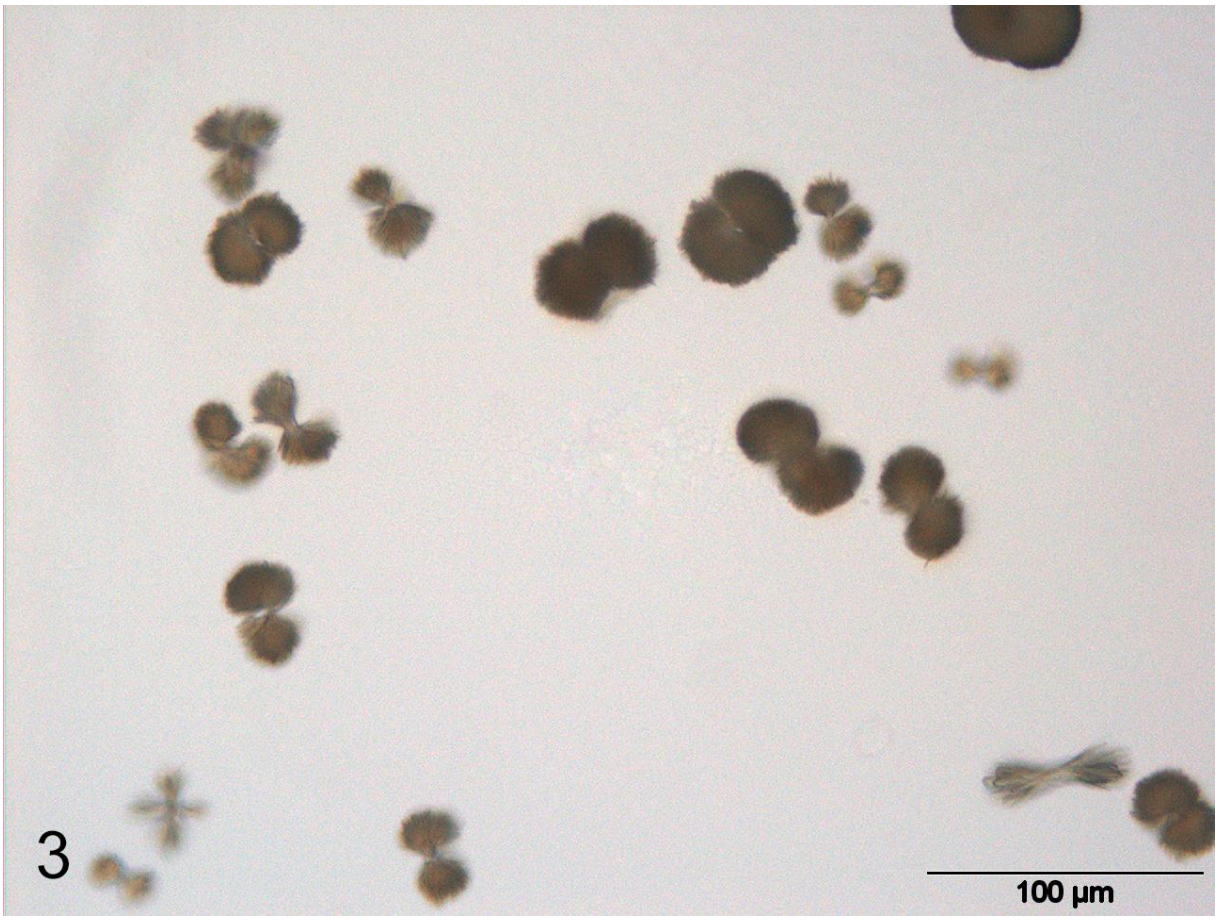

Figure 3. Urinary sediment examination showing 'bow-tie' morphology of urinary crystals from koalas with oxalate nephrosis.

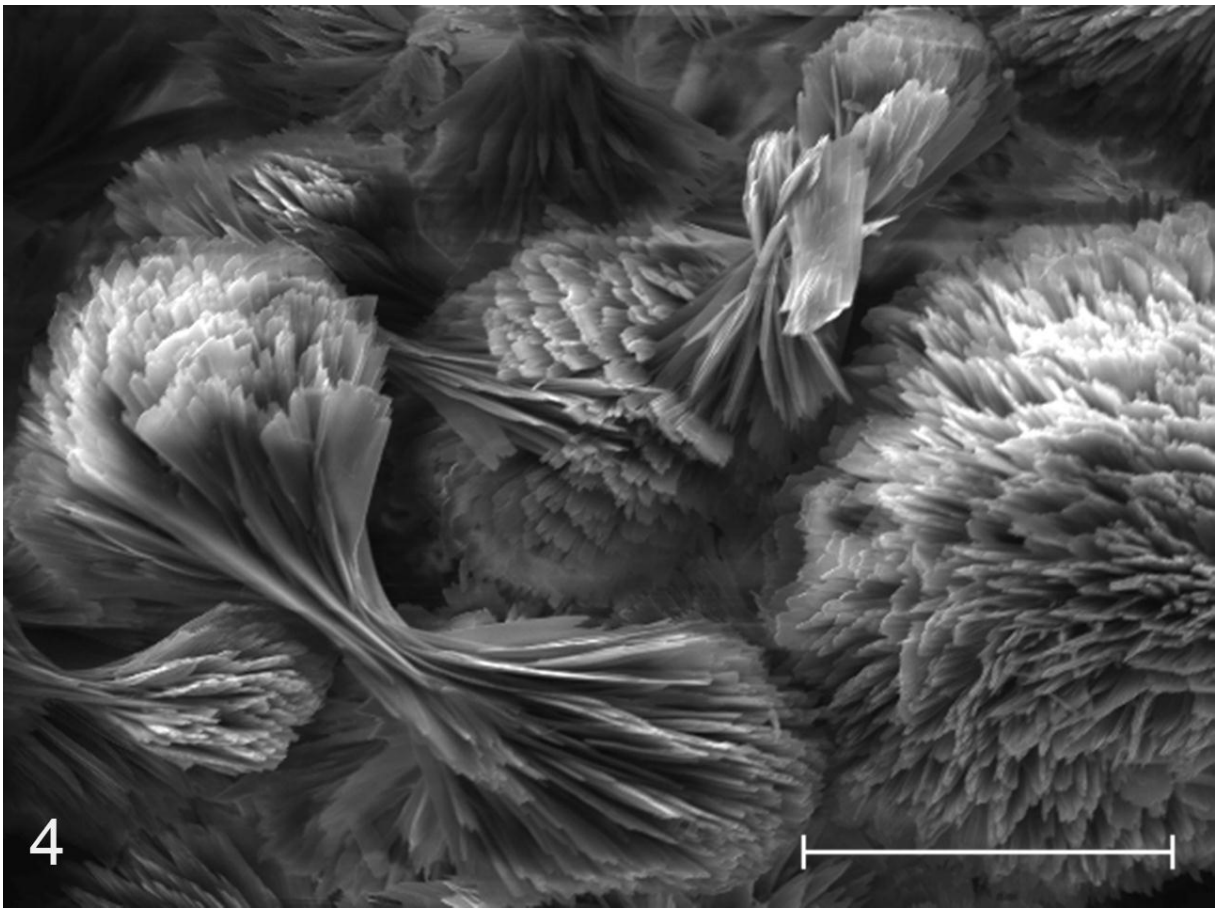

Figure 4. Scanning electron micrograph of urinary crystals from a koala affected by oxalate nephrosis showing 'bow-tie' and spherule morphology. Scale bar 20 µm.

3.5 DISCUSSION

This study found that almost all captive and rescued wild koalas from the Mount Lofty Ranges population in South Australia with gross or histological evidence of oxalate nephrosis showed clinicopathological findings consistent with renal dysfunction at euthanasia. This was characterised by azotaemia in conjunction with poorly concentrated urine of specific gravity <1.035. USG of affected koalas was shown to decrease with increasing renal cortical fibrosis, tubule dilation, intratubular inflammation and interstitial inflammation. These are the hallmark histopathological changes of oxalate nephrosis (Speight et al. 2013, Weiss et al. 2007) and show the association between decreasing ability to concentrate urine and progressive renal parenchymal damage.

All azotaemic koalas with oxalate nephrosis showed USG <1.035 which is consistent with renal insufficiency, based on the reference interval for USG in koalas (1.062-1.135) (Canfield et al. 1989a). The USG interval for koalas is similar to that found in the domestic cat, for which renal insufficiency is indicated by USG <1.034 when paired with azotaemia, and renal failure <1.012 (Lane et al. 1994, Osborne and Polzin 1991). In koalas, a case of renal failure was previously reported as USG ≤1.017 (Spencer and Canfield 1993), and based on this value, renal failure was present in 25% koalas with oxalate nephrosis in the current study. The low proportion of koalas in renal failure in this study may reflect euthanasia on welfare grounds at an earlier stage of renal disease, prior to isosthenuria occurring.

Koalas from the Mount Lofty region unaffected by renal oxalate crystal precipitation also showed a high overall prevalence of azotaemia with inadequately concentrated urine <1.035. Yet few showed USG <1.030 compared with the group of koalas with oxalate nephrosis, a significant difference between the two groups. Despite a lack of histological evidence of oxalate, the authors suggest that renal dysfunction in these koalas may be due to oxalate ion induced renal damage prior to precipitation of calcium oxalate crystals

(Hackett et al. 1994, Khan and Hackett 1993, Scheid et al. 1996). This is based on the high prevalence of oxalate nephrosis in the Mount Lofty population (Speight et al. 2013), and that renal disease is otherwise uncommon in koalas unless associated with clinical chlamydiosis (Canfield 1989).

Urinary oxalate: creatinine was found to be significantly higher in all South Australian koala groups compared with Queensland koalas, indicative of hyperoxaluria. Despite this, two Queensland koalas with oxalate nephrosis were excluded from the study, showing that the disease also occurs at low levels in this population, consistent with reports from wildlife veterinarians. Although not statistically significant, urinary oxalate for both Mount Lofty groups showed higher maximal values than in Kangaroo Island koalas, suggesting increased importance of high oxalate in Mount Lofty.

In addition, Mount Lofty koalas unaffected by renal oxalate crystal deposition showed a higher median urinary oxalate value than those with oxalate nephrosis, although this also was not statistically significant. This difference may be due to the precipitation of calcium oxalate in koalas with oxalate nephrosis causing decreased urinary oxalic acid concentration; or poor renal function causing decreased excretion of oxalate. In humans, excretion of oxalate and other metabolites has been shown to decrease as renal function deteriorates, leading to low urine values despite increased circulating oxalate (Asplin 2002).

Other plasma biochemical results were generally unremarkable in koalas affected by oxalate nephrosis, showing little evidence of concurrent health problems. High AST was most likely due to release from skeletal muscle in a delay from time of death to blood collection for some animals in the Mount Lofty groups, since ALT and GGT were not elevated as in liver disease, and haemolysis was shown not to affect results (Yucel and Dalva 1992).

Total protein and albumin in Mount Lofty koalas unaffected by renal oxalate crystal deposition showed only mild decreases from the normal adult interval and may reflect early

renal disease with glomerular involvement (Lulich and Osborne 1991). However, these results are more likely due to a high proportion of young koalas in the cohort with lower plasma protein levels (Canfield et al. 1989b), which would also explain the elevation in mean plasma phosphate above the adult reference interval into the juvenile range established by Canfield et al. (1989b).

Plasma urate was significantly higher in the two Mount Lofty groups compared with the values of Queensland and Kangaroo Island, but koalas with oxalate nephrosis showed a lower mean value for urine urate: creatinine than Mount Lofty koalas unaffected by renal oxalate crystal deposition. This may be due to urate precipitation as uric acid in koalas with oxalate nephrosis, since uric acid was found in a previous study to be present in renal deposits from affected koalas (Speight et al. 2013). Hyperuricaemia in ML koalas could be similar to that in humans, whereby plasma urate can be elevated due to renal insufficiency (Darmady and MacIver 1980).

Phosphate, which was also previously found in renal deposits of koalas with oxalate nephrosis (Speight et al. 2013), was similar in the plasma and urine of both Mount Lofty groups. Histological evidence of calcium phosphate deposition has previously been reported in the kidneys of Mount Lofty koalas with oxalate nephrosis (Speight et al. 2013), and in the current study, phosphate deposits were identified in the kidneys of both Mount Lofty and Queensland koalas unaffected by renal oxalate crystal deposition. The presence of renal phosphate deposits in all three koala groups supports the previous conclusion that the deposits are clinically insignificant (Speight et al. 2013), since the severity of renal histopathological changes varied between groups. Mount Lofty koalas unaffected by renal oxalate crystal deposition showed minimal renal inflammatory changes, whilst many Queensland koalas had interstitial nephritis and fibrosis, probably due to ascending infection secondary to urogenital chlamydiosis (Canfield 1989).

Kangaroo Island koalas showed a marginal elevation in median plasma calcium above the published reference interval. Hypercalcaemia has been previously reported in a study of Queensland koalas, in which it was suggested to be due to low calcitonin secretion by the thyroid gland (Lawson and Carrick 1998). However, Kangaroo Island koalas in the current study showed otherwise normal biochemistry, suggesting a healthy population and therefore the reference interval for calcium may be broader in South Australian koalas than that previously published. Also in this previous thyroid gland study, hypercalcaemia was proposed as a possible cause of oxalate nephrosis in koalas (Lawson and Carrick 1998), but this is not supported by results of the current study since koalas with oxalate nephrosis were not found to be hypercalcaemic or hypercalciuric.

Urine sediment examination showed that the majority of koalas with oxalate nephrosis produced urinary crystals with an identical unusual morphology, consisting of narrow serrated plates arranged in bow-tie and spherule formations, which are not normal components of koala urine (Canfield et al. 1989a). EDX and infrared spectroscopy analyses showed a composition of calcium oxalate, as well as uric acid and phosphate, with peaks identical to that of the renal deposit of these same koalas, which were analysed in a previous study (Speight et al. 2013). Unusual morphological appearances of calcium oxalate crystals have been described previously (Millan 1997), such as fan-shaped patterns due to crystal aggregation (Osborne and Stevens 1999), and wheat-sheaf forms with pulmonary aspergillosis in humans (Farley et al. 1985).

Whilst the urinary crystals found in koalas with oxalate nephrosis in the current study could have precipitated in the bladder, it is more likely that the crystals were formed in the kidney and flushed through to the bladder. This is supported by the observation of crystalline casts in a fresh urine sample, and the identical complex composition of the urinary crystals with those analysed from the kidney in the previous study (Speight et al.

2013). In addition, the morphology of some crystals seen in kidney tissue sections in the previous study occasionally showed a spiculated appearance and arrangement in both spherules and bowtie formations (Speight et al. 2013), similar to that seen in the urine in the current study. This pathognomonic urinary crystal morphology will be valuable for diagnosis of koalas affected by oxalate nephrosis, although it is recommended that repeated urine sampling is used to detect the crystals, since not all samples from affected koalas showed crystalluria in the current study.

This study has shown that nearly all koalas from the Mount Lofty population with oxalate nephrosis have renal insufficiency and therefore, the prevalence of renal dysfunction found in this population is likely to be similar to that of oxalate nephrosis (55%) (Speight et al. 2013), and much higher than previously estimated (11%) (Haynes et al. 2004). Renal dysfunction was also found in koalas without gross or histological evidence of renal oxalate crystals, but the majority of these koalas had USG > 1.030, whereas most with oxalate nephrosis showed USG < 1.030. Hyperoxaluria was also found in both Mount Lofty koalas with oxalate nephrosis as well as those unaffected by renal calcium oxalate deposition, which suggests that the majority of koalas in this population have increased oxalate levels. This may explain the occurrence of renal dysfunction in both groups of koalas, with renal damage occurring in affected koalas due to the oxalate crystals and in Mount Lofty koalas unaffected by calcium oxalate crystal deposition, due to acute toxic effects of oxalate ions prior to crystal formation (Hackett et al. 1994, Khan and Hackett 1993, Scheid et al. 1996). If this theory is correct, the majority of koalas in the Mount Lofty population could be regarded as being affected by oxalate nephrosis.

Hyperoxaluria also suggests that oxalate nephrosis in Mount Lofty koalas has a primary origin, rather than occurring secondary to renal failure, in which urinary oxalate usually decreases (Hodgkinson 1977). Also supportive of a primary pathogenesis is that

many young Mount Lofty koalas <2 years old are affected by oxalate nephrosis (Speight et al. 2013). One common primary cause of oxalate nephrosis in other herbivores is high oxalate intake in the diet (Maxie and Newman 2007). A possible dietary cause for oxalate nephrosis in Mount Lofty koalas is currently under investigation, whereby eucalypt leaf oxalate is being measured, since eucalypt preferences of koalas in South Australia differ from those in Queensland (Phillips 1990). Also, a recent study of oxalate nephropathy in captive marmosets implicated manna gum (*Eucalyptus viminalis*) as the cause (Vanselow et al. 2011), which is the preferred diet of SA koalas (Phillips 1990).

In addition, due to the low genetic diversity of SA koalas (Seymour et al. 2001), another potential aetiology for oxalate nephrosis is an inherited metabolic abnormality similar to primary hyperoxaluria in humans, in which excess oxalate is produced endogenously (Cochat et al. 2006). Other causes of oxalate nephrosis in mammalian species include ethylene glycol ingestion, oxalate overabsorption due to intestinal disease, lack of oxalate-degrading gastrointestinal bacteria and high intake of precursors such as glycolate, glycine and ascorbic acid (Allison and Cook 1981, Asplin 2002, Maxie and Newman 2007). The clinicopathological findings of the current study have established a framework for the current investigations into these possible causes of disease and will also assist veterinarians and clinical pathologists in the diagnosis of oxalate nephrosis in koalas.

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CHAPTER 4

Eucalyptus spp. leaf oxalate content and its implications for koalas (*Phascolarctos cinereus*) with oxalate nephrosis

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Submitted manuscript.

CONTEXTUAL STATEMENT

Oxalate nephrosis was shown to be a leading disease of the Mount Lofty koala population (**Chapter 2**), characterised by renal insufficiency and hyperoxaluria (**Chapter 3**). In other mammalian species, high dietary oxalate intake can lead to increased urinary excretion of oxalate and oxalate nephrosis (Maxie and Newman 2007). The diet of koalas consists primarily of eucalypt leaves, but little is known of their oxalate content.

Chapter 4 describes the oxalate content of several species of eucalypt eaten by koalas to assess their potential for causing oxalate nephrosis in koalas.

STATEMENT OF AUTHORSHIP: Chapter 4

***Eucalyptus* spp. leaf oxalate content and its implications for koalas (*Phascolarctos cinereus*) with oxalate nephrosis. Submitted manuscript.**

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4.1 ABSTRACT

Oxalate nephrosis is a leading disease of the Mount Lofty Ranges koala population in South Australia, but the cause is unclear. In other herbivorous species, a common cause is high dietary oxalate and therefore this study aimed to determine the oxalate content of eucalypt leaves. Juvenile, semi-mature and mature leaves were collected during spring from eucalypt species eaten by koalas in the Mount Lofty Ranges and compared with those from Moggill, Queensland, where oxalate nephrosis has lower prevalence. Total oxalate was measured as oxalic acid by high performance liquid chromatography. Results showed that oxalate content of eucalypts was low (<1% dry weight), but occasional leaf samples had higher oxalate levels of 4.68 - 7.51% dry weight. Mount Lofty eucalypts were found to be higher in oxalate than those from Queensland ($P<0.001$). In conclusion, dietary oxalate in eucalypt leaves is unlikely to be the primary cause of oxalate nephrosis in the Mount Lofty koala population. However, occasional samples showed higher oxalate levels which could cause oxalate nephrosis in individual koalas or worsen disease in those already affected. More studies on the seasonal variation of eucalypt leaf oxalate are needed to understand its role in the pathogenesis of oxalate nephrosis in koalas.

Short summary

Many koalas in the Mount Lofty Ranges population, South Australia are affected by oxalate nephrosis, in which calcium oxalate crystals are deposited in the kidneys. In other mammals, dietary oxalate can cause oxalate nephrosis, hence this study measured oxalate content in the eucalypt leaf diet of koalas. Results showed that eucalypt leaf oxalate content is low overall. Dietary oxalate is therefore unlikely to be the primary cause of oxalate nephrosis in Mount Lofty koalas and further research is underway to understand the pathogenesis.

4.2 INTRODUCTION

Oxalate nephrosis, in which calcium oxalate crystals are deposited in the kidneys, is common in the koala (*Phascolarctos cinereus*) population in the Mount Lofty Ranges in South Australia, but the cause is unclear (Speight et al. 2013). In other herbivores, oxalate nephrosis may be caused by ingestion of plants high in oxalic acid (Maxie and Newman 2007), which occurs primarily in the form of oxalate salts of calcium, potassium or sodium within the plants (McBarron 1977). Acute oxalate toxicity in livestock can occur from ingestion of plants >10% oxalic acid on a dry weight basis, such as halogeton (*Halogeton glomeratus*) and soursobs (*Oxalis pes-caprae*), and is characterised by hypocalcaemia and death (James and Panter 1993, McBarron 1977).

Sub-acute and chronic forms of toxicity have also been reported in sheep grazing soursobs over many months (Bull 1929), with animals showing weight loss, inappetance (Dodson 1959, McIntosh 1972), renal fibrosis and calcium oxalate deposition (James 1972, James and Panter 1993, Rahman et al. 2013). The severity of oxalate-induced disease from ingestion of plants depends on the rate and amount of consumption, concentration and form of oxalate in the plant, and the amount absorbed from the gastrointestinal tract (James and Panter 1993, Michael 1959).

Oxalate is absorbed across the mucosa of the stomach, small and large intestine into the bloodstream (Hatch and Freel 2005), and excreted by the kidneys (Massey 2007). The amount of oxalate absorbed from the gastrointestinal tract is dependent upon two factors, the extent of intraluminal binding with dietary calcium to form insoluble calcium oxalate, which is excreted in the faeces; and the degree of oxalate degradation by micro-organisms in the gastrointestinal tract (James 1972, James and Panter 1993), such as *Oxalobacter formigenes* in humans (Allison et al. 1985).

In a recent study, oxalate nephrosis was found to affect 55% of captive and rescued

wild koalas in the Mount Lofty Ranges (Speight et al. 2013). This prevalence is much higher than that reported in the eastern states of Australia, <3% in New South Wales koalas (Canfield 1989) and <12% in Moggill, Queensland (Speight et al. in press). In addition, significant hyperoxaluria in Mount Lofty koalas has been found to occur, compared with those from Moggill, Queensland in which urinary oxalate levels were found to be 5 -20 fold lower (Speight et al. in press). Hyperoxaluria is indicative of increased circulating and excreted oxalate, suggesting a primary cause of oxalate nephrosis in koalas, such as high dietary oxalate (Asplin 2002, Maxie and Newman 2007). A previous study in humans showed that high dietary oxalate results in significant hyperoxaluria, contributing up to 53% of urinary oxalate after ingestion of high oxalate-containing foods, and is a significant risk factor for calcium oxalate urinary stone formation (Holmes et al. 2001).

Koalas primarily eat *Eucalyptus* leaves, with tree preferences differing between populations across Australia (Phillips 1990). Koalas in South Australia have a strong preference for manna gum (*E. viminalis*), whereas those in Queensland favour forest red gum (*E. tereticornis*) (Phillips 1990). Little is known of the oxalate content of eucalypt leaves, with one source reporting 'immeasurably low' levels in a few leaf samples eaten by koalas (Dickson 1989). A study of karri (*E. diversicolor*) showed moderate levels of total oxalate in leaves (3.7-4.4% dry weight) (O'Connell et al. 1983), but this eucalypt species is not known to be eaten by koalas (Jackson et al. 2003). Recently, manna gum *(E. viminalis)* was implicated as the cause of oxalate nephropathy in seven laboratory common marmosets (*Callithrix jacchus)*, with 0.16% soluble oxalate on an approximate dry weight basis occurring in three samples of bark and wood, and 0.3% in two leaf samples of the supplied branch (Vanselow et al. 2011).

In koalas, high dietary oxalate has previously been suspected in isolated cases of oxalate nephrosis (Canfield and Dickens 1982, Ladds 2009). Therefore, the aim of this study

was to determine the oxalic acid content in eucalypt leaves eaten by Mount Lofty koalas in order to assess their potential for causing oxalate nephrosis.

4.3 MATERIALS AND METHODS

4.3.1 Leaf collection

Leaves were collected from eucalypt plantations used to feed captive and hospitalised wild koalas at two locations: Cleland Wildlife Park, Mount Lofty, South Australia (SA) and Moggill Koala Hospital, Moggill, Queensland (Qld). Four eucalypt species eaten by koalas were collected at each location. In SA, manna gum (*Eucalyptus viminalis*), river red gum (*E. camaldulensis*), SA blue gum (*E. leucoxylon*) and messmate stringybark (*E. obliqua)* (Phillips 1990); and in Qld, forest red gum (*E. tereticornis),* small-fruited grey gum *(E. propinqua),* tallow wood *(E. microcorys)* and red stringybark *(E. resinifera)* (Jackson et al. 2003).

For each eucalypt species, 'juvenile' new leaves, 'semi-mature' fully expanded young leaves and 'mature' fully expanded older leaves were collected. In Mount Lofty, leaf collection occurred in spring 2008 and 2009 from plantation trees (see Table 1) as well as full-grown trees in 2008 (n=9) and 2009 (n=12). In Moggill, leaf collection occurred in spring 2009 from plantation trees (see Table 2). Approximately fifteen leaves of each leaf type were collected from the canopy of each tree into envelopes in sealed plastic packets and stored in a chilled container until weighing. Leaves were weighed to the nearest milligram on a fine balance (Ohaus, New Jersey, USA) and dried in an oven at 40˚C for 7 days, so that a constant dry weight was reached without loss of essential oils (Ellis et al. 2002). Dried leaf samples were ground with a mortar and pestle and sieved using standard 25-mesh (Scienceware, Bel-Art products, Pequannock, USA) to obtain a consistent leaf particle size for oxalic acid analysis.

4.3.2 Oxalate measurement

Leaf samples of approximately 30 mg were weighed to 0.1 mg on a Mettler balance (Mettler, Zurich, Switzerland). Samples were incubated in 1 mL 0.01 M hydrochloric acid for 1 h at 60˚C to ensure complete solubilisation of the oxalate salt to form oxalic acid (Holmes and Kennedy 2000, James 1972). The samples were then centrifuged at 13,000g for 5 min and the supernatant removed for analysis. Total oxalic acid was measured by reverse phase high performance liquid chromatography. Prevail Organic Acid columns (Alltech, Deerfield, IL, USA) were used as the stationary phase with a guard column positioned ahead of the analytical column. The mobile phase, consisting of 97% 0.025M potassium dihydrogen phosphate buffer pH 2.5 and 3% acetonitrile (HPLC grade, Burdick & Jackson, Muskegon, MO, USA), was applied at a flow rate of 1mL min^{-1} for 6 minutes. Oxalic acid was detected at 210nm and the concentration was calculated from a calibration curve produced by plotting peak height versus concentration. The oxalic acid concentrations of samples were determined as follows:

$$\text{Oxalic acid } (\mu g\ g^{-1}) = \frac{\text{Peak height for assay x standard concentration x sample dilution}}{\text{Peak height for standard x sample weight (g)}}$$

The calibration curve was linear with a least squares regression of R^2= 0.99 (Figure 1a). Chromatograph output was processed using Delta Chromatography Data Systems software with the oxalic acid peak appearing approximately 3 min after injection (Figure 1b). The mean recovery of oxalic acid was 95.6 ± 9.8% (mean ± SD) and the within-sample variation 2.3%. Oxalic acid concentration is reported in the results as percentage on a dry weight basis (% DW).

Positive controls were prepared from 10 samples of soursob (*Oxalis pes-caprae*) and analysed as above. These showed an average total oxalic acid content of 7.2 ± 3.3% DW (mean ± SD), similar to that found in a previous study (8.5-14.5% DW) (Dodson 1959).

4.3.3 Data analysis

Eucalypt oxalic acid data normality and homogeneity were determined and data analysed with the nonparametric Kruskal-Wallis test with post hoc Mann Whitney U tests using SPSS software. P-values for individual leaf type analyses were adjusted by Holm's step-down Bonferroni correction for multiple comparisons (Holm 1979). Pairwise comparisons were analysed using nonparametric Mann Whitney U analyses.

Table 1. Eucalypt species and leaf age sampled for leaf oxalate analysis in Mount Lofty, South Australia in 2008 and 2009.

Species	Leaf age	2008	2009
E. viminalis	J	12	11
	SM	10	11
	M	11	12
E. camaldulensis	J	9	10
	SM	9	10
	M	7	10
E. leucoxylon	J	10	11
	SM	7	9
	M	8	11
E. obliqua	J	10	12
	SM	11	12
	M	9	12
Total tree number		**113**	**131**

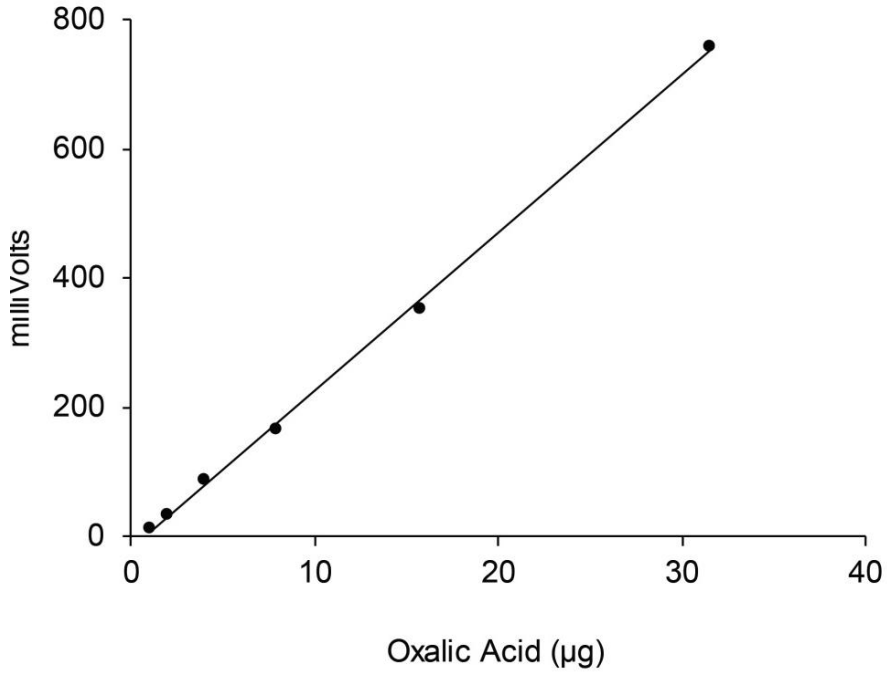
J=juvenile leaves, SM=semi-mature leaves, M= mature leaves.

Table 2. Eucalypt species and leaf age sampled for leaf oxalate analysis in Moggill, Queensland in 2009.

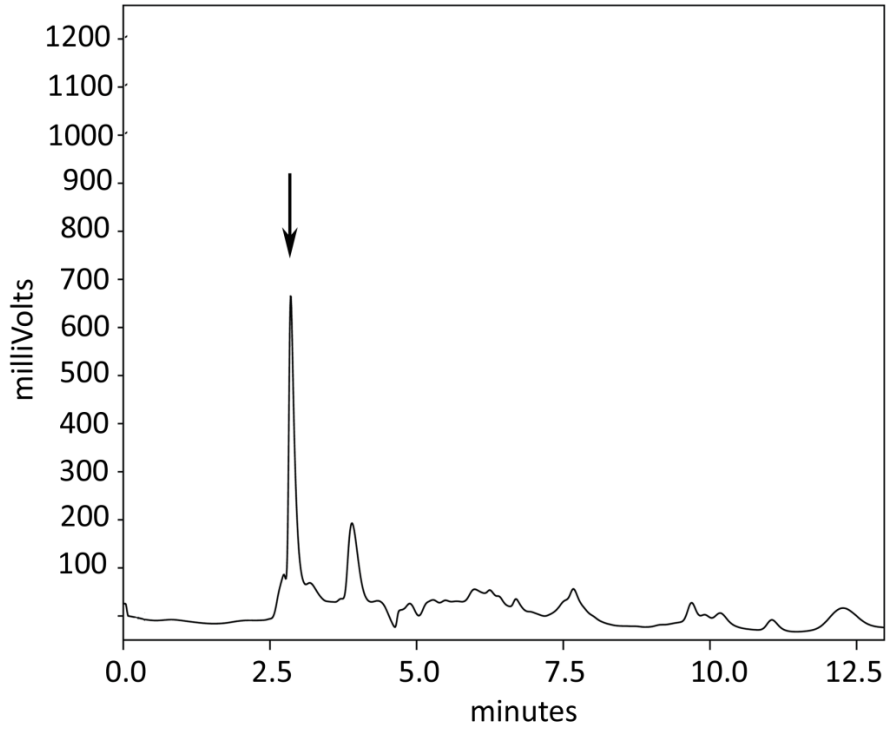
Species	
E. tereticornis	12
E. propinqua	12
E. microcorys	10
E. resinifera	14

Leaf age	
Juvenile	14
Semi-mature	15
Mature	19

Total tree number	**48**



a)



b)

Figure 1. a) Calibration curve for oxalic acid using high performance liquid chromatography. b) High performance liquid chromatogram of mature manna gum (*E. viminalis*) leaf sample showing elution of oxalic acid (arrow) at 3 minutes.

4.4 RESULTS

The majority of leaves from four Mount Lofty eucalypt species (*E. viminalis, E. camaldulensis, E. leucoxylon* and *E. obliqua*) showed low oxalic acid concentration of < 1% DW. In spring 2008, Mount Lofty eucalypts showed an overall oxalic acid concentration of 0.71 ± 0.55% DW (mean ± SD). Based on both eucalypt species and leaf age (Figure 2), semi-mature *E. camaldulensis* (river red gum) leaves had significantly higher levels of total oxalic acid (average 0.96% DW) than juvenile *E. camaldulensis,* semi-mature *E. viminalis* (manna gum) and juvenile and semi-mature *E. obliqua* (messmate stringybark) leaves ($P<0.0001$). Two samples of mature *E. obliqua* from plantation trees showed higher levels of total oxalic acid (7.51% and 5.22% DW), but mature *E. obliqua* leaves were not statistically higher in oxalic acid than other leaf types overall. One sample of mature *E. viminalis* leaves from a full-grown tree also showed a higher oxalic acid concentration of 4.68% DW. Based on leaf age only, mature eucalypt leaves had significantly higher oxalic acid content overall than semi-mature leaves ($P<0.05$). No significant differences were found between the four eucalypt species when leaf ages were pooled.

In spring 2009, Mount Lofty eucalypts showed an overall oxalic acid concentration of 0.64 ± 0.45% DW. No statistically significant differences were found between oxalic acid levels of individual leaf types (Figure 2). There were also no significant differences in oxalic acid content found between pooled data for eucalypt species or leaf age, indicating similar oxalic acid concentrations between all samples overall. The highest total oxalic acid concentration for 2009 was 2.54% DW for a sample of mature *E. viminalis* leaves from a plantation tree.

Juvenile leaves of full-grown trees in Mount Lofty were found to be significantly higher in oxalic acid than juvenile leaves of plantation trees ($P<0.05$). No significant differences were found between semi-mature and mature leaf types and when leaf ages

were pooled. There was also no statistically significant difference found between oxalic acid concentration of leaves from plantation and full-grown trees for pooled samples from 2008 and 2009.

Oxalic acid measurements in eucalypt leaf samples from the Mount Lofty Ranges during spring of 2008 and 2009 and from Moggill, Queensland in spring 2009, showed that overall, mean total oxalic acid content was statistically higher in Mount Lofty eucalypt leaves for both 2008 and 2009 than those from Queensland ($P<0.001$). Based on comparison of eucalypt species between locations (Figure 3), leaves of *E. resinifera* (red stringybark) in Queensland were significantly lower in oxalic acid content than all four species of eucalypt from Mount Lofty in 2008 ($P<0.001$) and lower than *E. camaldulensis* (river red gum) and *E. leucoxylon* (SA blue gum) from Mount Lofty in 2009 ($P<0.05$).

Queensland eucalypts (Figure 4) showed an overall mean oxalic acid concentration of 0.39 ± 0.14% DW. Based on eucalypt species, *E. microcorys* (tallow wood) leaves were significantly higher in oxalic acid overall than *E. propinqua* (small-fruited grey gum) ($P<0.05$), whilst *E. resinifera* leaves were statistically lower than the other three Queensland species ($P<0.05$). There were no overall significant differences based upon pooled leaf age data, and no leaf samples exceeded 0.95% DW total oxalic acid.

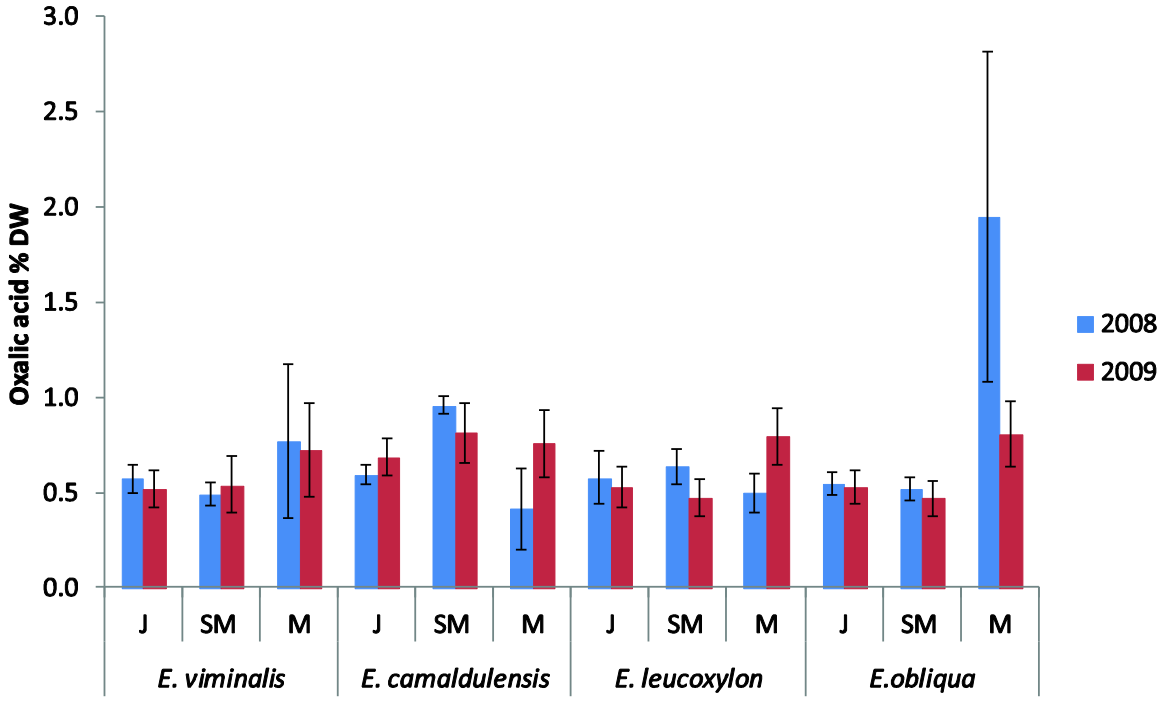


Figure 2. Mean oxalic acid content of juvenile (J), semi-mature (SM) and mature leaves (M) of eucalypt species at Mount Lofty, South Australia in spring 2008 and 2009. Error bars show SEM

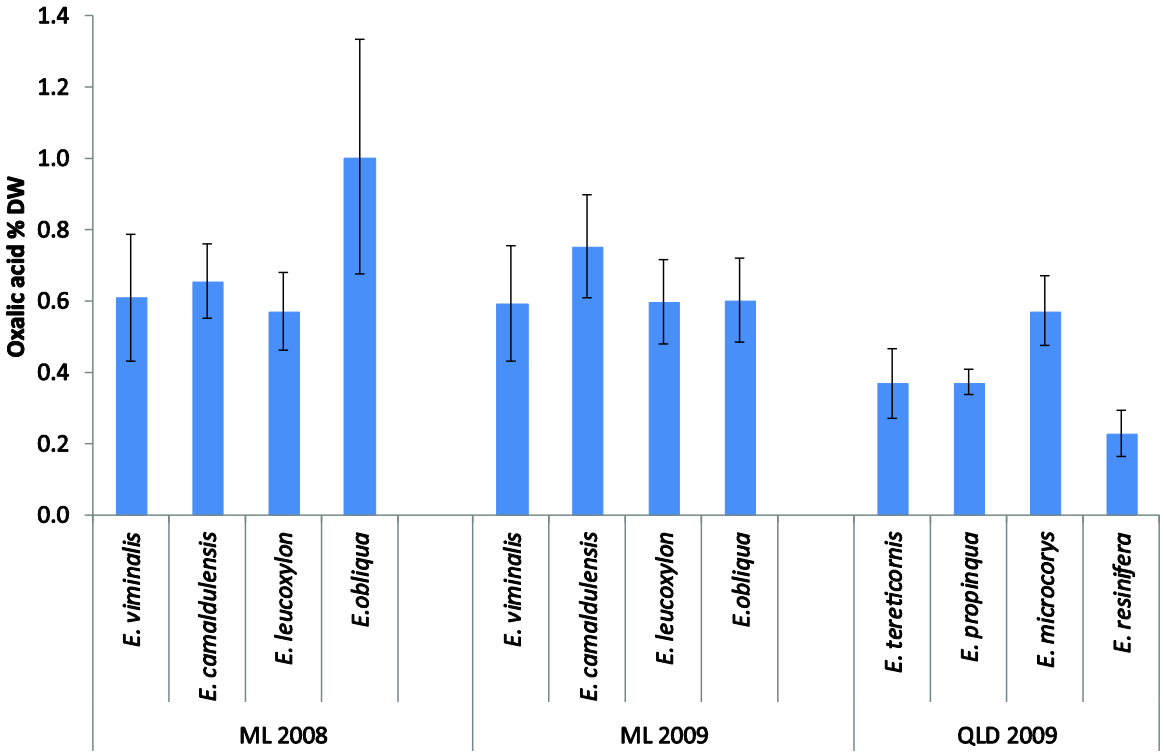


Figure 3. Mean oxalic acid content of eucalypt species from Mount Lofty (ML), South Australia in spring 2008 and 2009 and Moggill, Queensland (Qld) in spring 2009. Error bars show SEM

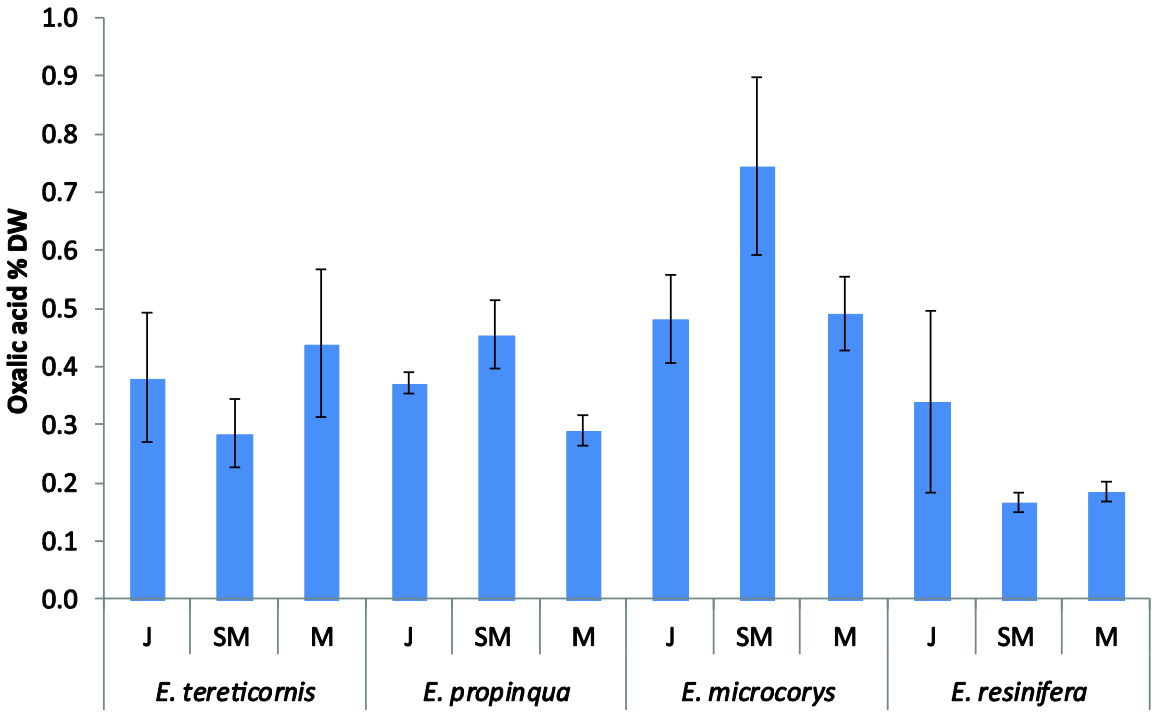


Figure 4. Mean oxalic acid content of juvenile (J), semi-mature (SM) and mature leaves (M) of eucalypt species at Moggill, Queensland in spring 2009. Error bars show SEM

4.5 DISCUSSION

Overall, total oxalic acid content of Mount Lofty eucalypt leaves collected in spring was found to be low, <1% DW, similar to that found in spinach and beetroot (Asplin 2002, Siener et al. 2006). This level of dietary oxalate is much lower than that of known toxic oxalate containing plants, such as soursobs (*Oxalis pes-caprae*), which are greater than 10% DW oxalate (McBarron 1977). Oxalate content was found to be similarly low for all four Mount Lofty eucalypt species, *E. viminalis, E. camaldulensis, E. leucoxylon and E. obliqua,* in both 2008 and 2009. Mature eucalypt leaves showed higher oxalate than semi-mature leaves for 2008, but not 2009. Based on both leaf species and age, semi-mature leaves of *E. camaldulensis* (river red gum) showed significantly higher oxalic acid content (mean 0.96% DW) than several other leaf types in the 2008 leaf collection, but this was not evident in 2009.

One sample of mature *E. viminalis* and two leaf samples of *E. obliqua* from Mount Lofty were found to have higher oxalate concentrations (4.68, 5.22 and 7.51 % DW respectively). The cause of these occasional high oxalate values is unclear, however a previous study of eucalypt responses to drought in the Mount Lofty Ranges showed *E. obliqua* was particularly susceptible to water stress (Sinclair 1980). Also, another study found that in response to drought stress, a fruiting tree species *Prunus persica* showed up to 10-fold increase in leaf oxalate to aid in osmotic adjustment and increase efficiency of water use (Arndt et al. 2000). Hence, the occurrence of high oxalate in individual samples of *E. obliqua* could possibly be due to oxalate accumulation following water stress in this eucalypt species, although whether a similar osmotic adaptation occurs in eucalypts is not known.

If koalas were to ingest a large quantity of eucalypt leaves that were high in oxalate, it is possible that oxalate nephrosis could occur. However as only occasional samples of eucalypt leaves were found with high levels, this would explain only sporadic cases of

oxalate nephrosis in individual koalas. Since over half of koalas in the Mount Lofty population are affected by oxalate nephrosis (Speight et al. 2013), but the majority of eucalypt leaves were found to contain only low levels of oxalate, oxalate nephrosis is unlikely to have a primary dietary cause in this koala population.

Despite this, Mount Lofty eucalypts were found to be higher in total oxalate than those in Moggill, Queensland, where there is a lower prevalence of oxalate nephrosis in koalas (Speight et al. in press). The reason for higher oxalate in Mount Lofty eucalypts compared with those in Moggill, Queensland could be related to climatic differences, whereby Mount Lofty has a wet winter and dry summer and Queensland has a wet summer and dry winter. The leaf collections in the current study were performed in spring, following high winter rainfall in Mount Lofty when water stress should be low, and in Queensland, following low winter rainfall when water stress may have been high. This suggests that the oxalate concentration found in Mount Lofty eucalypts, measured at a time of low water stress, may be an underestimate of maximal oxalate levels if eucalypts were to use oxalate as an osmotic adjustment during times of high water stress.

Due to the climatic differences, Mount Lofty eucalypts showed more new leaf growth than the Moggill eucalypts during spring. Yet juvenile and semi-mature leaves were not found to be higher in oxalate than mature leaves for any collection or location. The only significant difference in oxalate concentration based on leaf age was the finding that mature leaves from Mount Lofty were higher than semi-mature leaves in the 2008 collection, similar to the finding in karri (*E. diversicolor*) that mature leaves were highest in oxalate (O'Connell et al. 1983). Since mature leaves were collected at both Mount Lofty and Queensland, this would support the finding that Mount Lofty eucalypts are higher overall in oxalate compared with Queensland eucalypts. However, this difference in eucalypt leaf oxalate concentration

cannot explain the large variation in prevalence of oxalate nephrosis between the two locations, since all eucalypt species were found to be low in oxalate overall.

An alternative pathogenesis of oxalate nephrosis should be considered, and since koalas in South Australia have low genetic variability (Seymour et al. 2001), they may be at increased risk of an inherited abnormality of oxalate metabolism, similar to primary hyperoxaluria in humans (Cochat et al. 2006). In this disease, oxalate is overproduced endogenously due to liver enzyme dysfunction and results in increased oxalate excretion, causing renal calcium oxalate deposition (Cochat et al. 2006). In affected humans, dietary oxalate contributes further to hyperoxaluria and for this reason, patients are instructed to avoid high oxalate foods (Cochat et al. 2006).

Hence, if oxalate nephrosis in Mount Lofty koalas was caused by a similar inherited disease, ingestion of high oxalate eucalypt leaves would worsen the disease in affected koalas. Further research is underway to determine whether a disease similar to primary hyperoxaluria could explain the high prevalence of oxalate nephrosis in the Mount Lofty koala population. Also, investigation into the contribution of dietary oxalate is planned, to determine whether seasonal differences in eucalypt leaf oxalate exist between Moggill and Mount Lofty, particularly in summer, to better understand the pathogenesis of oxalate nephrosis in koalas.

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CHAPTER 5

Oxalate concentration in stomach contents of koalas with oxalate nephrosis

Speight KN, Haynes JI, Boardman W, Breed WG, Taggart D and Rich B.

Text in manuscript.

CONTEXTUAL STATEMENT

Oxalate nephrosis, a significant disease in the koala population of the Mount Lofty Ranges in South Australia (**Chapter 2**), has an unknown cause but is characterised by hyperoxaluria (**Chapter 3**). Dietary oxalate was investigated as a potential cause by analyses of eucalypt leaves eaten by koalas and was found to be low overall, <1 % oxalate on a dry weight basis (**Chapter 4**). However, koalas are fastidious in their choice of eucalypt leaves (Moore and Foley 2000) and hence it is unclear what oxalate levels occur in leaves which koalas consume.

Chapter 5 investigates the oxalate content of eucalypt leaves which are consumed by koalas by measurement of oxalate in stomach contents samples.

STATEMENT OF AUTHORSHIP: Chapter 5

Oxalate concentration in stomach contents of koalas with oxalate nephrosis
Text in manuscript.

Speight, K.N. (Candidate)
Designed project, performed analysis on all samples, interpreted data, wrote manuscript and acted as corresponding author
I hereby certify that the statement of contribution is accurate
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Haynes, J.I.
Supervised development of work, helped in data interpretation and manuscript evaluation
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Breed, W.G.
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Taggart, D.
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Rich, B.
Helped with experimental design, data interpretation and manuscript evaluation
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5.1 ABSTRACT

Koalas in the Mount Lofty Ranges population in South Australia have a high prevalence of oxalate nephrosis, with many young koalas affected. Oxalate nephrosis in these koalas is associated with hyperoxaluria, indicative of a primary aetiology such as dietary oxalate. A previous study has shown that eucalypt leaves in the Mount Lofty region are low in oxalate concentration overall. However, some individual leaf samples were found to contain potentially toxic levels, which could affect individual koalas. The aim of this study was to determine the oxalate concentration of consumed eucalypt leaves in stomach contents from Mount Lofty koalas with oxalate nephrosis and compare with that of koalas from Mount Lofty and Queensland unaffected by oxalate nephrosis. Soluble and insoluble oxalate concentration was measured using high performance liquid chromatography. Results showed that oxalate levels in stomach contents were low overall. Koalas with oxalate nephrosis had significantly lower soluble and total concentrations of oxalate in stomach contents compared with Queensland koalas ($P<0.05$). However, it was also shown that whilst a significant association between advancing age and increasing concentration of oxalate in stomach contents was found overall ($P<0.005$), some young Mount Lofty koalas with oxalate nephrosis had stomach contents high in oxalate concentration. These results suggest that whilst dietary oxalate is unlikely to be the primary cause of oxalate nephrosis in the majority of Mount Lofty koalas, young individuals may be more susceptible if they consume leaves high in oxalate concentration.

5.2 INTRODUCTION

The koala is a folivorous arboreal marsupial that primarily eats eucalypt leaves. Approximately 600 species of *Eucalyptus* trees are found in Australia, but relatively few of these are eaten regularly by koalas (Jackson et al. 2003, Tyndale-Biscoe 2005). In South Australia the preferred species of eucalypt eaten by koalas is the manna gum (*Eucalyptus viminalis*) (Phillips 1990), although it has been shown that individual koalas may have interspecific and intraspecific preferences for eucalypt trees (Moore and Foley 2000). The determinants of leaf selection by koalas has been extensively investigated and leaf nutrients such as nitrogen, as well as 'antinutrients' such as fibre and tannins, may be important factors in leaf choice (Hume and Esson 1993).

Koalas of the Mount Lofty population in South Australia have a high prevalence of oxalate nephrosis compared to those in the eastern states of Australia (Speight et al. 2013). Oxalate nephrosis in these koalas is associated with renal insufficiency and hyperoxaluria (Speight et al. in press), indicative of a primary aetiology. In other herbivores, ingestion of plants containing high levels of oxalate can cause oxalate nephrosis, and a previous study has shown that significantly higher oxalate concentrations occur in Mount Lofty eucalypts compared with eucalypt species eaten by koalas in Moggill, Queensland (Chapter 4), where oxalate nephrosis is at lower prevalence (Speight et al. in press). However oxalate levels in Mount Lofty eucalypts were found to be low overall, <1% on a dry weight (DW) basis (Chapter 4), compared with plants high in oxalate that are known to cause toxicity, such as soursobs >10% DW oxalate (James and Panter 1993, McBarron 1977). Yet some individual samples of eucalypt leaves from Mount Lofty, particularly mature *E. obliqua* (messmate stringybark), showed high values up to 7.5% DW (Chapter 4). Since koalas are known to be selective in their choice of leaves, the aim of this study was to measure the oxalate levels in stomach contents of Mount Lofty koalas with oxalate nephrosis to determine whether

leaves high in oxalate are consumed compared with koalas that are unaffected by oxalate nephrosis in Mount Lofty and Queensland.

5.3 METHODS

5.3.1 Stomach contents collection

To determine total and soluble oxalate content in consumed eucalypt leaves, stomach content samples were collected from koalas that were euthanased on welfare grounds at Cleland Wildlife Park, Mount Lofty, South Australia (SA) and Moggill Koala Hospital, Moggill, Queensland (Qld). Presence or absence of oxalate nephrosis was confirmed in all sampled koalas by renal histopathological examination (see Speight et al. 2013) which allowed classification into groups of koalas affected by oxalate nephrosis and those that were unaffected.

Approximately 1 gram of luminal stomach contents was sampled and stored at −70˚C until analysis. Samples were taken from 11 Mount Lofty koalas with oxalate nephrosis (5 male, 6 female; 5 rescued wild, 6 captive) and compared with 10 koalas unaffected by oxalate nephrosis from Mount Lofty (6 male, 4 female; 6 rescued wild, 4 captive), and 15 unaffected rescued wild koalas from Moggill, Qld (4 male, 11 female). Koalas were aged by tooth wear class (Martin 1981) (Table 1); the mean tooth wear class for Mount Lofty koalas with oxalate nephrosis was 2.1 ±1.5 (mean ±SD) or approximately 2 years old, for unaffected Mount Lofty koalas 2.4 ±1.4, approximately 2 - 3 years old, and Queensland koalas 6.1 ±0.9 or approximately 9 - 13 years old (Martin and Handasyde 1990, Young et al. 1996).

All samples were collected with approvals from the University of Adelaide Animal Ethics Committee, Department of Environment and Natural Resources (SA) and Department of Environment and Resource Management (Qld).

5.3.2 Oxalate measurement

Stomach content samples of approximately 15 mg were weighed on a balance (Mettler, Zurich). For each sample, soluble oxalate was extracted in 1 mL water for 1 hour at 60˚C and total oxalate in 1 mL 0.01 M hydrochloric acid (pH 2) under the same conditions. Stomach content oxalate concentration was measured in duplicate as oxalic acid using high performance liquid chromatography as previously described (Chapter 4) and reported on a dry weight basis. Variation between duplicates averaged 19% for soluble oxalate and 17% for total oxalate. Oxalate concentration was calculated from a calibration curve produced by plotting peak height against concentration (Figure 1). Least squares regression was performed to determine the slope, intercept and coefficient of determination.

5.3.3 Data analysis

Oxalic acid data were assessed for normality and homogeneity and then analysed with nonparametric Kruskal-Wallis and post hoc Mann Whitney U tests, with a level of significance of 0.05. Spearman's rank order correlation was used to detect significant relationships between stomach content oxalate concentration and koala tooth wear class.

Table 1. Tooth wear class of koalas.

	Tooth wear class[a]							
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**Total**
ON	6	2		2	1			11
ML	4	2		4				10
Qld				1	2	6	6	15

ON= Mount Lofty koalas with oxalate nephrosis; ML= Mount Lofty koalas unaffected by oxalate nephrosis; Qld= Queensland koalas unaffected by oxalate nephrosis. [a] Tooth wear class determined according to method of Martin (1981).

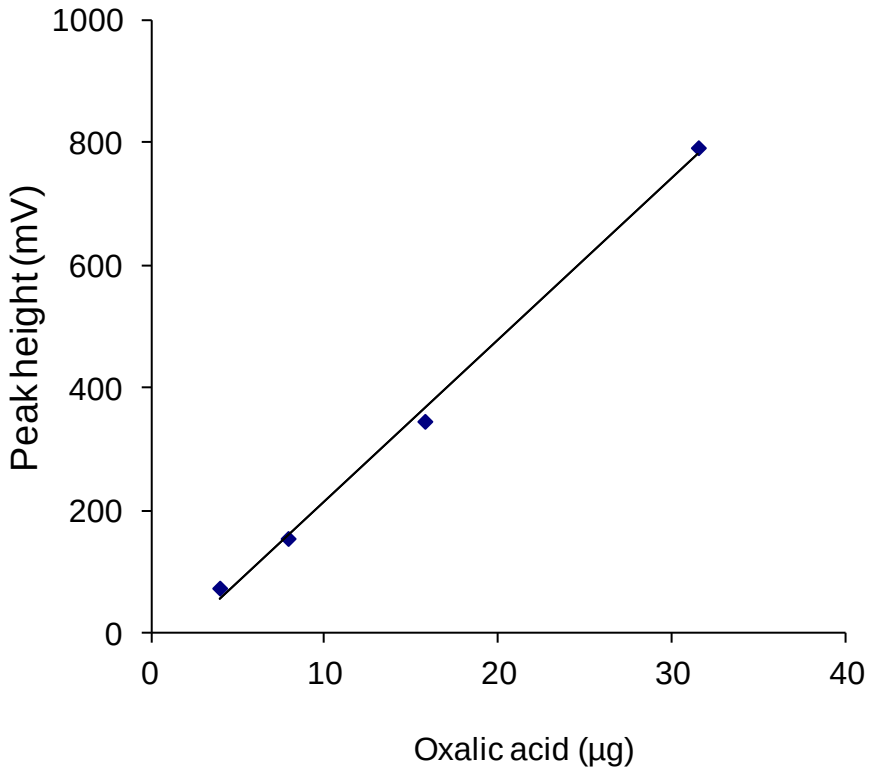


Figure 1. Calibration curve for oxalic acid using high performance liquid chromatography (R^2= 0.997).

5.4 RESULTS

Oxalate analysis of stomach contents showed low levels of total oxalate (<1.5% DW) and soluble oxalate (<0.5% DW). Mount Lofty koalas with oxalate nephrosis did not show the highest oxalate concentration in stomach contents; soluble oxalate concentration was significantly higher in Queensland koalas compared with Mount Lofty koalas with oxalate nephrosis, and also compared with Mount Lofty koalas unaffected by oxalate nephrosis ($P<0.005$) (Figure 2). Total oxalate concentration in stomach contents of Queensland koalas was also significantly higher than that of both groups of Mount Lofty koalas ($P<0.005$). The percentage of soluble oxalate to total oxalate in stomach content samples was similar between all three koala groups, at approximately 34% (Figure 3).

The highest soluble oxalate concentration found in stomach contents from a Mount Lofty koala with oxalate nephrosis was 0.14% DW, in an unaffected Mount Lofty koala 0.24% DW and in a Queensland koala 0.38% DW. The highest total oxalate concentration in stomach contents from a koala with oxalate nephrosis was 0.58% DW, in an unaffected Mount Lofty koala 0.57% DW and in a Queensland koala 1.44% DW. No significant differences in soluble or total oxalate concentration of stomach contents was found between male and female koalas, or based on captive or wild origin.

A significant correlation between advancing tooth wear class and increasing soluble oxalate concentration in stomach contents ($R=0.476$; $P<0.005$) and increasing total oxalate concentration in stomach contents ($R= 0.481$; $P<0.005$) was found overall for all three groups of koalas (Figure 4). Within both the Mount Lofty and Qld koala groups unaffected by oxalate nephrosis, this trend was shown by increasing soluble and total oxalate concentration in stomach contents with advancing tooth wear class. In contrast, for Mount Lofty koalas with oxalate nephrosis, young koalas in tooth wear class I showed a trend of higher soluble and total oxalate concentrations in stomach contents compared with those in more advanced tooth wear classes.

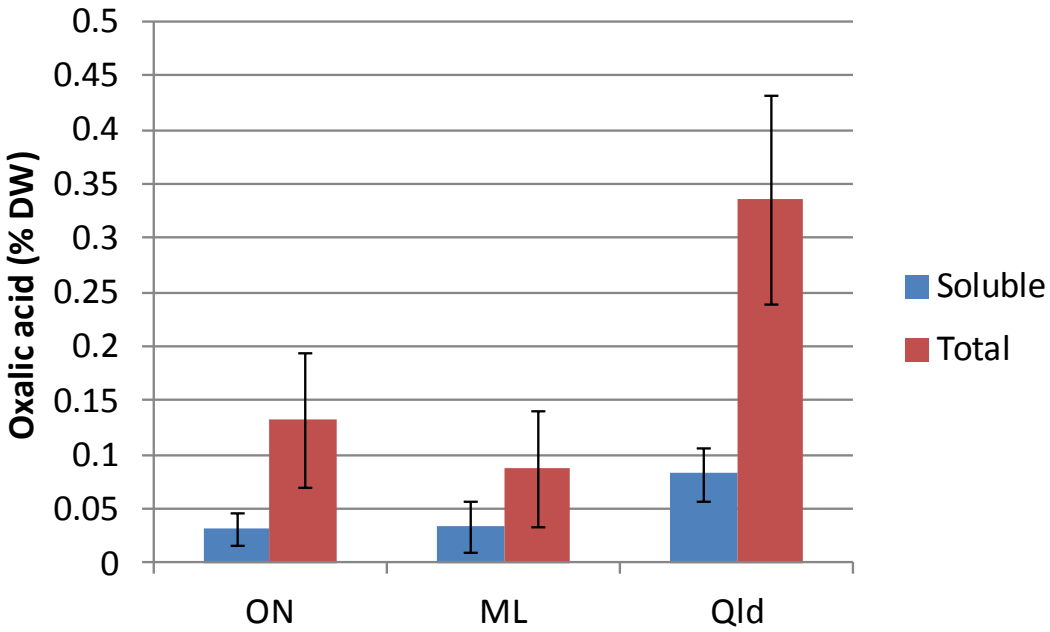


Figure 2. Comparison of soluble and total oxalic acid concentration in stomach contents of koalas from Mount Lofty, South Australia with oxalate nephrosis (ON), and unaffected koalas from Mount Lofty (ML) and Moggill, Queensland (Qld). Error bars show SEM.

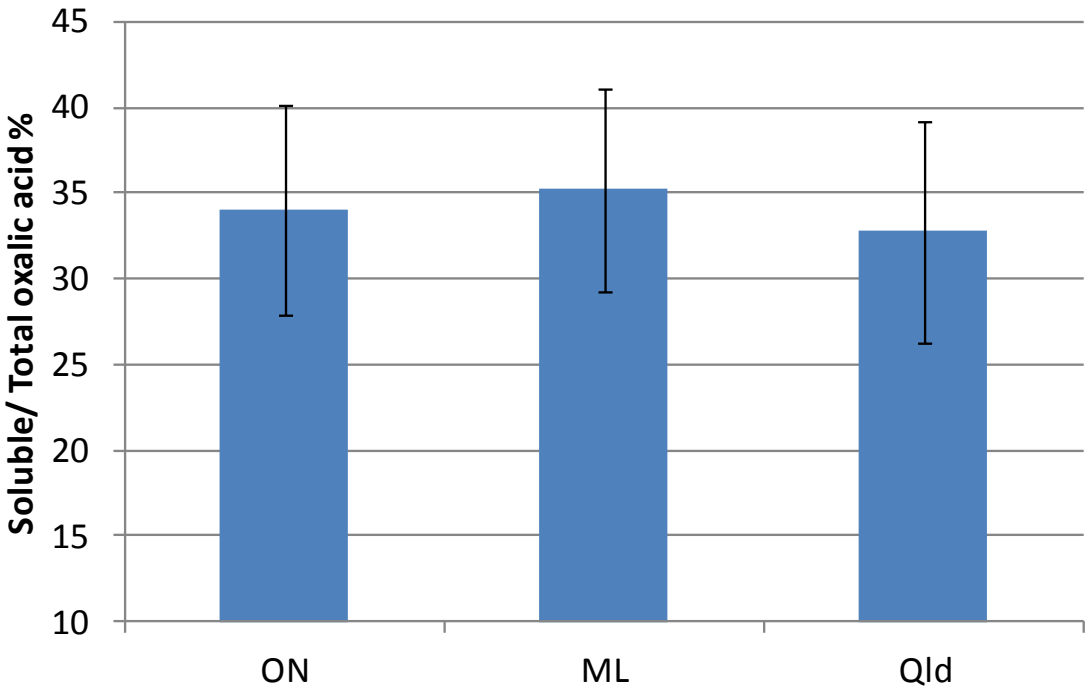
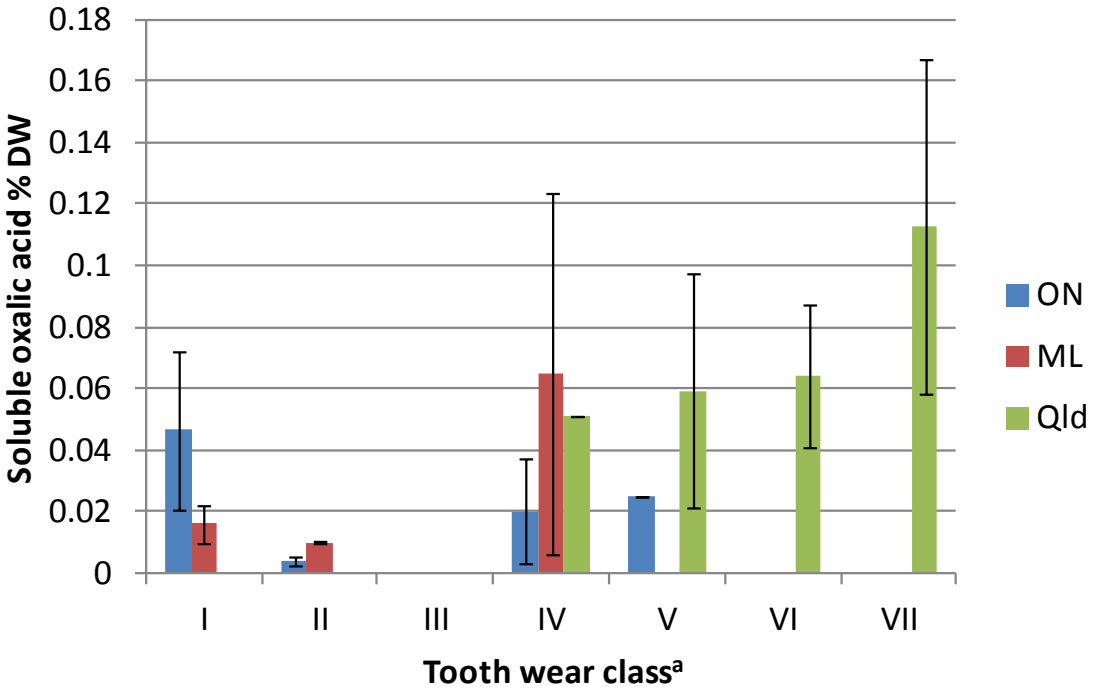
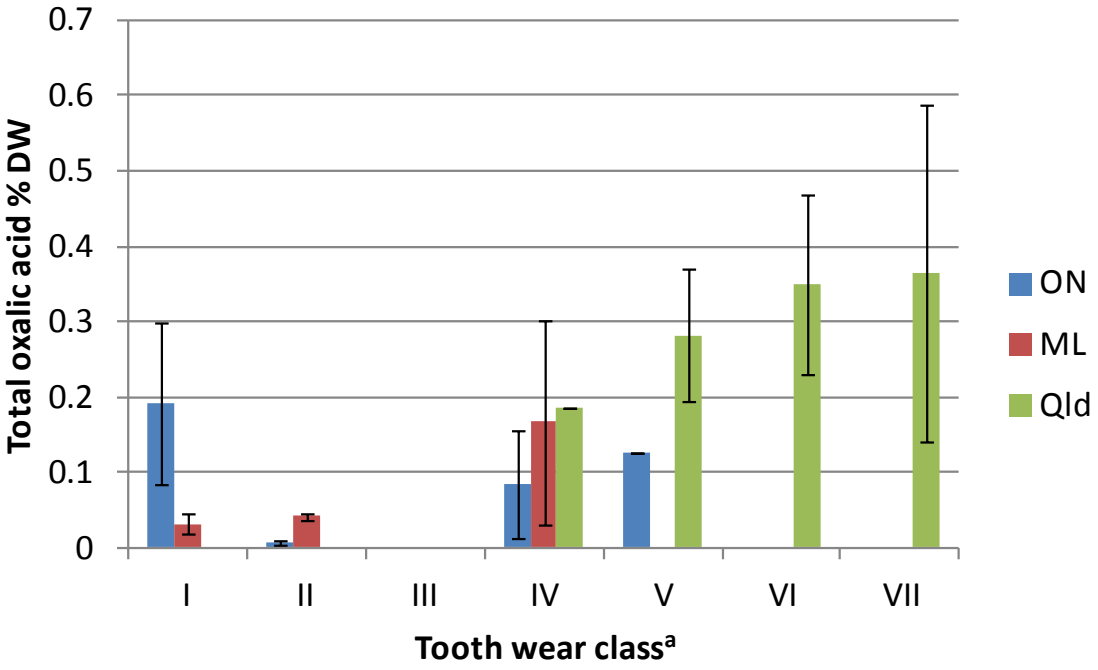


Figure 3. Proportion of soluble to total oxalic acid in stomach contents of koalas from Mount Lofty, South Australia with oxalate nephrosis (ON), and unaffected koalas from Mount Lofty (ML) and Moggill, Queensland (Qld). Error bars show SEM.



a)



b)

Figure 4. a) Soluble and b) total oxalic acid content of stomach contents based on tooth wear class for koalas from Mount Lofty, South Australia with oxalate nephrosis (ON), and unaffected koalas from Mount Lofty (ML) and Moggill, Queensland (Qld). Error bars show SEM. Absence of error bars indicates n=1. [a]Tooth wear class determined according to Martin (1981).

5.5 DISCUSSION

Oxalic acid analysis of stomach contents showed that Mount Lofty koalas with oxalate nephrosis did not appear to have consumed eucalypt leaves that were higher in oxalate content than koalas in Mount Lofty and Queensland which were unaffected by oxalate nephrosis. Queensland koalas showed the highest levels of both soluble and total oxalate concentration in stomach contents, suggesting that dietary eucalypts are higher in oxalate content in Queensland than those from Mount Lofty. However, these findings differ to those of a previous study of oxalate concentration in dietary eucalypt leaves which found that eucalypt leaves from Moggill, Queensland were significantly lower in oxalate content than those from the Mount Lofty region (Chapter 4). Based on the higher oxalate concentration found in Mount Lofty eucalypt leaves, as well as the high prevalence of oxalate nephrosis in Mount Lofty koalas, it was expected that the stomach contents of the Mount Lofty koalas with oxalate nephrosis would have higher oxalate concentration.

Despite this, the soluble and total oxalate levels of ingested eucalypt leaves in stomach contents from all three groups of koalas were low compared with known toxic high oxalate plants, such as the soursob (*Oxalis pes-caprae)* (James and Panter 1993, McBarron 1977). However, a recent study of oxalate nephropathy in a group of captive marmosets showed intoxication from ingesting *E. viminalis* (manna gum)*,* which was between 0.16 and 0.3% soluble oxalate DW (Vanselow et al. 2011). This is very similar to that found in the current study of koala stomach contents (0.14 - 0.38% DW). Hence it appears possible that this level of oxalate could be high enough to cause toxicity in some species of mammals.

Koalas are specialist folivores of eucalypts and hence should have evolved adaptations to cope with digestion and metabolism of leaf constituents. Oxalate has been shown to be present in many eucalypt species (Chapter 4), and therefore koalas are likely to possess mechanisms to cope with oxalate intake, as with other eucalypt compounds

(Stupans et al. 2001). In other herbivores it has been shown that oxalate-degrading bacteria occur in the rumen or hindgut (Allison and Cook 1981, Allison et al. 1985), and have the ability to adapt to different concentrations of ingested oxalate (James and Panter 1993). The koala has an enlarged hindgut (Hume 1982) and whilst little is known about the microflora of the koala gastrointestinal tract (Cork and Sanson 1990), it is likely that oxalate-degrading bacteria are present, as in other hindgut dominant herbivorous species such as horses, rabbits and guinea pigs (Allison and Cook 1981, Argenzio et al. 1988).

Therefore, given that low levels of total oxalate content were found in the majority of eucalypt leaves in the previous study (Chapter 4), it is suggested that oxalate toxicity may only occur in koalas if large amounts of high oxalate eucalypt leaves are ingested in a short period of time, as in other herbivores (James and Panter 1993). In the previous study, a sample of mature *E. obliqua* (messmate stringybark) showed a high total oxalate concentration of 7.5% DW, similar to that found in soursobs (Chapter 4). Therefore, it is possible that oxalate nephrosis in individual Mount Lofty koalas could be initiated by ingestion of a large amount of high oxalate eucalypt leaves during a single feeding session.

Soluble oxalate is more toxic than insoluble oxalate since it is unbound and therefore more easily absorbed across the gastrointestinal tract mucosa (McBarron 1977). However, insoluble oxalate becomes soluble in the stomach of monogastric species at pH 2 (Reyers and Naude 2012). In the previous study only total, and not soluble, oxalate was measured in eucalypt leaves, but it was found in the current study that the ratio of soluble to total oxalate was similar in koala stomach contents from both Mount Lofty and Queensland. This suggests that the amount of soluble oxalate in eucalypt leaves may be approximately 34% that of the total oxalate concentration. This would imply that in the previous study, the samples of mature *E. obliqua* that were 7.5% DW total oxalate may have contained 2.6% DW

soluble oxalate, a highly toxic concentration (McKenzie 2012). This ratio of soluble to total oxalate is similar to that found in other studies of plant oxalate (Siener et al. 2006).

Overall, increasing age of koalas was found to be associated with increasing oxalate concentration of stomach contents. This association was also found in unaffected koalas in Mount Lofty and Queensland, but not in the cohort of koalas with oxalate nephrosis. Two young koalas with oxalate nephrosis in tooth wear class I (<2 years old) showed higher oxalate concentration in stomach contents than that of the older koalas. This difference between groups of koalas may be explained by a large number of koalas of advanced age in the Queensland group compared with a large number of Mount Lofty koalas in tooth wear class I.

Oxalate nephrosis has been found to be common in young koalas (Speight et al. 2013), and ingestion of eucalypts relatively high in oxalate content could potentially cause oxalate nephrosis in young koalas if oxalate-degrading microflora were not well established. This could occur in young koalas that do not receive sufficient 'pap', a soft faeces originating in the caecum of the mother, which is rich in gastrointestinal microflora (Osawa et al. 1993). This occurs at approximately 200 days and prior to weaning from milk to eucalypt leaves at approximately 227 days (Blanshard and Bodley 2008).

It is unknown whether pap contains oxalate-degrading bacteria, but there is the potential that if there are insufficient pap microbes to colonise the caecum of the young koalas, and eucalypt leaves high in oxalate are ingested, less oxalate would be degraded and more absorbed into the bloodstream (Hatch and Freel 2005). This could partly explain why many young koalas are susceptible to oxalate nephrosis. Unfortunately there were no young koalas sampled from Queensland in the current study for comparison, however there were also no Mount Lofty koalas unaffected by oxalate nephrosis in tooth wear class I with stomach contents high in oxalate concentration.

In the Mount Lofty population, older koalas with oxalate nephrosis appeared to have lower oxalate concentration in stomach contents than the younger affected koalas. This may indicate that koalas with oxalate nephrosis learn aversion to leaves high in oxalate following their initial ingestion and subsequent renal damage. Moore and Foley (2000) propose this aversion theory, amongst others, in a review of the fastidious feeding habits of koalas, whereby individuals may learn from prior foraging experiences or from social interactions. Oxalate has been shown to cause learned feed aversion in sheep (Duncan et al. 1998), whilst in koalas it has been shown that they may be deterred by leaves high in a certain phenolic compound (Lawler et al. 1988), suggesting the ability of koalas to detect the constituents of leaves and make negative associations. Based on the results of the current study, it is recommended that captive young koalas are fed eucalypt leaves low in oxalate content, such as those of *E. leucoxylon* (SA blue gum), as found in the previous study (Chapter 4).

The results of the current study, in conjunction with the previous study on oxalate concentrations of various eucalypt species (Chapter 4), suggest that although oxalate levels of eucalypt leaves are low overall, there is the potential for oxalate nephrosis to be caused in individual koalas by ingestion of eucalypts which are high in oxalate. Young koalas may be more vulnerable, possibly due to decreased ability to microbially degrade oxalate in their gastrointestinal tracts. However, the current study is limited in measurement of oxalate levels in the stomach contents at the time of death, after the initiating event of oxalate nephrosis has already occurred. Whilst the finding of higher oxalate concentration in stomach contents of Queensland koalas than those from Mount Lofty was unexpected, similar stomach contents oxalate concentration was found in young koalas with oxalate nephrosis to that of older unaffected Queensland koalas. It is likely that the aetiology of oxalate nephrosis in koalas is complex, and koalas in the Mount Lofty region may have an inherited metabolic abnormality which causes increased endogenous production of oxalate,

such as in primary hyperoxaluria in humans (Asplin 2002). More studies are needed to increase the understanding of the pathogenesis of this disease and the importance of dietary oxalate as a predisposing factor.

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CHAPTER 6

Investigation of an inherited basis for oxalate nephrosis in koalas

Speight KN, Haynes JI, Boardman W, Breed WG, Taggart D, McCarthy P and Rich B.

Text in manuscript.

CONTEXTUAL STATEMENT

Oxalate nephrosis is a leading disease in the koala population of the Mount Lofty Ranges in South Australia (**Chapter 2**), but its cause remains unknown. Dietary oxalate in eucalypts has been found to be low overall (**Chapter 4 & 5**) and unlikely to be the primary cause of the high prevalence of oxalate nephrosis. Based on the low genetic diversity of this koala population (Houlden and St John 2000, Seymour et al. 2001) and the recent finding of hyperoxaluria (**Chapter 3**), it is possible that an inherited disease similar to primary hyperoxaluria in humans occurs. Also, the clinical condition of captive koalas with renal disease receiving pyridoxine therapy appears to improve (I. Hough, A. Sulley, pers.comm.), consistent with primary hyperoxaluria type I (Asplin 2002). In this disease, dysfunction of the pyridoxine-dependent liver enzyme, alanine: glyoxylate aminotransferase, causes overproduction of oxalate by the liver (Cochat 1999).

Chapter 6 investigates if a disease similar to primary hyperoxaluria type I is the cause of oxalate nephrosis in the Mount Lofty koalas.

STATEMENT OF AUTHORSHIP: Chapter 6

Investigation of an inherited basis for oxalate nephrosis in koalas.
Text in manuscript.

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Designed project, performed analysis on all samples, interpreted data, wrote manuscript and acted as corresponding author
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6.1 ABSTRACT

Koalas in the Mount Lofty Ranges region in South Australia have a high prevalence of oxalate nephrosis, for which the cause remains unclear. Eucalypt leaf analyses for dietary oxalate have shown low levels of oxalate overall, with only occasional leaf types containing toxic levels of oxalate; therefore high dietary oxalate is unlikely to be the primary cause of this disease. However, koalas from the Mount Lofty population have low genetic diversity, increasing the risk of an inherited disease. In humans a disease called primary hyperoxaluria type I occurs, in which oxalate is overproduced by the liver due to dysfunction of the pyridoxine-dependent enzyme alanine: glyoxylate aminotransferase (AGT). A disease similar to primary hyperoxaluria type I was also suggested by pyridoxine supplementation leading to clinical improvement in koalas with oxalate nephrosis. Therefore, this study aimed to determine AGT activity in koalas with oxalate nephrosis. Liver samples were obtained from koalas with oxalate nephrosis and compared with unaffected koalas from the Mount Lofty region, as well as with unaffected koalas from Moggill, Queensland, in which genetic diversity is high and the prevalence of oxalate nephrosis is low. Hepatic AGT activity was measured using a spectrophotometric assay. Results showed similar AGT activity between koalas with oxalate nephrosis and unaffected koalas from Mount Lofty and Queensland. AGT activity reference interval was established as 2.4-13.7 µmol pyruvate/h/mg protein (mean ± 2SD) in koalas, similar to that of healthy humans. Although AGT activity was not found to be decreased in koalas with oxalate nephrosis, a variant of primary hyperoxaluria type I may still occur whereby AGT activity is normal, but ineffective due to intracellular mistargeting. Further studies are needed to confirm a genetic basis for oxalate nephrosis in koalas.

6.2 INTRODUCTION

Koala (*Phascolarctos cinereus*) populations are widely distributed throughout eucalypt forests from the northeast to the southeast of Australia. Three subspecies or races of koalas are recognised on the basis of morphological, rather than genetic differences (Houlden et al. 1999), which include variations in body size and fur colour (Martin and Handasyde 1999). These three koala races are spread geographically, whereby *P. c. adjustus* is found in the northern regions (Queensland), *P. c. cinereus* in intermediate eastern areas (New South Wales) and *P. c. victor* in the south (Victoria and South Australia) (Martin and Handasyde 1999).

In South Australia, koalas were originally distributed in the lower southeast of the state but became extinct by the 1930s due to hunting, habitat loss, fire and disease (Phillips 1990, Robinson et al. 1989). The current koala populations of South Australia consist of koalas that were translocated from French Island, where a koala colony was established in the 1890s with only a few individuals from mainland Victoria (Jackson 2007, Lewis 1954). Approximately 18 koalas, some with pouch young, were introduced to Kangaroo Island from French Island between the years 1923 to 1925 and the population flourished (Phillips 1990). In the 1960s, small numbers of koalas were translocated from Kangaroo Island to various locations in mainland South Australia, including the Adelaide Hills and Ashbourne regions of the Mount Lofty Ranges, the Riverland, Eyre Peninsula and their original range in the lower southeast of the state (Phillips 1990, Robinson 1978). The koala population of the Mount Lofty Ranges is therefore primarily descended from Victorian koalas, and although there are records of individuals from Queensland or New South Wales (Robinson 1978), this has not been supported by genetic analyses (Seymour et al. 2001).

Low genetic variability is present in South Australian koala populations due to the bottleneck that has occurred as a result of these translocations of limited numbers of

individuals (Houlden et al. 1996, Phillips 1990). Evidence of reduced genetic variation has been found in koalas from French Island (Cristescu et al. 2009, Fowler et al. 1998, Houlden et al. 1996, Taylor et al. 1997), Kangaroo Island (Cristescu et al. 2009, Fowler et al. 1998, Houlden et al. 1996, Seymour et al. 2001), the Eyre Peninsula (Seymour et al. 2001) and the Mount Lofty Ranges (Houlden and St John 2000, Seymour et al. 2001). Studies have also found an increase in the prevalence of testicular aplasia in the koala populations on French Island, Eyre Peninsula (Seymour et al. 2001) and Kangaroo Island (Cristescu et al. 2009, Seymour et al. 2001). In contrast, koala populations in New South Wales (Fowler et al. 1998, Houlden et al. 1996) and southeast Queensland (Fowler et al. 1998, Fowler et al. 2000, Houlden et al. 1996) have been shown to have high genetic variation.

Despite this low genetic diversity, several koala populations in South Australia have been flourishing and as a consequence, koalas in Kangaroo Island are now regarded as an overabundant species (DEWHA 2009, Duka and Masters 2005). The Mount Lofty Ranges koala population is also considered robust and this is likely to be because both populations are relatively free of disease, particularly chlamydiosis, with zero prevalence in Kangaroo Island koalas and low clinical disease prevalence in the Mount Lofty population (Houlden and St John 2000, Polkinghorne et al. 2013). This contrasts with the declining koala populations in New South Wales and Queensland, where the high prevalence of chlamydiosis causes ocular and urogenital disease, leading to blindness and reduced fertility and fecundity (Polkinghorne et al. 2013, Timms 2005). Also, koala retrovirus (KoRV) infection has been found to occur in 100% of Queensland koalas compared with only 14.8% of Kangaroo Island koalas, in which there is also lower proviral load (Simmons et al. 2012). KoRV status in the Mount Lofty koala population is currently unknown, but is likely to be similar to that found in Kangaroo Island koalas.

Whilst infectious disease prevalence appears to be low in koalas from the Mount

Lofty population, approximately 55% are affected by oxalate nephrosis (Speight et al. 2013, Speight et al. in press). Affected koalas show clinical signs typical of renal failure such as polydipsia, polyuria and weight loss (Haynes et al. 2004), associated with high plasma urea and creatinine, poorly concentrated urine and hyperoxaluria (Speight et al. in press). In contrast, koala populations in the eastern states have a low prevalence of oxalate nephrosis, with <3% of koalas in New South Wales affected (Canfield 1987, Canfield 1989). Likewise, in Moggill, Queensland, only 2/19 koalas (12%) were found to be affected by oxalate nephrosis in a recent study (Speight et al. in press).

The cause of oxalate nephrosis in koalas is unknown and recent investigations have focussed on a dietary origin, since oxalate nephrosis in humans and animals can be caused by ingestion of foodstuffs containing high concentrations of oxalate, such as rhubarb leaves and soursobs (Maxie and Newman 2007). However, it has been found that most eucalypt leaves eaten by koalas contain only low levels of oxalate, <1% on a dry weight (DW) basis (Chapter 4). Whilst some mature leaves of *E. obliqua* (messmate stringybark) were shown to contain high levels of oxalate, up to 7.5% DW, a dietary cause for oxalate nephrosis in the Mount Lofty koala population is unlikely (Chapter 4). The overall low level of oxalate in eucalypt leaves suggests that the high prevalence of oxalate nephrosis in the Mount Lofty koala population may have a more complex pathogenesis. Due to the low genetic diversity of these koalas, it is therefore possible that the disease may be due to an inherited abnormality in oxalate metabolism.

In humans an inherited disease, called primary hyperoxaluria type I, causes endogenous overproduction of oxalate, leading to hyperoxaluria and oxalate nephrosis (Asplin 2002). Primary hyperoxaluria type I results from the dysfunction of the hepatic peroxisomal enzyme alanine:glyoxylate aminotransferase (AGT). In healthy individuals, AGT catalyses the transamination of glyoxylate to glycine (Birdsey et al. 2005), using pyridoxine

(vitamin B6) as a cofactor, with small amounts of oxalate produced as a by-product of the metabolic pathway (Cochat et al. 2006) (Figure 1a). With AGT dysfunction, glyoxylate accumulates and is converted to oxalate in the cytosol by the enzyme lactate dehydrogenase, with glycolate production also increased (Figure 1b) (Harambat et al. 2011, Raju et al. 2008, Robijn et al. 2011). This excess oxalate enters the bloodstream and is excreted by the kidney, leading to the precipitation of calcium oxalate crystals (Cochat 1999, Robijn et al. 2011).

Conservative treatment in humans includes pyridoxine supplementation to maximise the residual activity of AGT (Fargue et al. 2013), in conjunction with low oxalate intake in the diet and high water intake to increase urine volume (Asplin 2002, Cochat et al. 2012). Orthophosphate has also been found to be effective at preserving adequate renal function long-term, particularly when combined with pyridoxine (Milliner et al. 1994), however response to pyridoxine can be variable (Monico et al. 2005). The only life-saving cure for severely affected patients is a combined liver-kidney transplant (Hoppe et al. 2009, Watts et al. 1987).

In humans, AGT dysfunction most commonly occurs as a result of genetic mutations which cause a loss or decrease of enzyme function (Danpure 1993, Danpure et al. 1987, Hoppe et al. 2009, Tarn et al. 1997), but may also arise due to intracellular mistargeting of AGT with normal activity in some cases (Danpure 1993, Danpure et al. 1989, Danpure et al. 1994b). This enzyme mistargeting occurs because AGT has evolved to have association with different intracellular organelles of hepatocytes based on the variable dietary precursors of glyoxylate, glycolate in herbivores and hydroxyproline in carnivores (Birdsey et al. 2005, Danpure et al. 1994a). It has been found that AGT is associated with the peroxisomes in herbivores and the mitochondria in carnivores (Birdsey et al. 2005, Danpure et al. 1989).

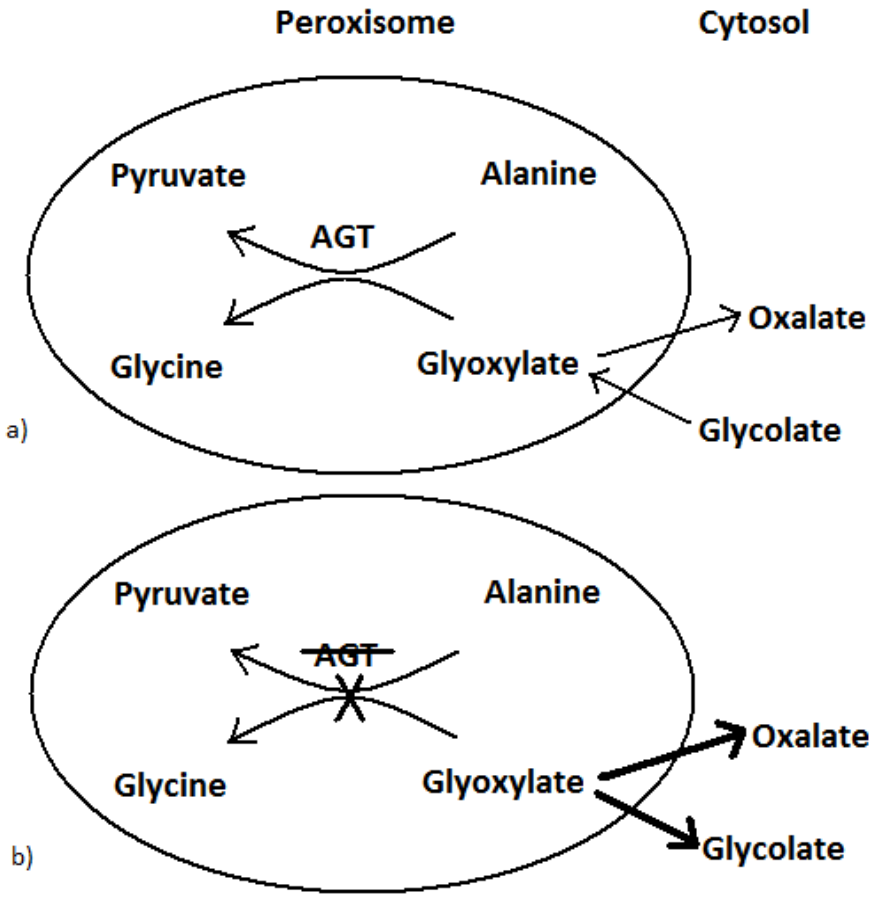


Figure 1. Enzymatic pathways for alanine glyoxylate: aminotransferase (AGT) occurring within the peroxisome in human hepatocytes in a) normal metabolism and b) primary hyperoxaluria type I, in which AGT dysfunction occurs causing overproduction of oxalate.

As part of a large study of the evolutionary adaptations of AGT to diet, it has been shown by immuno-electron microscopy that AGT was present in the hepatic peroxisomes of a koala (Danpure et al. 1994a). A peroxisomal AGT intracellular location would be expected due to their herbivorous diet of eucalypt leaves. However, immunoreactivity of the AGT protein was reported as 'low' and AGT enzyme activity was described as 'very low' (<0.8 µmol/h/mg protein) (Danpure et al. 1994a). Several other herbivorous mammals were also classified as having very low AGT activity and the authors suggested that the metabolic role of AGT may be reduced in these species, compared with those with high AGT activity.

An analogous disease to primary hyperoxaluria type I in humans has only been identified in one other species, the domestic canine. Two Tibetan spaniel pups affected by oxalate nephropathy (Jansen and Arnesen 1990) were found to have low AGT activity as well as low AGT protein immunoreactivity (Danpure et al. 1991), whilst another study found that seven affected Coton de Tulear pups had a mutation in the AGT gene (Vidgren et al. 2012). A disease similar to primary hyperoxaluria type I has also been suspected as the cause of oxalate nephropathy in a colony of the endangered Gilbert's potoroo (*Potorous gilbertii*) in Western Australia, however AGT dysfunction has not been established (D. Forshaw, 2013, pers. comm.).

The prevalence of oxalate nephrosis in Mount Lofty koalas has been found to be high, affecting both rescued wild and captive koalas (Speight et al. 2013). Since this koala population has low genetic diversity, an inherited condition such as primary hyperoxaluria is more likely, such as has been found in humans with high consanguinity (Kamoun and Lakhoua 1996) and purebred dog breeds (Danpure et al. 1991, Vidgren et al. 2012). Levels of hyperoxaluria found in Mount Lofty koalas are similar in magnitude, median 209 µmol oxalate/mmol creatinine (Speight et al. in press), to that found in children with primary hyperoxaluria, >100 µmol/mmol (Hoppe et al. 2009). In addition, the ratio of urinary calcium: oxalate was found to be less than 10: 1 (Asplin 2002, Robertson and Peacock 1980), approximately 4.5: 1 in koalas with oxalate nephrosis and 2: 1 in unaffected Mount Lofty koalas (Speight et al. in press), showing increased levels of oxalate in the urine. This is similar to that found in hyperoxaluric otters, in which the urinary calcium: oxalate ratio was determined to be 1: 1, and an inherited cause such as primary hyperoxaluria was suspected (Petrini et al. 1999).

Equal numbers of male and female koalas have been found to be affected by oxalate nephrosis, which would be expected with an autosomal recessive inherited disease similar to

primary hyperoxaluria. Also, many young koalas are affected (Speight et al. 2013), and the onset of primary hyperoxaluria is often in childhood (Asplin 2002). In addition, captive koalas at Cleland Wildlife Park appear to show clinical improvement with pyridoxine therapy (I. Hough, A. Sulley, pers. comm.), consistent with that found with decreased AGT activity in primary hyperoxaluria type I in humans (Asplin 2002, Cochat et al. 2012, Fargue et al. 2013). Therefore, this study investigates whether a disease similar to primary hyperoxaluria type I is the cause of oxalate nephrosis in koalas from the Mount Lofty region of SA by measurement of AGT activity.

6.3 METHODS

6.3.1 Sample collection

Liver samples were collected at necropsy from koalas that had died or been euthanased on animal welfare grounds. Koalas sampled from the Mount Lofty Ranges region included rescued wild koalas and captive koalas at Cleland Wildlife Park, whilst koalas sampled from Moggill, Queensland included rescued wild koalas admitted to Moggill Koala Hospital. Oxalate nephrosis was confirmed in 16 Mount Lofty koalas by renal histopathological examination. Thirteen koalas from Mount Lofty and 16 koalas from Moggill, Queensland were found to be unaffected by oxalate nephrosis and suitable as controls. Koalas were aged by tooth wear class (Martin and Handasyde 1990, Martin 1981) or from animal management records. See Table 1 for sex, age and origin of koalas as rescued wild or captive. Of the captive koalas with oxalate nephrosis, five were receiving regular pyridoxine supplementation. Also, three captive koalas found not to have oxalate nephrosis upon renal histopathological examination were also receiving pyridoxine therapy.

Liver samples were stored at -80˚C until analysis of the activity of alanine: glyoxylate aminotransferase (AGT). The interval from death to liver sample collection was recorded for

all koalas, with the majority (93%) of koala samples collected <24 hours after death to preserve liver enzyme activity as in Danpure and Jennings (1988). AGT activity in koala liver was determined to be stable up to 72 hours after death (see 'Quality control' section). The interval from death to liver sample collection was 1 - 48 hours (median 4 hours) for koalas with oxalate nephrosis and 1 - 48 hours (median 3 hours) for Mount Lofty koalas unaffected by oxalate nephrosis. The interval from death to liver sample collection was <1 hour for all Queensland koalas due to on-site necropsy facilities at Moggill Koala Hospital.

6.3.2 Measurement of AGT activity

Liver suspensions were prepared using a potter-elvehjem apparatus, in 1 mL phosphate buffer containing sucrose (Sigma-Aldrich, St Louis, USA) and pyridoxal-5-phosphate (Sigma-Aldrich, St Louis, USA) as described by Rumsby et al. (1997). The homogenised tissue was then sonicated on ice at 50W for 4 cycles of ten second bursts and thirty second rests with a Labsonic 1510 Probe sonicator (Braun, Germany) and centrifuged to obtain a supernatant for analysis (Rumsby et al. 1997).

AGT activity was measured by the method of Rumsby et al (1997), based on the assay conditions of Danpure and Jennings (1988) and Rowsell et al. (1972). 50 µL of sonicate was added to potassium phosphate buffer pH 7.4, containing the substrates glyoxylate (Sigma-Aldrich, St Louis, USA) and L-alanine (Sigma-Aldrich, St Louis, USA), and the co-factor pyridoxal-5-phosphate. Glyoxylate was initially excluded from control reactions. The reaction procedure was incubated at 37 ˚C for 60 minutes. The concentration of the end-product pyruvate (see Figure 1) was determined using the enzyme lactate dehydrogenase (LDH) and nicotinamide-adenine dinucleotide (NADH) (Sigma-Aldrich, St Louis, USA) with absorbance measured at 340 nm on a Cobas Bio analyser (Roche, Switzerland). Pyruvate concentration was calculated from a calibration curve produced by plotting absorbance versus standard

pyruvate concentrations. Least squares regression was performed to determine the slope, intercept and coefficient of determination (Figure 2a).

Liver sample protein was measured using the bicinchoninic acid (BCA) method of Smith et al. (1985), adapted from Lowry et al. (1951). Koala liver sonicates and standards of bovine albumin serum (Sigma-Aldrich, St Louis, USA) were diluted by adding 20 µL of sample to 980 µL deionised water, from which 20 µL was added to 400µL of Standard Working Reagent (1:20), containing bicinchoninic acid and copper sulphate (Smith et al. 1985). Reaction mixtures were incubated at 60 ˚C for 30 minutes and absorbance measured at 562nm on a Cobas Bio analyser (Roche, Switzerland) (Smith et al. 1985). Protein concentration was calculated from a calibration curve produced for bovine serum albumin by plotting absorbance versus concentration. Least squares regression was performed to determine the slope, intercept and coefficient of determination (Figure 2b). AGT activity was expressed as µmol pyruvate produced per hour per mg protein (Rumsby et al. 1997).

6.3.3 Quality control

To determine stability of the enzyme post mortem, AGT was measured in refrigerated liver samples at various times after death (5.5h, 7h, 12h, 18h, 24h, 48h, 60h and 72h) in a healthy male koala unaffected by oxalate nephrosis. AGT was found to be stable in refrigerated liver up to 72 hours post mortem (Figure 3), showing little decrease in activity over this time period ($R=0.048$; $P>0.05$) and <16% within sample variation. Also, AGT showed no decrease in activity in the group of koalas with oxalate nephrosis based on the time interval from death to liver sample collection up to 48 hours ($R=0.247$; $P>0.05$). Samples were prepared in duplicate for each koala, with <15% variation between duplicates. The averages of the duplicates were used in the final results.

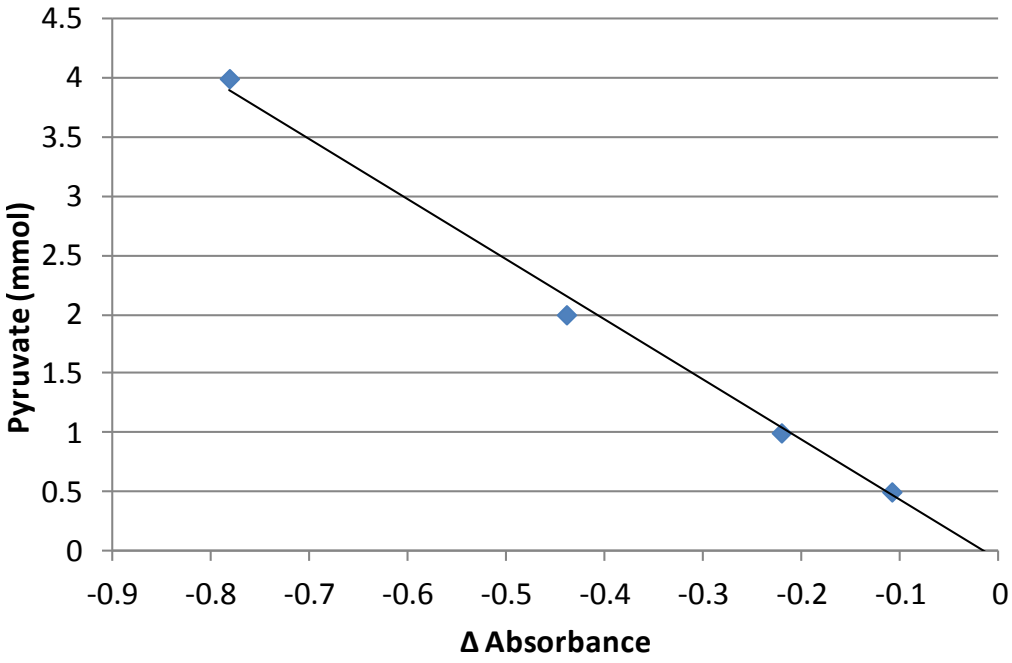
6.3.4 Data Analysis

Alanine: glyoxylate aminotransferase activity data were analysed using the nonparametric Kruskal-Wallis test with post hoc Mann Whitney U using SPSS software, with a level of significance of 0.05. Spearman's test was used to determine any association between alanine: glyoxylate aminotransferase activity and time interval of liver sample collection after death.

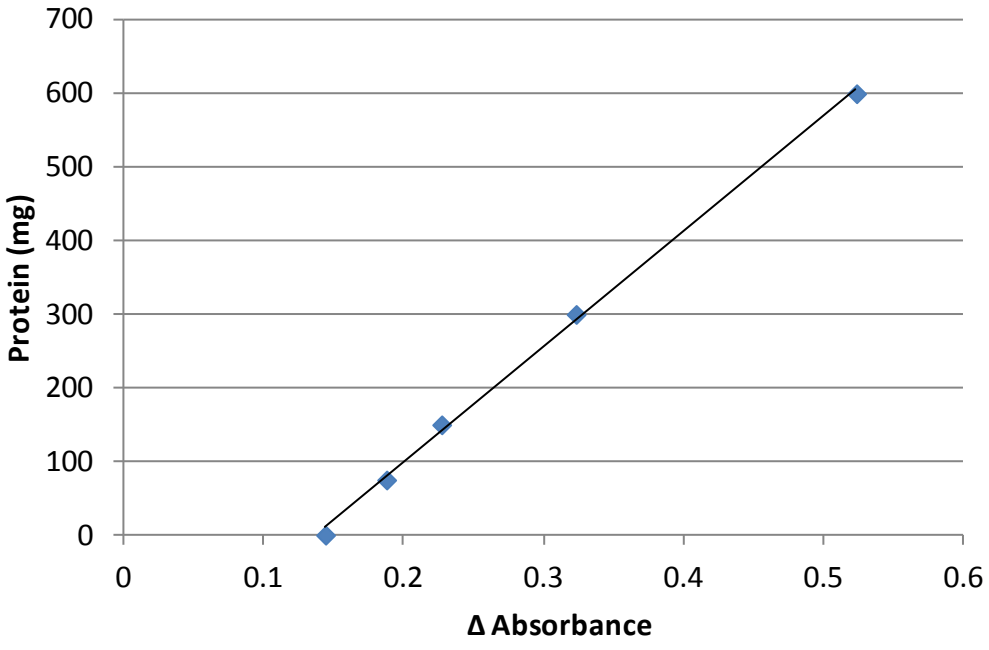
Table 1. Age, sex and origin of koalas

	Oxalate nephrosis	**Mt Lofty Ranges**	**Moggill**
TWC[a]	**SA**	**SA**	**Qld**
I	8	3	-
II	2	4	-
III	1	1	-
IV	3	4	2
V	1	-	7
VI	-	-	3
VII	-	-	4
unknown	1	1	-
TOTAL	**16**	**13**	**16**
Sex	6 M, 10 F	9 M, 4 F	4 M, 12 F
Origin	7 C, 9 W	3 C, 10 W	16 W

TWC= tooth wear class, M=male, F= female, C= captive koalas kept at Cleland Wildlife Park, Mount Lofty, SA; W= wild rescued koalas. [a] Tooth wear class determined according to method of Martin (1981).



a)



b)

Figure 2. a) Calibration curve for pyruvate (R^2=0.995), b) calibration curve for protein (R^2=0.998).

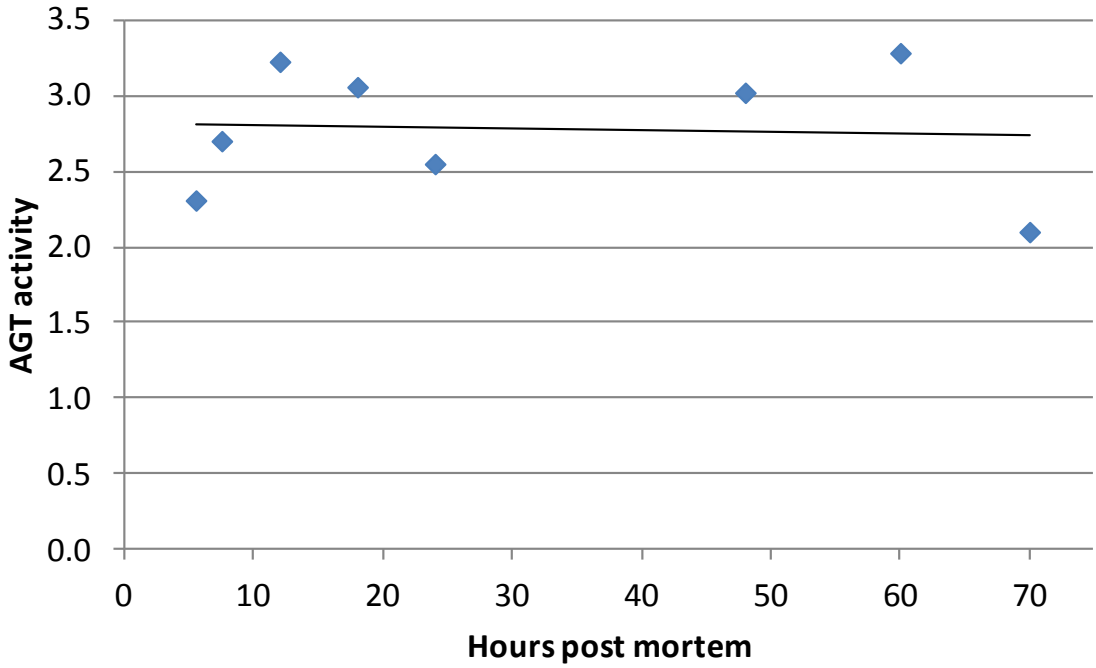


Figure 3. Alanine: glyoxylate aminotransferase (AGT) activity at various times post mortem in refrigerated liver samples from a healthy koala. AGT activity expressed as µmol pyruvate/hour/mg protein.

6.4 RESULTS

Activity of the enzyme alanine: glyoxylate aminotransferase showed no significant differences in liver samples from koalas with oxalate nephrosis and unaffected koalas from Mount Lofty, SA and Moggill, Queensland (Figure 4). AGT activity was 8.6 ± 0.8 µmol pyruvate/hour/mg protein (mean ± SEM) for koalas with oxalate nephrosis and 8.6 ± 0.7 µmol pyruvate/hour/mg protein in Queensland koalas, whilst unaffected Mount Lofty koalas showed an AGT activity of 6.8 ± 0.5 µmol pyruvate/hour/mg protein. There were no significant differences in AGT activity within each group based on sex or captivity status but overall, females had significantly higher AGT activity (8.8 ± 0.6 µmol pyruvate/hour/mg protein) than males (7.1 ± 0.5 µmol pyruvate/hour/mg protein) ($P<0.05$). There were also no significant differences found between koalas receiving vitamin B6 supplementation, both within the group of koalas with oxalate nephrosis and those that were unaffected. Overall mean AGT activity in koalas was determined to be 8.1 ± 2.8 µmol pyruvate/h/mg protein (reference interval 2.4 - 13.7 µmol pyruvate/h/mg protein; mean ± 2SD).

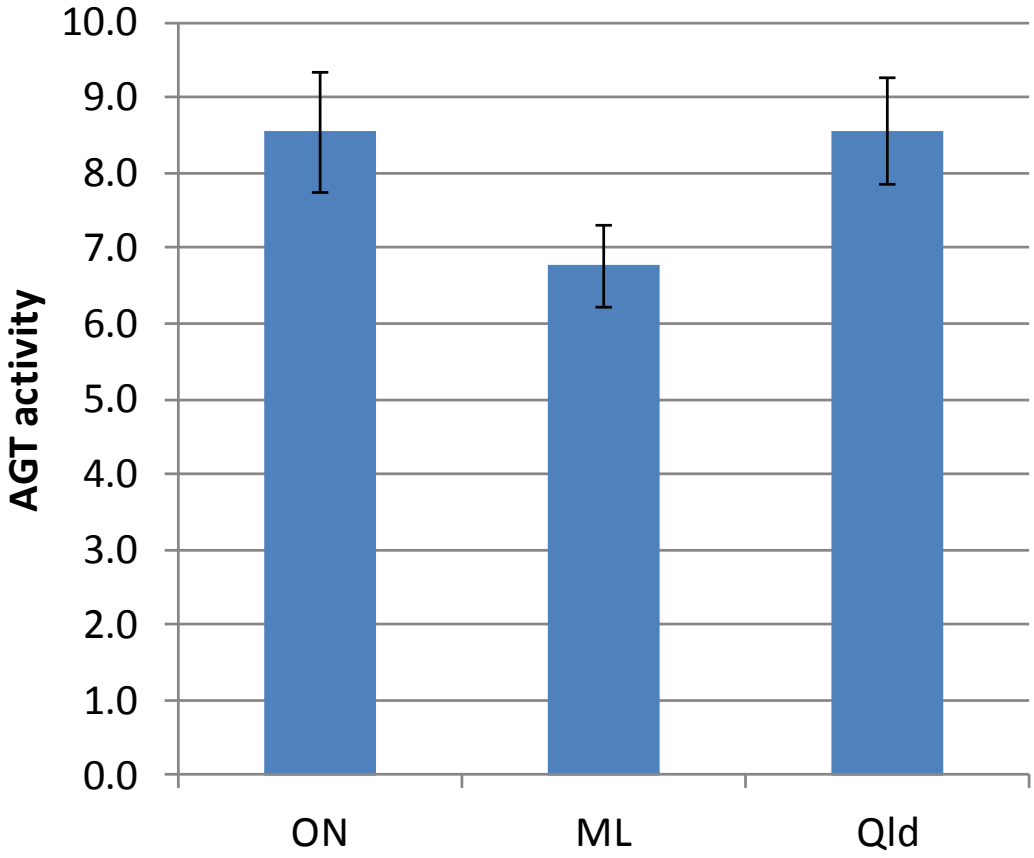


Figure 4. Mean alanine: glyoxylate aminotransferase (AGT) activity in koalas affected by oxalate nephrosis in Mount Lofty (ON), and unaffected koalas in Mount Lofty (ML) and Queensland (Qld). AGT activity expressed as µmol pyruvate/hour/mg protein. Error bars show SEM.

6.5 DISCUSSION

Activity of the hepatic enzyme alanine: glyoxylate aminotransferase (AGT) was found to be similar in koalas affected by oxalate nephrosis to those from Mount Lofty and Queensland that were unaffected by oxalate nephrosis. Overall AGT activity for all koalas (2.4 – 13.7 µmol/h/mg protein) was similar to that reported in healthy humans (range 3.25 – 8.99 µmol/h/mg protein) (Danpure and Jennings 1988). However, AGT activity was found to be much higher than that reported previously in the koala (<0.8 µmol/h/mg protein) (Danpure et al. 1994a), which may have been based upon one koala only.

The liver enzyme AGT did not show decreased activity in koalas affected by oxalate nephrosis, as would be expected in a disease similar to primary hyperoxaluria type I. In humans with primary hyperoxaluria type I, AGT activity has been found to be 0.27 - 1.32 µmol/h/mg protein, or 13% of normal values (Danpure and Jennings 1988). Another study found much higher AGT activity in healthy humans (17.9 – 38.5 µmol/h/mg protein), but showed a similar proportional decrease in AGT activity in affected patients (0.8 - 9.5 µmol/h/mg protein) (Rumsby et al. 1997). Likewise, in two young Tibetan spaniels found to have a disease similar to primary hyperoxaluria type I, AGT activity was 1.3 and 0.6 µmol/h/mg protein in the affected pups, decreasing from 4.3 µmol/h/mg protein in healthy littermates (Danpure et al. 1991).

These findings suggest that a disease similar to primary hyperoxaluria type I due to decreased AGT activity is not the cause of oxalate nephrosis in Mount Lofty koalas. This was an unexpected finding since wildlife staff have reported that captive koalas appear to show clinical improvement with pyridoxine (vitamin B6) therapy (I. Hough and A. Sulley, 2013, pers. comm.), the cofactor of AGT. Pyridoxine is a common medical therapy recommended for humans with primary hyperoxaluria type I due to low AGT activity, since the cofactor

allows the decreased levels of AGT to function at maximal capacity to reduce oxalate production by the liver (Cochat et al. 2012).

Whilst no decrease in activity of AGT in koalas with oxalate nephrosis compared with unaffected koalas was detected, a disease similar to primary hyperoxaluria type I may still be possible. This could occur due to enzyme mistargeting, whereby AGT activity is normal but the AGT enzyme is located at an abnormal location within the cell. This form of primary hyperoxaluria type I is relatively common in humans (Danpure et al. 1994b) but may be pyridoxine unresponsive since AGT is functioning normally (Cooper et al. 1988). The basis for this form of dysfunction is that the AGT reaction primarily occurs at the peroxisome organelles of the cell in herbivores, whereas in carnivores the reaction is mitochondrial (Birdsey et al. 2005). This difference has occurred due to an evolutionary divergence based on diet for the location at which precursors of glyoxylate, glycolate in herbivores and hydroxyproline in carnivores, are metabolised within hepatocytes (Birdsey et al. 2005, Danpure 1993). Therefore, AGT may be mistargeted to the mitochondria in species which should have peroxisomal reactions and vice versa, causing oxalate to be overproduced in spite of normal AGT activity (Danpure 1993, Danpure et al. 1994b, Lhotta et al. 1996).

Koalas, as herbivores, have been shown to have peroxisomal AGT (Danpure et al. 1994a). Therefore, koalas with oxalate nephrosis may have adequate AGT activity but at the incorrect location within their hepatocytes, leading to dysfunction of the metabolic pathway and overproduction of oxalate. Further studies need to be performed using immunolocalisation of AGT in hepatocytes of koalas with oxalate nephrosis, to determine whether it is peroxisomal or in an abnormal intracellular location, such as at the mitochondria (Cooper et al. 1988, Danpure et al. 1989, Danpure et al. 1994a).

To further investigate whether a disease similar to primary hyperoxaluria type I in koalas exists, urinary glycolate could also be measured since in addition to oxalate, it is

overproduced when AGT activity is decreased (Asplin 2002). In addition, assessment of levels of pyridoxine, the co-factor of AGT, may be important to determine if a deficiency exists and whether supplementation is required in affected koalas, as in humans (Cochat et al. 2012). Whilst many studies in humans have focussed on identification of mutations in the AGT gene leading to loss of function of this enzyme (Danpure 1993, Danpure et al. 1994b, Tarn et al. 1997, Williams et al. 2009), this would require extensive investigation in koalas to first determine the coding of the AGT gene in healthy animals.

Another possible inherited cause of oxalate nephrosis in koalas is primary hyperoxaluria type II, a rare form of the PH-disease complex in humans (Johnson et al. 2002, Mansell 1995). Primary hyperoxaluria type II is due to deficiency of the hepatic enzymes glyoxylate reductase and hydroxypyruvate reductase (GR/HPR) and also results in overproduction of oxalate by the liver (Giafi and Rumsby 1998, Mistry et al. 1988, Rumsby 2006). A disease similar to primary hyperoxaluria type II has been identified in cats (Blakemore et al. 1988, Danpure 1989, McKerrell et al. 1989). The levels of hyperoxaluria determined in these cats (17-523 µmol oxalate/mmol creatinine) (Blakemore et al. 1988) are similar to that found in koalas with oxalate nephrosis (36-1053 µmol oxalate/mmol creatinine) (Speight et al. in press). Urinary L-glycerate is also elevated in primary hyperoxaluria type II and can be measured to obtain a tentative diagnosis of disease, although liver enzyme activity is regarded as the conclusive test (Asplin 2002). Recently, primary hyperoxaluria type III has been identified as the dysfunction of hepatic enzyme 4-hydroxy-2-oxoglutarate aldolase, (or DHDPSL) and leads to accumulation of glyoxylate and hence oxalate, but its pathogenesis is still not well understood (Belostotsky et al. 2010, Hoppe 2012).

Whilst an inherited cause of oxalate nephrosis in koalas remains the most likely cause due to the low genetic variation of this koala population (Houlden and St John 2000,

Seymour et al. 2001), further studies are needed to confirm whether a disease similar to primary hyperoxaluria exists. If a disease similar to primary hyperoxaluria is found in affected koalas with oxalate nephrosis in the Mount Lofty koala population, the autosomal recessive inheritance pattern would suggest that many unaffected koalas are likely to be carriers. By comparison, oxalate nephrosis appears to occur only at low prevalence in the founding koalas in Kangaroo Island, found at 4% of 25 koalas in a recent study (Speight et al. in press), suggesting that a genetic mutation may have occurred in the Mount Lofty koalas since their translocation from Kangaroo Island in the 1960s, or that increased inbreeding in the Mount Lofty koalas has increased the prevalence of the disease.

Primary hyperoxaluria is a complex group of diseases for which new research is continually emerging to improve the understanding of pathogenesis and treatment options in humans. If this disease can be confirmed in koalas, short-term conservative management strategies could include high water intake and low dietary oxalate, and in the case of PH type I, pyridoxine therapy. Long-term strategies could include increasing genetic diversity in the population with introduction of unaffected individuals from the eastern states, as well as carefully managed captive breeding programs to ensure that future generations are free of the disease.

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CHAPTER 7

Seasonal variation in eucalypt leaf moisture and its implications for koalas (*Phascolarctos cinereus*) with oxalate nephrosis

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Submitted manuscript.

CONTEXTUAL STATEMENT

Koalas in the Mount Lofty population have a high prevalence of oxalate nephrosis (**Chapter 2**) associated with renal insufficiency and hyperoxaluria (**Chapter 3**). In humans with oxalate nephrosis, high water intake decreases the risk of further renal calcium oxalate deposition (Asplin 2002, Cochat et al. 2006). Koalas rely on the moisture of eucalypt leaves to maintain hydration (Degabriele et al. 1978), but the low rainfall and high temperatures in summer and autumn in the Mount Lofty region may increase the likelihood of dehydration. In addition, the Mount Lofty region has recently experienced a prolonged drought (CSIRO 2007).

Chapter 7 investigates the seasonal changes in moisture content of eucalypt leaves to determine if koalas in Mount Lofty are at higher risk of oxalate nephrosis due to dehydration.

STATEMENT OF AUTHORSHIP: Chapter 7

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7.1 ABSTRACT

Many koalas in the Mount Lofty Ranges population, South Australia are affected by oxalate nephrosis, which is characterised by renal calcium oxalate deposition and chronic renal dysfunction. Low water intake increases the risk of calcium oxalate precipitation. However, koalas primarily rely on the moisture content of eucalypt leaves to maintain hydration and therefore, if leaf moisture is low, koalas could become dehydrated and have increased occurrence of oxalate nephrosis. Moisture content was determined in juvenile, semi-mature and mature leaves from four species of dietary eucalypts in the Mount Lofty region and compared with those in Kangaroo Island and Moggill, Queensland. Leaf moisture was found to be lower overall in Mount Lofty eucalypts than those in Moggill for autumn 2010 ($P<0.05$). Seasonal comparisons of Mount Lofty eucalypts showed that juvenile and semi-mature leaves of *E. obliqua* (stringybark) were lowest in moisture content in summer ($P<0.05$), and leaves of *E. leucoxylon* (blue gum) were lowest in autumn ($P<0.001$). Findings of this study suggest that koalas in the Mount Lofty region are likely to experience dehydration in the hot summer/autumn period due to low eucalypt leaf moisture in conjunction with low rainfall and few water sources from which koalas can drink.

7.2 INTRODUCTION

Koalas (*Phascolarctos cinereus*) in the Mount Lofty region of South Australia (SA) have a high prevalence of oxalate nephrosis, in which calcium oxalate crystals are deposited in the kidneys (Speight et al. 2013). In contrast, koala populations in New South Wales and Moggill, Queensland have much lower occurrence of oxalate nephrosis (Canfield 1989, Speight et al. in press). In the Mount Lofty koalas, oxalate nephrosis is associated with renal insufficiency, with affected koalas showing increased thirst (Haynes et al. 2004) in conjunction with poorly concentrated urine (Speight et al. in press). The cause remains uncertain, but there may be an underlying inherited predisposition to oxalate nephrosis in this population, since the majority of eucalypt leaves have been found to contain low levels of oxalate, <1% on a dry weight basis (Chapter 4)

An inherited disease which causes oxalate nephrosis in humans is primary hyperoxaluria, which involves abnormalities of hepatic metabolic enzymes and has increased prevalence in inbred populations (Asplin 2002, Cochat et al. 2006). A disease similar to primary hyperoxaluria may be occurring in the Mount Lofty koalas, since the population has low genetic diversity following several bottlenecks prior to their introduction to the region in the 1960s (Houlden and St John 2000, Robinson 1978). However, ingestion of certain eucalypt leaves which are high in oxalate could also be a contributing factor since leaves of mature *E. obliqua* (messmate stringybark) were occasionally found to contain up to 7.5% oxalate on a dry weight basis (Chapter 4). This high oxalate concentration may have the potential to sporadically cause oxalate nephrosis in dehydrated koalas, as well as increase the severity of disease in those that are already affected.

In humans and domestic animals affected by oxalate nephrosis, it has been found that maintenance of adequate hydration is critical (Cochat et al. 2006), since insoluble calcium oxalate crystals are more likely to precipitate in highly concentrated urine (Asplin

2002, McIntosh 1978, Stevenson et al. 2003). Since oxalate nephrosis in koalas is associated with renal dysfunction (Speight et al. in press), there is a higher risk of dehydration due to the impaired ability of the kidney to conserve water. The Mount Lofty region in SA experienced a drought for the decade 1997-2006 (CSIRO 2007) and moisture content of eucalypt leaves is likely to have decreased during this time. Therefore koalas, which primarily rely on leaf moisture for their daily water intake, may become dehydrated and at higher risk of developing oxalate nephrosis.

Koalas are arboreal folivores and have a specialised diet of *Eucalyptus* leaves. Koala populations extend across eastern Australia, where optimal eucalypt forest habitat is located (Phillips 1990). Due to their extensive geographical range, dietary preferences for species of eucalypt differ considerably between populations (Jackson et al. 2003), with manna gum (*E. viminalis*) favoured by koalas in South Australia (Phillips 1990). In addition, koalas are highly selective browsers and may favour individual trees within their preferred eucalypt species, as well as particular leaves on one branch (see Moore and Foley 2000 for review). Koalas generally prefer young foliage to mature leaves (Nagy and Martin 1985, Ullrey et al. 1981) however, mature leaves still form a major component of the koala diet (Tun 1993 in Moore and Foley 2000). Many studies have investigated eucalypt leaf chemistry to determine koala leaf preference determinants, which include low fibre (Ullrey et al. 1981), low phenolics (Moore and Foley 2005) and high nitrogen (Ullrey et al. 1981).

High water content of leaves is also an important factor in leaf choice (Ellis et al. 1995, Hume and Esson 1993, Pahl and Hume 1990), since koalas obtain most of their moisture requirements from their diet (Degabriele et al. 1978). This water intake may also include droplets of water on the outer surface of the leaves following rainfall (Ellis et al. 2010) or from dew formation during the night and early morning (Degabriele et al. 1978). However, koalas may drink free water during periods of drought or episodes of illness

(Phillips 1990), and it has also been found that male koalas may be more likely than female koalas to increase their water intake by drinking from water sources, or selecting young leaves that are higher in moisture (Nagy and Martin 1985). Drinking water was found to contribute 26% of water intake in a study of captive koalas, both in summer and winter, whilst water within eucalypt leaves contributed approximately 45% (Degabriele et al. 1978).

The koala conserves water primarily by the production of dry faecal pellets following extensive water reabsorption from the distal colon, as well as by the production of low volumes of concentrated urine (Degabriele et al. 1978) with high urine specific gravity (Canfield et al. 1989). However it has been shown that the koala kidney does not have specialised adaptive features for a water-limited environment (Degabriele et al. 1978), suggesting that access to adequate moisture in the eucalypt leaves and habitat are required to maintain normal body hydration.

Water turnover experiments in koalas have concluded that eucalypt leaves should provide adequate water intake in a microenvironment with an ambient temperature <30˚C, and that evaporation accounts for the greatest water loss (Degabriele et al. 1978). Once temperatures are over 30˚C, evaporative water losses double for koalas and are almost six-fold at 40˚C (Degabriele and Dawson 1979). To minimise evaporative water losses during summer, koalas show behavioural adaptations to lower the temperature of their microenvironment by actively seeking shade during the day in large non-food trees with extensive canopy (Ellis et al. 2010). In addition, to meet their metabolic water requirements, koalas may vary their food tree preferences, choosing species with higher moisture content in summer (Ellis et al. 1995, Hindell and Lee 1987). In feeding trials, koalas have been shown to decrease intake of foliage which falls below a moisture threshold, estimated as 65% by Pahl and Hume (1990) and 55% by Hume and Esson (1993).

To determine whether Mount Lofty koalas with oxalate nephrosis are at higher risk of renal calcium oxalate deposition due to dehydration, this study compared the seasonal variation in moisture content of eucalypt leaves in the Mount Lofty region in SA that are eaten by koalas with those from Kangaroo Island in SA and Moggill in Queensland.

7.3 METHODS

7.3.1 Comparisons of eucalypt leaf moisture between locations

Eucalypt leaves were collected from two eucalypt plantations used to feed captive and hospitalised wild koalas: Cleland Conservation Park, Mount Lofty, South Australia (elevation 685m) and Moggill Koala Hospital, Moggill, Queensland (Qld), adjacent to the Brisbane River; as well as from full-grown trees from the Cygnet River area on Kangaroo Island (KI), South Australia. Leaves from four dietary species preferred by koalas were collected in each location (Jackson et al. 2003, Phillips 1990): manna gum (*Eucalyptus viminalis*), river red gum (*E. camaldulensis*), SA blue gum (*E. leucoxylon*) and messmate stringybark (*E. obliqua)* in the Mt Lofty Ranges and Kangaroo Island, SA; and forest red gum (*E. tereticornis),* small-fruited grey gum *(E. propinqua),* tallow wood *(E. microcorys)* and red stringybark *(E. resinifera)* in Qld.

For each eucalypt species, juvenile (new tips), semi-mature (newest fully expanded young leaves) and mature (fully expanded older leaves) leaf types were collected from the canopy circumference of up to 10 non-irrigated plantation trees and two full-grown trees in Mt Lofty, five full-grown trees on Kangaroo Island and five non-irrigated plantation trees in Qld. Up to 15 leaves of each leaf type were collected into individual envelopes in sealed plastic packets and stored in a chilled container until measurement of wet weight. Leaves were weighed to the nearest milligram on a fine balance (Ohaus, New Jersey, USA), and then dried at 40 ˚C to ensure loss of moisture without loss of eucalypt oils (Ellis et al. 2002). Leaf

sample dry weight was measured after seven days. Leaf samples were collected under research permits from the Department of Environment and Natural Resources (SA) and Department of Environment, Water and Resource Management (Qld).

Leaf collections focussed on the seasons following high and low rainfall periods. In South Australia the highest seasonal rainfall is in winter (June – August) and the lowest rainfall is in summer (December - February), whereas in Moggill, Queensland high rainfall occurs in summer and low rainfall in winter, so that monthly rainfall and monthly maximal temperatures follow a similar pattern throughout the year (BOM 2012) (Figure 1). The long-term average annual rainfall at each location is: Mt Lofty 1188 mm (Stirling, near Mt Lofty), Moggill, Queensland 878 mm (Ipswich, near Moggill) and Kangaroo Island 431 mm (Kingscote airport, near Cygnet River) (BOM 2012).

To determine leaf moisture content differences following the hot summer period, Mt Lofty and Moggill eucalypt leaf collections were compared for autumn 2010. In addition, to address the differences in rainfall patterns and allow direct comparison of leaf moisture between locations, leaf collections were grouped into 'dry season' and 'wet season'. The dry season leaf moisture comparison included summer (January) and autumn (April) collections for 2009 from Mt Lofty, summer (February) 2009 collection from KI and spring (October) 2009 collection from Qld. The wet season leaf moisture comparison included spring (October) 2008 and 2009 collections from Mt Lofty, spring (October) 2009 from KI and autumn (May) 2010 collection from Qld.

7.3.2 Seasonal changes in leaf moisture of Mount Lofty eucalypt species

Mount Lofty eucalypt leaf moisture was compared seasonally over a period of one year, winter (July 2008), spring (October 2008), summer (January 2009) and autumn (April 2009), to determine seasonal species differences. Annual Mt Lofty eucalypt leaf moisture for

autumn (April) was also compared over three years (2009 - 2011) to determine differences between the low rainfall summer/autumn periods of 2009 and 2010 and the high rainfall summer/autumn period of 2011, following a La Nina weather event (BOM 2012) (Figure 2).

7.3.3 Mount Lofty climate and oxalate nephrosis in koalas

The association between oxalate nephrosis in koalas in the Mount Lofty region and climatic factors such as rainfall and maximum temperature was determined from data collected from a cohort of rescued wild and captive koalas (n= 28), for which oxalate nephrosis was confirmed using histopathological examination (see Speight et al. 2013). From March 2008 to April 2010, the month in which koala death or euthanasia on welfare grounds occurred, due to oxalate nephrosis and associated renal dysfunction, was compared with the mean monthly rainfall and mean monthly maximum temperature at Cleland Conservation Park (BOM 2012). Month of death was also compared with cumulative monthly rainfall in the preceding one to three months. The association between Mount Lofty eucalypt leaf moisture and cumulative monthly rainfall for the previous one, two, three and six months was also determined.

7.3.4 Statistical analyses

Data were analysed using SPSS software, after determination of normality and homogeneity, with Kruskal Wallis analyses and post hoc Mann Whitney U tests, adjusted with Holm's stepdown Bonferroni procedure for multiple comparisons (Holm 1979). Spearman's test was used to determine the correlation between the incidence of deaths of koalas with oxalate nephrosis and monthly rainfall and maximal temperature and that between monthly rainfall and leaf moisture.

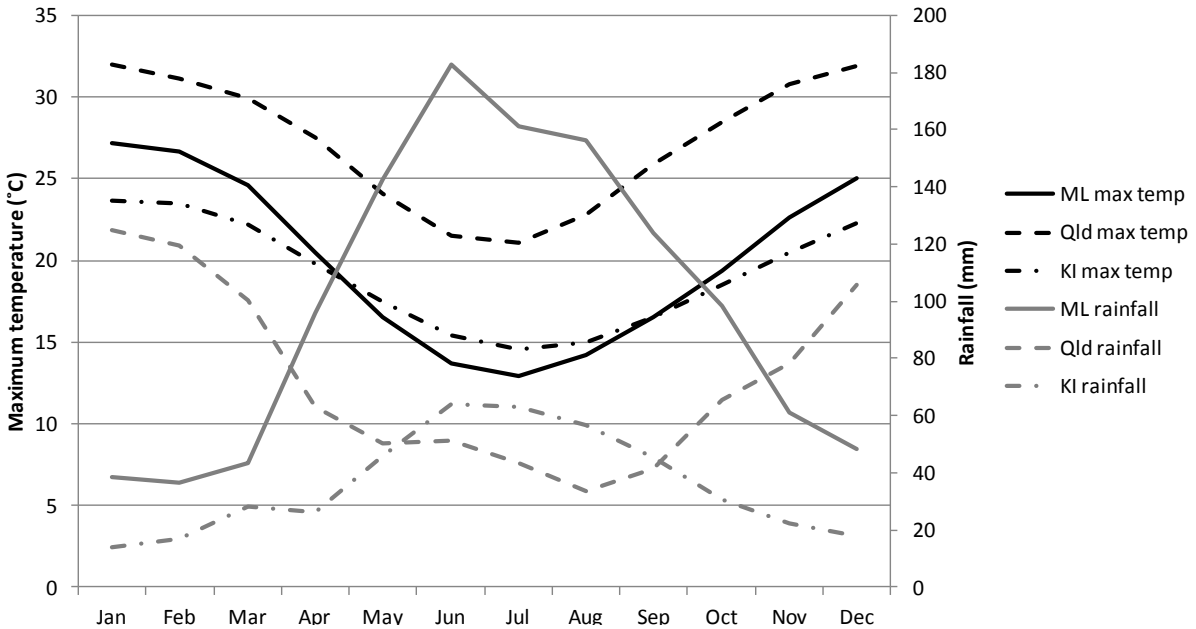

Figure 1. Comparison of long-term average monthly rainfall and mean monthly maximum temperatures at leaf collection sites in Mount Lofty and Kangaroo Island, South Australia and Moggill, Queensland (BOM 2012).

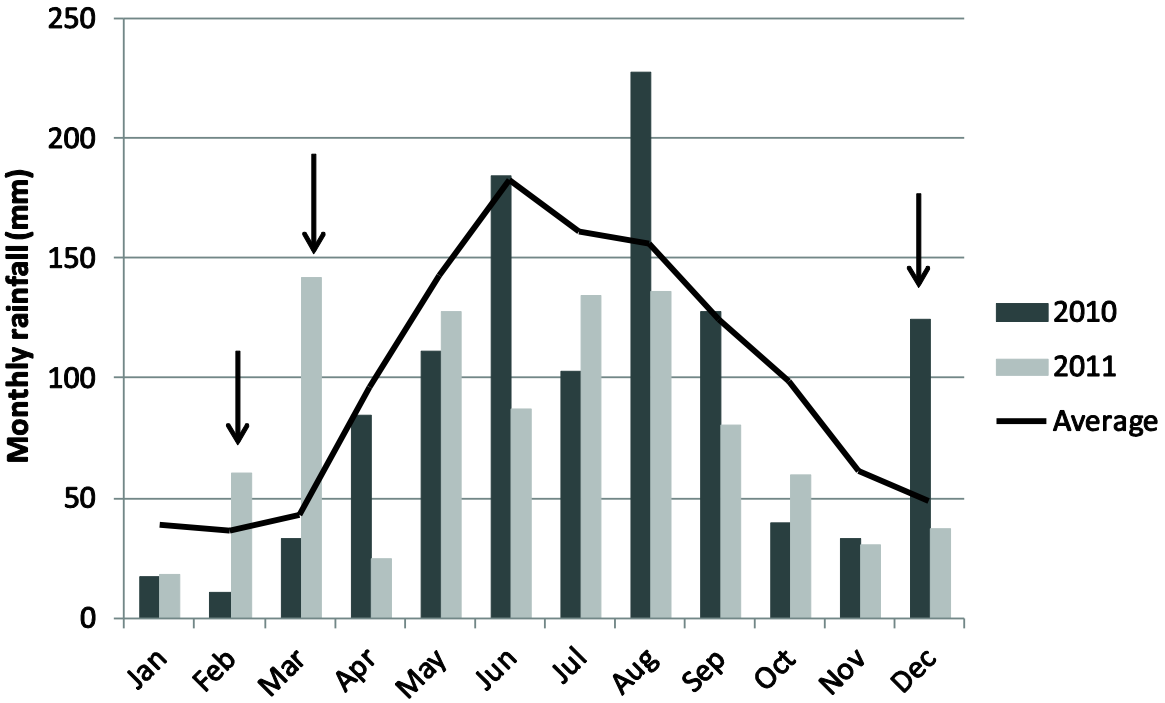

Figure 2. Monthly rainfall for Mount Lofty, South Australia for 2010- 2011. Arrows indicate high rainfall summer months above the long-term average due to a La Nina weather event (BOM 2012)

7.4 RESULTS

7.4.1 Eucalypt leaf moisture comparisons between locations

In autumn 2010, following the hot, dry summer period, leaves sampled from Mt Lofty eucalypts were found to be on average 3.5% lower in moisture content than those from Moggill, Queensland ($P<0.05$). Based on leaf age, semi-mature leaves from Mt Lofty had significantly lower moisture content than those from Moggill ($P<0.005$) (Figure 3), however there were no significant differences in moisture content of juvenile or mature leaves between the two locations.

When leaf collections in Mt Lofty, Kangaroo Island and Moggill were grouped by wet and dry season, it was found that eucalypt leaves collected during the dry season were on average 3% lower in overall moisture than those collected during the wet season ($P<0.05$). Juvenile and semi-mature leaves were both significantly lower in moisture content in the dry season ($P\leq0.005$), however moisture content of mature leaves was not significantly different between the wet and dry seasons.

Comparison of eucalypt leaf moisture across the three locations during the dry season (Figure 4a) showed that leaf moisture content from leaves collected from Mt Lofty trees was not significantly different from Moggill. Also, no differences were found between the moisture content of juvenile, semi-mature or mature leaves from Mt Lofty and Moggill in the dry season collections. Overall, leaves from Kangaroo Island eucalypts were the lowest in moisture content compared with those from Mt Lofty and Moggill ($P<0.001$). Juvenile leaves collected in summer from Kangaroo Island were lower in moisture content than juvenile leaves from both the summer and autumn Mt Lofty collections ($P\leq0.005$). Also, both semi-mature ($P<0.05$) and mature ($P<0.001$) leaves from Kangaroo Island were lower in moisture than both Mt Lofty collections and the spring collection in Qld. For both dry season Mt Lofty collections, overall moisture was lower in leaves from full-grown trees than those from plantation trees ($P<0.0005$).

Comparisons of wet season leaf moisture for Mt Lofty, Kangaroo Island and Moggill (Figure 4b) showed that leaf collections from Mt Lofty in spring 2008 and 2009 had significantly higher moisture than those collected in spring from Kangaroo Island ($P<0.001$). Juvenile Mt Lofty leaves for both years were higher than KI juvenile leaves ($P\leq0.005$), as were semi-mature leaves ($P\leq0.005$). Also, semi-mature eucalypt leaves from Mt Lofty in 2009 were significantly higher in moisture than semi-mature Qld leaves ($P<0.05$). Mature KI leaves were significantly lower in moisture than Qld and both Mt Lofty 2008 and 2009 leaves ($P<0.05$); whilst mature Mt Lofty 2008 leaves were significantly lower than those in Qld ($P<0.05$) and Mt Lofty 2009 leaves ($P<0.001$). For both wet season Mt Lofty collections, overall moisture was lower in full-grown trees than in plantation trees ($P<0.05$).

7.4.2 Seasonal changes in leaf moisture of Mount Lofty eucalypt species

Leaf moisture was compared from eucalypt leaves collected seasonally from the Mount Lofty site in winter 2008, spring 2008, summer 2009 and autumn 2009 (Figure 5). In winter, it was found that there were no statistically significant differences in overall leaf moisture content between the four eucalypt species. In spring, overall leaf moisture content was significantly higher in *E. camaldulensis* compared with the other eucalypt species ($P<0.05$). Also, *E. viminalis* showed higher leaf moisture content than *E. leucoxylon* ($P<0.05$). In summer, *E. obliqua* had significantly lower leaf moisture content overall than *E. viminalis* and *E. camaldulensis* ($P<0.05$). In autumn, *E. leucoxylon* showed highly significant decreases in overall leaf moisture compared with the other eucalypt species ($P<0.005$).

For juvenile leaves, there were no statistically significant differences in leaf moisture content in winter. In spring, juvenile leaves of *E. obliqua* were significantly lower in moisture content than those of *E. viminalis* and *E. camaldulensis* ($P<0.001$), and in summer juvenile leaves of *E. obliqua* were lower in moisture than those of the other eucalypt species

(P<0.05). Juvenile *E. leucoxylon* was significantly lower in leaf moisture than *E. camaldulensis* in spring (P≤0.005) as well as in summer (P<0.01). In autumn, juvenile leaves of *E. leucoxylon* showed highly significant decreases in leaf moisture compared with the other three eucalypt species (P<0.001).

For semi-mature leaves in winter, *E. obliqua* leaves were significantly higher in moisture content than those of *E. viminalis* (P=0.05) and *E. camaldulensis* (P<0.01). In spring, semi-mature leaves of *E. camaldulensis* were significantly higher in moisture than those of the other eucalypt species (P<0.005). Also, *E. viminalis* showed significantly higher semi-mature leaf moisture than *E. leucoxylon* and *E. obliqua* (P<0.05). In summer, *E. obliqua* was significantly lower in moisture content than semi-mature leaves of the other three species of eucalypt (P<0.05); whilst in autumn, semi-mature leaves of *E. leucoxylon* showed highly significant decreases in leaf moisture compared with the other eucalypt species (P<0.001). For mature leaves, there were no statistically significant eucalypt species differences in moisture content between the seasons.

Following increased rainfall during the summer of 2010-2011 at Mt Lofty due to a La Nina weather event, overall eucalypt leaf moisture content was found to be significantly increased in autumn 2011 compared with autumn 2009 and 2010 (P<0.001). Eucalypt leaf moisture content was on average 3.8% higher in autumn 2011 than in 2009, and 5.5% higher than in 2010 (Figure 6). Juvenile, semi-mature and mature leaves of 2011 were all significantly higher in moisture content than the same leaves in 2010 (P<0.001), but only semi-mature and mature leaves were higher in moisture content than those collected in 2009 (P<0.001). In addition, 2009 leaves were found to be significantly higher in moisture than 2010 (P<0.05), with semi-mature leaves collected in 2009 higher in moisture than those of 2010 (P<0.001).

7.4.3 Mount Lofty climate and oxalate nephrosis in koalas

Data suggested that there may be an association between low monthly rainfall and increased deaths or euthanasia of Mt Lofty koalas with oxalate nephrosis (Figure 7), but this was not statistically significant for monthly rainfall and the month of death ($R=-0.369$; $P=0.144$), or for cumulative monthly rainfall in the preceding one to three months. This is despite leaf moisture being significantly correlated with cumulative monthly rainfall for the two months preceding collection ($R= 0.829$; $P<0.05$). However, there was no statistical association found between leaf moisture and cumulative rainfall in the previous one, three or six months. A trend in the data also suggested an association between increased mean monthly maximum temperature and the month of death of koalas with oxalate nephrosis, but this was also not significant ($R=0.286$; $P=0.283$).

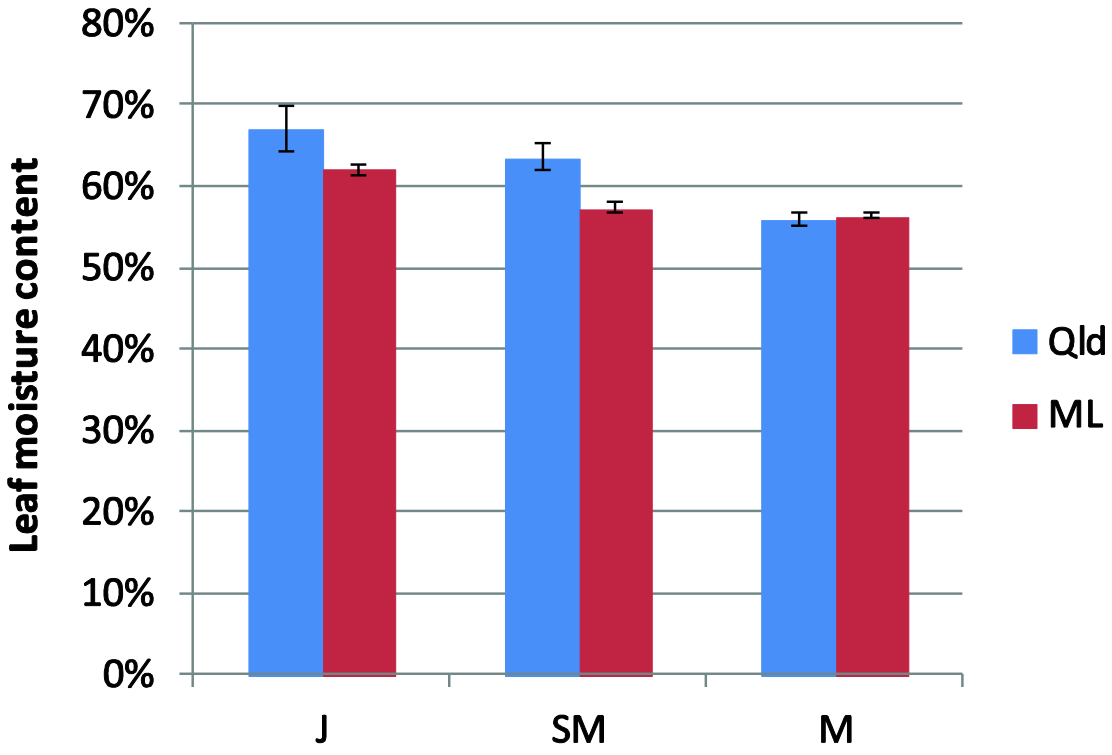

Figure 3. Leaf moisture in autumn 2010 for Mount Lofty (ML) and Moggill, Queensland (Qld) for juvenile (J), semimature (SM) and mature (M) eucalypt leaves. Error bars show SEM

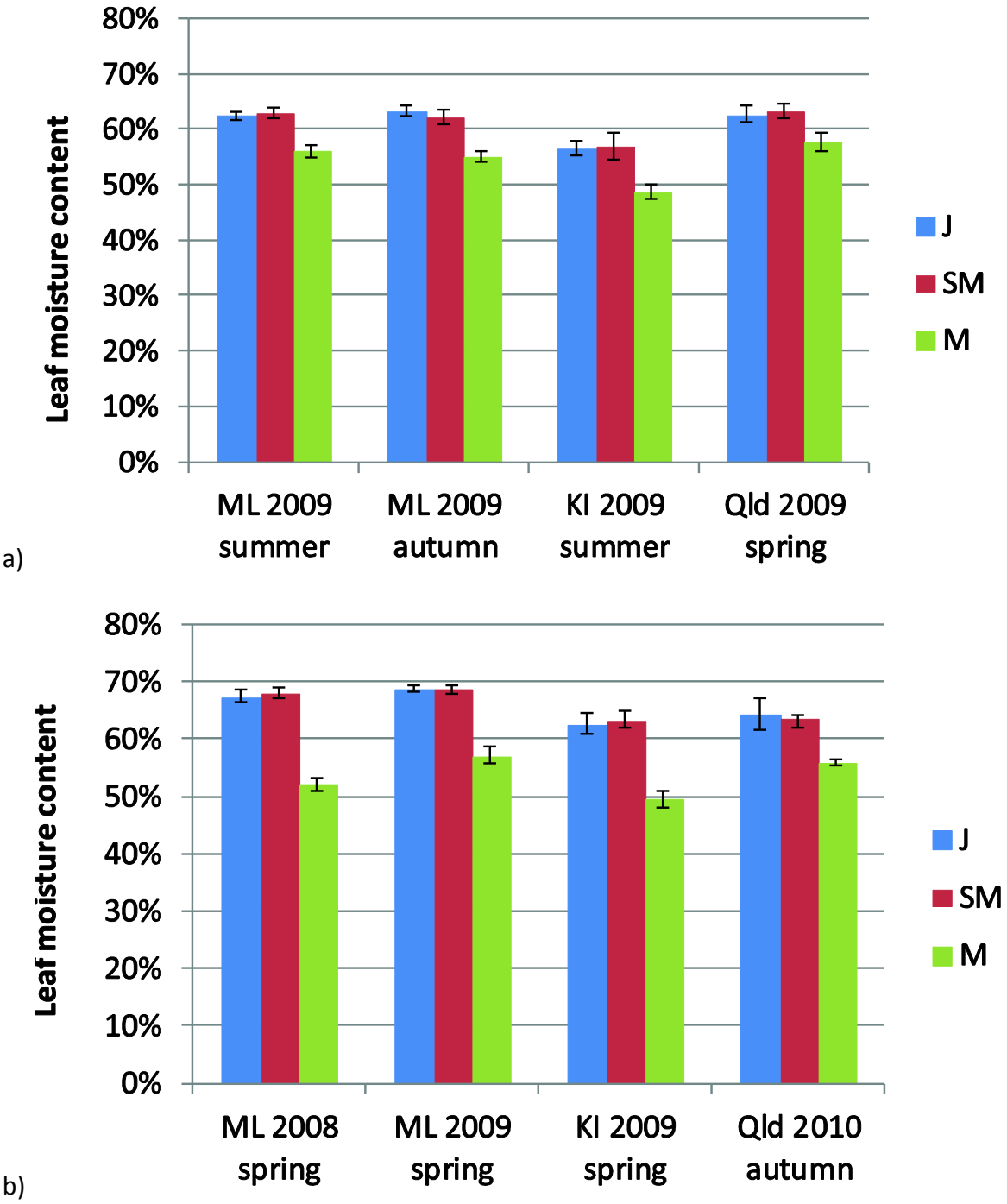

Figure 4. Leaf moisture in the a) dry season and b) wet season for Mount Lofty (ML) and Kangaroo Island (KI) in South Australia, and Moggill, Queensland (Qld) for juvenile (J), semimature (SM) and mature (M) eucalypt leaves. Error bars show SEM.

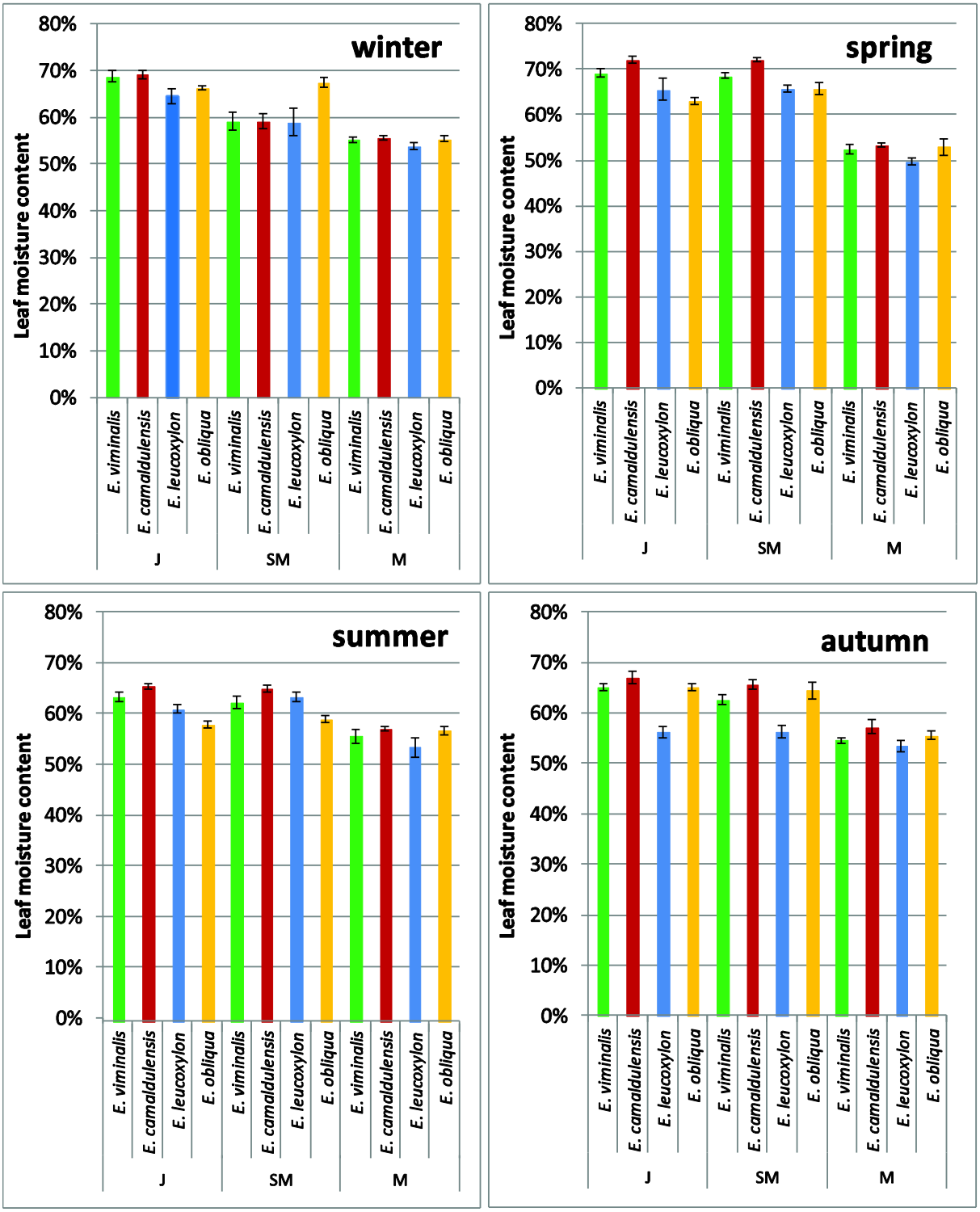

Figure 5. Mount Lofty eucalypt leaf moisture in winter, spring, summer and autumn for juvenile (J), semimature (SM) and mature (M) leaves. Error bars show SEM.

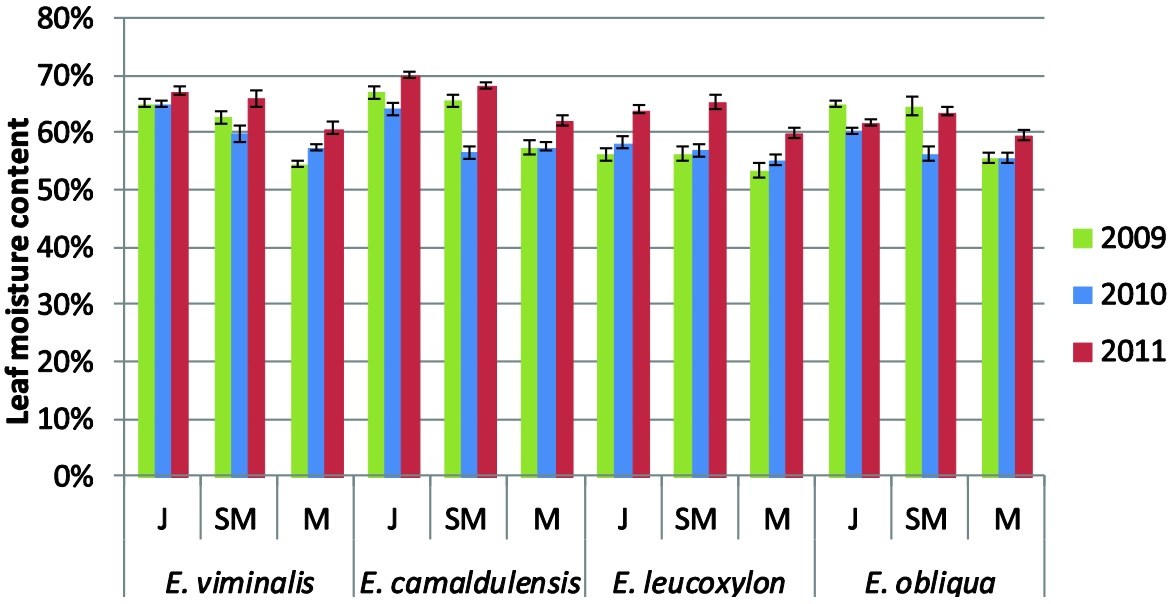

Figure 6. Annual leaf moisture in Mount Lofty eucalypts in autumn (April), comparing two low summer rainfall seasons at the beginning of 2009 and 2010 with a high summer rainfall season at the beginning of 2011, for juvenile (J), semimature (SM) and mature (M) leaves. Error bars show SEM.

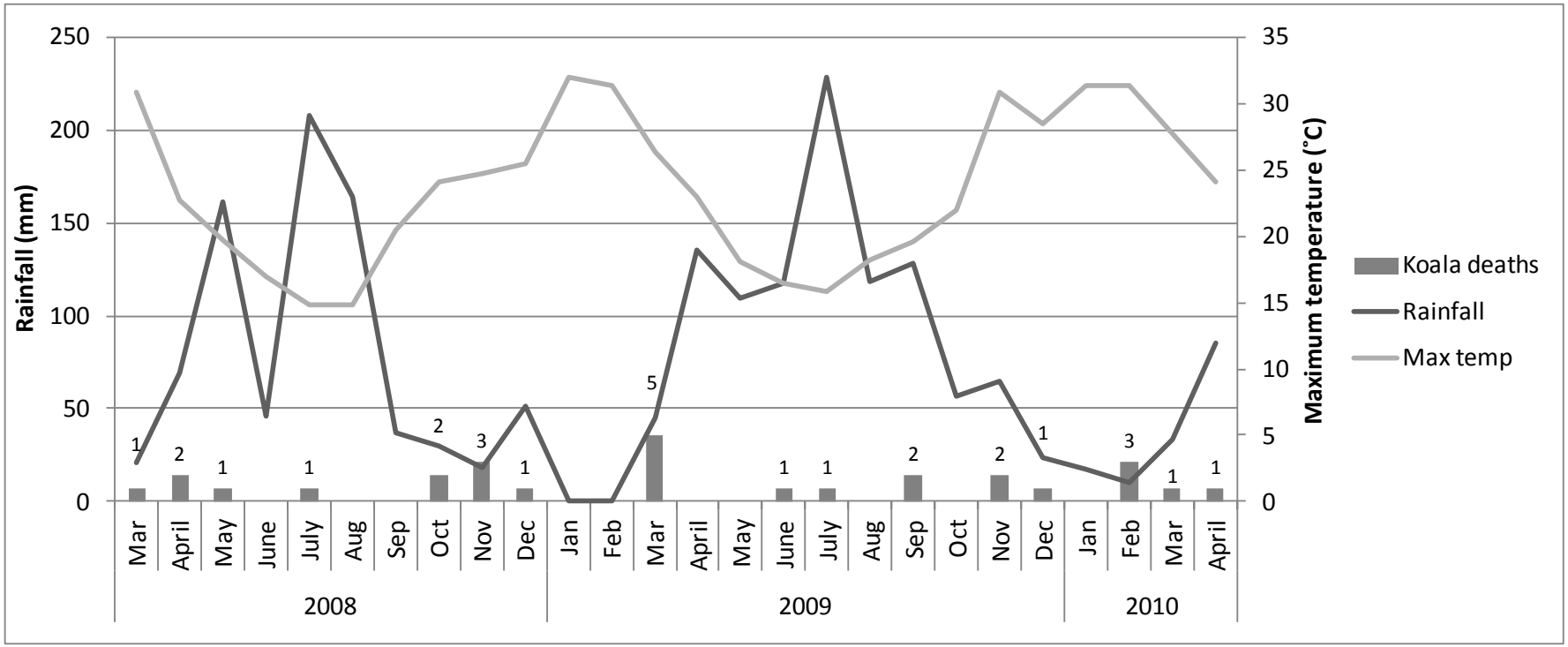

Figure 7. Deaths of captive and rescued wild Mount Lofty koalas with oxalate nephrosis (columns show koala numbers) and monthly rainfall and maximum temperature at Cleland Conservation Park (BOM 2012).

7.5 DISCUSSION

Eucalypt leaf moisture measured in autumn, following the hot summer months, was found to be significantly lower in Mount Lofty eucalypts in South Australia than those in Moggill, Queensland for 2010. Although both locations experience high daily maximum temperatures in autumn, in Mount Lofty this corresponds to a low rainfall period, whereas in Moggill, Queensland rainfall is highest during this time. Cumulative rainfall for the two months previous to leaf collection was found to be significantly correlated with leaf moisture. Therefore, lower eucalypt leaf moisture in Mount Lofty eucalypts during the hot, dry summer/autumn period could result in suboptimal water intake by koalas in this region. This could therefore lead to dehydration in koalas and increase the risk of development of oxalate nephrosis.

For the low rainfall 'dry season', eucalypt leaf moisture was found to be similar between Mount Lofty and Moggill, suggesting that when rainfall is at its lowest during the year, koalas in these two locations are ingesting leaves of similar moisture content. However, the dry season occurs in summer in the Mount Lofty region whereas it coincides with winter in Queensland, and therefore Mount Lofty koalas are likely to have increased water stress during the dry season due to the high ambient temperature. Whilst all eucalypt leaves in every collection were above the leaf moisture minimum level of 42.7% proposed by Degabriele *et al*. (1978) to maintain adequate koala water intake in a microenvironment up to 30˚C, temperatures in the Mount Lofty region in summer frequently exceed 35˚C. Hence the water requirements of Mount Lofty koalas would be increased in summer due to evaporative losses (Degabriele and Dawson 1979), suggesting that the maintenance water requirements of koalas may not be met by eucalypt leaf moisture during hot summer and autumn periods in this population.

Koalas may choose leaves with higher moisture content in hot weather (Nagy and Martin 1985), but this may not be possible for koalas in the Mount Lofty region. Whilst koalas rely on leaf moisture for most of their water requirements, rain and dew droplets on leaves may also be important for koala hydration (Degabriele et al. 1978). Wild koalas in the Mount Lofty region would appear to have little opportunity for increasing their water intake during the hot summer by this method due to the low rainfall during this time of year. In contrast, in southeast Queensland where high summer temperatures coincide with high rainfall, koalas would have adequate access to rain and dew droplet formation on leaves. These climatic differences suggest that the koala population in Mount Lofty experiences significantly greater water stress during the summer months than koalas in southeast Queensland.

In hot weather, koalas may also seek water sources from which to drink (Tyndale-Biscoe 2005), but wild koalas in the Mount Lofty Ranges region have access to few natural sources of water from which to drink and are therefore likely to experience thirst and dehydration during hot weather. During the summer months, Mount Lofty wildlife staff receive many reports of wild koalas seeking water in fishponds and swimming pools in suburban backyards. This is likely to be due to these climatic factors, whereby wild koalas are exposed to extended hot, dry weather with eucalypt leaves low in moisture content, little access to rain or dew formation on leaves and few natural water sources from which to drink.

These factors compound the problem of dehydration in both healthy and diseased wild Mount Lofty koalas. Healthy individuals may be more likely to develop oxalate nephrosis if they are eating particular eucalypt leaves which have higher levels of oxalate when they are dehydrated. Koalas which are already affected by oxalate nephrosis would be more likely to have increased oxalate crystal deposition and further decreases in renal

function, including production of dilute urine (Speight et al. in press), in a downward spiral of worsening dehydration. This pathogenesis explains the apparent increase in occurrence of oxalate nephrosis and renal failure in the Mount Lofty koalas during summer and the reported increases in number of koala deaths, and necessity for euthanasia on welfare grounds, at this time.

Captive koalas are more fortunate than wild koalas as they have access to water ad lib and the harvested eucalypt branches used as feed are placed in water to maintain leaf moisture (Blanshard and Bodley 2008, Ladds 2009). In addition, during summer the eucalypt leaves are sprayed with water, causing formation of droplets on the leaf surface and thereby increasing water intake by koalas. However, captive koalas may still become dehydrated in hot weather if they do not drink and wildlife staff report increased oxalate crystals in urine samples. This highlights the role of hot weather and dehydration in the pathogenesis of this disease in koalas.

Previous studies in Queensland have found that quality of habitat was an important predictor of koala mortality in drought and heatwaves, with trees located near permanent water sources associated with increased koala survival (Gordon 1988, Seabrook et al. 2011). This shows the importance of permanent water sources, such as major rivers, in providing adequate soil water for eucalypt trees to produce well hydrated leaves to meet koala water intake requirements during drought periods. However, few permanent water sources exist for Mount Lofty eucalypts, whereas in Moggill, eucalypts are located next to the Brisbane river and horizontal recharge of deep soil water would maintain adequate levels (Holland et al. 2006). Hence, Mount Lofty eucalypts are reliant on replenishment of soil water from rainfall, which occurs primarily during the cool winter months. During the low rainfall summer, Mount Lofty eucalypts are likely to experience increased water stress due to decreased soil moisture.

Kangaroo Island eucalypt leaves were also assessed for leaf moisture and were found to be lower than those from both the Mount Lofty region and Moggill, Queensland, which may be due to the lower annual rainfall for this region of Kangaroo Island (BOM 2012). However, the finding of low leaf moisture may also be because all sampled Kangaroo Island eucalypts were full grown, and it was found that leaves from full-grown trees were significantly lower in moisture content overall than those from plantation trees in the Mount Lofty collections. Despite the lower moisture content of eucalypt leaves, the koala population on Kangaroo Island is thriving (Duka and Masters 2005). Oxalate nephrosis has been shown to occur in this koala population, but is thought to be low in prevalence following a recent study showing normal kidney function in Kangaroo Island koalas (n=24) (Speight et al. in press).

Mount Lofty eucalypts showed significant variations in leaf moisture between species, leaf ages and seasons, except for mature leaves, in which there were no species differences in moisture. This suggests that Mount Lofty eucalypts maintain a threshold level of moisture for leaf metabolic processes all year round in mature foliage, which is similar to the leaf moisture minimum proposed by Degabriele *et al*. (1978). Also in winter, the season when leaves are at their oldest and most fibrous (Harrop and Degabriele 1978), all eucalypt species were similar in moisture overall. *E. viminalis* (manna gum), the preferred dietary species of Mount Lofty koalas showed average leaf moisture content across all seasons, indicating a relatively stable source of water for koalas.

High moisture content was found to occur in juvenile and semi-mature leaves of *E. camaldulensis* (river red gum) in spring. In a recent study, semi-mature leaves of *E. camaldulensis* were found to have higher oxalate concentration than some other leaf types (Chapter 4), which may indicate that oxalate accumulates in fast growing young eucalypt leaves. This has been previously suggested to occur in other young plants, with the amount

of oxalate decreasing with maturity (Maxie and Newman 2007). However this trend was not found overall for juvenile, semi-mature and mature leaves in the recent eucalypt oxalate study, since no differences were found in oxalate levels based on leaf age overall (Chapter 4).

In the current study, juvenile and semi-mature leaves of *E. obliqua* (messmate stringybark) were found to have lower moisture content in summer than other species of eucalypt in the Mount Lofty region, which supports the findings of previous studies that this eucalypt species is easily water stressed (Merchant et al. 2007, Sinclair 1980). Also, the highest levels of oxalate have been found in samples of mature *E. obliqua* (Chapter 4) and it is suggested that this accumulation of oxalate may be a response to drought stress, as has been found to occur in a fruiting tree species (Arndt et al. 2000).

Juvenile and semi-mature leaves of *E. leucoxylon* (SA blue gum) showed much lower moisture in autumn, which is consistent with anecdotal reports from staff at Cleland Wildlife Park that SA blue gum appears to become dry soon after harvesting during the hot months of the year. Koalas have been shown to prefer leaves that are higher in moisture in summer (Ellis et al. 1995), hence leaves of *E. obliqua* and *E. leucoxylon* may be more likely to be rejected by koalas on hot days compared with those of *E. viminalis* and *E. camaldulensis*.

An increase in leaf moisture was seen in all leaf types for all eucalypt species in the Mount Lofty region in April 2011 compared with April 2009 and 2010, following the high rainfall summer of 2010-2011 due to the effects of a La Nina weather event (BOM 2012). Prior to this event, SA had lower than average rainfall for the years 2002-2009 (BOM 2012), and rainfall 1997-2006 was 7% lower than the historical average in the eastern Mount Lofty Ranges (CSIRO 2007).

7.6 CONCLUSION

Results of this study have shown that koalas in the Mount Lofty region are likely to experience dehydration during the hot summer/autumn period caused by decreases in eucalypt leaf moisture due to low rainfall. Dehydration in koalas in the Mount Lofty region may also be exacerbated by a lack of dew and rainfall droplets on leaves or natural water sources available for wild koalas from which to drink. In the Mount Lofty koala population, which have a high prevalence of oxalate nephrosis, dehydration is likely to be a significant risk factor for development of this kidney disease or increasing the severity of renal calcium oxalate deposition in koalas that are already affected.

Despite this, oxalate nephrosis appears to be equally common in captive and rescued wild Mount Lofty koalas (Speight et al. 2013), even though captive individuals have water-sprayed eucalypt leaves and unlimited access to drinking water. This is likely to be because oxalate nephrosis in koalas may be caused by an underlying inherited condition, similar to primary hyperoxaluria in humans (Cochat et al. 2006), although this has yet to be confirmed. Hence, whilst koalas in other populations which experience hot, dry summers are also likely to suffer from dehydration in the absence of drinking water availability, with increased koala mortality as previously reported (Clifton 2010, Gordon 1988, Seabrook et al. 2011), they are not necessarily predisposed to developing oxalate nephrosis.

A key management strategy for oxalate nephrosis in humans is to increase water intake (Cochat et al. 2006). Since dehydration in Mount Lofty koalas is likely to be due to a combination of climatic factors and inadequate leaf moisture, management of this issue for wild koalas is difficult. Yet for captive and hospitalised koalas it is recommended that eucalypt plantations are irrigated to increase leaf moisture and that eucalypt species are selected for feeding based on seasonal fluctuations in moisture content as found in this study. Also, the practice of standing cut branches of gum in buckets of water, spraying leaves

with water and providing access to drinking water should continue to increase water intake by captive koalas. For wild koalas, the creation of artificial water sources from which koalas can drink during summer may provide relief from dehydration in the short-term, but long-term solutions for water management in the Mount Lofty region should also be considered.

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CHAPTER 8 GENERAL DISCUSSION

This study has shown that oxalate nephrosis is a significant disease of the koala population in the Mount Lofty Ranges region of South Australia, affecting 55% of 51 captive and rescued wild koalas. The prevalence of oxalate nephrosis in the Mount Lofty koalas is much higher than that reported in the eastern states of Australia, such as in New South Wales, where several surveys have found that <3% of koalas show renal calcium oxalate deposition (Canfield 1989, Connolly 1999). As koala populations in Queensland and New South Wales are listed as vulnerable (DEWHA 2009), in part due to high infection rates with chlamydiosis (Polkinghorne et al. 2013) and koala retrovirus (Simmons et al. 2012), the South Australian koala populations may become increasingly important in ensuring the longevity of this unique marsupial species. With limited knowledge of the diseases affecting South Australian koalas, this study contributes to the assessment of the current health status of the Mount Lofty koala population.

8.1 Pathological features of oxalate nephrosis in koalas

This study describes the gross and histopathological changes and clinicopathological features that occur in koalas with oxalate nephrosis, expanding upon a previous case report of the disease in one New South Wales koala (Canfield and Dickens 1982). The current study has found that deposition of calcium oxalate crystals can be identified in the majority of koalas at necropsy, most often in the papillary region, but histological examination is necessary for detection of milder cases. The crystalline deposition in the kidneys was confirmed to be composed of calcium oxalate using a combination of polarisation microscopy, alizarin red S staining, infrared spectroscopy and scanning electron microscopy with energy dispersive X-ray analysis (EDX). A common histochemical stain for

demonstrating calcium oxalate crystals, Pizzolato's silver nitrate and hydrogen peroxide method (Bancroft and Gamble 2002), which had previously been used in the kidneys of koalas (Canfield and Dickens 1982, Haynes et al. 2004), was found to be unreliable in the current study. However, by using the alizarin red S staining technique, an additional benefit was the ability to differentiate between calcium oxalate, calcium phosphate and calcium carbonate (Proia and Brinn 1985). This technique has been similarly used in investigations of renal crystal deposition in other animal species, such as dogs (Thompson et al. 2008), cats (Suzuki et al. 2012) and macaques (Skelton-Stroud and Glaister 1994, Yanai et al. 1995).

Renal deposits from all koalas were shown to have an identical composition by infrared spectroscopy, indicative of a similar pathogenesis. In addition to calcium oxalate, renal deposit analyses showed phosphate and uric acid to be present. Calcium phosphate deposition was also observed in kidney tissue sections from koalas with oxalate nephrosis (57%) as well as those unaffected by oxalate nephrosis from Mount Lofty (48%). This suggested that calcium phosphate was not directly associated with calcium oxalate deposition and it is likely that these basophilic concretions occur in koalas as an incidental finding, as in humans (Weiss et al. 2007). They may also occur secondary to renal damage, since 94% of Queensland koalas showed calcium phosphate deposition, which in 35% of cases occurred in conjunction with renal interstitial inflammation, probably as a result of urogenital chlamydiosis (Canfield and Spencer 1993).

Uric acid was identified consistently in renal deposits using infrared spectroscopy, yet was only visualised in unstained renal histological sections from two koalas with oxalate nephrosis. This suggests that specialised histological processing and staining techniques may be necessary to investigate this deposit further (Bancroft and Gamble 2002). Renal uric acid deposits may occur in koalas similar to that in humans, whereby decreased renal excretory ability leads to elevated levels of uric acid circulating in the bloodstream (Ohno 2011). This

pathogenesis is supported by the finding of hyperuricaemia in koalas with oxalate nephrosis, which otherwise occurs in conditions such as gout in humans (Asplin et al. 2000, Weiss et al. 2007).

Kidneys of koalas with calcium oxalate deposition showed histopathological features similar to that seen in humans (Chonko and Richardson 1994, Weiss et al. 2007). These included intratubular and interstitial inflammation, tubule epithelial necrosis, tubule dilation, glomerular atrophy, nephron loss and fibrosis associated with the crystals. Koalas with renal calcium oxalate deposition showed renal insufficiency, characterised by azotaemia in conjunction with poorly concentrated urine (Osborne and Polzin 1991), urine specific gravity (USG) <1.030. However renal failure, shown by isosthenuric urine (Lane et al. 1994, Osborne and Polzin 1991), was only diagnosed in three affected koalas, which may be due to early euthanasia of koalas on animal welfare grounds. Increasing severity of renal histopathological changes was correlated to decreasing USG, showing the association between the progression of oxalate-induced nephrosis and renal insufficiency. This renal dysfunction is likely to be caused by obstruction and damage to renal tubules by calcium oxalate crystals (Chonko and Richardson 1994, Jones and Hunt 1983, Weiss et al. 2007), as well as the toxic effects of oxalate ions (Hackett et al. 1994, Khan and Hackett 1993, Scheid et al. 1996).

The finding of oxalate nephrosis in Mount Lofty koalas provides an explanation for the high level of renal disease which has been reported in this koala population for over a decade, which had previously been thought to be associated with high dietary aluminium (Haynes et al. 2004). In this previous study, aluminium was shown to be present in the eucalypt leaves, and renal tubule cells and bone of koalas with renal disease (Haynes et al. 2004). However the significance of these findings are unclear given that in the current study, aluminium was not detected in renal tissue or deposits by X-ray analysis.

Hyperoxaluria was found to occur in Mount Lofty koalas, with 5 - 20 fold higher urinary oxalate levels compared with koalas from Moggill, Queensland. Both Mount Lofty koalas with oxalate nephrosis, as well as those with no histological evidence of renal calcium oxalate deposition, showed hyperoxaluria. In addition, the ratio of urinary calcium: oxalate was found to be less than 10: 1 (Asplin 2002, Robertson and Peacock 1980), approximately 4.5: 1 in koalas with oxalate nephrosis and 2: 1 in unaffected Mount Lofty koalas, showing increased levels of oxalate in the urine. This is similar to that found in hyperoxaluric otters, in which the urinary calcium: oxalate ratio was determined to be 1: 1 (Petrini et al. 1999). Mount Lofty koalas with no histological evidence of oxalate nephrosis but high urinary oxalate were also found to have renal insufficiency, which may indicate renal damage due to acute toxic effects of oxalate ions prior to crystal formation (Hackett et al. 1994, Khan and Hackett 1993, Scheid et al. 1996). This would suggest an even higher prevalence of oxalate nephrosis in Mount Lofty koalas.

Measurement of urinary oxalate is a commonly used diagnostic test in human medicine (Cochat et al. 2006, Harambat et al. 2011, Milliner 2005), for which many methodologies exist (Mazzachi et al. 1984, Zerwekh et al. 1983). In the current study, the combination of specimen storage at pH 5 in – 80 ˚C conditions, pre-analysis sample acidification and high performance liquid chromatography showed consistent analytical results with low variation. Whilst oxalate levels in the blood could not be measured due to the complexity of the method (Ladwig et al. 2005), the high urinary oxalate levels are indicative of the increased oxalate load of the Mount Lofty koalas since the kidneys are the primary route of oxalate excretion (Robijn et al. 2011).

Urinary crystals in koalas with oxalate nephrosis were found to be composed of calcium oxalate with uric acid and phosphate, and showed identical infrared spectra to those of the renal deposits. Crystals showed unusual spiculated bow-tie or spherule morphology,

which had not been previously reported in koala urine (Canfield et al. 1989a). In domestic animals and humans, calcium oxalate urinary crystals usually exhibit two specific morphologies based on whether they are the monohydrate or dihydrate form (Fogazzi 1996). Calcium oxalate monohydrate is typically envelope-shaped, whereas calcium oxalate dihydrate is dumbbell-shaped (Fogazzi 1996, Millan 1997, Osborne and Stevens 1999). The specific calcium oxalate form of the koala urinary crystals could not be determined by infrared spectroscopy as the diagnostic peaks occurring between 4000 and 2000 cm^{-1} were overlaid by peaks from the uric acid and phosphate.

Although unusual morphologies of calcium oxalate have been previously observed (Farley et al. 1985, Osborne and Stevens 1999), based on the urinary crystal composition analyses being identical to that of the renal deposits, it is likely that these urinary crystals originated in the kidney rather than forming within the bladder. Supportive of this is that many crystals were deposited within the collecting ducts of the renal papilla from which they could enter the lower urinary tract, and that morphologically the renal and urinary crystals were similar. Also, crystals were seen in cast formation in the urine of one koala, indicative of a renal origin. The unique morphology of the urinary crystals found in koalas with oxalate nephrosis may be regarded as pathognomonic for this disease in koalas and utilised as a diagnostic tool for veterinarians.

Oxalate nephrosis was found to occur in similar numbers of captive and rescued wild koalas, and also showed no predisposition based on sex. Oxalate nephrosis was identified in koalas of varying ages, including young koalas < 2 years old, showing that it is not an age-dependent disease. Koalas with oxalate nephrosis showed few signs of concurrent illness, except for poor body condition in the majority of koalas, which commonly occurs due to inappetance and weight loss associated with renal disease (Lane et al. 1994). Gastrointestinal disease, a common problem for captive koalas (Canfield 1990a), was found

in four (16%) captive koalas with oxalate nephrosis. Three of these were due to gastrointestinal torsion, which may be related to dehydration whereby luminal contents become dry and impacted (Blanshard and Bodley 2008). However this condition is unlikely to affect oxalate absorption, as found in chronic malabsorptive gastrointestinal diseases such as Crohn's in humans (Weiss et al. 2007). Furthermore, gastrointestinal torsion and/or impaction were also found in three captive koalas of 22 (14%) Mount Lofty koalas unaffected by oxalate nephrosis.

Similar to that found by previous studies (Canfield 1990a, Canfield 1987b, Weigler et al. 1987), trauma likely due to motor vehicle accident was the main cause of death or euthanasia in wild Mount Lofty koalas that were otherwise healthy and unaffected by oxalate nephrosis. Also, trauma was twice as common in male koalas compared with females, which is consistent with previous reports of increased male koala morbidity and mortality due to motor vehicle accident whilst roaming (Canfield 1987b). As found in previous studies (Canfield 1991, Weigler et al. 1987), there was no suggestion in the current study that koalas with underlying disease were at increased risk of motor vehicle accident.

In addition, oxalate nephrosis was identified in koalas from other populations in which it had not previously been reported in the scientific literature. Renal histological examination of koalas from Moggill, Queensland (n=19) showed a prevalence of 12%, which is higher than that observed in wild rescued koalas by wildlife veterinarians in Queensland (A. Gillett, 2013, pers. comm.). The main disease affecting Queensland koalas in this study was ocular and urogenital chlamydiosis, as expected based on previous studies (Polkinghorne et al. 2013). In Kangaroo Island, only one of 25 wild-caught koalas (4%) were found to have urinary crystals consistent with oxalate nephrosis, but this is likely to be a less sensitive diagnostic test than renal histological examination and may be an underestimate of true prevalence. Kangaroo Island koalas were shown to be otherwise healthy by their normal

plasma and urine biochemical values, which may explain the success of this abundant population (Duka and Masters 2005).

8.2 Investigation of the cause of oxalate nephrosis in koalas

The finding of hyperoxaluria in Mount Lofty koalas with oxalate nephrosis strongly suggested a primary pathogenesis, since increased concentration of oxalate in the body must have either an exogenous or endogenous source (Asplin 2002, Milliner 2005). Furthermore, the possibility of oxalate deposition occurring secondary to renal failure was found to be unlikely, since this usually results in decreased levels of urinary oxalate due to the inability of the kidney to perform normal excretory function (Chonko and Richardson 1994, Harambat et al. 2011, Hodgkinson 1977, Milliner 2005). A disorder of calcium homeostasis was also less likely as the cause of increased renal calcium oxalate deposition, due to the findings of hyperoxaluria and normocalcaemia in koalas with oxalate nephrosis, in conjunction with urinary calcium levels similar to that of unaffected koalas in Mount Lofty, Kangaroo Island and Queensland.

Hyperoxaluria was found to occur in koalas from both Mount Lofty and Kangaroo Island and due to the dietary similarities of these populations, whereby koalas from both populations prefer *E. viminalis* (manna gum) (Phillips 1990), a dietary cause of hyperoxaluria was possible. Also, an inherited cause of hyperoxaluria was considered possible, due to the low genetic variation of South Australian koalas following the bottleneck caused by translocations of only low numbers of founding animals (Robinson 1978, Seymour et al. 2001).

A dietary or inherited pathogenesis of oxalate nephrosis in Mount Lofty koalas was suggested further by the findings of lower levels of urinary oxalate in Queensland koalas, which also have a lower prevalence of oxalate nephrosis, browse different eucalypt species

(Jackson et al. 2003, Phillips 1990) and have higher genetic variation (Fowler et al. 2000). However, the higher prevalence of oxalate nephrosis in Mount Lofty koalas compared with those from Kangaroo Island is indicative of a difference between these two populations, which in the case of an inherited basis, may be due to a genetic mutation occurring in Mount Lofty koalas since the 1960s, when they were translocated from Kangaroo Island (Robinson 1978). Otherwise, the few founding koalas of the Mount Lofty population may have carried an inherited defect, thereby leading to a higher prevalence due to inbreeding.

In herbivores, ingestion of plants high in oxalate is the main cause of oxalate nephrosis (Maxie and Newman 2007), but in the current study eucalypt leaves were found to be low in oxalate overall (<1% DW). Also, there was no access for captive koalas to oxalate-containing toxic plants >10% DW, such as soursobs (McBarron 1977). However, some Mount Lofty eucalypt leaf samples of manna gum and messmate stringybark showed higher oxalate content, up to 7.5% DW, and also Mount Lofty eucalypts showed higher oxalate content overall compared with Queensland trees. This suggests that dietary oxalate may play an indirect role in the pathogenesis of oxalate nephrosis in Mount Lofty koalas, even if it is not the primary cause.

Dietary oxalate may contribute up to 53% of urinary oxalate in humans (Holmes et al. 2001) and patients with oxalate nephrosis are advised to avoid foods high in oxalate (Asplin 2002). Since koalas are selective in their choice of eucalypt leaves (Moore and Foley 2000) the oxalate content of the eucalypt leaves that koalas were consuming was also investigated. Oxalate concentration of stomach contents was found to be low overall, yet koalas from Moggill, Queensland showed higher oxalate levels in stomach content samples than Mount Lofty koalas. This may have been age-related since there was a significant association between increasing age and increasing stomach oxalate concentration overall, and the Queensland koalas were predominantly older animals. Yet young Mount Lofty koalas with

oxalate nephrosis showed a trend of higher oxalate intake compared with older koalas; this may indicate a learned aversion to high eucalypt leaf oxalate in the older koalas with oxalate nephrosis (Duncan et al. 1998, Moore and Foley 2000). A limitation of this analysis was that it only measured stomach content oxalate concentration at the time of koala death and does not necessarily reflect previous eucalypt intake. However, the results of the stomach contents analyses further confirmed that the ingestion of oxalate in eucalypt leaves is unlikely to be the cause of oxalate nephrosis in the Mount Lofty koala population.

The investigation of an inherited cause for oxalate nephrosis in the Mount Lofty koalas showed that alanine: glyoxylate aminotransferase (AGT), the deficient hepatic enzyme in primary hyperoxaluria type I in humans, had similar activity in koalas with oxalate nephrosis and unaffected koalas from Mount Lofty and Queensland. This spectrophotometric enzyme assay is performed at only a few medical laboratories worldwide (Milliner 2005), however the method of Rumsby et al. (1997) was able to be adapted successfully to koalas, which showed similar AGT activity overall to that of healthy humans (Danpure and Jennings 1988). It was hypothesised that Mount Lofty koalas with oxalate nephrosis would show decreased activity of AGT, since the co-factor of this enzyme, pyridoxine or Vitamin B6, appears to improve clinical condition in affected koalas at Cleland Wildlife Park (I. Hough and A. Sulley pers. comm.), as occurs in human patients with primary hyperoxaluria type I (Asplin 2002, Cochat et al. 2006, Fargue et al. 2013, Hagler and Herman 1973a). Also, the levels of hyperoxaluria found in Mount Lofty koalas was similar to that found in children and adults with primary hyperoxaluria (Hoppe et al. 2009).

However, primary hyperoxaluria type I remains a potential cause since this disease can be caused both by decreased activity of the enzyme, as well as by a variant in which AGT with normal activity mistargets to the mitochondria, rather than the peroxisomes (Danpure et al. 1989, Danpure et al. 1994b). Previously it has been shown that koalas, as herbivores,

have peroxisomal AGT (Danpure et al. 1994a), in comparison with carnivores in which it is mitochondrial, which relates to the main dietary precursors of glyoxylate being glycolate and hydroxyproline, respectively (Birdsey et al. 2005). Hence, Mount Lofty koalas may have this variant of primary hyperoxaluria type I, in which AGT activity is normal but the enzyme is located at an abnormal intracellular location and therefore ineffective. In addition, it is possible that primary hyperoxaluria type II, in which the liver enzymes glyoxylate reductase and hydroxypyruvate reductase (GRHPR) are deficient (Giafi and Rumsby 1998, Mistry et al. 1988), may be implicated in oxalate nephrosis in koalas, or even the recently described primary hyperoxaluria type III (Belostotsky et al. 2010, Hoppe 2012).

A conservative management tool for human patients with primary hyperoxaluria is to increase water intake (Borghi et al. 2006), since low urine volume increases urine saturation and the risk of calcium oxalate deposition (Asplin 2002, Stevenson et al. 2003). Koalas rely on the moisture content of leaves in conjunction with formation of water droplets on the leaf exterior, following rainfall (Ellis et al. 2010) or overnight condensation (Degabriele et al. 1978). However, the summer and autumn period in Mount Lofty has high daily temperatures and low rainfall (BOM 2012), potentially increasing the risk of dehydration in koalas. Eucalypt leaf moisture analyses showed that Mount Lofty eucalypts were lower in moisture in autumn than those in Queensland, which has a high rainfall summer. In particular, juvenile and semi-mature leaves of *E. obliqua* (messmate stringybark) and *E. leucoxylon* (SA blue gum) were lower than other leaf types in summer and autumn, respectively.

Whilst koalas are known to choose leaves higher in moisture during summer (Ellis et al. 1995, Hindell and Lee 1987) and decrease intake of leaves below a moisture threshold (Pahl and Hume 1990), koalas in Mount Lofty may not have the opportunity for this selective feeding, and there are limited natural water sources from which to drink. Hence, Mount Lofty koalas may experience dehydration during the hot, dry summer and this is likely to be a

predisposing factor for oxalate nephrosis in this population, since insoluble calcium oxalate crystals are more likely to precipitate in highly concentrated urine (Asplin 2002). Also, dehydrated individual koalas may be at increased risk of developing oxalate nephrosis if they ingest large amounts of eucalypt leaves high in oxalate, such as those found in manna gum and messmate stringybark.

The majority of animal species in which cases of oxalate nephrosis have been reported are found to have a dietary cause, including livestock (James 1972, McKenzie 2012); guinea pigs (Holowaychuk 2006) and marmosets (Vanselow et al. 2011). Cases of oxalate nephrosis in other marsupial species such as a scaly-tailed possum and a swamp wallaby have also been attributed to ingested plant oxalate (Ellis et al. 1983). However, the current study has not identified a dietary cause for the Mount Lofty koalas. Due to the low genetic diversity of the Mount Lofty koala population, an inherited basis for oxalate nephrosis remains the most likely cause and further research is clearly required. However, diagnosis of the primary hyperoxalurias is complex even in human medicine (Cochat et al. 2006, Harambat et al. 2011, Milliner 2005), and only two animal species, domestic dogs and cats, have been confirmed to be affected by this disease thus far (Danpure 1989, Danpure et al. 1991). Primary hyperoxaluria has also been suspected but not confirmed in the Gilbert's potoroo (D. Forshaw, 2013, pers. comm.), otters (Petrini et al. 1999) and Beefmaster calves (Rhyan et al. 1992). Hence, it is likely that a disease similar to primary hyperoxaluria occurs in the Mount Lofty koalas, but it is also possible that the pathogenesis is multifactorial, involving both an inherited predisposition as well as environmental factors.

8.3 Future directions

To test for the primary hyperoxaluria type I variant in koalas with oxalate nephrosis, immunolocation studies to identify whether enzyme mistargeting of alanine: glyoxylate

aminotransferase occurs need to be performed. Detection of urinary glycolate, an additional metabolite produced in primary hyperoxaluria type I in humans (Asplin 2002, Milliner 2005), would also aid in the diagnosis of this disease. Further research could include molecular studies to detect if mutations in the AGT gene (AGXT) have occurred in koalas with oxalate nephrosis (Cochat et al. 2012, Tarn et al. 1997, Williams et al. 2009). If these tests proved negative, primary hyperoxaluria type II or III could be investigated as the inherited basis of oxalate nephrosis in koalas.

To better understand the potential impact of dietary oxalate in koalas, further investigation of eucalypt oxalate and the gastrointestinal absorption of oxalate may be warranted. Seasonal variation of oxalate in eucalypt leaves may occur, whereby levels of this anti-nutrient are higher in summer months and have increased impacts on koala health at this time. Also, the detection of the presence of oxalate-degrading bacteria such as *Oxalobacter formigenes* (Allison and Cook 1981, Allison et al. 1985) in the hindgut of koalas would determine whether there is significant microbial breakdown of ingested oxalate. In addition, calcium levels in eucalypt leaves and faecal calcium oxalate levels would indicate the degree of oxalate binding that occurs in koalas, reducing the amount absorbed from the gastrointestinal tract (Borghi et al. 2006, James 1972).

8.4 Applicable research outcomes

The findings in this study have increased our understanding of the pathological changes that occur in oxalate nephrosis in koalas and will assist veterinarians and veterinary pathologists in diagnosis of affected animals. For histological detection of oxalate nephrosis in koalas, transverse and longitudinal kidney sections which include the papillary region are recommended, as in other species (Morawietz et al. 2004), since this was found to be the primary site for crystal deposition to occur. Also, urinalysis to detect the pathognomonic

crystals will be valuable both for clinicians and clinical pathologists for routine health screening and as a diagnostic test. The prevalence of oxalate nephrosis in the wild population of koalas in the Mount Lofty region could be investigated in the future by detection of these crystals in urine samples from animals captured in the field.

Whilst a primary cause has yet to be identified, it is clear that low moisture content of eucalypt leaves is likely to be a significant risk factor for Mount Lofty koalas and should be considered in the management of both captive and wild koalas. Since koalas do not often drink free water (Degabriele et al. 1978), captive animals may require veterinary intervention to provide fluid therapy. Irrigation of eucalypt plantations that are used to feed captive koalas may improve the moisture content of trees, and in Cleland Conservation Park these plantations are also accessible for wild koalas. Increasing the availability of drinking water for wild koalas by the creation of artificial waterholes may also decrease the prevalence of oxalate nephrosis, particularly during summer. If an inherited cause is shown to occur, long-term strategies could include introduction of healthy koalas, free of urinary oxalate crystals, koala retrovirus and chlamydiosis, from the eastern states to improve genetic diversity, as well as captive breeding programs with healthy koalas to eliminate the genetic defect.

8.5 Conclusions

This study has been the first to describe the pathological, histopathological and clinicopathological features of oxalate nephrosis in a population of koalas. It has also been the first to initiate an investigation into the possible causes of what is clearly a complex disease process. Findings of this study will assist wildlife veterinarians and veterinary pathologists in the diagnosis of oxalate nephrosis in koalas in the Mount Lofty region, as well as across Australia. Understanding the health status of the Mount Lofty koala population, of

which little was previously known, has been improved by this study and information on other koala populations such as those in Kangaroo Island, South Australia and Moggill, Queensland has also been provided. With the recent increase in awareness of the vulnerability of many koala populations throughout Australia, research on koala ecology, biology and health has become even more crucial to conservation and management programs for this iconic Australian marsupial.

APPENDIX 1: DETAILS OF KOALAS USED IN STUDY

APPENDIX 1.1

MOUNT LOFTY KOALAS WITH OXALATE NEPHROSIS EXAMINED AT NECROPSY

Study ID	Origin	Other ID	Sex	TWC	Necropsy findings
20080325K	W	-	M	3	renal deposition
20080401K	W	-	F	3	renal deposition
20080410K	W	-	F	4	renal deposition
20080528K	C	Penni	F	2	renal deposition
20080702K	C	Moolah	M	1	renal deposition
20081017K	C	Elton	M	1	renal deposition & GI- caecal torsion
20081020K	C	Bess	M	1	renal deposition
20081103K	C (AZ)	Lola	F	1	renal deposition & GI- mesenteric torsion
20081119K	W	-	M	3	GI- inflammation
20081125K	W	-	M	1	renal deposition
20081202K	C	Mia	F	1	renal deposition
20090303K	W	-	F	5	renal deposition
20090306K	C	Chilo	F	1	renal deposition
20090310K	W	-	M	1	renal deposition
20090323K	C	Lola	F	1	renal deposition & GI- caecal torsion
20090331K	W	-	M	4	NAD
20090625K	W	-	F	2	renal deposition
20090706K	C	Ned	M	3	GI- torsion
20090917K	C	Lara	F	1	renal deposition and pneumonia
20090920K	C	Bernard	M	1	renal deposition
20091106K	W	-	F	1	renal deposition
20091110K	W	-	M	4	renal deposition
20091204K	C	Hamish	M	1	renal deposition
20100204K	C	Nena	F	1	renal deposition
20100209K	W	-	M	NR	renal deposition
20100226K	W	-	M	NR	NAD
20100302K	C	Ivy	F	1	renal deposition
20100413K	C	Luna	F	1	renal deposition

C= captive koala kept at Cleland Wildlife Park; C (AZ) captive koala kept at Adelaide Zoo, W= wild rescued koala, M= male, F= female, NR= not recorded, GI= gastrointestinal, NAD= no abnormalities detected at necropsy, TWC= tooth wear class method of Martin (1981).

APPENDIX 1.2

MOUNT LOFTY KOALAS UNAFFECTED BY OXALATE NEPHROSIS EXAMINED AT NECROPSY

Study ID	Origin	Other ID	Sex	TWC	Necropsy findings
20080318N	C	Coco	F	1	pneumonia
20080625N	W	-	M	1	trauma- fractured femur
20080801N	W	-	F	1	pneumonia
20080902N	W	-	M	1	trauma- fractured shoulder
20081118N	W	-	M	3	trauma- head trauma
20081230N	W	-	M	1	NR
20081231N	W	-	F	1	trauma- fracture
20090211N	C	Stanley	MN	2	GI- mesenteric torsion
20090302N	W	-	M	4	trauma- shoulder, possible tumour
20090304N	W	-	M	2	pneumonia
20090308N	W	-	M	NR	pneumonia
20090401N	W	-	F	4	GI- inflammation
20090618N	C	Abby	F	4	GI- torsion
20090705N	C	L J	M	1	GI- torsion
20090928N	W	-	F	1	trauma- fracture
20091109N	W	-	F	1	trauma- fractured distal tibia
20091111N	W	-	M	NR	trauma- fractured carpus
20091122N	W	-	M	4	trauma- fractured femur
20091124N	W	-	M	2	trauma- head trauma
20100128N	C	Barnie	M	2	GI- caecal impaction
20100203N	C	Graham	M	1	NAD
20100216N	C	Sizzle	M	1	GI- caecal impaction
20101012N	W	-	M	NR	NR

C= captive koala kept at Cleland Wildlife Park, W= wild rescued koala, M= male, F= female, NR= not recorded, GI= gastrointestinal, NAD= no abnormalities detected at necropsy, TWC= tooth wear class method of Martin (1981).

APPENDIX 1.3

QUEENSLAND KOALAS UNAFFECTED BY OXALATE NEPHROSIS EXAMINED AT NECROPSY

Study ID	Origin	Sex	TWC	Necropsy findings
200909Q1	W	F	7	old & poor body condition
200909Q2	W	F	7	pouch infection
200909Q3	W	F	7	blind due to chlamydiosis
200909Q4	W	F	4	dog attack
200909Q5	W	F	2	trauma- fractured femur
200909Q6	W	F	6	ocular and reproductive tract chlamydiosis
200909Q7	W	M	7	blind due to chlamydiosis
200909Q8	W	M	5	cystitis due to chlamydiosis & poor condition
200909Q9	W	F	6	cystitis & nephritis due to chlamydiosis
200912Q10	W	F	4	cystitis due to chlamydiosis & poor condition
200912Q11	W	M	6	conjunctivitis due to chlamydiosis & poor condition
200912Q12	W	F	7	cystitis due to chlamydiosis & poor condition
200912Q13	W	F	5	smoke inhalation & stress
200912Q14	W	F	6	trauma
200912Q15	W	F	6	nephritis & cystitis due to chlamydiosis
200912Q16	W	M	6	trauma, conjunctivitis & cystitis due to chlamydiosis
200912Q17	W	M	6	cystitis due to chlamydiosis & poor condition
200912Q18	W	F	7	dog attack & cystitis due to chlamydiosis
200912Q19	W	F	7	conjunctivitis & cystitis due to chlamydiosis

W= wild rescued koala, M= male, F= female, TWC= tooth wear class method of Martin (1981).

Grey shading indicates koalas that were found to have histological evidence of renal calcium oxalate deposition and were excluded from further analyses.

APPENDIX 1.4

KANGAROO ISLAND KOALAS SAMPLED FOR BLOOD AND URINE

Study ID	Origin	Sex	TWC
KI 1	W	F	4
KI 2	W	F	4
KI 3	W	F	4
KI 4	W	F	4
KI 5	W	F	3
KI 6	W	F	4
KI 7	W	F	1
KI 8	W	F	4
KI 9	W	F	6
KI 10	W	F	4
KI 11	W	F	4
KI 12	W	F	3
KI 13	W	F	3
KI 14	W	F	7
KI 15	W	F	4
KI 16	W	M	4
KI 17	W	M	4
KI 18	W	M	4
KI 19	W	M	4
KI 20	W	M	4
KI 21	W	M	5
KI 22	W	M	4
KI 23	W	M	4
KI 24	W	M	3
KI 25	W	M	4

W= wild rescued koala, M= male, F= female, TWC= tooth wear class method of Martin (1981).

Grey shading indicates the koala that was found to have urinary crystals similar to that found in koalas with oxalate nephrosis and was excluded from further analyses.

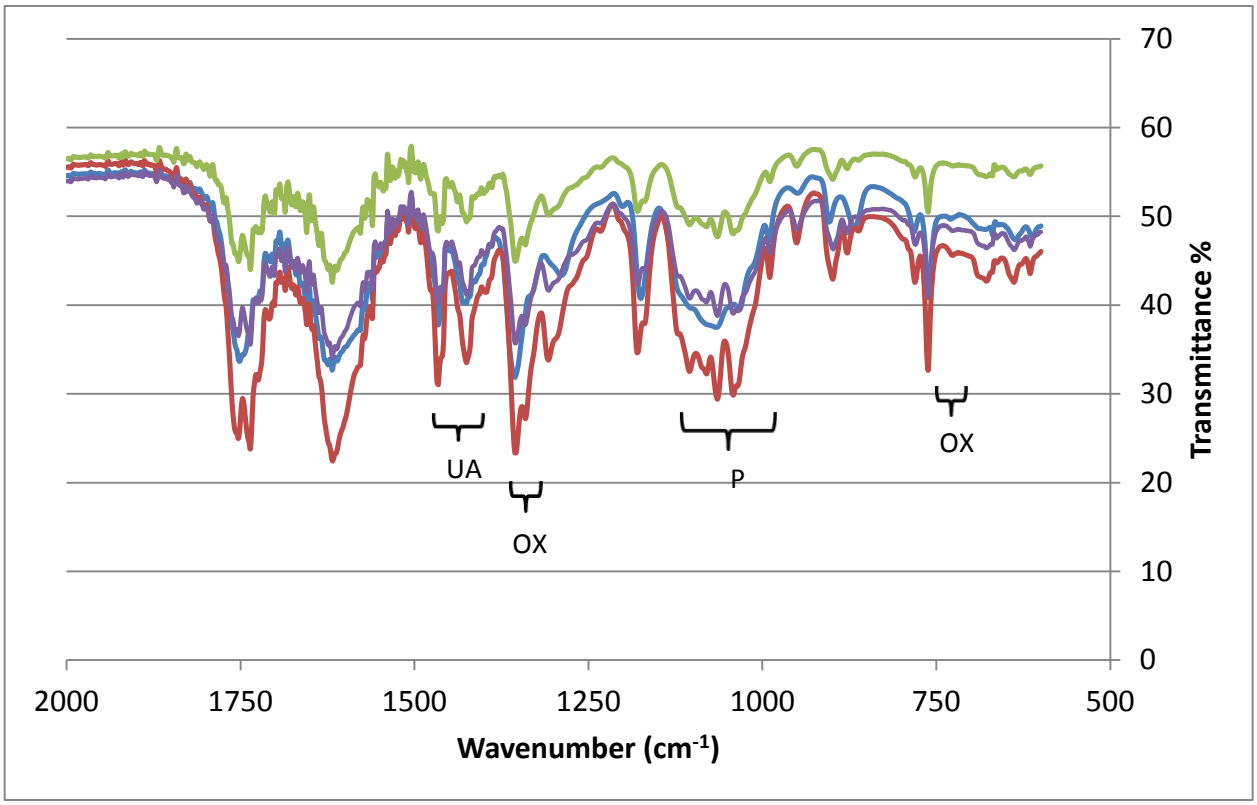
APPENDIX 1.5

CAPTIVE MOUNT LOFTY KOALAS WITH URINE CRYSTALS CONSISTENT WITH OXALATE NEPHROSIS

Name	Origin	Sex	Year of birth	Age at sampling
Osmond	C	M	1997	13 years
Rusty	C	F	1998	12 years
Kirra	C	F	2002	8 years
Yarrabee	C	M	2005	5 years
Audrey	C	F	2008	2 years
Ivy	C	F	2009	1 year

C= captive koala kept at Cleland Wildlife Park, M= male, F= female

APPENDIX 2: INFRARED SPECTRA OF URINE CRYSTALS FROM KOALAS WITH OXALATE NEPHROSIS



Infrared spectra for urinary crystals from 4 koalas with oxalate nephrosis (3 captive, 1 wild) showing peaks of oxalate (OX), phosphate (P) and uric acid (UA).

APPENDIX 3: EXAMPLES OF JUVENILE, SEMI-MATURE AND MATURE EUCALYPT LEAVES

Leaves of *E. obliqua* (messmate stringybark). Scale 10mm.

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