Using haematology and biochemistry to investigate the health and evolutionary biology of eastern grey kangaroos (*Macropus giganteus*)

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For Marino, Theresa Brandimarti and Frodo

Statement of originality

I declare that this thesis is the result of my own work and has not been submitted for another degree or qualification. All information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given at the end of the thesis.

Signed:

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Date: 28/2/2021

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Thesis abstract

The clearing and development of land for agriculture, housing and industry has detrimental and long-lasting impacts on natural ecosystems and biodiversity. Yet land clearing for urban development is accelerating world-wide. Arguably, all of the Earth's surface has been modified in some way, and Australia has one of the fastest rates of land clearance. Australia's shrinking wilderness is reducing the abundance of fauna while also threatening the health and welfare of species that remain abundant. There is a growing body of research focussed on the conservation and management of threatened species and populations of animals in decline, yet issues associated with common native species can often be overlooked. The eastern grey kangaroo (*Macropus giganteus*, hereafter kangaroo) is a large common macropod that can reach high densities and monopolize resources to the detriment of other species. Despite their apparent success, kangaroo populations are susceptible to food shortages, increased infectious disease risk and motor vehicle collisions; all of which reduce health and negatively impact their welfare. Developing a broad understanding of the drivers of kangaroo health is required to establish a benchmark to study poor health, however existing information is not always species-specific or applicable across populations. Experimental studies and customised tools which can measure aspects of kangaroo health can aid our understanding of how variations in biotic and abiotic factors can influence individual animals. Such tools could also be employed to enhance our understanding of the evolution of host-fitness trade-offs now and into the future.

This thesis aims to develop an evidence-based tool that characterises kangaroo health throughout their geographic range and investigates the factors associated with variable blood parameters and health status at the individual and population level. To measure health, species-specific haematological reference intervals (RI) were developed using blood samples collected from up to 245 animals from four sites across New South Wales (NSW) and the Australian Capital Territory (ACT). A novel machine learning method was then applied to an additional seven sites to evaluate the importance of biotic and abiotic factors in determining health variables. Results showed that

abiotic factors (site, rainfall, temperature and season) are critical determinants of kangaroo health outcomes. A health investigation was then performed, utilising the developed RIs, on a population of kangaroos in which health and welfare issues became increasingly apparent during the study period. This kangaroo population was from Look At Me Now Headland (LAMN) in NSW and was found to have widespread disease (parasitism and non-regenerative anaemia) and nutritional deficiencies hypothesised to stem from: high population density (5.4 individuals ha^{-1}), prolonged drought and reduction of grazing habitat due to ongoing development. The risk factors associated with parasitism were explored by examining potential selection pressures driving the evolution of kangaroos.

Host parasite and disease susceptibility varies due to an individual's sex. The sex differences in disease dynamics of wildlife populations are often overlooked in epidemiological studies. This thesis aimed to examine the influence of sex using a manipulative experiment that evaluated whether testosterone suppression improved health parameters, reduced parasite burdens and altered movement patterns in male kangaroos. While there was no effect of testosterone on these factors during the ten-week suppression period, a longer duration of suppression may be required to observe changes in parasite burdens. Further research is recommended, particularly as testosterone suppression may have applications in reducing aggressive behaviour toward humans, which is commonly reported.

This thesis develops a tool to assess health of kangaroos and examines the physiological role of sex in influencing health and parasite dynamics. Utilisation of the RI to assess health in a case-study population highlighted the challenges faced by kangaroos in urbanising areas and suggests that ongoing health and disease monitoring, and potential management intervention, may be necessary to ensure population persistence and individual welfare in isolated peri-urban kangaroo populations. In the longer-term, local and regional planning must consider habitat connectivity for kangaroo populations, in order to prevent overabundance and enhance positive outcomes for the health and welfare of the species.

A note on the style and layout of this thesis

This thesis is presented as a series of chapters and published papers. Chapters 2-4 are published papers, accepted for publication on the 8th of July 2020, 30th of March 2021 and 5th of July 2021 respectively. Published manuscripts are listed in the following section. A single reference list is provided at the end of this thesis.

Manuscripts included in this thesis

Brandimarti, M. E., R. Gray, G. Coulson, J. Cripps, M. Wilson, C. Death, M. Snape, C. Wimpenny, F. R. O. Silva, E. Miller, E. Scanes, D. Spielman, G. Thomas, C. A. Herbert. 2020. Reference intervals for parameters of health of eastern grey kangaroos (*Macropus giganteus*) and management implications across their geographic range. *Wildlife Biology* doi:wlb.00692. **(Chapter 2)**

Brandimarti, M. E., R. Gray, F. R. O. Silva, and C. A. Herbert. 2021. Kangaroos at maximum capacity: health assessment of free-ranging eastern grey kangaroos on a coastal headland. *Journal of Mammalogy* doi.org/10.1093/jmammal/gyab022. **(Chapter 3)**

Brandimarti, M. E., R. Gray, Z. J. Hilton, T. Keeley, K. P. Murray, and C. A. Herbert. 2021. The effect of testosterone suppression on health and parasite burden in male eastern grey kangaroos (*Macropus giganteus*). *Australian Mammalogy* doi.org/10.1071/AM21017. **(Chapter 4)**

Author contributions

Confirmation of co-authorship of published work

Author contributions to Chapter 2

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Author contributions

M. E. Brandimarti, R. Gray and C. A. Herbert conceived and designed the study. M. E. Brandimarti, F. R. O. Silva, G. Thomas, C. A. Herbert, G. Coulson, J. Cripps, M. Wilson, C. Death, D. Spielman and E. Miller collected the data. M. E. Brandimarti performed all statistical analyses with guidance from E. Scanes and R. Gray. M. E. Brandimarti wrote the manuscript. All co-authors reviewed drafts of the manuscript and provided feedback and revisions.

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

(All authors have signed a copy of the above and this is available upon request)

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M. E. Brandimarti, R. Gray and C. A. Herbert conceived and designed the study. M. E. Brandimarti, F. R. O. Silva, C. A. Herbert and R. Gray collected the data. M. E. Brandimarti performed all statistical analyses. M. E. Brandimarti wrote the manuscript. All co-authors reviewed drafts of the manuscript and provided feedback and revisions.

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

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M. E. Brandimarti, C. A. Herbert and R. Gray conceived and designed the study. M. E. Brandimarti, Z. J. Hilton, 'Kangaroo' Phil Murray and C. A. Herbert collected the data. M. E. Brandimarti and Z. J. Hilton, supported by T. Keeley conducted laboratory analyses developed by T. Keeley. M. E. Brandimarti statistically analysed the data and wrote the paper with contributions from all authors on the writing and revision of draft versions of the manuscript.

(All authors have signed a copy of the above and this is available upon request)

In addition to the statements above, in cases where I am not the corresponding author of a published item, permission to include the published material has been granted by the corresponding author.

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Attesting author contribution statement

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

X

Catherine Herbert Associate Professor

Date 26/02/2021

Abbreviations

ACT	Australian Capital Territory
AGC	Anglesea Golf Course
AM	Ainslie Majura Kangaroo Management Unit
BACI	Before-After-Control-Impact
BRB	Biased random bridge utilisation distributions
CI	Confidence intervals
CL	Calga
cm	Centimetre
CV	Coefficient of variation
CW	Cowan
DP	Darlington Park
EPG	Parasite worm eggs per gram of faeces
glmm	Generalised linear mixed effect regression model
GnRH	Gonadotropin-releasing hormone
GPS	Global positioning system
Ha	Hectares
HCT	Haematocrit
HGB	Haemoglobin
HP	Heritage Park
HPA	Hypothalamic-pituitary-adrenal
ICHH	Immunocompetence handicap hypothesis
Kangaroo	Eastern grey kangaroo
kg	Kilograms
LAMN	Look At Me Now Headland
lmm	Linear mixed effect model
m	Metre
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume

mg	Miligram
mL	Millilitre
mm	Millimetre
n	Sample size
NBGC	Nelson Bay Golf Course
ng	Nanogram
NRBC	Nucleated red blood cell count
NSW	New South Wales
PA	Portland Aluminium
PLT	Platelet
QLD	Queensland
RBC	Red blood cell
RI	Reference intervals
SEM	Standard error of the mean
SS	Serendip Sanctuary
TP	Total protein
UN	United Nations
USYD	The University of Sydney
Vic	Victoria
VPDS	Veterinary Pathology Diagnostic Services
WBC	White blood cell
WHP	Woodlands Historic Park
YAF	Young-at-foot
µl	Microlitre

Overview of thesis chapters

This thesis investigates factors associated with the health of eastern grey kangaroos (kangaroos hereafter), a common macropod which has seemingly adapted successfully to living amidst urban Australia. It presents a practical tool that veterinarians and wildlife managers can use to assess kangaroo health and provides insight into the welfare and life history strategy of this iconic animal across multiple populations.

Chapter 1 provides a review of the impacts of urbanisation on wildlife species in Australia, including the population trends and health effects of urbanisation on kangaroos. This chapter summarises the existing scientific knowledge of kangaroo biology and their life history strategy, highlighting the role of parasitism as a selective factor driving evolution. A diagnostic framework is then provided for common parameters of health in kangaroos, which forms the basis for developing reference intervals (RI) for the species, as reported in Chapter 2. Chapter 1 explores the animal welfare paradigm, focussing on how to measure welfare in a wild species. Lastly, Chapter 1 briefly explores kangaroo management in Australia and presents the broad aims of the thesis.

There is no established, evidenced-based methodology for quantifying the health of a kangaroo population, therefore, Chapter 2 describes species-specific haematological, serum protein and glucose RIs developed from healthy kangaroos. This provides veterinarians and wildlife managers with an evidence-based tool to monitor health, identify disease and contribute to animal welfare assessments in other free-living kangaroo populations. This chapter reports independent means from 11 discrete populations of kangaroos with varying density, body condition, locations and management interventions and determines the influence of biotic (sex and animal maturity) and abiotic (season, site, rainfall, temperature and laboratory) factors on health parameters. This chapter was published in *Wildlife Biology*.

Chapter 3 employs the RIs developed in Chapter 2 to investigate the health status of a high-density (5.4 individuals per ha) population of kangaroos at Look At Me Now Headland (LAMN) in New South Wales (NSW). This population has been impacted by ongoing urbanisation and prolonged drought.

Parasite counts (faecal egg counts, ticks and mites), trace element and heavy metal concentrations and haematological values from this population are compared to lower density populations and management recommendations are made. Before this study, the health and welfare of this population was unknown, and this study provided an opportunity to test the application of the RI tool in a real-world context. This chapter was published in the *Journal of Mammalogy*.

Males and females allocate energy differently, with males often investing less energy into their immune system compared to females. The influence of sex on disease transmission and infection within wildlife populations is relatively unknown but could be important in understanding sex-specific responses to anthropogenic disturbance. Chapter 4 examines the role of sex-specific hormones (testosterone) on haematological parameters, parasite burden and movement in kangaroos. This study demonstrated that a novel gonadotropin-releasing hormone (GnRH) vaccination was effective at reducing testosterone concentration in adult male kangaroos. This chapter described the influence of testosterone in determining fitness and parasite burden in a marsupial species and provides insights on the drivers of life history strategy in kangaroos. This chapter was accepted for publication in *Australian Mammalogy*.

Chapter 5 presents the general discussion and conclusions for the thesis, including 'real world' applications of the research and management implications.

General introduction

1.1. Impact of urbanisation on Australia's ecosystems and native fauna

Humans have modified almost 75% of the earth's surface (UN 2020) and are consuming natural resources at an unprecedented rate (Sanderson et al. 2002). Clearing land for agricultural production, industry and urban infrastructure development degrades ecosystems in various ways and in varying magnitudes, often with devastating consequences for native fauna (Seto et al. 2011; Taylor-Brown et al. 2019). Combating the threat of human actions on global species extinction rates is recognised by the United Nations (UN) as one of 17 Sustainable Development goals (UN 2020). Both the UN and the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services (IPBES) recognise that global land clearing is one of the largest single threats to biodiversity, while also one of the strongest drivers of climate change (IPBES 2019; UN 2020; IPCC 2020). Australia is clearing land at one of the fastest rates globally (WWF 2017), removing or degrading natural habitat and replacing it with agricultural, urban or industrial development. The Australian agriculture industry currently accounts for 58% of Australian land use (excluding timber production) and continues to expand (ABARES 2021). These anthropogenic changes result in irreversible ecosystem change that has immediate and long-term implications for remaining endemic fauna. In disturbed ecosystems, animals can either adapt to changes and persist in the ecosystem, move elsewhere or become restricted to habitat islands, or undergo local extirpation (Garden et al. 2006). Species with the capacity to adapt to disturbance often have a similar suite of characteristics resulting in ecological communities becoming increasingly similar over time. This process, termed 'biotic homogenisation', is considered a key threat to maintaining global biodiversity (Olden etal. 2016). The capacity to adapt (or not) depends upon the nature and magnitude of the disturbance interacting with a species life history, sensitivity to disturbance, interactions with other species and ability to move within or out of the affected area (Tischendorf et al. 2003). Land clearing is a type of disturbance that irreversibly changes ecosystems by reducing and fragmenting available habitat, and

it is therefore recognised as one of the key factors in fauna population declines (Koskimäki et al. 2014).

1.1.1. Habitat loss, fragmentation and modification

Habitat loss and fragmentation have decreased the abundance of fauna populations and their geographic distribution globally (Lino et al. 2019). Land clearing changes the landscape by reducing the availability of resources for native fauna, including shelter from predators (Doherty et al. 2015). The physical act of clearing alone directly harms fauna through mechanical force trauma and death, notwithstanding the psychological stress (Finn and Stephens 2017) and forced dispersal that can ensue. If animals respond to land clearing by moving to a new environment, they are often met with the same threats present in the cleared land itself, such as food shortages and aggressive conspecifics (Finn and Stephens 2017). For example, species that are mobile may be driven away by local fauna which increases the risks of stress, starvation and predation (Neldner et al. 2017). Species of animals that can persist in habitat patches but perceive the built environment as unsuitable are termed 'matrix sensitive' (Garden et al. 2006). These species face the long-term consequences of disconnected habitat and fragmentated populations; some of which include loss of genetic diversity and 'edge effects' (Garden et al. 2006).

A habitat 'edge' is the boundary between two ecosystems where inherent environmental conditions influence ecological processes and species abundance (Tanner 2005). Greater fragmentation of ecosystems result in a higher proportion of edges and greater 'edge effects' (Ranta et al. 1998), altered fauna assemblages and often homogenisation of species. Some species such as the endemic noisy miner (*Manorina melanocephala*) are considered generalists and prosper on 'edge' habitat (Garden et al. 2006). Generalist species are tolerant to edge habitat because of their ability to adapt to considerable environmental change (Callaghan et al. 2019). Despite favouring generalist species, habitat edges have increased predation rates, invasion of introduced species, parasitism of vulnerable animals and altered flora communities (Ruzicka et al. 2010). For squirrel gliders (*Petaurus*

norfolcensis) in southeast Queensland (QLD), changes in forest composition in and near edges affect their feeding and nesting requirements making this species sensitive to urban environments (Brearley et al. 2011). Urban sensitive species cannot live in urban landscapes or even in remnant patches of forest due to their limited dispersal ability that is incompatible with their often specialised diets (Garden et al. 2006). In squirrel gliders, stress levels, measured using cortisol concentrations in hair, were highest in gliders occupying edge habitats adjacent to major roads, and lowest in interior habitats (Brearley et al. 2012). Thus, for species that require interior habitat, habitat fragmentation and increased edge effects can introduce additional threats to their persistence.

1.1.2. Reduced genetic diversity

Lack of physical connectivity between sub-populations of animals results in loss of genetic diversity and an increase in genetic differentiation because gene flow is interrupted (Keyghobadi et al. 2005). The long-term effects of genetic disconnect are considerable. A recent meta-analysis (Lino et al. 2019) determined that mammalian populations occupying areas of high habitat fragmentation had reduced heterozygosity, allelic diversity and allelic richness. An example of genetic drift has been shown in the common ringtail possum (*Pseudocheirus peregrinus*) whereby, possums occupying remnant vegetation patches had extensive genetic differentiation and loss of genetic diversity when compared to possums inhabiting continuous forest (Lancaster et al. 2016). Together these characteristics can result in inbreeding depression, reduced fitness and can erode resilience to disease threats, reducing the evolutionary potential of a population and increasing the likelihood of extinction (Amos and Balmford 2001; Spielman et al. 2004).

1.1.3. Increased threat of introduced species

The introduction of new species which become invasive is another primary threat to global species decline (UN 2020). Invasive species and habitat modification work synergistically in circumstances where land clearing and habitat fragmentation opens up the landscape to invasive species which

may be more generalist and better suited to the modified environment (Grarock et al. 2014; Doherty et al. 2015). In Australia, the introduced common myna (*Acridotheres tristis*) is an example of a generalist that thrives in both urban and vegetated habitat (Grarock et al. 2012; Grarock et al. 2014). The common myna is a 'passenger species' which takes advantage of habitat modification and can aggressively displace smaller native bird species (Grarock et al. 2012; Grarock et al. 2014). The common myna can occupy cavity nesting sites of both small and large birds such as the sulphur-crested cockatoo (*Cacatua galerita*) and laughing kookaburra (*Dacelo novaeguineae*), in addition to mammalian species (Grarock et al. 2012; Grarock et al. 2014). Invasive species may capitalise on human resource subsidies obtained from the surrounding matrix such as food, but also from infrastructure like roads (May and Norton 1996; Doherty et al. 2015). In Australia, introduced predators such as feral cats (*Felis catus*) and foxes (*Vulpes* vulpes) use roads and forested roadsides to their advantage as they facilitate hunting, movement and the invasion of new environments that were previously inaccessible to them (May and Norton 1996).

1.1.4. Alteration of fire regimes

Fires are integral to ecosystem function, driving evolutionary adaptations of Australian plants and animals (Enright and Thomas 2008; Bowman et al. 2011), and have been used by indigenous communities for approximately 45,000 years (Enright and Thomas 2008). Land use and habitat fragmentation have a direct influence on the incidence and intensity of large fires by altering fuel loads and the distribution of assets (Williams and Bradstock 2009). During large fires, protection of human life and property is prioritised (understandably) over that of threatened species populations and biodiversity (Wintle et al. 2020). Biodiversity loss is further sustained by post-fire effects, such as food shortages and increased predation rates, contributing to localised fauna population declines (Robertson 2009; Wintle et al. 2020). Wildlife species with limited distribution, low fecundity, and poor dispersal ability are particularly vulnerable (Woinarski and Recher 1997).

1.1.5. Urban impacts

Artificial light and noise pollution occasioned by urbanisation of wilderness has the capacity to impact animal behaviour, physiology and reproduction across a range of species (Newport et al. 2014). For seasonal breeders, such as the tammar wallaby (*Notamacropus eugenii*), light pollution affects reproductive cues and has the potential to alter population dynamics (Robert et al. 2015). Research comparing two populations of tammar wallabies exposed to different levels of artificial light at night showed that elevated light masked the ambient light cues that trigger blastocyst reactivation (Robert et al. 2015). The population that was exposed to elevated light levels showed delayed birth dates of up to ten weeks with flow-on effects for timing of maternal investment with environmental conditions (Robert et al. 2015).

Wildlife road mortality is becoming increasingly recognised as a major contributor to species declines globally (Taylor-Brown et al. 2019; Englefield et al. 2020). While Australia does not have a national database on roadkill (Englefield et al. 2020) a study by Macquarie University and WIRES New South Wales (NSW) estimated that 7,000 animals per day are killed on roads in NSW alone (Ramp 2004). As cities and road infrastructure expand, these figures are increasing. Wildlife road mortality will likely constitute one of the most prominent threats to wild populations of many taxa (Taylor-Brown et al. 2019).

The impact of road infrastructure is not confined to wildlife road mortality alone. Roads fragment habitat and can split large populations into small sub-populations with reduced connectivity (Forman and Alexander 1998). Roads can also alter movement behaviour and act as a barrier to home range use, dispersal or migration with flow on effects for social organisation and reproduction (Taylor and Goldingay 2010). Therefore, roads further fragment the landscape, increasing the vulnerability of wildlife populations to key threatening processes.

1.2. Australia's kangaroos

Habitat loss in Australia has detrimentally affected many faunal species' distribution and has interacted with other threats to cause species extinction in some circumstances (Woinarski et al. 2015). However, this has not been the case for some of Australia's most iconic species. Kangaroos, wallabies, and pademelons make up the Macropodidae family (Dawson 2012). The three largest members of this family are the eastern grey kangaroo *(Macropus giganteus*), western grey kangaroo (*Macropus fuliginosus*) and the red kangaroo (*Osphranter rufus)* (Caughley et al. 1987), all of which are abundant throughout Australia. The habitat range of the red kangaroo is typically inland Australia in the arid and semi-arid zone, extending to the Western Australian coast (Caughley et al. 1987). The grey kangaroos typically occupy wetter climates (Caughley et al. 1987). Western grey kangaroos are distributed across the south of Australia, in areas that receive winter rainfall, whereas eastern grey kangaroos occupy the eastern coast and southern areas of Australia with consistent, summer dominated rainfall (Poole et al. 1982; Caughley et al. 1987). The range of each species overlaps to a degree with the other two (Caughley et al 1987; Dawson 2012).

Eastern grey kangaroos are the most abundant of the three species, and high kangaroo densities now occur subsequent to forest clearing, improved pasture for grazing livestock, provision of artificial water points, cessation of hunting by Indigenous Australians and removal of natural predators (Coulson 2008). The distribution of kangaroos along the eastern coast of Australia coincides with higher human population densities and oftentimes kangaroos occur in the peri-urban space, utilising places such as golf courses, sports fields, parks and reserves (Herbert et al. 2006). Their apparent success has polarised community views on kangaroos, including how, and if, they should be managed (Descovich et al. 2016). Kangaroos are regarded as Australia's unofficial national icon, generating tourism income domestically and internationally (Higginbottom et al. 2004). But when overabundant, they can be perceived as pests and alike other abundant wildlife (e.g. white tailed deer, *Odocoileus virginianus Zimm.*) are subject to mitigation strategies such as culling or fertility control (Herbert 2004; Schmit et al. 2020).

Despite being the most abundant of the three species, eastern grey kangaroos show limited dispersal and are more sedentary compared to red kangaroos (Jarman and Taylor 1983; Dawson 2012). Dawson (2012) reports events where many eastern grey kangaroos have died near dried-up water sources, with adequate food and water available only kilometres away. Eastern grey kangaroos also show lower survivorship compared to other species, such as the red kangaroo, living much shorter lifespans (Dawson 2012). These factors, unique to eastern grey kangaroos demonstrate that under certain environmental conditions, eastern grey kangaroos may display different species-specific responses compared to other macropod species.

1.2.1. Biology of eastern grey kangaroos

Eastern grey kangaroos (hereafter kangaroo) are gregarious herbivores (Taylor 1984), forming social, open membership groups (Jarman and Coulson 1989) which come together primarily at dawn and dusk to graze. Kangaroos are sexually dimorphic in body size and employ a polygynandrous mating system, whereby males and females have multiple mating partners (Montana et al. 2020). Male's fiercely compete for access to females and in captive populations, often the largest, dominant males monopolise females and sire most offspring (Miller et al. 2010). However, in the wild, siring success is often determined by a male's mating opportunity with females (e.g. whether their home range overlaps) (Montana et al. 2020). Like most marsupials, kangaroos have a short gestation period of approximately 36 days and give birth to a single young, typically between December and March (Poole 1973; Herbert et al. 2006). Pouch young is defined here as a young that is less than 320 days old (approximately) and has not yet permanently exited the pouch. After the first young vacates the pouch, females usually give birth to a second young; therefore, kangaroos typically have one young annually (Poole et al. 1982). However, in mid-late lactation, when the pouch young is greater than 188 days old, some kangaroos may come into oestrus and mate, with the resulting conceptus stored as a blastocyst in utero in 'embryonic diapause' until their current young reduces their suckling intensity (Clark and Poole 1967).

1.2.2. Population trends

Like many large grazers, wild kangaroo numbers fluctuate depending on 'bottom-up' pressures such as food availability, but also from 'top-down' effects such as predators or motor vehicle collisions (Descovich et al. 2016). In the Australian Capital Territory (ACT) kangaroo populations across nature reserves and adjacent lands are flourishing and regular culling is required to mitigate the impacts of excessive grazing (ACT Government 2020). In the peri-urban space, kangaroos may reach high densities due to low predation rates and provisioned food and water resources (Herbert 2004). These populations can be impacted over the longer term by the expansion of built infrastructure which reduces habitat and may isolate populations of kangaroos, particularly along the east coast of Australia (Descovich et al. 2016). With movement obstructed by infrastructure such as motorways and housing developments, these populations can become isolated and susceptible to local decline. Peri-urban kangaroos have already declined in many parts of south-eastern QLD (Brunton et al. 2019). Declines in peri-urban kangaroo populations may occur as a result of cumulative urban pressures (Brunton etal. 2019) causing reduced health outcomes associated with motor vehicle collisions, increased stress levels, malnutrition and density related diseases.

1.2.2.1. Peri-urban kangaroo management

Overabundant wildlife can harm individual animals, populations and their habitats causing a shift in resource availability, structural modification of vegetation and altered species interactions (Garrott et al. 1993). Defining a population as 'overabundant' can be divisive as it tends to involve arbitrary, value laden judgements (Garrott et al. 1993). Populations are generally considered overabundant if they reduce natural flora and fauna diversity, impact human life or livelihood and/or affect the fitness of the overabundant species (Herbert 2004). These reasons along with economic gain through commercial harvesting underpin the rationale for managing kangaroo populations. The decision to manage kangaroo populations in Australia, and the techniques used, are determined separately for each population. Factors that may be considered include; location (rural or peri-

urban), their perceived, or actual impact and the management goal (Descovich et al. 2016). Management is predominately suppressive and is often achieved through the lethal techniques of non-commercial shooting (culling) and commercial harvesting, with alternatives such as reproductive control, deterrents, or translocation employed (Herbert 2004; Descovich et al. 2016). However, translocation is often deemed inappropriate given the associated animal welfare impacts, the challenge of locating a suitable release site and the high cost (Cowan et al. 2020).

For high-density peri-urban kangaroo populations, a combination of management techniques is often employed (DTMS 2010). In some cases, the density of kangaroos is initially determined using methods such as direct observation counts or sweep counts, then non-commercial culling is performed to reduce the density of animals (DTMS 2010). To reduce the population growth rate, culling may be performed annually, or if deemed feasible and cost-effective, fertility control may be used (DTMS 2010). Given the gradient of views on kangaroo culling and management and whether the desired management objective can be achieved, fertility control for peri-urban kangaroo management may be advocated for preferentially to lethal methods (DTMS 2010; Descovich et al. 2016). Fertility control targets primarily female kangaroos using surgical techniques, hormonal manipulation and immunocontraception to reduce fecundity (Hinds and Tyndale-Biscoe 1994; Nave et al. 2002). Despite its attractiveness, uncertainty remains regarding the number of animals that should be treated, the welfare concerns for delivery of fertility control and what the impacts are on population demography, survivorship, immigration and emigration (Herbert et al. 2010; Hampton et al. 2015; Descovich et al. 2016).

Kangaroo management involves a wide variety of stakeholders and differing state or territory legislation, opinions, concerns, beliefs and values about kangaroo management (Sharp et al. 2014; Sharp and McLeod 2020). Therefore, clear, evidence-based decision making is critical when deciding to intervene and manage kangaroo populations. Despite the propensity for high-density populations to have nutritional stress, density related disease and mass mortality events, there is no established,

evidenced-based methodology for quantifying the health of a kangaroo population (Harvey et al. 2020).

1.3. Kangaroo health and welfare

Animal welfare is an interface of ethics and science that is far from settled. Humans appear to have always valued animals as more than objects, with records of human and animal companionship going back thousands of years in Australian indigenous cultures (Howe 1993), and respect for animals was encouraged by ancient western philosophers including Pythagoras (Steiner 2005). The field of animal welfare is constantly changing to meet the demands of both the ever-evolving community attitudes towards welfare, and advances in scientific understanding of what constitutes welfare (Mellor 2017). In its most general state, animal welfare depicts how an animal experiences life through its physical and mental state (Beausoleil et al. 2018). Therefore, as noted by Broom (1986); 'welfare is a wide term that embraces both the physical and mental well-being of the animal'. To more explicitly define welfare The 'Five Freedoms' paradigm was formulated by the Farm Animal Welfare Council (FAWC 1992). The Five Freedoms consider five targets of animal welfare to facilitate robust animal welfare assessment with consideration of the subjective experiences of animals, their health status and behaviour (FAWC 1992). The Five Freedoms include: freedom from hunger and thirst; freedom from discomfort; freedom from pain, injury and disease; freedom to express normal behaviour; and freedom from fear and distress (FAWC 1992). While these freedoms are an excellent target for animals under the direct care of humans, it is obvious that their application to wild animals is limited, especially when 'natural' processes (e.g. predation and parasitism) are considered (Brakes 2019).

The Five Freedoms paradigm has evolved in line with attitudes and scientific evidence (Webster 1994). However, the Five Freedoms were not designed to measure welfare, rather a target to aim for. To address this gap, Mellor (2017) suggested the Five Domains Model which originates from the Five Freedoms paradigm, but was designed to allow for a structured and systematic model to

measure welfare. Specifically, the Model focuses attention on welfare-significant internal states via Domains 1 to 3, which are labelled 'Nutrition', 'Environment' and 'Health', and on welfare-significant external circumstances via Domain 4, which is labelled 'Behaviour' (Mellor 2017). Any associated affective experiences can then be attributed to Domain 5, which is labelled 'Mental State' (Mellor 2017). This model's greatest strengths are the separation of those factors which can be objectively measured in Domains 1-3 (Mellor 2017). However, like the Five Freedoms, the Five Domains paradigm cannot easily be applied to wild animals (Brakes 2019).

Animals cope by responding in ways that attempt to correct a particular challenge, including the function of body repair systems, immune defence, emergency physiological responses and different behavioural responses (Broom et al. 1993). Health, including disease, is the 3rd Domain of welfare and is therefore, an integral component of welfare. The onset of disease can be triggered by an inadequate immune response, which may have occurred due to stress, adverse environmental or housing conditions, infant separation or overcrowding; all of which reduce animal welfare (Broom et al. 1993).

The measures which directly relate to health include the prevalence of disease, immunosuppression, reduced growth and body condition, reduced reproduction, cellular change which indicates system failure or aging and reduced life expectancy (Broom et al. 1993). Despite the lack of specific studies in wild species, it is well established that susceptibility to disease increases with adverse environmental conditions or the presence of stimuli that increase stress. The definition of welfare in this thesis (as outlined by Broom et al. 1993 and Mellor 2017) 'scales up' the Five Domains paradigm to wild animals, and centres upon the concept that coping with the environment requires a response (physiological, behavioural etc.) from the animal, and that the response either allows the animal to cope (successful response) or not to cope. When animals can't cope, welfare is reduced.

1.3.1. Stress

Stress is defined as 'an environmental effect on an individual which overtaxes its control systems and results in adverse consequences and eventually reduced fitness' (Broom et al. 1993). It has also been defined as a physiological response which activates the hypothalamic-pituitary-adrenal (HPA) axis (Hing et al. 2016; Broom et al. 1993). Activation of the HPA axis aims to restore homeostasis and results in the secretion of glucocorticoids (stress response) which can last several minutes or hours (Sapolsky et al. 2000). However, chronic activation of the HPA axis and the associated elevation of glucocorticoids can have negative effects on an individual's health and fitness (Sheriff et al. 2009).

The behavioural and physiological indicators of stress have been used to assess welfare in kangaroos. For example, the welfare implications of visitation impact on kangaroos housed in free-ranging exhibits was measured using behavioural indicators and faecal glucocorticoid concentration (Sherwen et al. 2015). A recent study by Brunton et al. (2020) found that kangaroos in south-eastern QLD living on private land currently undergoing development had higher stress levels, as measured by faecal glucocorticoid metabolites, than their non-urban counterparts.

The impact of prolonged stress can affect the ability of individuals to mount an immune response to disease and can reduce survival and reproduction in birds (Wingfield 1988; Sapolsky 1990). Therefore, it is likely that chronic stress in kangaroos as a result of continual urbanisation could impact health because the effect of glucocorticoids on white blood cell populations (e.g. reducing the number of circulating lymphocytes) increases susceptibility to disease (Strandin et al. 2018). Health, including the absence of disease, is the 3rd Domain (Five Domains; Mellor 2017) of welfare and is therefore a critical aspect of an animal's welfare.

1.3.2. Body condition

Peri-urban kangaroos are often confined to remnant habitat islands, bound by highly modified landscapes (Henderson et al. 2018a). These populations have the potential to grow to unsustainable densities, exceeding the carrying capacity of their environment. As density increases, overgrazing

can occur which reduces pasture biomass and the health condition of kangaroos (Moss and Croft 1999). Kangaroo mass mortality events have been reported in many parts of Australia (WHA 2018) and nutritional stress is recognised as one of the primary drivers or a contributing factor (WHA 2018). For example, over 300 unwell and dead kangaroos were recorded across reserves in NSW and the ACT in winter and early spring in 2015. The aetiology of these mortalities was multifactorial. However, restricted food availability due to high-density, malnutrition and starvation were identified as key factors (Grillo et al. 2015). Clinical signs of poor body condition include reduced fat cover and muscle mass of the vertebral spinous processes and hip bones (Johnston et al. 1997; Edwards et al. 2013). In adult animals, weight loss indicates severe conditions that negatively impact animal welfare. Body condition scoring can provide evidence of a lack of food or emaciation, and is used in a range of species as an indicator of welfare. Animals affected by malnutrition and starvation are also increasingly vulnerable to parasitism and disease, as energy is diverted away from the immune system (Holmes 1993). Therefore, it is likely that nutritional stress has a synergistic effect by combining with other biological or environmental stressors, such as parasites, to hasten the decline in kangaroo health.

1.3.3. Disease

High-density kangaroo populations have greater intra-specific contact rates compared to lower density populations, facilitating enhanced transmission of parasites and other infectious agents of disease (Gortázar et al. 2006). Overgrazing and heavy faecal contamination of pasture are also risk factors for increased exposure to infectious disease, both of which are associated with high-density (Carboni and Tully Jr 2009). While examples of disease associated with high-density in macropods are too numerous to detail, several of the most common, such as ‘lumpy jaw’, anaemia and coccidiosis are briefly described here and in section 1.2.3.

Oral necrobacillosis (‘lumpy jaw’) is a relatively common bacterial disease of overcrowded captive kangaroo populations (Vogelnest and Portas 2008; Rendle et al. 2020). Changing environmental conditions, consuming inappropriate vegetation and traumatic facial injury can predispose

individuals to the development of 'lumpy jaw' (Vogelnest and Portas 2008). Affected animals present with chronic osteomyelitis associated with soft tissue inflammation and infection of the mandible and is often associated with draining sinus tracts, swelling around the face and jaw and anorexia (Vogelnest and Portas 2008; Borland et al. 2012). Chronic cases can be associated with bone proliferation and remodelling, and animals often die from starvation (Vogelnest and Portas 2008). At Serendip Sanctuary in Victoria, 54% of natural kangaroo mortalities in 2006 showed signs of 'lumpy jaw' as a result of nutritional stress within the population (Borland et al. 2012).

Anaemia can also develop in high-density kangaroo populations with nutritional stress (Grillo et al. 2015; Portas and Snape 2018), or specific nutritional deficiencies. Anaemia is a deficiency of circulating red blood cells associated with haemorrhage, increased breakdown or inadequate production of new red blood cells that has many underlying causes (Radostits et al. 2007). Anaemia is detected by performing a full blood count. If there are too few red blood cells or if there is abnormal morphology, reduction in the oxygen carrying capacity of blood occurs, resulting in clinical signs of lethargy, weakness, shortness of breath and pale mucous membranes, among others. Iron deficiency anaemia is caused by insufficient absorption of iron in the gastrointestinal system or as a consequence of haemorrhage (Weiss and Wardrop 2011). Other causes of anaemia include blood loss due to chronic parasitism, gastrointestinal ulcers and neoplasia (Weiss and Wardrop 2011). Therefore, high-density populations with limited food availability and high levels of parasite transmission have a greater risk of developing anaemia through these mechanisms.

Coccidiosis is caused by protozoal infection due to *Eimeria* species (Arundel et al. 1977; WHA 2011). Coccidiosis typically affects juvenile animals, causing a range of signs such as lethargy, poor body condition, dehydration, abdominal discomfort, gastrointestinal bleeding and death (Vogelnest and Portas 2008). High-density kangaroo populations with nutritional stress during wet conditions are particularly susceptible to coccidiosis as damp conditions assist survival of infective oocytes in the environment (Barker et al. 1972; Arundel et al. 1977; Vogelnest and Portas 2008). Occasional

outbreaks with high mortality rates have occurred in free-ranging kangaroo populations, however outbreaks are more common in captive populations (Vogelnest and Portas 2008).

1.3.4. Welfare in the context of this thesis

This thesis focuses on parameters of health, the 3rd Domain within the Five Domains Model. As a part of welfare, health can be viewed as a gradient from healthy (functioning immune system and able to cope with environmental stress such as pathogens) to unhealthy (animal is immunocompromised and unable to overcome threats). These states have implications for an animal's welfare (Figure 1.1).

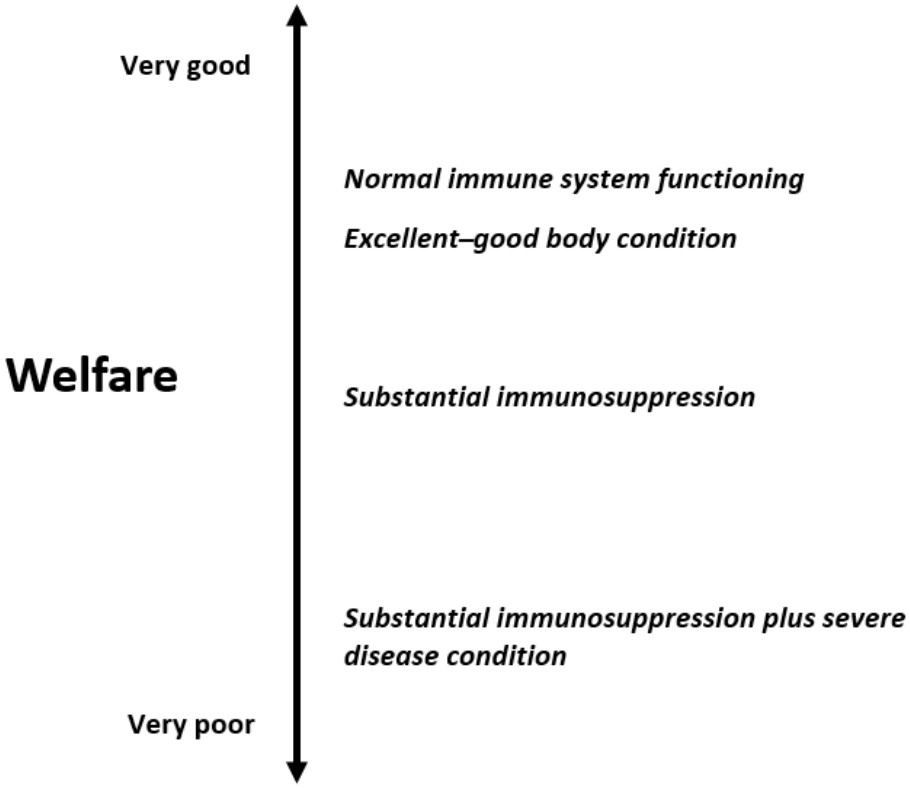

Figure 1.1 Scale of welfare levels for kangaroos using measurements of immune system function, disease susceptibility and body condition. In this figure disease condition refers to underlying disease (e.g. anaemia or parasitism). Immunosuppression was measured in this thesis using haematological, serum protein and micronutrient parameters (for example N:L ratio) but can also be derived from nutritional stress. Adapted from Broom et al. 1993.

This gradient view allows for the development of health measures such as haematological reference intervals (RIs) that aim to measure and 'scale' disease states on a continuous rather than binary

(discrete) scale. By using a gradient of health, a more detailed and accurate measure of health that is environmentally relevant can be achieved (Nordenfelt 2011).

In the context of health, efforts are made to improve the welfare of companion, laboratory, and farm animals through veterinary interventions, but for wild animals' acts of veterinary intervention to improve an individual's ability to cope with a disease are not always possible or appropriate (given societies differing attitudes towards wildlife) and is certainly less common (Kirkwood and Sainsbury 1996; Stephen and Wade 2018). Despite this, given the large effect that human activity has on wild populations, particularly the species that co-exist with humans, it is important to accurately measure animal welfare in free-ranging animals (Beausoleil et al. 2018; Stephen and Wade 2018). Measuring the affective state (e.g. loneliness or isolation), behavioural (e.g. movement or exploration) and long-term indicators of welfare (e.g. life expectancy and reproductive success) require long term monitoring of wild populations, are subjective, logistically expensive (in time and money) and difficult to achieve with accuracy (e.g. animal behaviour can change in the presence of researchers) (Broom et al. 1993; Mellor 2017). Therefore, measuring welfare by focusing on quantifiable aspects, such as measures of health through blood analysis, condition scoring or measuring parasite levels, is an efficient way to infer a wild animal's welfare at a given point in time.

Some have suggested applying more human centred ideas of welfare such as the 'salutogenesis concept' which focuses on building individual animal welfare through providing the resources for successful populations (Stephen and Wade 2018). Using these ideas in the context of the Five Domains Paradigm, particularly Domains 1-3, animal welfare can be a measurable characteristic that indicates how an animal, and its population, is coping within the environment (Broom et al. 1993).

1.3.5. Parasitism

Parasites can regulate wildlife populations by directly altering fitness, causing disease and reducing survival (Tompkins and Begon 1999; Tompkins et al. 2011). Parasites also exert evolutionary pressure on wildlife populations, driving life history strategy and shaping the physiology of species

(Grenfell and Gulland 1995). Kangaroos host a large variety of parasitic species, which have a range of impacts such as death, interference with feeding and reproduction, or no clinical effect (Arundel et al. 1990; Cripps et al. 2014). The next two sub-paragraphs describe common internal (endoparasites) and skin (ectoparasites) of kangaroos, and discusses their impact on kangaroo health.

1.3.5.1. Endoparasites

Kangaroos carry diverse and often large helminth communities which fluctuate depending on seasonality, locality and host-specific factors (sex, maturity, body condition) (Beveridge and Arundel 1979; Cripps et al. 2015). Descriptive surveys of parasitic fauna in kangaroos in Victoria (VIC, Australia) have shown that nematodes are the dominant helminth species, followed by cestodes (Beveridge and Arundel 1979; Aussavy et al. 2011). Table 1.1 lists common, well-tolerated nematode species found in kangaroos. It has been demonstrated in subadult kangaroos treated with an anthelmintic (to remove parasites) compared to a *Control* group, that large burdens of nematodes (including species listed in Table 1.1) caused negligible changes in most health and growth parameters (Cripps et al. 2014). *Control* kangaroos showed lower concentrations of the protein albumin compared to the *Treated* animals, and this likely reflects the presence of more pathogenic nematode species (such as *Globocephaloides trifidospicularis* and *Macropostrongyloides baylisi*) but at low levels (Cripps et al. 2014). There was a trend shown for increased weight gain and skeletal growth in *Treated* kangaroos, possibly indicating some subclinical effects of parasitism in the *Control* group, however these results were not significant (Cripps et al. 2014). Similarly, another study investigating foraging strategy of adult female kangaroos between *Treated* (anthelmintic treated) and *Control* groups demonstrated that foraging behaviour (e.g. faecal aversion) did not differ depending on parasite burdens (Cripps et al. 2016). These studies suggest that kangaroos can tolerate gastrointestinal parasite infections and may compensate for their potential cost (Cripps et al. 2014).

Table 1.1 Examples of common, well-tolerated nematode species found in eastern grey kangaroos (*Macropus giganteus*). The location within the body of kangaroos is described alongside clinical features and their impacts.

Nematode species	Location	Clinical features and impacts
Labiostrongylus spp.	Stomach	Adult worms are large (≤12cm) and present in high numbers[a,b] Larvae numbers peak in Autumn Larvae can penetrate mucosa, causing ulceration and inflammation[c]
Cloacina spp.	Stomach	High number of different species present[a] (up to ten species in a single individual host[d] Adult numbers peak in March and October[c]
Macropoxyuris spp.	Intestine	Small worm species, present in high numbers[a] Non-pathogenic[a]
Rugopharynx australis	Stomach	Small worm species, present in high numbers[a] Non-pathogenic[a]
Dirofilaria roemeri	Kneejoint	Filaroid nematode Can provoke inflammatory lesions[e] Non-pathogenic in some individuals[e]
Progamotaenia festiva	Bile duct	Individual cestode can reach 34cm[f] 20 worms found in a single individual[f] Mild lesions of biliary tract, increased mucus secretion and thickened walls of hepatic duct[f]

[a](Beveridge and Arundel 1979), [b](Vogelnest and Portas 2008), [c](Arundel et al. 1990), [d](Beveridge 1982), [e](Spratt and Varughese 1975), [f](Beveridge and Presidente 1978)

Despite the ability to co-exist and host diverse burdens of helminths, high burdens of certain species can cause significant pathological changes and have the potential to shape life history strategy. Table 1.2 includes examples of pathogenic nematodes found in kangaroos and describes their location and main clinical impacts. Notably, *G. trifidospicularis* is highly pathogenic at high burdens, particularly in subadult kangaroos, and this species is commonly recorded as a cause of kangaroo mortality (Arundel et al. 1990; WHA 2018). *Globocephaloides trifidospicularis* are blood feeders and cause anaemia, which can be characterised by reductions in total protein, haemoglobin and haematocrit concentration in the blood as worm burdens increase (Arundel et al. 1990). Anaemia can occur annually in populations at high-density and with heavy infestations (Arundel et al. 1977; Arundel et al. 1990; Vogelnest and Portas 2008). *Globocephaloides trifidospicularis* numbers peak in August, and subadult kangaroos then shed the eggs from June to October; eggs survive in the environment to infect the next years susceptible animals (Arundel et al. 1990). Therefore, this nematode species may play a regulatory role in kangaroo populations (Hudson et al. 1998; Tompkins and Begon 1999).

Table 1.2. Examples of pathogenic nematode species found in eastern grey kangaroos (*Macropus giganteus*). Anatomical location of the parasite and clinical features and their impacts are included.

Nematode species	Location	Clinical features and impacts
Globocephaloides trifidospicularis	Small intestine	Blood feeder Heavy burdens can cause anaemia and mortality[a,b,c] Subadults particularly susceptible[b]
Rugopharynxrosemariae	Stomach	Larvae form small crateriform nodules and hypertrophicgastritis on stomach wall[d] Subadults exposed in Autumn can develop extensive lesions and high burdens Adults can develop resistance[d]
Strongyloides spp.	Stomach	Larvae bury into mucosa causing hyperaemia, oedma and gastritis[a,b] Mortality reported in captive animals[c] Can be tolerated in some individuals[c]
Fasciola hepatica (Common liver fluke)	Liver	Trematode introduced to marsupials from livestock[e] Found typically in kangaroos when domestic ruminants and intermediate host (*Lymnaea tomentose*) are present[c,f] Highly pathogenic, causing marked hyperplasia, inflammationof biliary mucosa, erode the bile duct epithelium and may cause mortality[c,d] Can be tolerated by some animals[c]

[a](Arundel et al. 1977), [b](Arundel et al. 1990), [c](Vogelnest and Portas 2008), [d](Beveridge and Presidente 1978), [e](Beveridge and Spratt 2015), [f](Spratt and Presidente 1981)

1.3.5.2. Ectoparasites

Macropods can host a range of ectoparasites, including flies, ticks, lice, mites and fleas (Vogelnest and Portas 2008). The degree of pathogenicity of ectoparasites depends on the level of parasite burden and species, in addition to the hosts health status. Mild infestations of ectoparasites can result in no clinical signs or pruritus; however heavier burdens can cause ulceration, alopecia and anaemia (Vogelnest and Portas 2008).

Ticks are commonly recovered from the ears, eyes, axilla and inguinal region of kangaroos (Brandimarti et al. 2021[thesis Chapter 3]). Kangaroos can tolerate tick parasitism, including species such as *Amblyomma triguttatum*, which similar to most tick species, can cause localised inflammation, craters in the skin and dermatitis in severe cases (Vogelnest and Portas 2008). However, heavy tick burdens, particularly of the species *Ixodes holocyclus*, can cause paralysis and respiratory failure in kangaroos (Vogelnest and Portas 2008). Heavy tick burdens also contribute to anaemia in many species (Neilson and Mossman 1982; Martin 1985; Pfäffle et al. 2009), including koalas (*Phascolarctos cinereus*) heavily infested with *Ixodes tasmani* (Obendorf 1983). One of the main risks associated with ticks is their role as vectors in transmitting disease. Babesiosis is a tick-borne disease caused by an intraerythrocytic protozoa that can cause significant disease in kangaroos (Dawood et al. 2013; Donahoe et al. 2015). Once infected, kangaroos can be asymptomatic while others have severe haemolytic anaemia, neurological signs and altered mentation, including depression (Dawood et al. 2013). Babesiosis is transmitted in the saliva of ticks during feeding, and a number of tick species can transmit the disease, including *Haemaphysalis* and *Ixodes* (Donahoe et al. 2015). Hence, high tick burdens, particularly in immunocompromised hosts, are a risk factor for developing mild to severe disease.

1.4. Parameters of health in kangaroos

This thesis develops a standardised tool for assessing health via the development of haematological and serum protein RIs which wasn't previously available to veterinarians and animal managers. Standardised tools previously employed to assess health in kangaroos include body condition scoring and the evaluation of kidney fat or bone marrow reserves (Shepherd 1987; Moss and Croft 1999; Fletcher 2007). Body condition scoring is a highly subjective measurement, often differing between scorers and has limited repeatability across populations. Similarly, the evaluation of fat and bone marrow reserves has limited applications in population comparisons, and these methods may lack general public support due to the requirement of killing animals. Destructive sampling techniques also restrict data collection to one timepoint, such that revaluation of individuals is not possible. Therefore, the current methodologies lack accuracy and repeatability, which are both crucial when justifying potential management interventions for kangaroo populations with poor health outcomes. Condition indices, such as the 'scaled mass index' (Cripps et al. 2014) have proved to be objective and repeatable, yet their comparative application across populations has not been tested and further research is required for this method to be reliably used.

In veterinary medicine, haematological parameters of health are commonly employed to screen and provide diagnostic information for the evaluation of individual and population health status (Schalm et al. 1975; Radostits et al. 2007). For an accurate interpretation of animal health, the history and clinical examination findings are considered along with the patient's haematological blood parameters and how they compare with those of a reference population (Friedrichs et al. 2012). Derived from a reference population, RIs comprise of 95% healthy, well-defined animals (Friedrichs et al. 2012) and they vary depending on an animal's sex, age and environment. Hence, partitioning based on age, sex and season is often required for accurate interpretation (Paltrinieri et al. 2014). When describing animal health utilising RIs, it is critical that an accurate haematological RI are used because health and disease status are usually determined by comparison with 'normal' values

(Walton 2001).

Haematological RIs are commonly used in domestic animals. For wildlife the use of RIs is dramatically increasing as their broad application is desirable for monitoring wildlife, particularly threatened species (Warren et al. 2015). Blood samples are repeatable across populations, minimally invasive and do not affect the survival of individuals (apart from capture associated mortality), making repeated measurements possible (Clark 2004). Therefore, the development of haematological RIs for parameters of health in kangaroos provides a tool for government agencies, wildlife carers and veterinarians to make highly contentious decisions about the health status of a kangaroo population and determine whether management interventions are required. The use of repeatable, objective and informative health parameters across different populations allows for long-term monitoring, creating benefits to the health and welfare of animals. The RI developed in this thesis can be used to detect immunosuppression and provide evidence of disease. Immunosuppression generally includes the modification and reduced efficacy of all steps of the immune response, including the maturation, selection and proliferation of lymphocytes and the activation of inflammatory cells (Griffin 1989). If an individual is immunosuppressed and cannot adequately respond to pathogens, or if the animal has a disease that reduces body condition, growth, reproduction, or survival then the animal is showing physiological indicators of compromised welfare.

Routine haematological parameters can be used to identify disease and understand animal physiology include the total white blood cell count and a differential count of the five white blood cells (neutrophil, lymphocyte, eosinophil, monocyte and basophil); erythrocyte indices (red blood cell count and morphology); and immune system indicators measured from whole blood, and serum protein. These parameters are presented in Table 1.3, with examples of some diagnostic implications for clinical changes in each parameters baseline. This list is not exhaustive.

Table 1.3. Common haematological, glucose and serum protein parameters, their function and diagnostic value of clinical changes. Effect indicates whether a parameter is higher or lower than baseline (or reported) values. Where applicable, diagnostic descriptions are given for eastern grey kangaroos (*Macropus giganteus*).

Parameter (units)	Function	Effect	Diagnostic indicator
Red blood cell count (10^{12}/L)	Major component of blood. Transports O_2 to tissues and removes CO_2 [a]	↑	Dehydration[a]
			Captive populations with high protein diets or free-ranging animals when nutrient content of plants is high[b,c]
		↓	Anaemia[d]
			Kangaroos may be lethargic and self-isolate[e]
White blood cell count (10^9/L)	Total of five types (below). Control inflammation and pathogen defence[a]	↑	Infections[f] Inflammation Stress Adrenalin Regenerative anaemia (neutrophils)
		↓	Poor energetic condition[g], immunosuppression
Neutrophil count (10^9/L)	Ingest and breakdown pathogens[a]	↑	Bacterial infections (e.g. oral necrobacillosis)[c] Inflammation[h] Stress, fear and pain[a,i] Metabolic dysfunctions[h]
		↓	Increased risk of infection
Lymphocyte count (10^9/L)	Make antibodies, regulate WBC, mediate cytotoxicity, immunological memory[a]	↑	Acute infection and immunostimulation[a,h] Fear and pain in marsupials[a]
		↓	Stress[i] Poor energetic condition[g], neoplasia[h]
Eosinophil count (10^9/L)	Primarily parasite and allergen inflammation[a]	↑	Prolonged parasitic contact and parasite shedding[h,j] Allergic responses
			Inflammation[k]
		↓	Stress[h]
Monocyte count (10^9/L)	Phagocytosis and breakdown of material[a]	↑	Tissue damage, chronic inflammation and infection(often bacterial)[h,l]
		↓	Clinically insignificant

Parameter (units)	Function	Effect	Diagnostic indicator
Basophil count (10^9/L)	Least common WBC. Likely role in inflammation and cytotoxicity[a]	↑	Tissue damage, inflammation, parasite and non-parasite infection[h]
		↓	Clinically insignificant
Glucose (mmol/L)	Energy source	↑	Stress response[h] diabetes mellitus
		↓	Septicaemia, poor nutrition[h]
Haemoglobin (g/L)	Present in RBC. O_2 carrying capacity of blood[a]	↓	Anaemia, iron deficiency[h]
Haematocrit (l/L)	Total RBC volume	↑	Dehydration (Increased water loss or decreased water intake[a] High protein diets (captivity)[b]
		↓	Anaemia[a]
Platelets (10^9/L)	Aggregate to minimise blood loss	↑	Acute blood loss, iron deficiency anaemia[o]
		↓	Haemorrhagic diathesis (slow blood clotting)
Nucleated red blood cell count (10^9/L)	RBC with a nucleus, undergoing development[a]	↑	Healthy in koalas[d] and neonate marsupials such as the quokka (*Setonix brachyurus*)[Q] and common brushtail possum (*Trichosurus vulpecula*)[R] Regenerative anaemia, poisoning, bone marrow hypoxia or erythroleukaemia[a]
		↓	Non-regenerative anaemia, bone marrow dysfunction[f]
Reticulocyte count (g/L)	Immature RBCs in final stage of differentiation[p]	↑	Bone marrow responding to anaemia. May not be enough to overcome a disorder[a]
		↓	Non-regenerative anaemia[f] Bone marrow disease, reduced iron availability or chronic disease[m]
Albumin (g/L)	Most abundant serum protein	↑	Dehydration[a]
		↓	Splenic contraction following anaemia[a]

Parameter (units)	Function	Effect	Diagnostic indicator
Total protein (g/L)	Total albumin and globulin concentration	↑	Necrosis, inflammation[h] Dehydration[a,e]
		↓	Liver damage, pregnancy[h] Splenic contraction following anaemia[a]
Globulin (g/L)	Immunoglobulins, enzymes, carrier proteins and complement	↑	Inflammation and infection[n] Prolonged immunostimulation

[a](Clark 2004), [b](Amin et al. 2007), [c](Brandimarti et al. 2020 [thesis Chapter 2)), [d](Canfield et al. 1989), [e](Brandimarti et al. 2021 [thesis Chapter 3]), [f](Thrall et al. 2012), [g](Owen and Moore 2006), [h](Maceda-Veiga et al. 2015), [i](Davis et al. 2008), [j](Obendorf 1983), [k](Jain 1993), [l](Gilot-Fromont et al. 2012), [m](Nemeth 2008), [n](Serrano et al. 2018), [o](McMichael et al. 2015), [p](Koepke and Koepke 1986), [Q](Yadav 1972), [R](Calvert et al. 1994).

1.5. Kangaroo life history theory and immunocompetence

The immune system is an important fitness trait which has evolved to protect hosts from pathogens and enables them to combat disease (Maligoli and Ottaviani 2014). For animals to cope with their environment, they require an immune system which functions optimally to allow an animal to effectively respond to pathogens (Broom et al. 1993). As previously discussed, poor body condition and the effects of glucocorticoids can reduce an animal's ability to mount an optimal immune response (Holmes 1993; Broom et al 1993), however, there are also host-specific evolutionary drivers which can affect immune function (Folstad and Karter 1992). Life history trade-off theories propose that an individual must choose to invest energetic resources between competing traits (Partridge and Sibly 1991; Quesnel et al. 2018) and this can result in intra-specific variation in disease susceptibility. The immune system requires energy to function and hosts must allocate this energy at the expense of other biological systems (Maligoli and Ottaviani 2014). The immunocompetence handicap hypothesis (ICHH) proposes that optimal immune function comes at a cost to an individual's reproductive success (Folstad and Karter 1992). The ICHH centres upon the role of testosterone, the predominant reproductive hormone in males, and its capacity to both enhance and reduce an individual's fitness (Folstad and Karter 1992). Whilst testosterone stimulates the development of sexual signals (e.g. colourful plumage, large antlers, body size) and sperm production, which improve a male's chance at siring offspring, it has been suggested that testosterone directly impairs immune function and is energetically costly to produce (Folstad and Karter 1992), such that males must forgo optimal immune defence in order to invest in reproduction and pass on their genes to the next generation. This thesis investigates whether testosterone influences the capacity of an animal to cope with its environment by measuring immunoparameters and parasite levels in male kangaroos. The study design used to test the ICHH is novel and seeks to examine whether males with high levels of testosterone have sub-clinical changes in health parameters – the trade-off. All animals have a biological basis that underpins their physiology and behaviour. It is important to understand the evolution of trade-offs between immune function and

other physiological traits (e.g. reproductive strategies) to enhance our understanding of factors that influence kangaroo health, and intra-specific variations therein. Utilising tools such as haematological RIs also present biologists with a unique opportunity to perform manipulative experiments that can measure fitness-related and immunological traits and provide insight into long-standing evolutionary theories.

1.5.1. Immunosuppressive effect of testosterone

Research in birds, reptiles and mammals has demonstrated that testosterone can directly alter haematological parameters, and that males experience higher parasite loads compared to female conspecifics (Poulin 1996; Schalk and Forbes 1997; Klein 2000). These observations are supported by clinical research which found that the immune system is altered following hormone replacement therapies or gonadectomy (Grossman 1985). For example, the mass of primary and secondary lymphoid organs increases following castration of male rats (Grossman 1985; Grossman 1990). Additionally, female rats have been reported to have higher levels of immunoglobulins compared to male conspecifics (Klein 2000). This change may have occurred due to hormone receptors present on lymphoid organs, which respond to sex hormones such as testosterone (Schuurs and Verheul 1990).

Manipulative experiments have established that immunological parameters respond to changes in the concentration of testosterone. For example, lymphocyte counts and immunoglobulin concentration declined in birds after artificially increasing testosterone levels (Zuk 1990; Zuk et al. 1995). The dose amount, and the frequency of administration of testosterone also alters parameters of the immune system. Short-term, repetitive doses of testosterone reduce humoral and cell-mediated immunity in mice (Inman 1978; Grossman 1984; Ahmed et al. 1985) while continuous, low doses of testosterone reduce natural killer cell activity and antibody-dependant, cell-mediated cytotoxicity (Hou and Wu 1988). Studies of parasitism in several species highlight the role of testosterone in reducing immunocompetency and fitness traits. For example, testosterone

administration in female and castrated male mice increased their parasite burden, parasite egg production and infection duration thereby influencing individual host survival (Alexander and Stimson 1988; Nakanishi et al. 1989). Conversely, castration in mice reduced host susceptibility to parasites and delayed parasite reproductive output, minimising the risk and success of parasitic infection in the host (Noble and Noble 1961). Similarly, male barn swallows (*Hirundo rustica*) with increased testosterone during the breeding season had increased parasite burdens compared to females (Zuk 1990).

Despite the evidence suggesting that testosterone can suppress and alter the immune system, there is also research that suggests there is no effect, or at least implicates other mechanistic pathways as responsible for the reduced immunocompetency typically seen in males. Recent work on the impact of testosterone on innate and adaptive immunity in humans determined that testosterone had no immunomodulatory properties and did not suppress immunofunction (Nowak et al. 2018). Similarly, testosterone-induced immunosuppression was not demonstrated in adult chickens (*Gallus gallus domesticus*) but was observed in young birds (Schuurs et al. 1992). This may be due to the absence of the bursa of Fabricius in adult birds, a lymphoid organ that regresses at sexual maturity. The inconclusive literature highlights that further research should be conducted on the effects of testosterone on the immune system (Owen-Ashley et al. 2004). Other androgen metabolites, such as dehydroepiandrosterone (DHEA) and dihydrotestosterone (DHT), may also have immunosuppressive effects. However, like testosterone, their role is conflicting as both metabolites have been found to stimulate the immune system (Owen-Ashley et al. 2004).

1.5.2. Indirect effects of testosterone

Testosterone has been associated with indirect immunosuppressive effects, often as a precursor hormone to a cascade of biological events. For example, testosterone increases glucocorticoids in song sparrows (*Melospiza melodia*), resulting in stress related immunosuppression (Owen-Ashley et al. 2004). As discussed previously, it can also inhibit an animal's fitness by diverting resources away

from immune defence and active elimination of disease threats. Prolonged exposure to testosterone can affect the allocation of energy to the immune system by increasing the activity demands for metabolism and the rate of protein catabolism in muscle cells (Gray et al. 1990; Lynn et al. 2000). Prolonged exposure to testosterone using implants in birds reduces body mass and fat stores compared to non-exposed animals (Wingfield 1984). This suggests that testosterone plays an indirect role in increasing energy utilisation for metabolic activity and protein and fat catabolism, therefore limiting the energy available for immune function.

1.5.3. Immunosuppression in marsupials

Marsupials evolved in isolation from eutherians and although similarities in the architecture of their immune systems exist, there are differences which are still being explored (Belov et al. 2007). Despite a lack of research on the impact of testosterone on the immune system of marsupials, there are a few landmark and extreme examples. Complete mortality of males after mating is commonplace in several small dasyurid and didelphid species (Oakwood et al. 2001). Observational and experimental research has shown that total post-mating mortality (semelparity) occurs in some dasyurid and didelphid species due to increased glucocorticoids, which are required to sustain males during the mating period (Bradley et al. 1980; Bradley 1987). Increased glucocorticoids in the red-tailed phascogale (*Phascogale calura*) and brown antechinus (*Antechinus stuartii*) have been shown to reduce immunocompetence and immunoglobulin levels in males during the mating period (Bradley et al. 1980; Bradley 1987). This immunosuppression then gives rise to increased levels of parasitism with subsequent disease in both species (Bradley et al. 1980; Bradley 1987). This phenomenon among a few species of marsupials demonstrates a possible indirect link between testosterone and immunological fitness. Yet, little is known about the duality of testosterone in iteroparous marsupial species, and it is unclear whether parasitism is an influential pressure arising from immunosuppression broadly.

1.6. Conclusions

Conservation research and management prioritises the eradication of introduced species and the preservation of threatened species in order to prevent biodiversity loss (Garrott et al. 1993; Subroy et al. 2018). However non-threatened species are also impacted by anthropogenic changes to the environment, through local population declines and/or compromised health and welfare (Brunton et al. 2019). Kangaroos are a large, iconic native macropod common in peri-urban areas where land clearing is most rapid. High-density populations of kangaroos are also vulnerable to mass mortality events and compromised welfare (WHA 2018). Further research on high-density kangaroo populations is needed to understand and manage the threats they pose to biodiversity (Howland et al. 2014), but also the threats posed to the kangaroos themselves. Evidence-based methods to monitor the health of kangaroos and understand their unique biology are increasingly needed to inform population management strategies in the face of further habitat decline and a changing climate.

1.7. Thesis aims and structure

This thesis aims to provide a tool to assess kangaroo health and establish whether population management is necessary to improve welfare, on the grounds that animals are deemed unresponsive or incapable of appropriately responding to their environment and the stressors therein. In addition to the development of haematological RIs, two case studies are presented which use the developed RI as a method of evaluating health in a high-density kangaroo population, and for examining the relationship between testosterone and health parameters in free-ranging male kangaroos, thereby enhancing our understanding of intra-specific variations in disease susceptibility.

Chapter 2 of this thesis develops haematological RIs, unique for males and females at different stages of maturity and across seasons. It then compares these values with site-specific mean haematological values from 11 populations of kangaroos and determines the influence of biotic (sex

and sexual maturity) and abiotic (season, site, rainfall, temperature and laboratory) factors.

Chapter 3 describes a health survey from a high-density population of kangaroos which have been impacted by ongoing urbanisation and drought. This chapter utilises the described RIs to inform decision makers of the health and welfare parameters of kangaroos at the LAMN headland. The results guide management interventions aimed toward maximising animal welfare, highlighting the importance of evaluating populations compared to a 'healthy' baseline to better target management needs and outcomes.

Chapter 4 examines the effect of sex on animal health and parasite burden. By investigating the interaction of testosterone (the male reproductive hormone) with host immunological health and agents of disease, the influence of sex on disease dynamics in wildlife populations can be further understood. Kangaroos exhibit a large sexual dimorphism in body size, and to maintain a larger physical size, male kangaroos must likely have energetic trade-offs (Jarman 2000; Folstad and Carter 1992). Additionally, kangaroos have a large, diverse community of parasites. It is important to understand all aspects of wildlife disease transmission to inform decision makers on animal health, but also to understand the drivers of species evolution which may influence the population in the short term.

Lastly, Chapter 5 discusses the key research findings of this thesis, including the real-world application of haematological RIs to kangaroo populations, making a general conclusion regarding the health of high-density, peri-urban kangaroo populations. This chapter also discusses the challenges presented in Chapter 4 and suggests improvements on experimental design.

Reference intervals for parameters of health of eastern grey kangaroos (*Macropus giganteus*) and management implications across their geographic range

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2.1 Abstract

Reference intervals (RIs) describe baseline parameters of healthy animals, providing a powerful tool for wildlife managers to monitor health, identify disease and assess animal welfare. This paper reports haematological, glucose and serum protein RIs for one of Australia's most iconic and managed mammals, the eastern grey kangaroo (*Macropus giganteus*). Blood samples (n = 514) were collected from 11 populations of eastern grey kangaroos, across much of their geographic range. A species-level RI was initially established based on samples collected from four sites (n = 245) and was further partitioned based on significant differences associated with sexual maturity and season. Unique population means were established from a further seven sites to investigate the importance of biotic (sex and sexual maturity) and abiotic (season, site, rainfall, temperature and laboratory) factors on kangaroo health parameters. Random forest analysis of health parameters revealed that abiotic factors (site, rainfall, temperature and season) were largely responsible for differences in haematological, glucose and serum protein values. Sex was found to have no influence, while sexual maturity and laboratory of analysis had moderate effects. Based on these findings, interpretation of individual and population haematological and serum protein values requires careful consideration of

the timing of sample collection, environmental conditions, and sexual maturity. When assessing kangaroo health, the relevant sexual maturity RI must be considered initially. For populations with similarities to those described (e.g. high density or captive populations) users should also consider site-specific mean haematological and serum protein values. The RIs reported are valuable when establishing the health status of kangaroo populations. Furthermore, understanding the influence of biotic and abiotic factors will improve the utility of these RIs to assess health, disease status and improve welfare in eastern grey kangaroos.

2.2 Introduction

Haematological and serum protein parameters are routinely evaluated to assess health and detect disease in a wide range of species (Thrall 2012). Individual blood values can be interpreted objectively and accurately by comparing with a reference interval (RI) developed from a 'healthy' cohort of the same species (Clark 2004; Friedrichs et al. 2012). Reference intervals are increasingly used to monitor the health of wildlife, particularly in threatened species (Peck et al. 2015; Warren et al. 2015; Woolford et al. 2020) because sampling can be repeated with minimal impact on an animal's survival (Clark 2004). Reference intervals are also helpful in assessing dietary shifts, nutritional stress and can predict survival when reintroducing or translocating animals (Mathews et al. 2006; Maceda-Veiga et al. 2015). Changes in serum protein concentrations can indicate and differentiate between different parasite infections (Kaymaz et al. 1999) or can reflect energy and protein deficiencies (Robert and Schwanz 2013), assisting in diagnosis and management of animal health.

Kangaroos are a common marsupial found throughout Australia. Eastern grey kangaroos (*Macropus giganteus*) are one of the largest kangaroo species, with adult males weighing up to 85 kg (Coulson 2008). Eastern grey kangaroos occupy grassy woodlands throughout eastern and south-eastern Australia. Artificially high kangaroo densities now occur subsequent to forest clearing, improved pasture and removal of natural predators (Banks et al. 2000; Descovich et al. 2016). These high

density kangaroo populations are subject to increased risk of density dependent diseases, starvation and mass mortality (Portas and Snape 2018). Kangaroo body condition can be determined by palpating and visually assessing tail circumference/fat cover, evaluating kidney fat or bone marrow reserves, or by body condition scoring (Shepherd 1987; Moss and Croft 1999; Fletcher 2007). However, these options are destructive and/or subjective, and can have limited repeatability. Body condition indices using standardised residuals (King et al. 2011; Quesnel et al. 2017) or the 'scaled mass index' (Cripps et al. 2014; Gélin et al. 2015) can be derived from the ratio of body mass to skeletal length (Moss and Croft 1999). Condition indices are non-destructive, objective and repeatable, but like all current body condition assessments their comparative application across sites has not been tested. The management and harvesting of kangaroo populations can be controversial issues in Australia (Descovich et al. 2016). One of the rationales for kangaroo management is to minimise harm to the health and welfare of individual kangaroos in dense populations (Herbert 2004). Therefore, developing an objective tool to assess kangaroo health and welfare would be useful in kangaroo management.

There are no published haematological or serum protein RIs developed from free-ranging eastern grey kangaroos. Although several authors (Clark 2004; Vogelnest and Portas 2008; Wilcox et al. 2011; Cripps et al. 2014; Green-Barber et al. 2018) have published haematological values for kangaroos, it may be inappropriate to apply them to other populations, as they could reflect local variation rather than provide baseline values for the species (Presidente 1978). In addition, some published haematological values have been developed under non-standard circumstances. For example; samples from captive populations, samples collected within a short timeframe or samples lacking individual and population demographics, such as sex, sexual maturity or season. Furthermore, blood values reported to date are based on sample sizes (maximum n = 53 from previous studies) well below those recommended for reliable RI development (≥ 120) (Friedrichs et al. 2012). Additionally, the sources of biological and laboratory variabilities need to be examined to develop RIs as recommended by the American Society for Veterinary Clinical Pathology (ASVCP)

(Friedrichs et al. 2012). Other recommendations for developing RIs include representative sampling across different demographic classes of the population, including only healthy individuals, and standardised sample collection, handling and processing consistent for the species (Friedrichs et al. 2012). Given the often contentious nature of kangaroo management in Australia, it is important to ensure that RIs for the species are robust and that the potential effects of biotic and abiotic factors on RIs are well understood. This will enable wildlife managers to incorporate haematological parameters into evidence-based decisions on the health status of kangaroo individuals and populations.

This study provides comprehensive haematological RIs for the eastern grey kangaroo (hereafter kangaroo) and RIs for serum total protein, albumin, globulin and glucose concentrations. This study also consolidates independently collected mean haematological, glucose and serum protein values from 11 discrete populations of kangaroos spanning more than 1000 km of the species' geographical range (Figure 2.1) determined over several years. The influence of biotic (sex and sexual maturity) and abiotic (season, site, rainfall, temperature and laboratory) factors on haematological, glucose and serum protein parameters are also examined using a novel statistical approach.

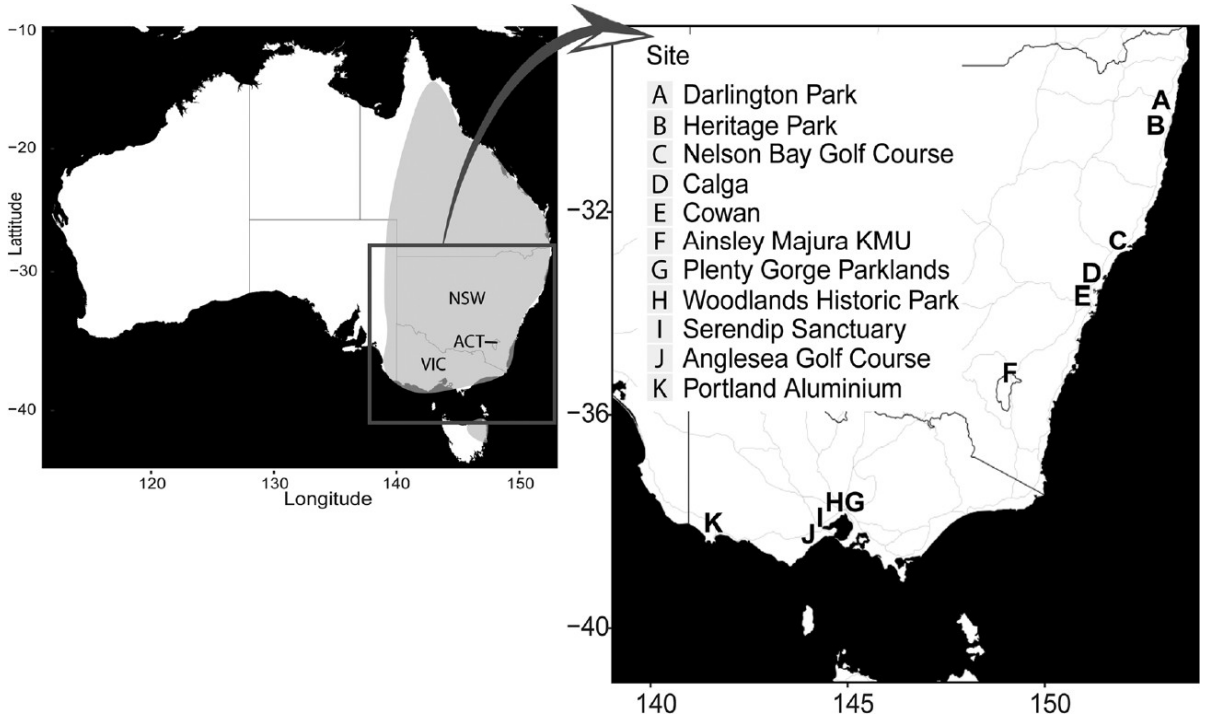


Figure 2.1 Geographic location of sampled eastern grey kangaroo (*Macropus giganteus*) populations in New South Wales (NSW), Nelson Bay Golf Course, (NBGC), Darlington Park (DP), Heritage Park (HP), Cowan (CW) and Calga (CL); Australian Capital Territory (ACT), Ainslie Majura Kangaroo Management Unit (KMU) (AM); Victoria (Vic), Anglesea Golf Course (AGC), Serendip Sanctuary (SS), Woodlands Historic Park (WHP), Portland Aluminium (PA). The grey shaded area represents the approximate distribution of eastern grey kangaroos. Populations were sampled from 2006 to 2019. The map of Australia is sourced from Stamen Design, under CC BY 3.0. Data by OpenStreetMap, under ODbL.

2.3 Material and methods

2.3.1 Data origin and site-specific details

Blood samples were collected from kangaroos at 11 sites across the species' geographic range (Table 2.1, Figure 2.1). To meet the criteria for developing RIs, Nelson Bay Golf Course (NBGC), Darlington Park (DP), Heritage Park (HP) and Ainslie Majura Kangaroo Management Unit (AM) were selected as reference sites because only healthy animals were sampled, each site had a large and representative sample size (both males, females, adults and subadults) and all samples were analysed at the same laboratory as recommended by the ASVCP (Friedrichs et al. 2012). Blood samples (n = 245) were collected from these four reference sites between 2015 and 2019. However, not every parameter was analysed for each blood sample, so the sample size for some variables differ.

Separate from the RIs developed, site-specific mean haematological, glucose and serum protein values for the species are described by combining our reference sites with data collected independently by several researchers across Australia. Data from these additional sites were collected for disease investigations, management interventions and contraceptive efficacy trials. These sites are Anglesea Golf Course (AGC; Cripps et al. 2013; Cripps et al. 2014; Cripps et al. 2016; Wilson and Coulson 2016), Serendip sanctuary (SS; Borland et al. 2012; Wilson et al. 2013), Woodlands Historic Park (WHP; Coulson 2001; Coulson et al. 2008), Plenty Gorge Parklands (PGP; Wilson et al. 2013), Portland Aluminium (PA; Death et al. 2017), Cowan (CW; Miller et al. 2017) and Calga (CL) (Table 2.1, Figure 2.1).

Table 2.1 Kangaroo site, management and clinical history information from 11 discrete populations of eastern grey kangaroos (*Macropus giganteus*) across their geographic range in Australia; Nelson Bay Golf Course, (NBGC), Darlington Park (DP), Heritage Park (HP), Ainslie Majura Kangaroo Management Unit (KMU) (AM), Anglesea Golf Course (AGC), Serendip Sanctuary (SS), Woodlands Historic Park (WHP), Portland Aluminium (PA), Cowan (CW) and Calga (CL).

Nelson Bay Golf Course (NBGC)		
Sampling details	Years sampled	2015-2019
	Sample size	≤ 83
	Laboratory	Veterinary Pathology Diagnostic Services (VPDS)
Capture/sampling type		Immobilised using Zoletil
Site information	Description	Peri-urban free-ranging population on a 20 ha^{-1}coastal golf course
	Habitat type	Pastoral grasses and dry sclerophyll vegetation
	Latitude & Longitude	-32.728°S, 152.150°E
	Clinical history	Good condition. Occasional known incidence of disease (trauma, lumpy jaw, tape worm). Primary known cause of death: motor vehicle collision (MVC)
Kangaroo management	Population density	1.88 (2015) to 1.21 (2018) individuals ha^{-1}
	Population trend	Declining
	Management intervention	Fertility control (Deslorelin contraceptive implants) applied in 2013 to 39 females
Darlington Park (DP)		
Sampling details	Years sampled	2017-2019
	Sample size	≤ 59
	Laboratory	VPDS
	Capture/sampling type	Immobilised using Zoletil
Site information	Description	Semi-rural free-ranging coastal population. Animals based in caravan park, golf course, private farmland and coastal bushland
	Habitat type	Wet and dry sclerophyll forest, ornamental grasses and grazing pasture
	Latitude & Longitude	-30.048°S, 153.191°E

	Clinical history	Good condition. Primary known cause of death is MVC
Kangaroo management	Population density	1.44 individuals ha^{-1}
	Population trend	Static
	Management intervention	Fertility control (Deslorelin contraceptive implants)applied in 2017 to 22 females
Heritage Park (HP)		
Sampling details	Years sampled	2017
	Sample size	≤ 64
	Laboratory	VPDS
	Capture/sampling type	Immobilised using Zoletil
Site information	Description	Semi-rural free-ranging population. Animals based in a newly developed housing estate, historically agricultural land
	Habitat type	Grazing pasture/grasslands and wet and dry sclerophyll forests
	Latitude & Longitude	-30.183°S, 153.149°E
	Clinical history	Animals in good condition. Occasional reports ofsubadult kangaroo mortality. MVC is the primary known cause of death
Kangaroo management	Population density	1.23-1.52 individuals ha^{-1} (Henderson et al. 2018b)
	Population trend	Increasing
	Management intervention	Fertility control (Deslorelin contraceptive implants) applied in 2017 to 45 females
Ainslie Majura Kangaroo Management Unit (KMU) (AM)		
Sampling details	Years sampled	2018
	Sample size	≤ 28
	Laboratory	VPDS
	Capture/sampling type	Cull
Site information	Description	Semi-rural free-ranging population within Mount Ainslie Nature Reserve
	Habitat type	Natural temperate grassland and box-gum grassy woodland

	Latitude & Longitude	-35.274°S, 149.165°E
	Clinical history	Good condition based on the amount of renal fat collected from fresh carcasses
Kangaroo management	Population density	1.69 individuals ha^{-1} (before 2018 cull)
	Population trend	NA
	Management intervention	Culling conducted in 2018
Anglesea Golf Course (AGC)		
Sampling details	Years sampled	2008-2012
	Sample size	≤ 60
	Laboratory	IDEXX
	Capture/sampling type	Immobilised using Zoletil
Site information	Description	Peri-urban, free-ranging population on a 73 ha^{-1} golf course
	Habitat type	Pastoral grasses and dry sclerophyll woodland with s shrub understorey
	Latitude & Longitude	-38.406°S, 144.171°E
	Clinical history	Good condition. Occasional known incidence of disease (trauma, lumpy jaw). Primary cause of death is MVC
Kangaroo management	Population density	2.0 (winter) to 3.6 (summer) individuals ha^{-1}
	Population trend	Essentially stable
	Management intervention	Fertility control applied to adult females in 2008-2011: 24 treated with Deslorelin implants and 24 with Levonorgestrel implants. Anthelmintic treatments (Ivermectin, Moxidecin, Albendazole) administered to 40 adults in 2010-2011. Albendazole administered to 42 juveniles in 2012

Serendip sanctuary (SS)		
Sampling details	Years sampled	2007-2009
	Sample size	≤ 80
	Laboratory	IDEXX and Gribbles
	Capture/sampling type	Immobilised using Zoletil or cull
Site information	Description	Peri-urban, free-ranging population in a 250 ha^{-1} nature reserve with bird breeding and display areas
	Habitat type	Improved pasture, revegetated woodland, remnant dry sclerophyll woodland and ephemeral wetlands
	Latitude & Longitude	-38.003°S, 144.411°E
	Clinical history	Poor condition. Inadequate food, high incidence of MVC and extremely high prevalence of oral necrobacillosis (lumpy jaw)
Kangaroo management	Population density	1.1–2.6 individuals ha^{-1}
	Population trend	Declining
	Management intervention	Extensive culls conducted in 2007 and 2009. Fertility control (Deslorelin implants) applied to 18 adult females in 2007-2008
Woodlands Historic Park (WHP)		
Sampling details	Years sampled	2008
	Sample size	≤ 19
	Laboratory	IDEXX
	Capture/sampling type	Cull
Site information	Description	Peri-urban population in a 300 ha^{-1} predator-proof enclosure
	Habitat type	Lowland native grassland and dry sclerophyll woodland with grassy or shrubby understorey
	Latitude & Longitude	-37.643°S, 144.531°E
	Clinical history	Generally good condition.
Kangaroo management	Population density	1.6 individuals ha^{-1}
	Population trend	Fluctuating

	Management intervention	Regular culls conducted. Fertility control (Levonorgestrel implants) applied to 25 adult females in 1999-2000
Plenty Gorge Parklands (PGP)		
Sampling details	Years sampled	2007
	Sample size	≤ 8
	Laboratory	IDEXX
	Capture/sampling type	Immobilised using Zoletil
Site information	Description	Peri-urban, free-ranging population in a 1355 ha^{-1}reserve
	Habitat type	Retired pasture, wetlands and dry sclerophyll woodland
	Latitude & Longitude	-37.629°S, 145.113°E
	Clinical history	Generally good condition
Kangaroo management	Population density	0.6 individuals ha^{-1}
	Population trend	Probably increasing
	Management intervention	Fertility control (Deslorelin implants) applied to 11 adult females in 2007-2008
Portland Aluminium (PA)		
Sampling details	Years sampled	2010-2013
	Sample size	≤ 74
	Laboratory	Gribbles
	Capture/sampling type	Immobilised using Zoletil or cull
Site information	Description	Peri-urban, free-ranging population in a 450 ha^{-1} buffer zone around an aluminium smelter
	Habitat type	Improved pasture (grazed by cattle), hardwood plantation, wetlands, coastal heathland and shrubland
	Latitude & Longitude	-38.383°S, 141.623°E
	Clinical history	Chronic fluoride exposure resulting in varying degrees of dental and skeletal fluorosis in most individuals; skeletal and dental lesions more severe in older cases, however generally good animal welfare and body condition, with

		minimal changes to other body systems noted on necropsy
Kangaroo management	Population density	~0.3 individuals ha^{-1}
	Population trend	Fluctuating
	Management intervention	Regular culls conducted. Fertility control (Levonorgestrel implants) applied to 18 adult females in 1999
	Cowan (CW)	
Sampling details	Years sampled	2006-2007
	Sample size	≤ 22
	Laboratory	IDEXX
	Capture/sampling type	Immobilised using Zoletil
Site information	Description	Captive population at The University of New South Wales (UNSW) Research Facility. Colony was enclosed within 9 ha^{-1} of natural bushland
	Habitat type	Dry sclerophyll forest and improved pasture
	Latitude & Longitude	-33.594°S, 151.156°E
	Clinical history	Good condition
Kangaroo management	Population density	NA
	Population trend	NA
	Management intervention	Supplementary feeding, bi-monthly intestinal parasite treatment (Equiban)
	Calga (CL)	
Sampling details	Years sampled	2007
	Sample size	< 5
	Laboratory	IDEXX
	Capture/sampling type	Immobilised using Zoletil
Site information	Description	68 ha^{-1} predator proof sanctuary of natural bushland
	Habitat type	Dry sclerophyll forest
	Latitude & Longitude	-33.424°S, 151.223°E
	Clinical history	Good condition
Kangaroo management	Population density	NA

Population trend	NA
Management intervention	Supplementary feeding

2.3.2 Sample collection

This work was conducted with Animal ethics approval from the University of Sydney, permit number: 2016/1062 and 2015/917. Animal collection was performed under relevant permits from all New South Wales (NSW), Victoria and ACT governments (scientific license number: SL100961 and SL102148).

Kangaroos were immobilised or opportunistically sampled from culling operations. Immobilised kangaroos were sedated using Zoletil 100® (Virbac Pty. Ltd, Milperra, Australia) at a fixed dosage of 125 mg for females or subadult males and females, and 250 mg for adult males. Zoletil was delivered via pole syringe or dart gun (X-Calibre, Pneudart Inc, Williamsport, PA, USA), as a 1 mL injection intramuscularly. Young-at-foot received a dose of approximately 65 mg as a 0.5 mL injection. Once immobilised kangaroos were transferred into a capture bag and transported to an onsite processing area (<1 km from all capture locations). During immobilisation, sex, pes and leg length (Poole et al. 1982), weight (Wedderburn ® Digital Hanging Scales, Model: WS603) and female reproductive status (pouch young, lactating, young-at-foot, non-reproductive) were recorded. Female maturity was determined by teat eversion (adult female) and reproductive status (presence of a pouch young). Male maturity was determined by leg length; > 52.3 cm were deemed sexually mature (Poole 1973; Poole et al. 1982). Each kangaroo was examined clinically for visible injuries and body condition. Up to 6 mL of whole blood was collected from the lateral caudal vein, approximately 30 min after immobilisation usinga 20-22 gauge butterfly catheter attached to a 5 mL syringe (Luer slip, Terumo Australia Pty Limited, Macquarie Park).

Kangaroos from culling operations were dispatched by authorised shooters and transported to a central processing site on a trailer bed. Fresh carcasses were randomly selected and processed immediately.

Whole blood was collected by cardiac venipuncture using an 18 gauge needle and attached 5 mL syringe. Most blood samples were collected within 5 mins of death; the maximum time for blood

collection post-death was 30 mins. For both immobilised and culled animals, blood was immediately transferred into a 5 mL BD Vacutainer® SST™ II Advance tube (Becton Dickinson and Company, North Ryde, Australia) and 1.3 mL EDTA tube (Becton Dickinson and Company, North Ryde, Australia). Whole blood was gently inverted and stored on an ice brick prior to processing. Whole blood was allowed to clot, then centrifuged for 10 mins at 3000 rpm (LW Scientific E8C-08AF-150P Porta-Fuge Portable 12 Volt Centrifuge). Serum was stored at -20°C after separation. Two blood smears were freshly made using whole blood preserved in EDTA. Blood smears were air dried, fixed in methanol and stained with Diff Quik (Lab Aids Pty Ltd). Whole blood preserved in EDTA was mixed with Streck Cell Preservative (1:1 ratio, Streck, Omaha, NE USA) and stored at 4°C prior to analysis.

2.3.3 Haematological and glucose analyses for the reference populations

Once collected, EDTA whole blood samples preserved in Streck were analysed using a Sysmex XN1000i automated haematology analyser (Roche diagnostics, Australia) at the Veterinary Pathology Diagnostic Services (VPDS), Sydney School of Veterinary Science, The University of Sydney, NSW within seven days of blood collection. Quality control of the analyser is maintained internally every day, and externally cyclically, by relevant quality assurance programs under the Royal College of Pathologists Australasia (RCPAQAP). The following parameters were determined: haematocrit (HCT; L/L), haemoglobin (HGB; g/L), total red blood cell count (RBC; $x10^{12}$/L), total white blood cell count (WBC; $x10^{9}$/L) platelet count (PLT; $x10^{9}$/L) and nucleated red blood cell count (NRBC; $x10^{9}$/L). Values were doubled to correct for dilution with cell preservative. Mean cell volume (MCV; fL; (HCT/1000)/RBC), mean cell haemoglobin (MCH; pg; (HGB/RBC)) and mean corpuscular haemoglobin concentration (MCHC; g/L; (HGB/HCT)) were calculated manually. Haemolysed samples collected from shot individuals were excluded from analysis.

One hundred WBCs were differentiated on stained blood smears to determine the percentage of neutrophils, lymphocytes, eosinophils, monocytes and basophils. Absolute WBC counts were determined by multiplying the percentage of each WBC type determined on the blood smear (%) by the total WBC count determined by the automated analyser ($x10^{9}$/L). Blood glucose concentration

was determined immediately after collection in the field. One to two drops of fresh blood was applied to a glucose strip (Freestyle optimum glucose strips, Abbott, Alameda, California) and blood glucose determined using a hand-held glucose monitoring device (Freestyle Optium Neo Blood Glucose Monitoring System, Abbott, Alameda, California).

2.3.4 Serum protein analysis for the reference population

Frozen serum was thawed at room temperature prior to analysis. Albumin (g/L), total protein (TP; g/L) and globulin (g/L) concentrations were determined using the Konelab Prime 30i analyser (Thermo scientific, Australia) at VPDS, within seven days of blood collection. Relevant external quality control measures were undertaken regularly (Randox, County Antrim, United Kingdom).

2.3.5 Statistical analyses

2.3.5.1 Species-level reference intervals of health parameters

Data analysis followed the methods outlined by the 2012 ASVCP guidelines for the development of RIs (Friedrichs et al. 2012). RIs were developed using R version 3.5.3 statistical software (R core team 2017) in the 'referenceIntervals' version 1.1.1 R package (Finnegan 2015). Outliers were identified and removed using Horn's algorithm (using Tukey's interquartile fences on a Box-Cox transformation) or Cook's distance (for datasets containing zeros). Identified values using Cook's distance were confirmed visually as true outliers by evaluating a histogram of the data.

Based on the recommendations by the ASVCP (Friedrichs et al. 2012), non-parametric methods with 95% confidence intervals (CI) were used where n > 120 reference samples were available, or if alternative methods could not be applied. With > 40 and < 120 reference samples, robust methods with 90% CIs with bootstrapping (Friedrichs et al. 2012) were used on symmetrical data. For asymmetric data, normality was assessed. If data were normally distributed, parametric analysis was performed. If data were not normally distributed, non-parametric methods were used and the mean (± standard deviation; SD) and median presented. When > 20 and < 40 reference samples were

available, normally distributed data were analysed using parametric methods, and non-normal data were analysed using robust methods; minimum and maximum values presented. When < 20 reference samples were available, only the mean value (± SD) is presented. Basophil and NRBC counts contained too many zero values to calculate a RI; instead a range of observed values is presented.

Species-level RIs were created by combining data from both sexes (male and female), maturity (subadult, adult), sites (DP, HP, NBGC and AM) and seasons (summer, autumn, winter and spring). To minimise overrepresentation of a subgroup into the RI, each sex/maturity/site and season combination was sampled equally (where possible). Statistical criteria to aid partitioning was employed for each factor (sex, season and sexual maturity). Normally distributed data (albumin, total protein and globulin) were partitioned using distance between reference limits of the subgroup distributions (Lahti et al. 2002). Non-normal data were partitioned based on the proportion of observations in the distributions of the subgroups that fell outside of the combined RIs that were ≥ 4.1% or ≤ 0.9% (Lahti et al. 2004). Maturity RIs (subadult and adult) included reference samples from both sexes, sites and seasons. Seasonal RIs were created only for summer, autumn and winter due to the low sample size (n < 20) for animals sampled in spring.

2.3.5.2 Site-specific mean health parameters

Mean values (± SD) were determined for each site using all data (including sex, maturity, season and sampling years).

2.3.5.3 Influence of biotic and abiotic factors on reference intervals and site-specific means

2.3.5.3.1 Significance and effect size of biotic and abiotic factors

Effect size (ES) was used to examine the size of the difference between two groups (Nakagawa and Cuthill 2007). ES benchmark values of 0.2, 0.5 and 0.8 were selected to indicate small, medium and large effect size respectively (Cohen 1977). ES were established using the ‘effsize’ and ‘MBESS’ R

packages (R version 3.5.3). For biotic factors with two sets of observations (male vs female and subadult vs adult), ES was calculated using Cohen's *d* statistics (Durlak 2009), as the data contained both continuous and categorical data (Nakagawa and Cuthill 2007). A *d* value of 0.5 can be interpreted as the group means differing by 0.5 SDs. An ES of zero indicates there is no effect, whilst either a low or upper CI of zero (or near zero) indicates the subgroup mean differences are quite small. The ES for season was calculated using *eta* squared (η^2) which can be employed when there are more than two sets of observations (Lakens 2013; Maher et al. 2013). η^2 benchmark values of 0.01, 0.06 and 0.14 indicate small, medium and large ES respectively (Maher et al. 2013). A η^2 of 0.14 for season means that 14% of the total variance can be accounted for by season. CIs were calculated for both *d* and η^2 ES point estimates. Negative ES indicate a decrease in the parameter of interest, for example lower RBC count in female kangaroos, while positive ES indicate an increase. To facilitate comparison of ES among all variables, ES estimates (*d* or η^2) and 95% CIs of *d* and η^2 are depicted in a forest plot for each selected factor. If the ES was categorised as medium to large, factorial significance was also calculated. To establish factorial significance, a Shapiro-Wilk normality test was initially performed for each haematological, glucose and serum protein parameter, within each factor. Where parameters were normally distributed ($P > 0.05$), the parameters were assessed using one-way analysis of variance (ANOVA). For parameters that were not normally distributed ($P < 0.05$), either a square, log or cube root transformation was performed before analysis using ANOVA. If a normal distribution of the data could not be achieved (on either native or transformed data) the non-parametric Kruskal-Wallis (KW) test was applied. The significance between sites was established using the same methodology.

2.3.5.3.2 Random forest model of factors influencing parameters of health

To explore the effects of biotic and abiotic factors on the haematological, glucose and serum protein values of kangaroos, random forest (RF) models were used to analyse parameters from all 11 sites. RF models have high accuracy with complex datasets, can incorporate multiple predictor variables, automatically handle missing data, and are easy to apply and interpret (Cutler et al. 2007). RF

models are also robust to collinearity, which precluded the use of linear models on this dataset. 18 models, one for each parameter, were created using the 'randomForest' R package. Each factor (site, season, sex, sexual maturity, laboratory, rainfall and temperature) was selected to train the model based on their known influence on haematological and serum protein parameters (Pacioni et al. 2013; Fancourt and Nicol 2019). Average monthly rainfall and temperature data were obtained from the nearest weather station (BOM 2020) for the month preceding collection for each site. Samples were analysed by three commercial laboratories (VPDS, IDEXX Laboratories (IDEXX), NSW and Victoria (Vic) and Gribbles Veterinary Pathology (Gribbles), Vic), so laboratory was used as a factor in the RF model. RF models were grown using 1000 trees, with each tree using a bootstrap sample of 66% of the data. The number of variables tested at each split (mtry) was set at seven. Percentage of variation explained (PVE), and root mean square error (RMSE) were calculated for each model using 'out of the bag' predictions. A randomisation technique was used to test whether the model was significantly different to that of a random model (Murphy et al. 2010). Biotic and abiotic factors for each model were randomised and re-run 1000 times. The PVE from both the real (non-random data) model and the random model were compared and a *P* value generated (Evans and Cushman 2009; Murphy et al. 2010) to evaluate model significance. Finally, the percent increase in mean square error (%IncMSE) was used to determine the importance of each selected biotic and abiotic factor on each haematological, glucose and serum protein parameter (Breiman 2001; Evans and Cushman 2009). The %IncMSE provides a measure of how much the predictive ability of the model is reduced when the effect of a certain parameter is excluded. To demonstrate the collective importance of each factor, the number of times a factor occurred in the top three %IncMSE for each parameter was recorded and summed to create a rank score (ascending in importance). To examine the effect of temperature and rainfall on haematological, glucose and serum protein values, partial dependence plots (PDPs) were generated for all parameters with a PVE greater than 50. Predicted effects from models lower than a PVE of 50 were not used in this analysis due to their potential lack of reliability (Evans et al. 2011).

2.4 Results

2.4.1 Species-level reference intervals of health parameters

Species-level haematological, glucose and serum protein RIs for the reference population are presented in Table 2.2. Most parameters for each factor satisfied statistical criteria recommended for partitioning datasets into selected factors (Lahti et al. 2002; Lahti et al. 2004). Maturity- and season-specific RIs are presented in Tables 2.3-2.4 respectively and are described in more detail in section 3.3.

Table 2.2 Non-parametric haematological, glucose and serum protein reference intervals for free-ranging eastern grey kangaroos (*Macropus giganteus*) sampled from reference sites; Nelson Bay Golf Course, (NBGC), Darlington Park (DP), Heritage Park (HP) and Ainslie Majura Kangaroo Management Unit (KMU) (AM) from 2015 to 2019.

Parameter	Units	n	Lower reference interval (CI)	Upper reference interval (CI)	Mean (SD)	Median
Red blood cell count	10^{12}/L	227	1.52 (1.34-1.7)	4.83 (4.58-5.49)	2.99 (0.87)	2.92
White blood cell count	10^9/L	223	2.79 (2.16-3.5)	13.1 (12.08-14.36)	7.1 (2.46)	6.9
Glucose	mmol/L	133	1.84 (1.7-2.2)	7.06 (5.6-8.9)	3.65 (1.21)	3.5
Neutrophil count	10^9/L	197	0.49 (0.45-0.68)	5.17 (4.87-5.7)	2.47 (1.16)	2.32
Lymphocyte count	10^9/L	194	1.56 (1.34-1.99)	8.3 (7.61-8.75)	3.85 (1.63)	3.47
Eosinophil count	10^9/L	196	0.04 (0)	1.39 (1.29-1.41)	0.56 (0.36)	0.51
Monocyte count	10^9/L	196	0 (0)	0.52 (0.45-0.65)	0.13 (0.15)	0.08
Basophil count	10^9/L	195	0 (0)[b]	0.09 (0.07-0.1)[b]	0.01 (0.02)	0
Haemoglobin	g/L	226	96.7 (92-102)	164 (158-170)	129.02 (16.7)	128
Haematocrit	L/L	233	0.14 (0.09-0.16)	0.41 (0.4-0.45)	0.27 (0.08)	0.27
Mean corpuscular volume	fL	232	62.15 (59.18-63.4)	107.91 (105.51-108.99)	91.12 (11.46)	94.35
Mean corpuscular haemoglobin	pg	232	22.42 (20.77-24)	74.02 (67.82-76.12)	44.9 (11.86)	43.22
Mean corpuscular haemoglobin conc.	g/L	234	343.37 (327.59-349.36)	810.71 (718.31-1156.86)	497.72 (139.3)	468.77
Platelet	10^9/L	218	66.95 (60-85)	286.1 (258-312)	155.94 (48.91)	151.5
Nucleated red blood cell count	10^9/L	71	0 (0)[b]	0.14 (0.14-0.16)[b]	0.03 (0.04)	0.02
Albumin	g/L	100	18.06 (16.11-19.89)	42.59[a] (40.92-44.23)	29.91 (6.14)	
Total protein	g/L	55	54.19 (51.79-56.46)	77.37[a] (75.35-79.8)	65.55 (5.68)	
Globulin	g/L	55	22.55 (20.54-24.57)	41.55[a] (40.1-43.19)	31.67 (4.67)	

CI, confidence interval; SD, standard deviation. [a] Calculated using robust methods; [b] Range of observed values

Table 2.3 Maturity-specific haematological, glucose and serum protein reference intervals for adult and subadult free-ranging eastern grey kangaroos (*Macropus giganteus*) sampled from reference sites; Nelson Bay Golf Course, (NBGC), Darlington Park (DP), Heritage Park (HP) and Ainslie Majura Kangaroo Management Unit (KMU) (AM) from 2015 to 2019.

Parameter	Units	Maturity	n	Lower reference interval (CI)	Upper reference interval (CI)	Mean (SD)	Minimum	Maximum	Median
Red blood cell count	10^{12}/L	Adult	178	1.49[b] (1.34-1.65)	4.51[b] (4.32-4.78)	2.87 (0.78)			2.82
		Subadult	54	0.46[a] (0-1.14)	6.27[a] (5.61-6.97)	3.53 (1.41)			
White blood cell count	10^{9}/L	Adult	173	1.78[b] (1.38-2.96)	11.88[b] (10.72-12.8)	6.93 (2.28)			6.96
		Subadult	53	2.28[b] (1.4-2.56)	15.09[b] (14.83-16.62)	7.37 (3.26)			6.46
Glucose	mmol/L	Adult	98	1.09[a] (0.71-1.52)	5.47[a] (5.03-5.91)	3.43 (1.09)			
		Subadult	35	1.31[a] (0.37-2.24)	6.77[a] (5.89-7.78)	4.28 (1.32)	2.5	8.9	
Neutrophil count	10^{9}/L	Adult	155	0.62[b] (0.45-5.24)	5.24[b] (4.99-5.74)	2.6 (1.15)			2.51
		Subadult	42	0[a] (0-0.19)	4.1[a] (3.53-4.7)	2 (1.08)			
Lymphocyte count	10^{9}/L	Adult	161	0.74[b] (0.63-1.51)	7.59[b] (6.95-8.31)	3.52 (1.53)			3.36
		Subadult	40	0.09[a] (0-1.24)	8.56[a] (7.44-9.82)	4.63 (2.04)	1.34	9.2	
Eosinophil count	10^{9}/L	Adult	155	0.04[b] (0-0.07)	1.39[b] (1.28-1.44)	0.56 (0.36)			0.51
		Subadult	39	0[a] (0)	1.1[a] (0.9-1.28)	0.56 (0.36)	0	1.44	
Monocyte count	10^{9}/L	Adult	155	0[b] (0)	0.43[b] (0.35-0.53)	0.11 (0.12)			0.07
		Subadult	40	0[b] (0)	0.66[b] (0.65-0.8)	0.19 (0.19)	0	0.66	0.15
Basophil count	10^{9}/L	Adult	155	0[b,d] (0-0.08)	0.08[b,d] (0.07-0.1)	0.01 (0.02)			0
		Subadult	40	0[b,d] (0)	0.09[b,d] (0.09-0.13)	0.01 (0.03)	0	0.09	0
Haemoglobin	g/L	Adult	175	98.8[b] (92-106)	164[b] (158-170)	129.58 (16.4)			128
		Subadult	51	88.28[a] (80.99-95.48)	162.52[a] (154.8-1713)	125.27 (18.27)			
Haematocrit	L/L	Adult	179	0.15[b] (0.1-0.16)	0.41[b] (0.39-0.43)	0.27 (0.07)			0.26
		Subadult	54	0.12[a] (0.08-0.16)	0.47[a] (0.44-0.5)	0.29 (0.09)			
Mean corpuscular volume	fL	Adult	178	61.82[b] (58.27-63.4)	108.35[b] (105.56-109.86)	92.77 (10.66)			95.23
		Subadult	54	60.95[a] (54.91-65.65)	112.17[a] (106.59-116.52)	85.73 (12.42)			
Mean corpuscular haemoglobin	pg	Adult	179	29.94[b] (24.69-32.86)	75.62[b] (69.51-86.76)	47.21 (11.58)			45.4
		Subadult	53	15.06[a] (10.28-19.02)	60.72[a] (56.44-65.15)	38.31 (11.22)			
Mean corpuscular haemoglobin concentration	g/L	Adult	180	352.13[b] (343.95-364.53)	793.64[b] (718.31-1156.86)	509.79 (131.72)			482.74
		Subadult	54	315.47[b] (293.16-322.74)	1132.14[b] (984.29-1602.43)	457.46 (156.72)			418.9
Platelet	10^{9}/L	Adult	168	74.38[b] (58-94)	259.55[b] (238-318)	155.74 (43.97)			152
		Subadult	51	11.43[a] (0-33.92)	278.87[a] (247.78-310.4)	154.12 (65.1)			
Nucleated red blood cell count	10^{9}/L	Adult	45	0[b,d] (0)	0.16[b,d] (0.15-0.18)	0.04 (0.04)	0	0.16	0.02
		Subadult	26	0[b,d] (0)	0.06[b,d] (0.06-0.07)	0.02 (0.02)	0	0.06	0.02
Albumin	g/L	Adult	68	16.28[a] (13.99-18.55)	42.27[a] (40.23-44.48)	28.97 (6.46)			
		Subadult	32	22.32[c] (19.88-24.75)	41.53[c] (39.09-43.96)	31.92 (4.9)	17	40.7	
Total protein	g/L	Adult	31	59.14[a] (58.65-59.14)	80.99[a] (80.99-85.42)	67.14 (5.5)	59.14	80.99	
		Subadult	26	48.75[c] (44.66-52.84)	77.85[c] (73.76-81.94)	63.3 (7.42)	45.7	73.88	
Globulin	g/L	Adult	30	24.35[a] (21.85-26.66)	41.76[a] (39.98-43.79)	32.8 (4.15)	25.12	40.03	
		Subadult	26	19.63[c] (16.74-22.53)	40.24[c] (37.34-43.14)	29.94 (5.26)	20.38	39.39	

CI, confidence interval; SD, standard deviation. [a]Calculated using robust methods; [b]Calculated using non-parametric methods; [c]Calculated using parametric methods; [d] Range of observed values

Table 2.4 Seasonal haematological, glucose and serum protein reference intervals for free-ranging eastern grey kangaroos (*Macropus giganteus*) sampled from reference sites; Nelson Bay Golf Course, (NBGC), Darlington Park (DP), Heritage Park (HP) and Ainslie Majura Kangaroo Management Unit (KMU)(AM) from 2015 to 2019.

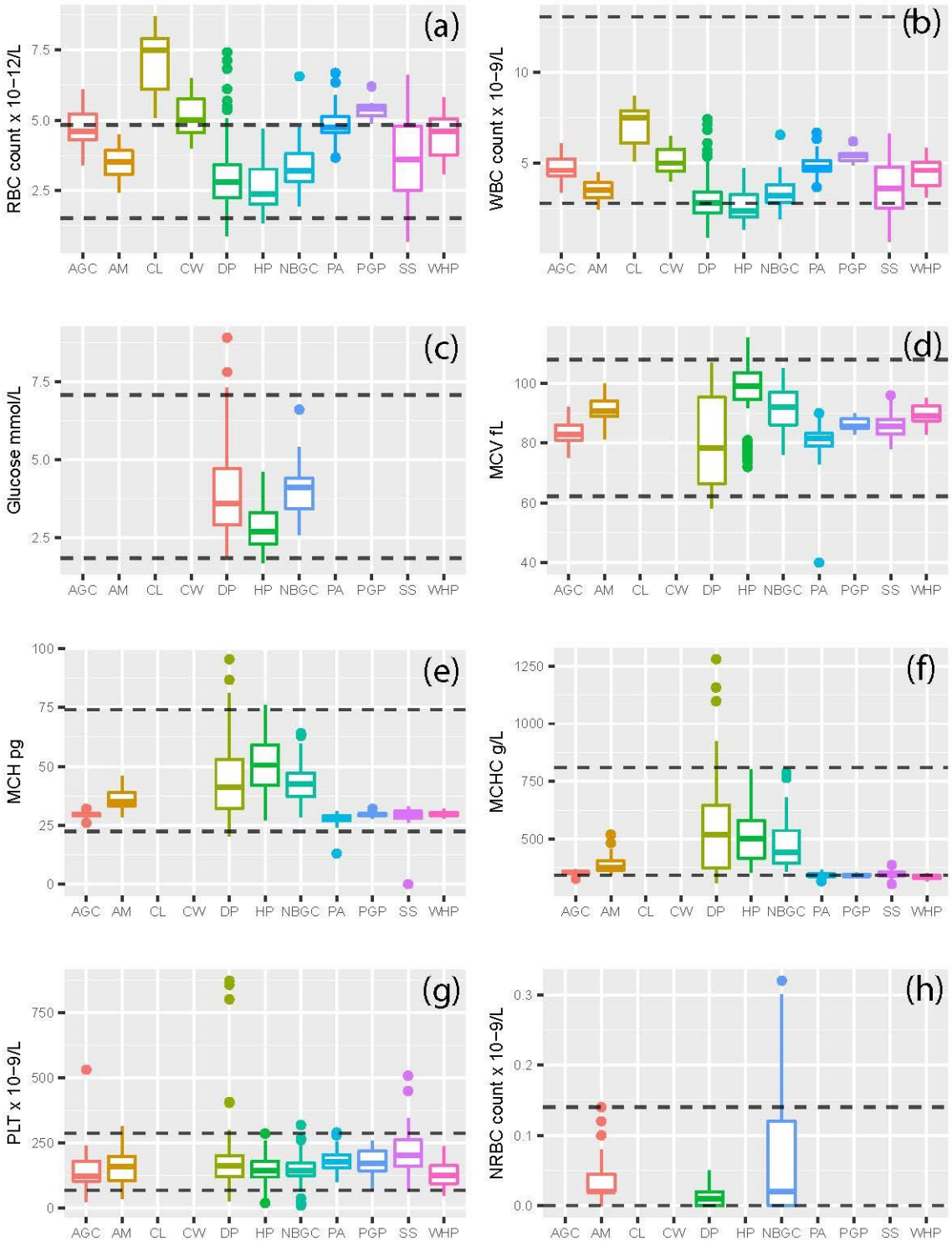
Parameter	Units	Season	n	Lower reference interval (CI)	Upper reference interval (CI)	Mean (SD)	Minimum	Maximum	Median
Red blood cell count	10^{12}/L	Summer	47	1.88[a] (1.68-2.04)	3.59[a] (3.43-3.76)	2.74 (0.41)			
		Autumn	101	0.7[a] (0.27-1.1)	5.52[a] (5.08-5.99)	3.3 (1.21)			
		Winter	63	1.43[a] (1.15-1.67)	4.82[a] (4.55-5.12)	3.13 (0.84)			
		Spring	19			2.18			
White blood cell count	10^{9}/L	Summer	49	2.66[a] (1.91-3.42)	10.97[a] (10.22-11.84)	6.84 (2.04)			
		Autumn	101	0.01[a] (0-0.9)	13.74[a] (12.69-14.8)	7.07 (3.44)			
		Winter	61	3.21[a] (2.56-3.79)	9.95[a] (9.33-10.63)	6.72 (1.65)			
		Spring	17			7.1			
Glucose	mmol/L	Summer	49	1.69[a] (1.29-2.1)	5.53[a] (5.03-5.95)	3.72 (0.94)			
		Autumn	44	0.25[a] (0-1.13)	7.02[a] (6.18-7.97)	3.87 (1.65)			
		Winter	23	1.7[c] (1.33-2.07)	4.2[c] (3.83-4.58)	2.95 (0.64)	1.7	4.3	
		Spring	17			3.86			
Neutrophil count	10^{9}/L	Summer	47	0.91[b] (0.58-0.92)	5.66[b] (5.61-6.2)	2.76 (1.23)			2.39
		Autumn	94	0[a] (0)	5.08[a] (4.65-5.54)	2.41 (1.39)			
		Winter	49	0[a] (0-0.47)	4.62[a] (4.01-5.33)	2.44 (1.15)			
		Spring	13			2.4			
Lymphocyte count	10^{9}/L	Summer	47	1.03[a] (0.62-1.55)	5.49[a] (5.05-5.98)	3.28 (1.09)			
		Autumn	90	0.81[b] (0.32-0.83)	8.74[b] (8.27-9.29)	4.17 (2.02)			3.76
		Winter	49	1.54[b] (1.1-1.58)	7.26[b] (6.91-8.76)	3.5 (1.29)			3.17
		Spring	11			3.77			
Eosinophil count	10^{9}/L	Summer	44	0[a] (0-0.03)	1.09[a] (0.96-1.23)	0.52 (0.29)			
		Autumn	91	0.02[b] (0-0.04)	1.37[b] (1.34-1.45)	0.54 (0.38)			0.44
		Winter	47	0[a] (0)	1.34[a] (1.17-1.54)	0.58 (0.38)			
		Spring	12			0.55			
Monocyte count	10^{9}/L	Summer	45	0[b] (0)	0.45[b] (0.44-0.56)	0.11 (0.12)			0.08
		Autumn	90	0[b] (0)	0.53[b] (0.34-0.62)	0.13 (0.16)			0.07
		Winter	47	0[b] (0)	0.22[b] (0.22-0.23)	0.07 (0.08)			0.04
		Spring	12			0.34			
Basophil count	10^{9}/L	Summer	42	0[b,d] (0)	0.01[b,d] (0.01-0.03)	0 (0)			0
		Autumn	90	0[b,d] (0)	0.05[b,d] (0.02-0.07)	0.01 (0.02)			0
		Winter	45	0[b,d] (0)	0.1[b,d] (0.1-0.11)	0.01 (0.03)			0
		Spring	12			0.02			
Haemoglobin	g/L	Summer	46	93.26[a] (86.92-99.17)	153.07[a] (146.45-159.62)	124.04 (14.64)			
		Autumn	99	94.04[a] (89.69-97.95)	154.86[a] (150.11-159.52)	125.39 (15.18)			
		Winter	62	104.23[a] (98.91-109.54)	169.27[a] (164.13-174.2)	136.55 (16.01)			
		Spring	16			123.5			
Haematocrit	L/L	Summer	49	0.12[a] (0.1-0.14)	0.33[a] (0.3-0.35)	0.23 (0.05)			
		Autumn	102	0.14[a] (0.12-0.16)	0.44[a] (0.42-0.46)	0.29 (0.07)			
		Winter	63	0.16[a] (0.13-0.18)	0.44[a] (0.42-0.47)	0.3 (0.07)			

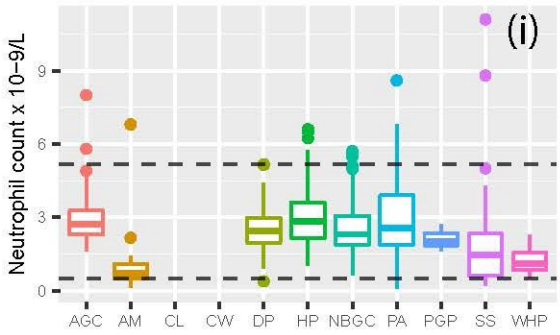
		Spring	19			0.2			
Mean corpuscular volume	fL	Summer	49	62.2[a] (56.57-69.76)	111.86[a] (108.31-115.94)	84.15 (12.19)			
		Autumn	101	62.48[b] (60.29-64.45)	108.33[b] (107.66-110.93)	91.69 (11.71)			94.42
		Winter	63	81.61[a] (78.27-84.87)	112.1[a] (109.61-114.74)	95.89 (7.53)			
		Spring	19			90.4			
Mean corpuscular haemoglobin	pg	Summer	49	29.18[a] (26.25-31.7)	62.6[a] (59.41-66.72)	46.34 (8.02)			
		Autumn	102	21.08[b] (19.89-21.68)	75.57[b] (75.03-80.81)	42.5 (14.1)			39.21
		Winter	64	28.48[c] (25.49-31.48)	61.88[c] (58.88-64.87)	45.18 (8.52)			
		Spring	19			57.99			
Mean corpuscular haemoglobin concentration	g/L	Summer	49	341.79[a] (298.79-379.42)	770.6[a] (728.26-816.97)	558.82 (105.27)			
		Autumn	102	331.31[b] (320.66-354.43)	714.02[b] (628.03-755.66)	454.42 (112.24)			404.3
		Winter	64	378.46[b] (373.17-382.62)	686.92[b] (683.36-755.6)	469.74 (81.05)			441.77
		Spring	19			666.8			
Platelet	10^9/L	Summer	44	95[b] (77.75-96)	311.5[b] (305-376.5)	159.77 (44.63)			150
		Autumn	100	36.62[a] (20.48-52.55)	265.68[a] (248.24-284.19)	154.98 (57.35)			
		Winter	57	73.23[a] (60.83-86.23)	209.94[a] (196.2-224.86)	141.3 (33.79)			
		Spring	19			148.47			
Nucleated red blood cell count	10^9/L	Summer	17			0.04			0.02
		Autumn	42	0[b,d] (0)	0.08[b,d] (0.08-0.1)	0.02 (0.02)			0.02
		Winter	4			0.01			
		Spring	6			0.01			
Albumin	g/L	Summer	28	23.73[c] (21.1-26.35)	43.1[c] (40.47-45.72)	33.41 (4.94)	25	41.06	
		Autumn	28	21.63[c] (18.95-24.31)	43.39[c] (38.71-44.07)	31.51 (5.04)	16	39	
		Winter	33	16.01c (13.77-18.25)	33.94[c] (31.7-36.17)	24.97 (4.57)	16	34.66	
		Spring	8			32.72			
Total protein	g/L	Summer	25	53.68[c] (50.19-57.17)	78.01[c] (74.52-81.5)	65.85 (6.21)	50.57	76.62	
		Autumn	16			65.25			
		Winter	6			65.17			
		Spring	8			65.34			
Globulin	g/L	Summer	27	21.71[c] (18.88-24.53)	42.17[c] (39.34-44.99)	31.94 (5.22)	20.38	40.03	
		Autumn	18			32.04 (32.38)			
		Winter	6			31.4			
		Spring	6			30.82			

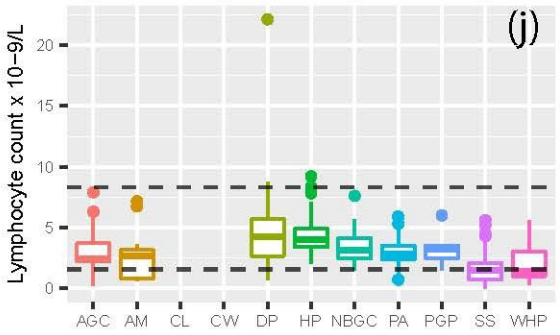
CI, confidence interval; SD, standard deviation. [a]Calculated using robust methods; [b]Calculated using non-parametric methods; [c]Calculated using parametric methods; [d] Range of observed values

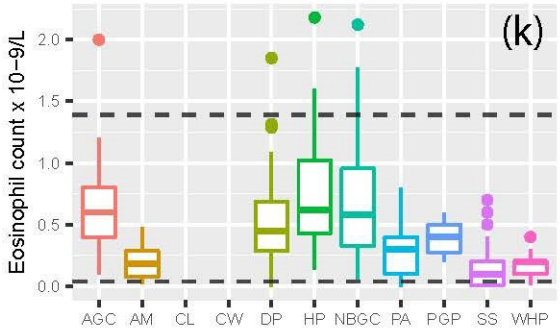
2.4.2 Site-specific mean health parameters

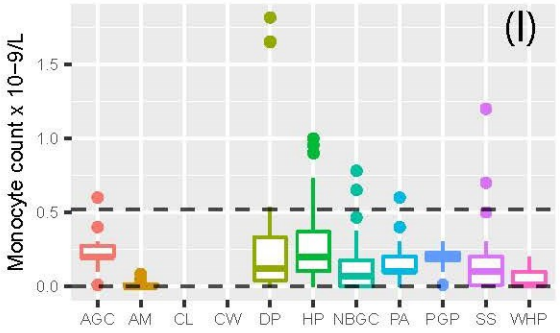
Site-specific mean haematological, glucose and serum protein parameters are presented as descriptive box plots in Figure 2.2 and described in Table 2.5. Values were included from a maximum of n = 269 individuals. Differences among site means were dependent on the parameter examined.

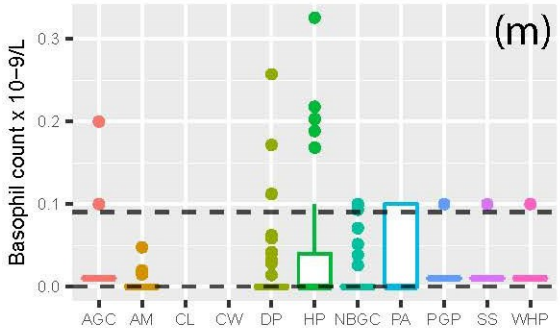

(a)
RBC count x 10−12/L
7.5
5.0
2.5
AGC AM CL CW DP HP NBGC PA PGP SS WHP
(b)
WBC count x 10−9/L
10
5
AGC AM CL CW DP HP NBGC PA PGP SS WHP
(c)
Glucose mmol/L
7.5
5.0
2.5
AGC AM CL CW DP HP NBGC PA PGP SS WHP
(d)
MCV fL
100
80
60
40
AGC AM CL CW DP HP NBGC PA PGP SS WHP
(e)
MCH pg
100
75
50
25
0
AGC AM CL CW DP HP NBGC PA PGP SS WHP
(f)
MCHC g/L
1250
1000
750
500
AGC AM CL CW DP HP NBGC PA PGP SS WHP
(g)
PLT x 10−9/L
750
500
250
0
AGC AM CL CW DP HP NBGC PA PGP SS WHP
(h)
NRBC count x 10−9/L
0.3
0.2
0.1
0.0
AGC AM CL CW DP HP NBGC PA PGP SS WHP

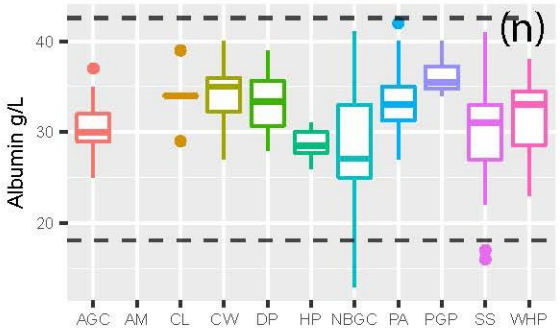

(i)
Neutrophil count x 10−9/L
0
3
6
9
AGC AM CL CW DP HP NBGC PA PGP SS WHP

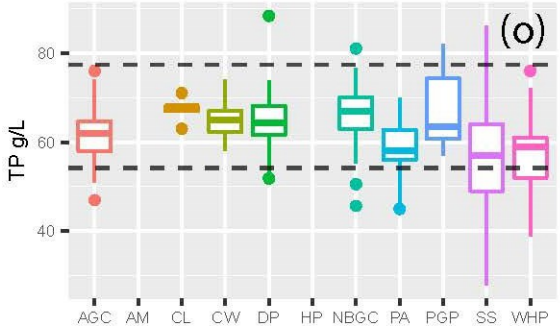

(j)
Lymphocyte count x 10−9/L
0
5
10
15
20
AGC AM CL CW DP HP NBGC PA PGP SS WHP

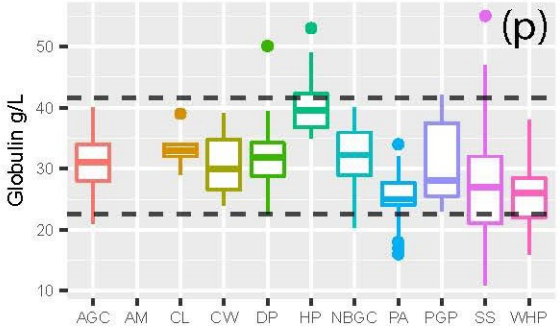

(k)
Eosinophil count x 10−9/L
0.0
0.5
1.0
1.5
2.0
AGC AM CL CW DP HP NBGC PA PGP SS WHP

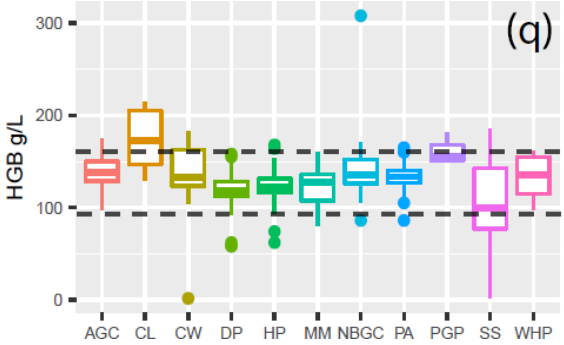

(l)
Monocyte count x 10−9/L
0.0
0.5
1.0
1.5
AGC AM CL CW DP HP NBGC PA PGP SS WHP


(m)
Basophil count x 10−9/L
0.0
0.1
0.2
0.3
AGC AM CL CW DP HP NBGC PA PGP SS WHP


(n)
Albumin g/L
20
30
40
AGC AM CL CW DP HP NBGC PA PGP SS WHP


(o)
TP g/L
40
60
80
AGC AM CL CW DP HP NBGC PA PGP SS WHP


(p)
Globulin g/L
10
20
30
40
50
AGC AM CL CW DP HP NBGC PA PGP SS WHP


(q)
HGB g/L
0
100
200
300
AGC CL CW DP HP MM NBGC PA PGP SS WHP

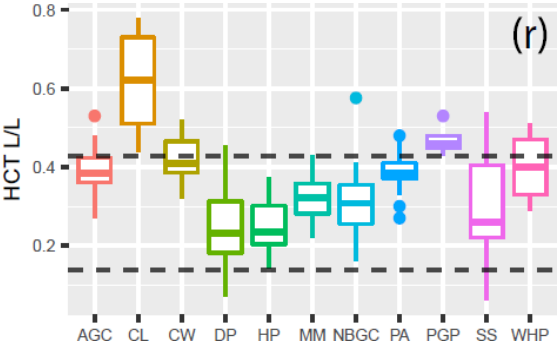

(r)
HCT L/L
0.2
0.4
0.6
0.8
AGC CL CW DP HP MM NBGC PA PGP SS WHP

Figure 2.2 Box and whisker plots of site-specific mean haematological, glucose and protein values for eastern grey kangaroos (*Macropus giganteus*) from 11 populations; Nelson Bay Golf Course, (NBGC), Darlington Park (DP), Heritage Park (HP), Ainslie Majura Kangaroo Management Unit (KMU) (AM), Anglesea Golf Course (AGC), Serendip Sanctuary (SS), Woodlands Historic Park (WHP), Portland Aluminium (PA), Cowan (CW) and Calga (CL). Box plots display the median, with the lower and upper limits of the box corresponding to the 25th and 75th percentiles. The upper whisker extends to the largest value no further than 1.5 times the interquartile range. The lower whisker extends to the smallest value at most 1.5 times the interquartile range. Data beyond the end of the whiskers are outliers and are plotted individually. Dashed lines indicate upper and lower reference intervals (RI) as calculated from the reference sites. (a) Red blood cell count (RBC), (b) White blood cell count (WBC), (c) Glucose, (d) Mean corpuscular volume (MCV), (e) Mean corpuscular haemoglobin (MCH), (f) Mean corpuscular haemoglobin concentration (MCHC), (g) Platelets (PLT), (h) Nucleated red blood cell count (NRBC), (i) Neutrophil count, (j) Lymphocyte count, (k) Eosinophil count, (l) Monocyte count, (m) Basophil count , (n) Albumin, (o) Total protein (TP), (p) Globulin, (q) Haemoglobin (HGB), (r) Haematocrit (HCT).

Table 2.5 Site-specific mean haematological, glucose and serum protein values for eastern grey kangaroos (*Macropus giganteus*) from 11 populations; Nelson Bay Golf Course, (NBGC), Darlington Park (DP), Heritage Park (HP), Ainslie Majura Kangaroo Management Unit (KMU) (AM), Anglesea Golf Course (AGC), Serendip Sanctuary (SS), Woodlands Historic Park (WHP), Portland Aluminium (PA), Cowan (CW)and Calga (CL).

Variable	Unit	*P*	Site	n	Mean (SD)	Minimum	Maximum
Red blood cell count	10^{12}/L	<0.001	NBGC	82	3.29 (0.69)		
			DP	54	3.05 (1.17)		
			HP	64	2.38 (0.59)		
			AM	28	3.5 (0.59)	2.44	4.5
			AGC	55	4.66 (0.6)		
			SS	80	3.67 (1.41)		
			PA	56	4.83 (0.42)		
			WHP	19	4.43		
			PGP	8	5.4		
			CW	19	5.11		
			CL	5	7.06		
White blood cell count	10^{9}/L	<0.001	NBGC	81	6.65 (1.69)		
			DP	57	7.76 (2.98)		
			HP	64	8.31 (2.34)		
			AM	28	3.38 (1.85)	0.86	8.32
			AGC	59	6.57 (1.89)		
			SS	80	3.54 (3.06)		
			PA	56	6.45 (1.9)		
			WHP	19	3.73		
			PGP	8	6		
			CW	19	7.34		
			CL	5	6.36		
Glucose	mmol/L	<0.001	NBGC	33	3.96 (0.72)	2.6	5.4
			DP	57	3.98 (1.42)		
			HP	42	2.9 (0.72)		
Neutrophil count	10^{9}/L	<0.001	NBGC	80	2.59 (1.09)		
			DP	44	2.48 (0.84)		
			HP	50	3.07 (1.2)		
			AM	23	0.82 (0.44)	0.17	2.15
			AGC	58	2.81 (0.72)		
			SS	56	1.86 (1.96)		
			PA	58	3.01 (1.46)		
			WHP	19	1.22		
			PGP	8	2.09		
Lymphocyte count	10^{9}/L	<0.001	NBGC	81	3.35 (1.1)		
			DP	45	4.36 (2.05)		
			HP	50	4.23 (1.65)		
			AM	25	2.52 (1.68)	0.63	7.16
			AGC	59	2.67 (1.18)		
			SS	55	1.73 (1.28)		
			PA	57	3.01 (0.94)		

			WHP	19	2.22		
			PGP	8	2.83		
Eosinophil count	10^9/L	<0.001	NBGC	77	0.62 (0.35)		
			DP	46	0.52 (0.36)		
			HP	49	0.69 (0.37)		
			AM	24	0.19 (0.13)	0.02	0.44
			AGC	60	0.52 (0.29)		
			SS	50	0.1 (0.11)		
			PA	55	0.26 (0.17)		
			WHP	18	0.15		
			PGP	8	0.39		
Monocyte count	10^9/L	<0.001	NBGC	78	0.09 (0.1)		
			DP	44	0.17 (0.17)		
			HP	46	0.18		
			AM	24	0.01 (0.02)	0	0.05
			AGC	57	0.22		
			SS	54	0.1 (0.11)		
			PA	57	0.14		
			WHP	19	0.07		
			PGP	8	0.21		
Basophil count	10^9/L	<0.01	NBGC	75	0 (0.01)		
			DP	45	0.01 (0.02)		
			HP	46	0.02 (0.03)		
			AM	24	0 (0.01)	0	0.02
			AGC	59	0.03 (0.04)		
			SS	43	0.01 (0)		
			PA	17	0.04		
			WHP	18	0.01		
			PGP	8	0.01		
Haemoglobin	g/L	<0.001	NBGC	81	138.27 (15.26)		
			DP	52	118.35 (11.6)		
			HP	58	123.52 (11.56)		
			AM	28	123.79 (19.44)	80	160
			AGC	55	136.91 (16.28)		
			SS	80	107.13 (41.23)		
			PA	57	132.68 (10.4)		
			WHP	19	132.84		
			PGP	8	160		
			CW	18	141.61		
			CL	5	174		
Haematocrit	L/L	<0.001	NBGC	82	0.3 (0.07)		
			DP	59	0.25 (0.09)		
			HP	64	0.24 (0.06)		
			AM	28	0.32 (0.05)	0.22	0.43
			AGC	52	0.39 (0.04)		
			SS	67	0.3 (0.12)		
			PA	56	0.39 (0.03)		
			WHP	19	0.4		

			PGP	8	0.47		
			CW	19	0.43		
			CL	5	0.62		
Mean corpuscular volume	f/L	<0.001	NBGC	83	91.9 (6.98)		
			DP	59	80.39 (14.73)		
			HP	64	100.68 (5.17)		
			AM	28	91.37 (4.35)	81.36	100
			AGC	54	82.72 (3.4)		
			SS	56	85.95 (3.93)		
			PA	57	81.23 (3.53)		
			WHP	19	89.84		
			PGP	8	86.38		
Mean corpuscular haemoglobin	pg	<0.001	NBGC	83	43.17 (7.53)		
			DP	59	43.28 (16.94)		
			HP	64	54.11 (10.38)		
			AM	28	35.68 (4.23)	28.64	46.1
			AGC	53	29.45 (0.97)		
			SS	55	29.69 (1.68)		
			PA	59	27.97 (1.43)		
			WHP	19	30.05		
			PGP	8	29.63		
Mean corpuscular haemoglobin concentration	g/L	<0.001	NBGC	83	473.46 (99.57)		
			DP	59	539.88 (206.47)		
			HP	64	537.28 (98.35)		
			AM	28	390.36 (42.79)	343.95	518.25
			AGC	55	355.2 (9.48)		
			SS	54	345.72 (11.84)		
			PA	58	342.52 (7.35)		
			WHP	19	335.42		
			PGP	8	343.25		
Platelet	10^9/L	<0.001	NBGC	75	150.03 (33.44)		
			DP	54	169.04 (72.24)		
			HP	63	147.94 (44.45)		
			AM	28	156.43 (71.42)	36	312
			AGC	53	144.32 (56.44)		
			SS	29	217.55 (79.87)	76	449
			PA	21	187.1 (44.08)	136	290
			WHP	19	129.79		
			PGP	8	173		
Nucleated red blood cell count	10^9/L	<0.001	NBGC	17	0.04		
			DP	25	0.01 (0.01)		
			AM	26	0.03 (0.03)	0	0.1
Albumin	g/L	<0.001	NBGC	70	28.4 (6.5)		
			DP	30	33.45 (3.06)	27.92	39
			AGC	57	29.89 (2.97)		
			SS	79	29.9 (4.68)		
			PA	73	33.74 (3.35)		

			WHP	19	31.68		
			PGP	8	36.13		
			CW	22	34.41 (3.46)	27	40
			CL	5	34		
Total protein	g/L	<0.001	NBGC	27	66.31 (6.31)	50.57	76.62
			DP	27	65.24 (4.56)	55.13	73.88
			AGC	60	61.47 (5.51)		
			SS	79	56.42 (10.59)		
			PA	74	58.76 (5.55)		
			WHP	18	56.5		
			PGP	8	67		
			CW	22	65.05 (4.1)	58	74
			CL	5	67.4		
Globulin	g/L	<0.001	NBGC	27	31.94 (5.22)	40.03	5.22
			DP	29	31.03 (4.57)	22.51	39.39
			AGC	57	30.85 (4.39)		
			SS	79	26.39 (7.45)		
			PA	68	25.34 (2.87)		
			WHP	19	25.84		
			PGP	8	30.88		
			CW	22	30.64 (4.33)	24	39
			CL	5	33.4		

SD, standard deviation

2.4.3 Influence of biotic and abiotic factors on reference intervals and site-specific means

2.4.3.1 Significance and effect size of biotic and abiotic factors

Several haematological and serum protein parameters varied with the effects of sex, season and sexual maturity (Table 2.6). The ES of sex and maturity on all variables combined ranged from -0.76 to 0.42 (Table 2.6, Figure 2.3 (a) & (b)). Seasonal ES on all variables ranged from 0 to 0.34 (Table 2.6, Figure 2.4). Mean ES were both positive and negative for sex and maturity, but always positive for season.

Table 2.6 Mean and 95% confidence intervals of effect sizes (ES, Cohen's d and eta squared (η^2)) of the factors sex, season and sexual maturity on haematological and serum protein values. Significance values from one-way analysis of variance (ANOVA) for effects of sex, season and sexual maturity for each parameter for free-ranging eastern grey kangaroos (*Macropus giganteus*) sampled from reference sites; Nelson Bay Golf Course, (NBGC), Darlington Park (DP), Heritage Park (HP) and Ainslie Majura Kangaroo Management Unit (KMU) (AM) from 2015 to 2019.

Parameter	Sex					Season					Maturity				
	ES	**ED**	**Low CI**	**Upper CI**	***P***	**ES**	**ED**	**Low CI**	**Upper CI**	***P***	**ES**	**ED**	**Low CI**	**Upper CI**	***P***
Red blood cell count	-0.27	small	-0.54	-0.01	**0.02**	0.14[a]	large	0.07	0.23	**<0.001**	-0.49	small	-0.8	-0.18	**0.02**
White blood cell	0.04	negligible	-0.22	0.3	0.75	0[a]	none	0	0	0.98	-0.23	small	-0.54	0.07	0.1
Glucose	-0.75	medium	-1.11	-0.4	**<0.001**	0.05[a]	medium	0	0.14	**0.04**	-0.76	medium	-1.16	-0.36	**<0.01**
Neutrophil count	-0.08	negligible	-0.36	0.2	0.56	0.02[a]	small	0	0.06	0.3	0.49	small	0.14	0.83	**<0.01**
Lymphocyte count	0.07	negligible	-0.21	0.35	0.6	0.03[a]	small	0	0.08	0.12	-0.65	medium	-1	-0.31	**<0.001**
Eosinophil count	-0.19	negligible	-0.87	0.49	0.51[b]	0[a]	none	0	0.01	0.46[b]	-0.13	negligible	-0.99	0.73	0.41[b]
Monocyte count	-0.23	small	-0.5	0.05	0.1	0.07[a]	medium	0	0.13	**<0.01**	-0.25	small	-0.59	0.1	0.31
Basophil count	0.2	small	-0.08	0.48	0.75[b]	0.03[a]	small	0	0.08	0.06[b]	-0.12	negligible	-0.46	0.22	0.41[b]
Haemoglobin	-0.09	negligible	-0.35	0.17	0.47	0.09[a]	medium	0.02	0.15	**<0.001**	0.27	small	-0.03	0.58	**<0.01**
Haematocrit	-0.16	negligible	-0.42	0.1	0.16	0.24[a]	large	0.14	0.32	**<0.001**	-0.25	small	-0.55	0.06	0.93
Mean corpuscular volume	0.29	small	0.03	0.55	**<0.001[b]**	0.11[a]	large	0.05	0.2	**<0.001[b]**	0.62	medium	0.31	0.94	**<0.001[b]**
Mean corpuscular haemoglobin	0.28	small	0.02	0.55	**0.02**	0.14[a]	large	0.07	0.23	**<0.001**	0.74	medium	0.42	1.05	**<0.001**
Mean corpuscular haemoglobin concentration	0.14	negligible	-0.12	0.4	0.23	0.25[a]	large	0.15	0.33	**<0.001**	0.52	medium	0.21	0.83	**0.03**
PLT	0	none	-0.26	0.26	0.98	0.05[a]	medium	0.01	0.11	**0.01**	0.12	negligible	-0.19	0.43	0.97
NRBC count	-0.26	small	-0.74	0.22	0.28	0.08[a]	medium	0	0.2	0.08	0.1	negligible	-0.38	0.58	0.91
Albumin	-0.1	negligible	-0.5	0.3	0.51	0.34[a]	large	0.21	0.48	**<0.001**	-0.49	small	-0.92	-0.06	**<0.001**
TP	0.04	negligible	-0.5	0.59	0.87	0[a]	none	0		0.2	0.58	medium	0.04	1.12	**<0.01**
Globulin	-0.09	negligible	-0.64	0.46	0.72	0[a]	none	0	0.01	0.96	0.66	medium	0.11	1.21	**<0.01**

Bold indicates significant effect of <0.05; [a]ES calculated using eta squared (η^2); [b]Significance values from Kruskal-Wallis (KW) test (non-normal distribution). ES, effect size; ED, effect description; CI, confidence intervals; *P*, significance.

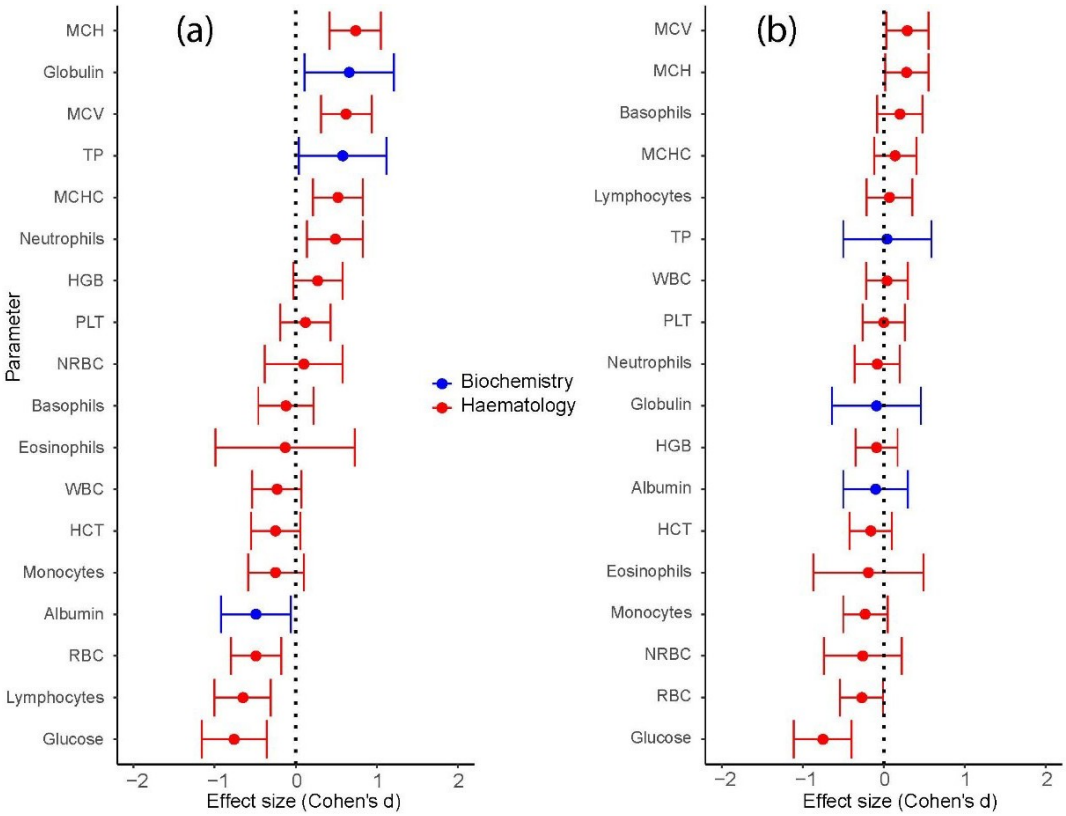


Figure 2.3 Forest plot of the effect size (Cohen's d and 95% confidence intervals) of (a) sexual maturity and (b) sex on haematological, glucose and serum protein values from the reference population (Nelson Bay Golf Course, NBGC; Darlington Park, DP; Heritage Park, HP; Ainslie Majura Kangaroo Management Unit (KMU) (AM)) of wild eastern grey kangaroos (*Macropus giganteus*). ES of 0.2, 0.5 and 0.8 indicate small, medium and large effect sizes respectively (Cohen 1977). (a) Negative Cohen's d indicates adults are lower than subadults for that parameter. Positive Cohen's d indicates adults are higher than subadults for that parameter. (b) Negative Cohen's d indicates females are lower than males for that parameter. Positive Cohen's d indicates females are higher than males for that parameter.

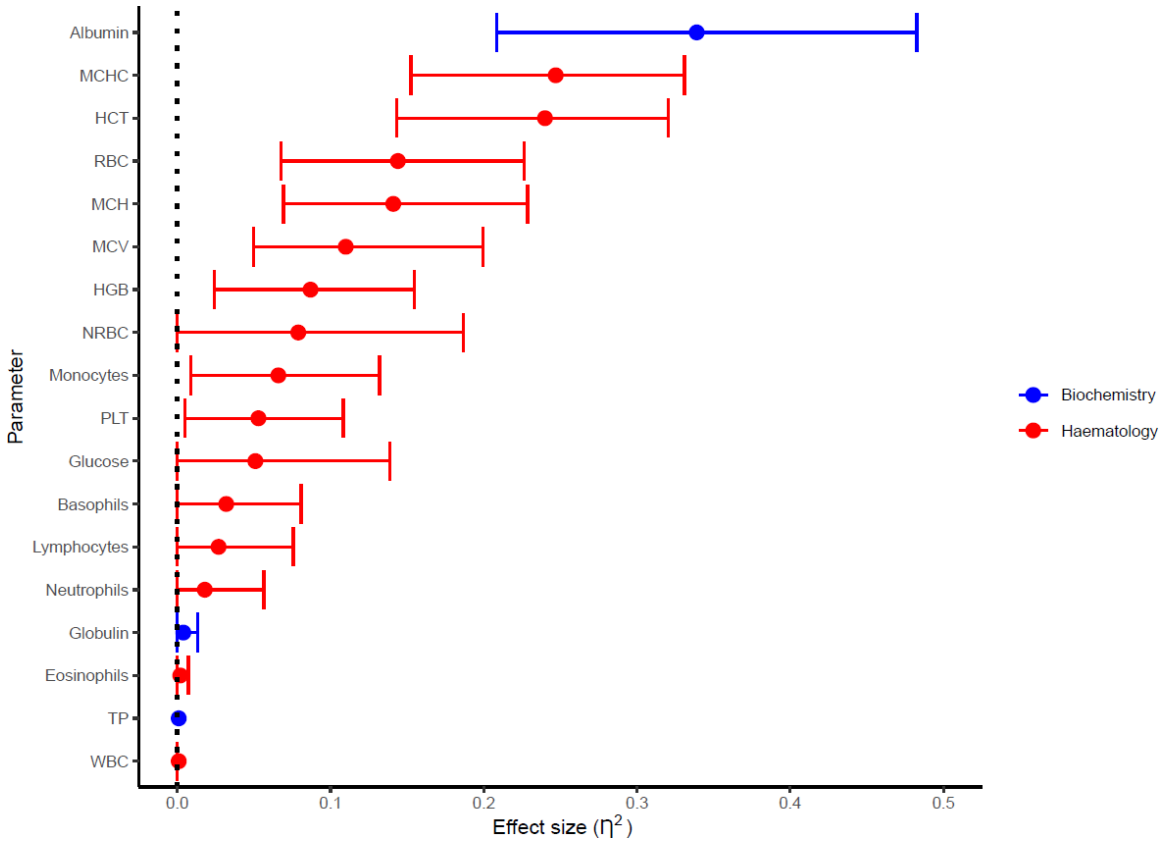


Figure 2.4 Forest plot of the effect size (ES, eta squared (η^2) and 95% confidence intervals) of season on haematological, glucose and serum protein values from the reference population (Nelson Bay Golf Course, NBGC; Darlington Park, DP; Heritage Park, HP; Ainslie Majura Kangaroo Management Unit (KMU) (AM)) of free-ranging eastern grey kangaroos (*Macropus giganteus*). ES of 0.01, 0.06 and 0.14 indicate small, medium and large effect sizes respectively (Maher et al. 2013).

2.4.3.1.1 Sex

The ES of sex on variables was negligible for most parameters analysed, while a small effect of sex was seen for RBC count, monocyte count, basophil count, MCV, MCH and NRBC count. The ES of sex on glucose concentration (Table 2.6, Figure 2.3b) was medium and shown to be significantly higher in males compared to females (ANOVA: $F_{1,127} = 20.91$, $P < 0.001$, Table 2.6), with means of 4.06 ± 0.96 mmol/L and 3.31 ± 1.3 mmol/L respectively. Due to the overall small effect of sex on haematological and protein parameters, RIs were not partitioned by sex.

2.4.3.1.2 Maturity

Adults had significantly lower glucose concentrations and lymphocyte counts than subadults (ANOVA: $F_{1,127} = 10.83$, $P < 0.01$ and $F_{1,197} = 12.52$ $P < 0.001$ respectively, Table 2.6), with a medium ES. Adults had significantly higher MCV (ANOVA: $F_{1,228} = 32.73$, $P < 0.001$), MCH (ANOVA: $F_{1,228} = 17$, $P < 0.001$), MCHC (ANOVA: $F_{1,228} = 4.72$, $P = 0.03$), TP (ANOVA: $F_{1,53} = 12.06$, $P < 0.01$) and globulin concentrations (ANOVA: $F_{1,51} = 18.98$, $P < 0.01$) than subadults, with a medium ES (Table 2.6). For all remaining parameters, there was a small ES of maturity, with no significant differences. Based on these results, RIs were partitioned by maturity and are presented in Table 2.3.

2.4.3.1.3 Season

Several variables were affected by season (Table 2.4). This effect was large and significantly different for RBC count (ANOVA: $F_{3,228} = 4.7$, $P < 0.001$), HCT (ANOVA: $F_{3,228} = 24.15$, $P < 0.001$), MCV (ANOVA: $F_{3,228} = 11.06$, $P < 0.001$), MCH (ANOVA: $F_{3,228} = 13.76$, $P < 0.001$), MCHC (ANOVA: $F_{3,228} = 25.26$, $P < 0.001$) and albumin concentration (ANOVA: $F_{3,94} = 19.01$, $P < 0.001$) (Table 2.6, Figure 2.4). Glucose, monocyte count, HGB, PLT and NRBC count were also dependent on season, with a medium ES (Table 2.6, Figure 2.4). Glucose (ANOVA: $F_{3,127} = 2.82$, $P = 0.04$), monocyte count (ANOVA: $F_{3,197} = 4.77$, $P < 0.01$), HGB (ANOVA: $F_{3,228} = 7.44$, $P < 0.001$) and PLT (ANOVA: $F_{3,228} = 4.26$, $P = 0.01$) were significantly different across seasons (Table 2.6). There was a small effect on neutrophil counts, lymphocyte counts and basophil counts, but there was no effect of season on WBC, eosinophil counts, TP and globulin concentrations. Based on these results, RIs and mean blood parameters

were partitioned by season (Table 2.4).

2.4.4 Random forest model of factors influencing parameters of health

Abiotic factors were the most important drivers of RF predictions of haematological, glucose and serum protein values across all 11 sites (Figure 2.5). Site was consistently the most important predictor, followed by rainfall, temperature, season, laboratory, maturity and sex (ranked 0). RBC count and derived parameters (HCT, MCV, MCH and MCHC) were best explained by biotic and abiotic factors compared to all other parameters, as indicated by a greater PVE (Table 2.7). All models were significant($P < 0.05 – 0.001$) except for NRBC count (Table 2.7). HGB, MCHC and PLT count have large RMSEs indicating a high error rate within the model (Table 2.7). PDPs were generated for RBC count and derived parameters (HCT, MCV, MCH and MCHC) as the PVE was greater than 50. PDPs showed a pattern of decreasing RBC count with increasing rainfall up to 100 mm, then an increase in RBC count. Increasing rainfall had a decreasing effect on HCT and an increasing effect on MCV, MCH and MCHC. Increasing temperature also had a decreasing effect on RBC count to 22°C, where RBC counts then increased. There were no simple patterns for the effect of temperature on HCT, MCV, MCH and MCHC.

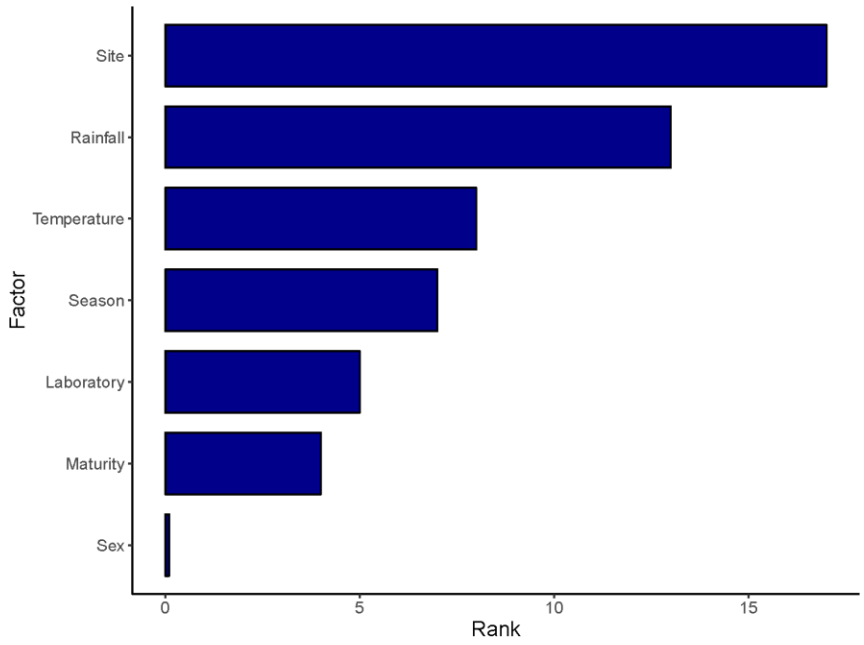


Figure 2.5 Importance rank of biotic and abiotic factors driving random forest (RF) predictions of haematological, glucose and serum protein parameters from 11 eastern grey kangaroo (*Macropus giganteus*) populations; Nelson Bay Golf Course, (NBGC), Darlington Park (DP), Heritage Park (HP), Ainslie Majura Kangaroo Management Unit (KMU) (AM), Anglesea Golf Course (AGC), Serendip Sanctuary (SS), Woodlands Historic Park (WHP), Portland Aluminium (PA), Cowan (CW) and Calga (CL).

Table 2.7 Random forest (RF) model validation metrics exploring the effects of biotic and abiotic factors on haematological, glucose and serum protein values of 11 populations of eastern grey kangaroos (*Macropus giganteus*); Nelson Bay Golf Course, (NBGC), Darlington Park (DP), Heritage Park (HP), Ainslie Majura Kangaroo Management Unit (KMU) (AM), Anglesea Golf Course (AGC), Serendip Sanctuary (SS), Woodlands Historic Park (WHP), Portland Aluminium (PA), Cowan (CW) and Calga (CL). The percentage of variation explained (PVE) indicates how well 'out of the bag' predictions explain the variance of the training dataset. Root mean square error (RMSE) indicates how well our RF predicts test set outcomes. The PVE from both the real (non-random data) model and the random model were compared and a P value generated to evaluate model significance (Evans and Cushman 2009; Murphy et al. 2010).

Parameter	**PVE**	***P***	**RMSE**
Red blood cell count	59.8	**<0.001**	0.81
White blood cell count	35.4	**<0.001**	2.38
Glucose	29.1	**<0.001**	0.54
Neutrophil count	10	**<0.001**	1.24
Lymphocyte count	14.3	**<0.001**	1.58
Eosinophil count	22.3	**<0.001**	0.32
Monocyte count	11.2	**<0.001**	0.19
Basophil count	-2.3	**<0.001**	0.04
Haemoglobin	28.7	**<0.001**	23.04
Haematocrit	53.4	**<0.001**	0.07
Mean corpuscular volume	54.4	**<0.001**	6.26
Mean corpuscular haemoglobin	70.3	**<0.001**	6.36
Mean corpuscular haemoglobin concentration	66.5	**<0.001**	69.33
Platelet	-4.3	**<0.05**	79.58
Nucleated red blood cell count	-22.3	NS	0.054
Albumin	39.7	**<0.001**	3.23
Total protein	26.9	**<0.001**	5.74
Globulin	34.8	**<0.001**	4.33

PVE, percentage of variation explained; RMSE, root mean squared error; *P*, significance.

2.5 Discussion

This study establishes RIs for several parameters of health for free-ranging kangaroos and investigated the significance of abiotic and biotic factors influencing these values. This study is novel as it develops the first RI for haematological, glucose and serum protein parameters for free-ranging kangaroos and characterises these values across multiple populations throughout a large portion of the species geographical range. Of the biotic factors considered, sex had a negligible effect on most of the parameters and was ranked zero in importance from the RF model across sites. This is consistent with previous investigations that found no effect of sex on WBC counts in kangaroos and agile wallabies (*Notamacropus agilis*) (Presidente 1978; Stirrat 2003). However, glucose concentrations were lower in female compared to male kangaroos, as reported in this species (Green-Barber et al. 2018) and in angora rabbits (*Oryctolagus cuniculus*) (Cetin et al. 2009). In addition, the sex-specific glucose RI for female kangaroos is much wider than for males, demonstrating that normal female glucose levels fluctuate more than in males. This is an interesting finding, as maternal glucose has been correlated with offspring sex determination (Helle et al. 2008) and there is evidence of condition-dependent sex bias in young kangaroos (Gall-Payne et al. 2015). Higher glucose concentration in males could also be attributed to a stress response (Peck et al. 2015). It is well known amongst social, sexually dimorphic animals that maintaining social dominance is a physiologically demanding activity (Creel 2005), thereby enhancing circulating glucose levels in response to glucocorticoids. We did not consider the effect of female reproductive status on blood parameters because the effects of pregnancy and lactation are unclear in other species (Harvey et al. 1994; Alonso et al. 1997; Harewood et al. 2000), and sex was ranked poorly in our RF model. However, we recognise that the long lactation period in kangaroos could affect some haematological, glucose and serum protein concentrations.

The immune system undergoes ontogenesis (Sidman et al. 1987), with many haematological and some serum protein parameters increasing with age (Fancourt and Nicol 2019). Consistent with this,

maturity was partitioned in our RI because it was determined as moderately important in our RF model, with adults having significantly higher MCV, MCH, MCHC, TP and globulin concentrations than subadults. Partitioning based on maturity also satisfied statistical criteria. Similar findings have been demonstrated in other marsupial species. For example, TP increases with age in the tammar wallaby (*Notamacropus eugenii*), short-eared mountain possum (*Trichosurus caninus*) and southern hairy-nosed wombat (*Lasiorhinus latifrons*) (Barnett et al. 1979; McKenzie et al. 2002; Woolford et al. 2020); and in small mammals, RBC values are higher in subadults when compared with adults (Sealander 1964). The significantly lower values for glucose concentrations and lymphocyte counts in adults is likely related to decreased glucose metabolism and the output of T lymphocytes as the immune response declines with age (Clot et al. 1978; Defronzo 1981; Linton and Dorshkind 2004). Given the observed maturity differences in blood parameters, it is important that subadult individuals are clinically evaluated with RIs specific to this age cohort.

While the purpose of this study was to develop haematological, glucose and serum protein RIs for kangaroos, the effect of analytical laboratory on these parameters was also assessed. Analysis of blood data by three different commercial laboratories showed that laboratory was more important than biological factors (sex and sexual maturity) but less important than environmental factors (site, rainfall and temperature) on haematological, glucose and protein parameters. This has implications for the interpretation of health parameters depending on the scale of the investigation. For assessing population health across sites or seasonal changes in blood parameters, laboratory is of lesser importance as environmental factors have the largest impact. However, when comparing individuals based on biological factors, differences between laboratories could mask underlying biological differences. There are many potential sources of difference among commercial laboratories: instrumentation, laboratory environmental conditions, quality control (calibration and standard operating procedures) and technician variation in manual determinations, for example, of differential WBC counts (Flatland et al. 2010). As a result of these differences, when monitoring intra-individual and biological factors it is recommended that the same laboratory is used.

Site was consistently the most important predictor of haematological, glucose and serum protein parameters, most likely because these parameters are highly sensitive to season, local environmental cues, intrinsic population characteristics, genetics and other non-climatic variables (Argente et al. 2014). The consistently large relative importance of rainfall compared to temperature and season suggests that from all factors modelled, rainfall drives the variation among sites. Due to limitations within our dataset, we cannot account for other sources of variation that might contribute to the importance of site; such as habitat type, food and water availability. Our PDPs show that for some haematological parameters, rainfall and temperature have a strong influence. When there is < 100 mm of rainfall in the month prior to sampling, the RBC count and HCT are high, likely due to increased water loss or decreased water intake and subsequent haemoconcentration (Clark 2004). RBC count then begins to decline as rainfall increases, until 100mm. The relationship in RBC count reverses above 100 mm of rainfall, where RBC counts begin to increase. A similar pattern was found for temperature: there is an initial decrease in RBC count in cooler temperatures, then above 22°C the RBC count increases. These highly seasonal effects on RBC counts have been shown in several species of dasyurids and echidnas (Andersen et al. 2000; Clark 2004) and in macropods. In wallaroos (*Osphranter robustus*), rainfall was shown to influence the nutrient content of plants, particularly proteins, which is correlated with increased RBC parameters (Ealey and Main 1967). MCV, MCH and MCHC were also shown to increase in response to increasing rainfall. Seasonal variation was evident in most of the haematological, glucose and serum protein parameters analysed, highlighting the importance of establishing season-specific RIs. If haematological parameters are being used to evaluate changes in condition of a population over time, it is recommended that samples are collected at the same time of year, to coincide with similar weather patterns. Specifically, RBC, HCT, MCV, MCH, MCHC, albumin, glucose, monocyte count, HGB, PLT and NRBC count are the parameters most affected by season. These effects are likely to become more pronounced as Australia's climate changes. This study was conducted through an extended period of drought in eastern Australia (CSIRO 2018). Australia's climate is predicted to become warmer and

drier over the next decade, with rainfall occurring in increasingly isolated and sporadic events (CSIRO 2018). Our results suggest that this changing climate will likely impact these indicators of kangaroo health.

As site was identified as the most important predictor of changes in haematological, glucose and serum protein values in this study, blood samples were analysed from a range of unique site-specific populations which varied in population density, presence of endemic disease (e.g. high fluoride exposed animals, parasitism and oral necrobacilliosis), food availability (inadequate to abundant) and water resources (e.g. artificial water resources at golf courses). Variations in haematological, glucose and serum protein parameters, due to environmental, parasitic and biological factors have been described in many wild mammalian species (Lepitzki and Woolf 1991; Spencer and Speare 1992). It is likely that these intrinsic population-based factors, in addition to environmental factors, are responsible for differences in haematological, glucose and serum protein values. Environmental factors such as rainfall, temperature and their seasonal changes, directly influence parasite load, reproductive stressors and nutrient supply, through food availability and quality (Chaplin and White 1972; Ellis et al. 1977; Magona and Musisi 2002). These factors influence an individual's haematological values accordingly. For example, increased numbers of haematophagus gastrointestinal nematodes can result in seasonal variation in RBC counts, corresponding to seasonal variation in parasite burden (Pacioni et al. 2013).

The RIs were developed from sampling chemically restrained kangaroos from the lateral caudal vein. However, animals from AM (n = 28) were bled within 30 minutes post-mortem by direct cardiac venipuncture. Differences in haematological, glucose and serum protein parameters due to post-mortem and venipuncture site have been reported (Maceda-Veiga et al. 2015). Specifically, MCV may increase immediately after death, as water temporarily moves from the extracellular to the intracellularfluid compartment (Hodgkinson and Hambleton 1969). HCT and glucose concentration also show variability between white tailed deer (*Odocoileus virginianus*) bled immediately after death versus animals bled 30 minutes later (Wesson III et al. 1979). However, comparisons between

AM and sites where chemical restraint was used show that MCV and PCV were not significantly different. For this reason, it was considered appropriate to include data from AM in the RI.

Most haematological, glucose and serum protein parameters determined from each site in this study had trends consistent with the developed RI. Most values fell within the RI or overlapped the upper or lower limits of the range; however, some key differences were seen. Previous reports in macropods have indicated that kangaroos have higher neutrophil compared to lymphocyte counts (ISIS 2002; Vogelnest and Portas 2008; Green-Barber et al. 2018). In this study, except for kangaroos sampled at two sites (AGC and SS) kangaroos had higher lymphocyte compared to neutrophil counts, consistent with a single report from four individual kangaroos (Spencer et al. unpublished data, cited in (Clark 2004)) and large grazing ruminants (Jones and Allison 2007). Higher neutrophil counts at SS could be evidence of inflammatory demand (Maceda-Veiga et al. 2015) as a result of the extremely high prevalence of lumpy jaw at this site or a corticosteroid-mediated stress response in unhealthy individuals. Alternatively, a higher lymphocyte count could indicate greater immunostimulation at most of the sites sampled or an adrenalin-mediated physiological lymphocytosis (Clark 2004). This is a significant finding when interpreting blood values for kangaroos.

Some site-specific differences in haematological, glucose and serum protein parameters could be attributed to captivity. For example, at CL and CW animals receive supplementary food, whilst CW also received intestinal parasite treatment. Both sites had higher RBC counts and HCT, and kangaroos sampled at CL had higher HGB concentrations. Captive diets high in protein are beneficial to production of RBCs (Amin et al. 2007), while elimination of internal and external parasitism would reduce the likelihood of anaemia (reduced HCT, RBC count and HGB) and protein loss (Blackburn et al. 1992).

Previously reported mean RBC and HCT counts from captive colonies are also higher than the RI reported (Spencer et al. unpublished data, cited in (Clark 2004); ISIS 2002). The variation in these parameters of health could also be attributed to site-specific influences, such as rainfall and temperature, as environmental factors were important predictors of haematological, glucose and

serum protein parameters in our RF models.

Our findings have informed some guidelines for assessing kangaroo health. Managers should aim to capture a representative sample of the population. A targeted approach should be taken where managers survey populations within a given timeframe and analyse samples as a batch at one laboratory, rather than relying on opportunistic sampling of individuals at different time points and using different laboratories. Health surveillance should also be conducted within a season, and maturity-specific RIs used for the relevant subgroups. Most analytical laboratories offer a 'profile' of tests which include most of the haematological and serum protein parameters described in this study. The RI developed in this study can be used as an aid to evaluate the health status of captive populations of kangaroos given their species-specificity. However, caution needs to be employed in the interpretation of individual results due to the inherent differences between captive and wild animals such as levels of parasitism and nutritional differences. For example, in this study, captive populations (CL and CW) had higher RBC, HCT and HGB counts compared to wild populations. For captive populations of kangaroos in countries with extreme climatic variability, consultation with the season-specific RI could be advantageous. Ideally, a RI for captive individuals comprising greater than 120 healthy individuals, would be considered the most appropriate RI for health evaluation of captive individuals (Friedrichs et al. 2012).

2.6 Conclusion

This study highlights the importance of both biotic and abiotic factors on the establishment of RIs for parameters of health. Use of these RIs in differing contexts therefore needs to be undertaken with caution. In this study, site and specific environmental factors inherent in different sampling locations were shown to be the most important factors affecting these parameters of health in kangaroos. Because Australia is becoming increasingly hotter and drier, health parameters in kangaroos will likely be increasingly affected by rapid changes in climate. There is an imperative to study more populations of kangaroos in poor condition, to discern the role of abiotic factors compared to other

causes of disease. Additionally, consideration of the effect of season on haematological, glucose and serum protein values, timing of sample collection throughout the year should be considered for meaningful comparisons among individuals, sites or time points. Importantly, attempting to compare samples from markedly different sites, samples analysed at different laboratories, or samples from populations with different age structures and disease status could mean that important fluctuations are masked.

Based on the findings in this study, it is recommended that the species-level RI be used with caution, and that maturity-specific RIs established in this study be used preferentially to inform clinical evaluation of kangaroos. We also recommend consulting site-specific population means, when specific site attributes are of interest; for example, when population density is high, for captive populations, or for populations with endemic disease. Understanding the plethora of factors that can influence haematological and serum protein values will improve the utility of developed RIs, providing wildlife managers and veterinarians with a robust tool to assess population health and improve the management and welfare standards for kangaroos in Australia.

Kangaroos at maximum capacity: health assessment of free-ranging eastern grey kangaroos on a coastal headland

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3.1 Abstract

On the Australian east coast, sprawling urban development is fragmenting the landscape and native wildlife habitats. The impact of rapid urbanisation on wildlife health is largely unknown. This study surveyed the health of a high-density eastern grey kangaroo (*Macropus giganteus*) population (5.4 individuals per ha^{-1}) impacted by urban encroachment and prolonged drought. Blood parameters (haematological and serum protein), trace element and heavy metal concentrations and parasite counts (faecal worm egg counts, ticks and mites) are reported for a sample of ≤ 54 kangaroos at Look At Me Now Headland, New South Wales, Australia. These parameters were compared to lower density kangaroo populations from other sites in New South Wales. We found the health and welfare of this population to be severely compromised, with non-regenerative anaemia and nutritional deficiencies evident. Our results indicate that high-density kangaroo populations isolated by urban encroachment are at significant health risk. To prevent further decline in this population's health, we discuss management strategies that could be employed, concurrent with ongoing health and disease monitoring, to mitigate the poor health outcomes in this population. We conclude that it is essential to retain habitat connectivity when altering land use in areas with resident kangaroo populations if managers are to maintain healthy populations.

3.2 Introduction

Humans have changed the natural environment and altered the dynamics of wildlife populations (Fosteret al. 2002). This can lead to an increase in density of some species (Ditchkoff et al. 2006), up to the point at which they are considered 'overabundant'. These populations reduce natural diversity, impact human life or livelihood and/or affect the fitness of individuals within the overabundant population itself (Herbert 2004). While most studies have focused on the negative impacts of overabundant specieson the ecosystem they inhabit, less is known about the impacts on the overabundant species themselves.

Overabundant populations are often visually perceived as flourishing, and the immediate impacts that increasing density can have on animal health, welfare and long-term population viability are often overlooked (Garrott et al. 1993). High-density populations are exposed to an array of physiological stressors that can affect an individual's diet, reproductive success, disease status, welfare and survival (Scott 1988; Gortázar et al. 2006). Animals at high-density can over-exploit food resources (McCarthy 1996) causing malnutrition, starvation and mortality. This can increase their susceptibility to disease and parasitism, resulting from greater intraspecies contact and opportunities for disease transmission (Gortázar et al. 2006). For urbanised native species, these stressors are exacerbated by habitat fragmentation, infrastructure expansion and motor vehicle collisions (Brunton et al. 2019). As the urban environment expands and continues to distort and disrupt native populations and their habitats, there is a growing need to elucidate the indirect costs of these changes to wildlife health, welfare and population resilience.

Wildlife health surveillance is increasingly important due to ongoing biodiversity loss and increasing threats of zoonotic disease (Ryser-Degiorgis 2013; Han et al. 2016). Wildlife health investigations may require species-specific baseline health parameters for long-term health monitoring of populations. If species-specific baseline haematology and biochemical reference intervals (RI) are known, the potential to gather physiological data and identify disease can be improved (Maceda-

Veiga et al. 2015). The number of individuals in a population that fall outside of the RI provides a quantifiable measure of the impact of parasites, disease and physiological stressors, such as malnutrition (Huber et al. 2017). Blood samples can also enable measurement of stress-related parameters, which are intimately linked to animal health and welfare (Huber et al. 2017). Classical measures of stress include glucocorticoid concentration (Sheriff et al. 2011), however there is a mounting body of evidence advocating the superiority of using the immune system as an indicator of stress (Davis et al. 2008; Huber et al. 2017). Specifically, the neutrophil to lymphocyte ratio (N:L) can be used as a proxy measure for stress as a result of characteristic changes in the white blood cell (WBC) profile within 4-8 hours (h) of exposure to a stressor (Davis et al. 2008; Schultze 2010; Huber et al. 2017). The occurrence of declining health status and/or increased incidence of disease and biomarkers of stress are sensitive indicators of environmental and ecological change for a species (Scott 1988) and can be used to direct management efforts. This is particularly important for overabundant native species in which management often involves controversial techniques such as culling (Descovich et al. 2016).

Eastern grey kangaroos (*Macropus giganteus*, kangaroos hereafter) are a common macropod species with a wide geographic distribution along eastern and south-eastern Australia (Coulson 2008). Overabundant kangaroo populations commonly occur on the urban fringes (peri-urban areas) because large scale movement of animals is uncommon and largely constrained by infrastructure development (Coulson et al. 2014). Kangaroos have been able to persist in these often isolated pockets of habitat because they actively use urban green spaces, remnant forests and land cleared for livestock grazing (Coulson et al. 2014). Despite the apparent success of kangaroos, peri-urban landscapes are becoming increasingly fragmented and populations isolated (Brunton et al. 2019). Kangaroo 'die off' events have been described in overabundant kangaroo populations in the Australian Capital Territory (ACT) (Portas and Snape 2018) with subadult animals primarily affected and at greater risk of starvation when compared to adults. Blood sampling revealed anaemia and hypoalbuminaemia in a number of animals, whilst necropsy investigations reported minimal fat

reserves in affected individuals (Portas and Snape 2018). Kangaroo populations at unsustainably high densities are also at risk of disease outbreaks, such as oral necrobacillosis ('lumpy jaw'), which can cause severe emaciation and death by starvation (Borland et al. 2012). However, apart from isolated 'die off' events and accounts of disease in enclosed populations, the potential health impacts on peri-urban dwelling kangaroos is largely unknown (Brunton et al. 2019).

This study describes the health status and density of a population of peri-urban kangaroos from a coastal headland in New South Wales (NSW) facing ongoing habitat fragmentation, human encroachment and affected by prolonged drought (NSW DPI 2020). This paper reports and compares the results of haematological and serum protein analyses, parasite counts (gastrointestinal worms, ticks and mites) and trace element and heavy metal concentrations from kangaroos at Look At Me Now Headland (LAMN) and lower density kangaroo populations elsewhere. Blood parameters of kangaroos sampled at LAMN are compared to established RIs for the species (Brandimarti et al. 2020 [thesis Chapter 2]). In addition, the influence of biotic (sex and maturity) and abiotic (season, site and rainfall) factors are examined.

3.3 Materials and methods

3.3.1 Primary study site (LAMN)

The study site, LAMN (-30.177°S, 153.189°E) is a headland bordering the coastal town of Emerald Beach, 15 km north of Coffs Harbour, NSW (Figure 3.1). The headland is part of the Moonee Beach Nature Reserve and is a key area for the conservation of endangered *Themeda*-grassland and threatened flora species like *Zieria prostrata*; but it is also home to a number of free-ranging macropods, including kangaroos and red-necked wallabies (*Notamacropus rufogriseus*) (Hunter and Hunter 2019; Hendersonet al. 2018b). Previous bi-monthly direct counts of kangaroos conducted in 2016 identified LAMN as a 'kangaroo hotspot' with between 2.25 to 4.87 individuals ha^{-1} (Henderson et al. 2018b).

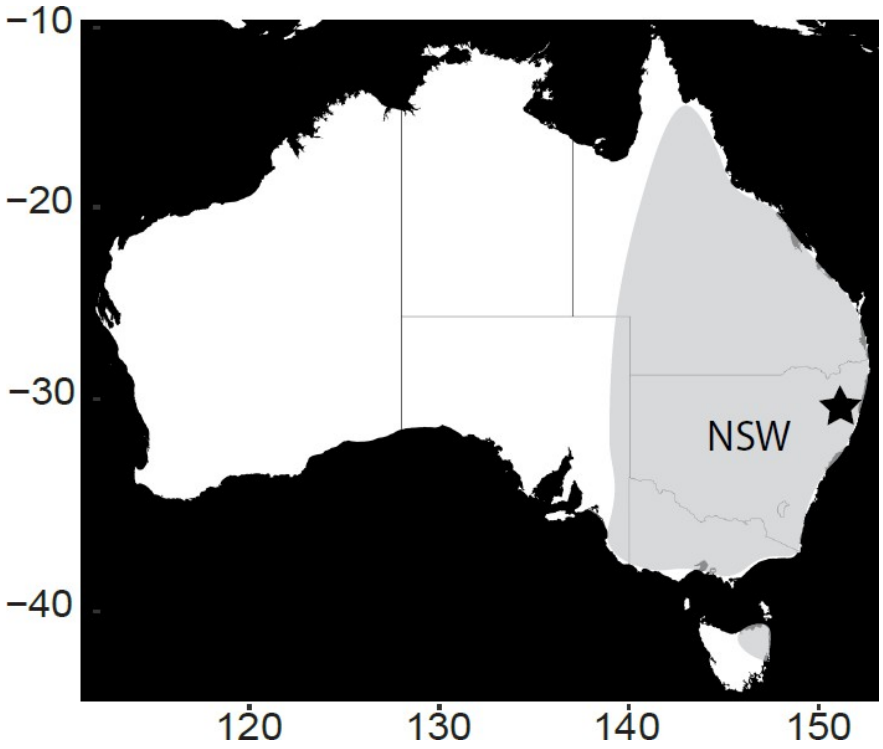

Figure 3.1 Location of the eastern grey kangaroo (*Macropus giganteus*) population sampled from 2017 to 2019 at Look At Me Now Headland (LAMN, star) in New South Wales (NSW), Australia. Degrees of latitude on the y axis and longitude on the x axis. Grey shading represents the approximate distribution of eastern grey kangaroos in Australia. Map sourced from Stamen Design, under CC BY 3.0. Data by OpenStreetMap, under ODbL.

3.3.2 Population counts at LAMN

The total population counts undertaken in this study in February 2018 and February 2019 were a continuation of a previous study (Henderson et al. 2018b). A similar methodological approach was adopted to permit comparison of counts over time. Counts were undertaken in the late afternoon or early morning, an optimal time of increased kangaroo activity. A total count approach (Inwood et al. 2008) was adopted in which at least two people simultaneously surveyed on foot from walking tracks on both LAMN and the adjacent Damerells Headland, and the adjoining walking tracks, within a 30 min timeframe (Figure 3.2). All kangaroos and red-necked wallabies were counted, including young-at-foot (YAF) and excluding dependant pouch young (solely in the pouch). Three counts were conducted on consecutive days in 2018 and 2019 to determine the precision of the count. Total counts were divided by the size of the survey area in hectares (Ha), measured using Google Earth to give an average animal density over the study timeframe. Precision estimates are presented alongside the means as the coefficient of variation (*CV*).

Figure 3.2 Foot based eastern grey kangaroo (*Macropus giganteus*) count transect of Damerells and Look At Me Now Headland, New South Wales (NSW), Australia. The existing count transect (Hendersonet al. 2018b) is yellow; the red line denotes the additional transect line utilised in this study.

3.3.3 Sample collection and parasitological analyses

Adult and subadult kangaroos (n = 21 males and n = 33 females) were immobilised at LAMN between 2017 and 2019 using Zoletil 100 (Virbac Pty. Ltd, Milperra, NSW, Australia) with a fixed dose of 65 mg for YAF, 125 mg for females or subadult males and females, and 250 mg for adult males. A 1 mL (or 0.5 mL for YAF) intramuscular injection of Zoletil was administered using a pole syringe or CO_2-powered projector (X-Calibre, Pneudart Inc, Williamsport, PA, USA). Kangaroos were followed from the time of drug administration to the time of immobilisation and then transferred into a soft cloth capture bag for transportation to an onsite processing area, within one km of the capture location. Sex, pes and leg length (Poole et al. 1982), body mass (to nearest 0.01 kg, Wedderburn ® Digital Hanging Scales, Model: WS603) and female reproductive status (teats not everted (subadult), pouch young present, lactating but no young present in the pouch (dependent YAF), or teats everted but no pouch young (non- reproductive but sexually mature)) were recorded during processing. Male maturity was determined by leg length; male kangaroos with a leg length > 52.3 cm were deemed sexually mature (Poole 1973; Pooleet al. 1982). While immobilised, each kangaroo was given a unique combination of coloured tags in their left/right ear ('mini' and 'button' sheep tags applied using the identiplier applicator (Allflex Australia Pty Ltd, Capalaba, Queensland, Australia) to allow identification of each animal. Kangaroos were examined clinically for visible injuries and body condition was scored as 'poor', 'thin', 'good', or 'excellent', based on palpation of the fat cover and muscle mass of the vertebral spinous processes and hip bones (Johnston et al. 1997; Edwards et al. 2013). Muscle mass on the rump was also visually assessed and recorded for animals that were in 'thin' or 'poor' condition. All body condition assessments were performed by the same observer (RG). A subjective scoring system was used to assess condition at LAMN because it is non-destructive and can be used to compare animals across populations for future investigations. Tick counts (n = 287 individuals, including lower density populations for comparison) were made at six locations on the body (inguinal, axilla, bilateral eyes and ears). An 8 cm x 8 cm square quadrant was placed under either the left or right axilla and left or right inguinal region and

all ticks within the quadrant were counted. Bilateral counts of ticks on the perimeter of the eye and on the inner and outer surface of the pinna were also performed. Total tick count per kangaroo was obtained by summing the number of ticks from all six sites. The presence or absence of mites was recorded following examination of the ears, eyes, axilla, inguinal and pouch (females) regions (n = 325, including lower density populations for comparison). Additional anecdotal observations were reported, including any associated skin lesions, typically noted as crusting of the skin, swelling, redness and/or discharge. Heart rate, respiratory rate, capillary refill time and the colour of oral and urogenital mucous membranes were recorded throughout the period of sedation. Up to 6 mL of whole blood was collected from the lateral caudal vein, approximately 30 min after administration of Zoletil 100, using a 23 gauge butterfly catheter (Surflo winged infusion set, Terumo Australia Pty Limited, Macquarie Park, NSW, Australia) attached to a 5 mL syringe (Luer slip, Terumo Australia Pty Limited, Macquarie Park). Blood was immediately transferred into a 5 mL serum tube (BD Vacutainer ® SST™ II Advance tube, Becton Dickinson and Company, North Ryde, NSW, Australia), 1.3 mL EDTA tube (Becton Dickinson and Company, North Ryde, Australia) and trace element tube (BD Vacutainer ® K2 EDTA, Becton Dickinson and Company, North Ryde, Australia). One to two drops of fresh blood were applied to a glucose strip (Freestyle optimum glucose strips, Abbott, Alameda, CA) and blood glucose determined using a hand-held glucose monitoring device (Freestyle Optium Neo Blood Glucose Monitoring System, Abbott, Alameda, CA). Whole blood was gently inverted and stored on an ice brick in an insulated box prior to processing. Whole blood in serum tubes was centrifuged for 10 min at 3000 rpm (LW Scientific E8C- 08AF-150P Porta-Fuge Portable 12 Volt Centrifuge). Serum was stored at -20 °C after separation. Two blood smears were made within 1 h of blood collection using whole blood preserved in EDTA. Blood smears were air dried, fixed in methanol and stained with Diff Quik (Lab Aids Pty Ltd) for differential WBC counts or brilliant cresyl blue (Sigma-Aldrich) for reticulocyte counts. 200 µL of whole blood preserved in EDTA was mixed with 200 µL of Streck Cell Preservative (Streck, Omaha, NE USA) and stored at 4 °C prior to automated haematological analysis. After processing, kangaroos were placed in a quiet location within observation distance from the

processing site and monitored remotely for up to 2.5 h from time of immobilisation. If animals were mobile before 2.5 h, kangaroos were followed at a distance for ongoing observation. During processing or recovery time a fresh faecal sample was usually obtained from each individual from the substrate around the recovery site. If an animal did not defecate during recovery, a faecal sample could be collected opportunistically while the animal was grazing. Faecal samples were stored on an ice brick in an insulated box or in a fridge prior to processing (within 48 h). Gastrointestinal worm burdens were estimated (n = 418, including lower density populations for comparison) using quantitative estimates of faecal worm eggs per gram of faeces (EPG). To determine EPG, a modified McMaster technique was employed whereby three grams of faeces were placed in 60mL of saturated salt solution (Gibbons et al. 2005). After homogenisation a sieve was inserted, and a sample was loaded into two chambers of a McMaster slide. All worm eggs were counted, and the number of eggs multiplied by 40 (1:20 dilution) to obtain an EPG.

All animal captures followed the American Society of Mammalogists (Sikes et al. 2016) guidelines and received animal ethics approval from The University of Sydney, 2016/1062 and 2015/917. Animal collection was performed under relevant permits from all NSW, Victorian and ACT governments (scientific license number: SL100961 and SL102148).

3.3.4 Automated haematological and protein analyses

EDTA whole blood samples preserved in Streck were analysed using a Sysmex XN1000i automated haematology analyser (Roche diagnostics, Australia) at the Veterinary Pathology Diagnostic Service (VPDS), The University of Sydney, Camperdown, NSW, Australia within seven days of blood collection (after Brandimarti et al. 2020 [thesis Chapter 2]). The following parameters were determined: haematocrit (HCT; L/L), haemoglobin (HGB; g/L), total red blood cell count (RBC; $x10^{12}$/L), total WBC count ($x10^{9}$/L), platelet count (PLT; $x10^{9}$/L) and nucleated RBC count (NRBC; $x10^{9}$/L). Values were doubled to account for dilution with cell preservative and any haemolysed samples were excluded from the study. Mean cell volume (MCV; fL; (HCT/1000)/RBC), mean cell haemoglobin (MCH; pg;

(HGB/RBC)) and mean corpuscular haemoglobin concentration (MCHC; g/L; (HGB/HCT)) were calculated manually. One hundred WBCs were differentiated on stained blood smears to determine the percentage of neutrophils, lymphocytes, eosinophils, monocytes and basophils. Absolute counts for each WBC were determined by multiplying the percentage of each WBC type (% from the smear) by the total WBC count determined by the automated analyser ($x10^9$/L). The relative proportion of N:Ls were determined by dividing absolute neutrophil counts by absolute lymphocyte counts. Reticulocyte counts (RET; $x10^9$/L) were performed by estimating the number of reticulocytes per 1000 RBCs on prepared blood smears.

Frozen serum was thawed at room temperature before analyses. Albumin (g/L), total serum protein (TSP; g/L) and globulin (g/L) concentrations were determined using the Konelab Prime 30i analyser (Thermo scientific, Australia) at VPDS, within seven days of blood collection.

3.3.5 Trace element and heavy metal analyses

Blood trace element and heavy metal concentrations were determined in whole blood (n = 41, including lower density populations for comparison) using the Ultra-Mass Spectrometer System (ICP-MS, Varian Australia Pty Ltd Mulgrave, Victoria) at a commercial laboratory (Department of Chemical Pathology, Royal Prince Alfred Hospital, Camperdown, Sydney, NSW). Blanks were regularly analysed during the sample run to ensure no contamination. Precision and accuracy of analyses were determined by comparing results against certified values (if available), determined using standard material Normal Range Trace Element Serum Toxicology Control (UTAK Lot # 66816, UTAK Labs Inc, Valencia, Ca 91355) as per laboratory standard operating procedures.

3.3.6 Comparison study sites

An additional larger dataset was utilised to compare health parameters from a range of sites. This larger dataset contains observations from up to five comparative kangaroo populations, described briefly below and previously by Brandimarti et al. (2020, [thesis Chapter 2]). Nelson Bay Golf Course (NBGC) (-32.728°S, 152.150°E) is a peri-urban free-ranging population in Port Stephens, NSW with a

population density ranging from 1.88 (2015) to 1.21 (2018) individuals ha^{-1}. Vegetation is dominated by pastoral grass species and dry sclerophyll forest. Samples were collected from NBGC between 2015 and 2019. Darlington Park (DP) (-30.048°S, 153.191°E) is a free-ranging coastal population in Arrawarra, NSW with a density of 1.44 individuals ha^{-1}. The site is a mixture of caravan park, golf course, private farmland and coastal bushland. Samples were collected at DP between 2017 and 2019. Heritage Park (HP) (-30.183°S, 153.149°E) is a semi-rural free-ranging population inhabiting a new housing estate and private farmland in Moonee Beach, NSW, with a density of 1.23 to 1.52 individuals ha^{-1} (Henderson et al. 2018b). The vegetation at HP consists of grazing pasture, ornamental grasses and wet and dry sclerophyll forests. Samples from HP were collected in 2017. Ainslie Majura Kangaroo Management Unit (AM) (-35.274°S, 149.165°E) is a semi-rural free-ranging population located within Mount Ainslie Nature Reserve, Canberra, ACT. The kangaroo density in the broader management area was 1.69 individuals ha^{-1}. This site is subject to ongoing population management via routine culling, due to the impacts of high-density kangaroos on natural values. The vegetation at AM is dominated by natural temperate grassland and box-gum grassy woodland. Sampling was conducted in 2018. Woolgoolga (WGA) (-30.102°S, 153.185°E) is a semi-rural free-ranging population bordering the town of Woolgoolga, NSW. Vegetation at WGA consists of pastoral grass species and dry sclerophyll forest. The population density at this site is unknown, however the density of a nearby population (Safety beach golf course) is 1.57 to 2.32 individuals ha^{-1} (Henderson et al. 2018b). Samples were collected from WGA in 2019.

3.3.7 Statistical analyses

3.3.7.1 Comparison of blood analytes to species-specific reference intervals and rainfall

Blood analytes were partitioned based on age (adult and subadult) as recommended for interpretation of species-specific RIs (Brandimarti et al. 2020 [thesis Chapter 2]). For both subgroups, each analyte from an individual was compared to the upper and lower limit of the relevant RI (Brandimarti et al. 2020 [thesis Chapter 2]). Each value was classified as residing within the RI range

or residing above or below the upper and lower limit. The number of animals in each classification was converted to a percentage of the total sample group at LAMN. Analytes with < 2.5% (non-parametric 95% confidence intervals, *CIs*) or < 5% (robust 90% *CIs*) of the population falling outside the central RI could indicate random error and are not uncommon, even for healthy populations (Friedrichs et al. 2012; Brandimarti et al. 2020 [thesis Chapter 2]). Blood analytes were described using median values with 95% *CIs* to allow for comparison between subgroups and because most analytes were not normally distributed (Shapiro-Wilk normality test). All further analyses were conducted using R version 3.5.3 statistical software (R core team 2017). To determine the effect of rainfall on blood parameters linear mixed effect models (lmm) using the 'lmer' function in the 'lme4' package were used (with year and animal ID as random factors), and rainfall as a fixed factor. Rainfall was selected due to its role in driving variation of blood parameters (Brandimarti et al. 2020 [thesis Chapter 2]). Accumulated rainfall (mm) data from the month of sample collection were obtained from the nearest weather station (BOM 2020). Where parameters were normally distributed a one-way analysis of variance (ANOVA) function in the 'car' package was then used to test (Type II Wald Chi-squared) analysis of deviance on models to determine *P* values. For parameters that were not normally distributed, either a square, log or cube root transformation was performed before analysis using ANOVA. If normality could not be established a Kruskal-Wallis test was applied.

3.3.7.2 Reticulocyte count and N:L ratio analyses

Raw reticulocyte counts are reported because counts were too low for descriptive statistics. To determine the effects of biotic and abiotic factors on N:L ratios (n = 286, including lower density populations for comparison) lmm were used. Prior to analysis a log transformation was performed and normality and collinearity assessed using the Shapiro-Wilk normality test and variance inflation factor (<2), respectively. The initial factors for all models were selected from relevance in the literature (Turneret al. 2012; Fancourt and Nicol 2019) and previous work (Brandimarti et al. 2020

[thesis Chapter 2]). Factors for N:L ratio model selection are presented in Table 3.1. A stepwise method was used to select fixed factors for the model, with the best fit used for final analyses. Factors were added individually, and the model fit was assessed using Akaike Information Criterion. Residual plots were evaluated to ensure random distribution of error. The ANOVA function was then used to test (Type II Wald Chi-squared) analysis of deviance on models to determine *P* values. For significant factors with more than one level, estimated marginal means were compared to determine significance among levels using the 'emmeans'package.

Table 3.1 Model factors for parasite abundance (faecal worm egg counts and ticks), presence-absence (mites) and relative proportion of neutrophils to lymphocytes (N:L ratio) in eastern grey kangaroos (*Macropus giganteus*) from Look At Me Now Headland (LAMN), Nelson Bay Golf Course (NBGC), Darlington Park (DP), Heritage Park (HP) and Ainslie Majura Kangaroo Management Unit (AM). A stepwise method was used to select fixed factors.

Response variable	**Abiotic fixed effects**	**Biotic fixed effects**	**Random effects**
Log_N:L	Site and rainfall	Maturity and sex	ID[b], year
EPG[a]	Site and rainfall	Maturity and sex	ID[b], year
Total tick burden	Site	Sex	ID[b], year
Mite prevalence	Site, rainfall and season	Sex	ID[b], year

[a]eggs per gram of faeces, [b]animal identification

3.3.7.3 Trace element and heavy metal analyses

Trace and heavy metal analytes were described using median values with 95% *CIs* because most analytes were not normally distributed (Shapiro-Wilk normality test). Values from the sample group at LAMN were qualitatively compared to two separate populations of low-density kangaroos (WGA and NBGC). Statistical comparisons were not performed due to the small sample size at these additional sites (WGA = 2 and NBGC = 5).

3.3.7.4 Parasitological analyses

To ensure observations were random before proceeding to analyses, separate time series plots of EPG and total tick counts were visually examined and determined to be random. Initial exploration of parasite data revealed that the variance was considerably higher than the mean, precluding the

use of a Poisson distribution on this dataset (Ver Hoef and Boveng 2007). To determine the biotic and abiotic factors driving parasite abundance, analyses of EPG and total tick counts were conducted using generalised linear mixed effect regression models (glmm) using the 'glmer.nb' function in the 'lme4' package. To determine mite presence-absence, a glmm was used with a binomial distribution and log link function. Prior to all analyses collinearity was assessed using variance inflation factor and found to be < 2. The stepwise method of model selection, ANOVAs and estimated marginal means (where appropriate) were repeated as above to generate a *P* value. Selected factors for EPG, tick and mite analyses are presented in Table 3.1.

3.4 Results

3.4.1 Population count at LAMN

The mean kangaroo density at LAMN in February 2018 and February 2019 was 5.4 (*CV* = 11.3%) individuals ha^{-1}. The mean red-neck wallaby density was 0.8 (*CV* = 57.3%) individuals ha^{-1}.

3.4.2 Haematological, glucose and serum protein analyses

3.4.2.1 Comparison of blood analytes to species-specific reference intervals and rainfall

All values were within the species-specific RI for glucose concentration and MCV. As such, these analytes will not be discussed further. There was a greater overall percentage of adults compared to subadults with blood analytes outside the RI limits (Figure 3.3A and 3.3B). More than 90% of adults were within the RI for absolute neutrophil, basophil, lymphocyte, eosinophil, monocyte count, NRBC count, WBC count and albumin concentration. However, many adults were below the RI for HGB concentration (26%), RBC count (48%) and HCT (72%); and above for PLT count (28%), TSP concentration (30%), MCH (46%), MCHC (72%) and globulin concentration (74%). More than 90% of subadults were within the RI for NRBC count, absolute neutrophil, lymphocyte and basophil count. However, many subadults were below the RI for HGB concentration (21%) and HCT (29%); and above the RI for PLT count (13%), MCHC (17%), TSP concentration (17%), MCH (50%) and globulin

concentration (58%). Maturity-specific sub-group median values (95% *CIs*) are presented alongside RI upper and lower limits for the LAMN population (Table 3.2).

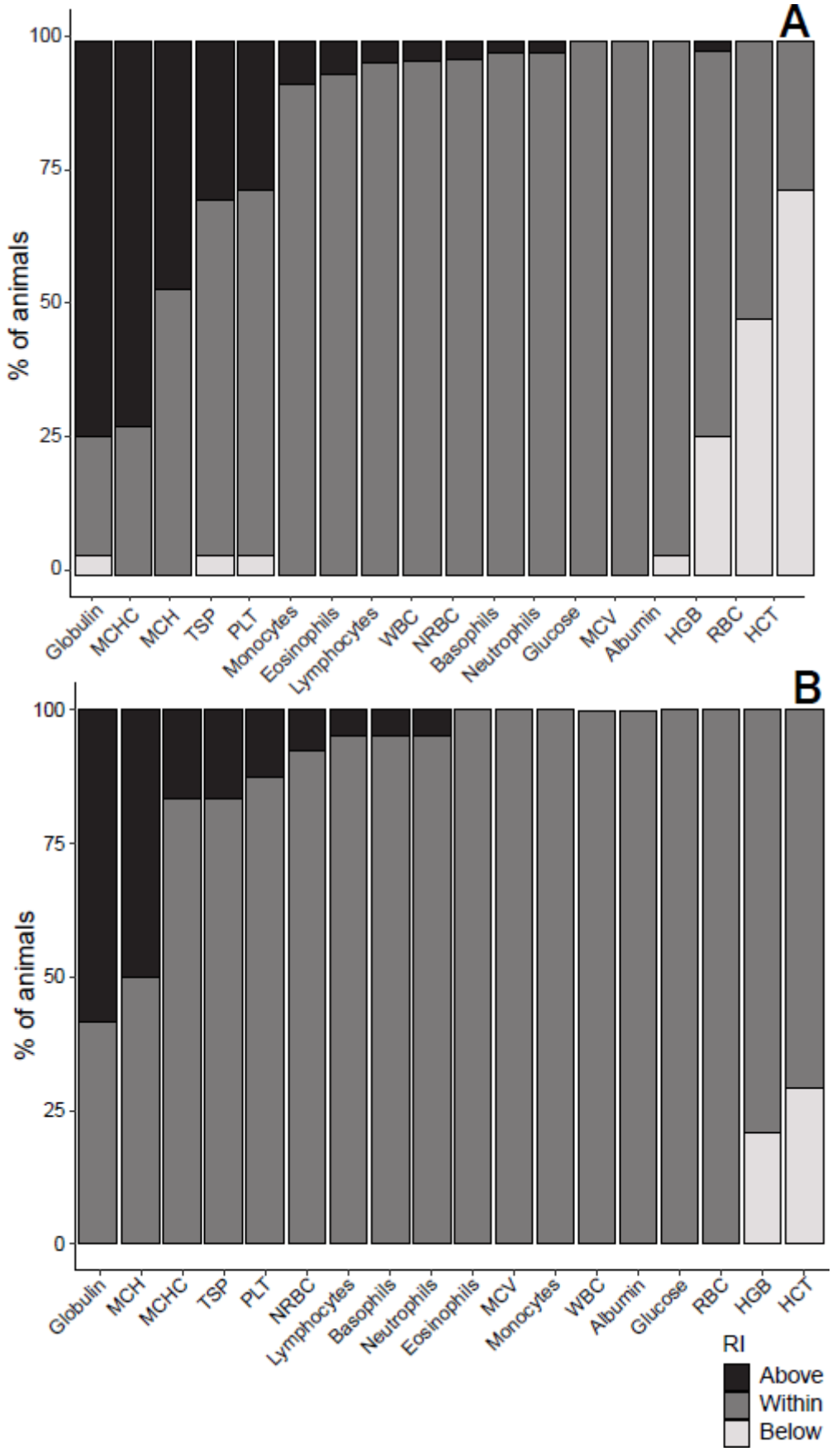

Figure 3.3A and B. Histograms of the percentage of (A) adult and (B) subadult eastern grey kangaroos (*Macropus giganteus*) whose blood analytes reside within, above or below the upper and lower limits of the population reference interval (RI) for each blood analyte (Brandimarti et al. 2020 [thesis Chapter 2]).The percentage has been determined from the sample group ((A) n ≤ 54 and (B) n ≤ 24) at Look At Me Now Headland (LAMN), New South Wales (NSW), Australia from 2017 to 2019. Dark grey indicates the percentage of animals sampled within the RI, black and white represents the percentage of animals above or below the reference limits respectively.

Table 3.2 Median (95% confidence intervals, CIs) subadult and adult eastern grey kangaroo (*Macropus giganteus)* blood results from the sample group at Look At Me Now Headland (LAMN), New South Wales(NSW), Australia from 2017 to 2019. Upper and lower population reference interval (RI) limits are provided for comparison (Brandimarti et al. 2020 [thesis Chapter 2]).

Parameter	Units	Maturity	n	Median (95% CIs)	Reference interval (n)
Red blood cell count	$x10^{12}$/L	Adult	54	1.5 (1.4, 1.7)	1.49-4.51 (178)
		Subadult	24	1.8 (1.5, 2.2)	0.46-6.27 (54)
White blood cell count	$x10^{9}$/L	Adult	54	6.9 (6.4, 7.5)	1.78-11.88 (173)
		Subadult	24	6.5 (5.6, 7.6)	2.28-15.09 (53)
Glucose	mmol/L	Adult	52	3 (2.8, 3.2)	1.09-5.47 (98)
		Subadult	22	3.6 (3.3, 4)	1.31-6.77 (35)
Neutrophil count	$x10^{9}$/L	Adult	50	2.8 (2.5, 3.2)	0.62-5.24 (155)
		Subadult	21	1.8 (1.5, 2.4)	0-4.1 (42)
Lymphocyte count	$x10^{9}$/L	Adult	50	3.3 (3, 3.7)	0.74-7.59 (161)
		Subadult	21	4.1 (3.4, 5.1)	0.09-8.56 (40)
Eosinophil count	$x10^{9}$/L	Adult	50	0.7 (0.5, 0.8)	0.04-1.39 (155)
		Subadult	21	0.4 (0.2, 0.5)	0-1.1 (39)
Monocyte count	$x10^{9}$/L	Adult	50	0.2 (0.1, 0.3)	0-0.43 (155)
		Subadult	21	0.2 (0.1, 0.3)	0-0.66 (40)
Basophil count	$x10^{9}$/L	Adult	50	a	0 -0.08 (155)
		Subadult	21	a	0-0.09 (40)
Haemoglobin	g/L	Adult	54	114 (108, 120)	98.8-164 (175)
		Subadult	24	105 (97, 112)	88.28-162.52 (51)
Haematocrit	L/L	Adult	54	0.1 (0.1, 0.1)	0.15-0.41 (179)
		Subadult	24	0.2 (0.1, 0.2)	0.12-0.47 (54)
Mean corpuscular volume	fL	Adult	54	84 (82.2, 85)	61.82-108.35 (178)
		Subadult	24	81.3 (79.3, 83.6)	60.95-112.17 (54)
Mean corpuscular haemoglobin	pg	Adult	54	78 (71, 86)	29.94-75.62 (179)
		Subadult	24	62.2 (52.1, 74)	15.06-60.72 (53)
Mean corpuscular haemoglobin concentration	g/L	Adult	54	944 (858, 1038)	352.13-793.64 (180)
		Subadult	24	754 (639, 907)	315.47-1132.14 (54)
Platelet	$x10^{9}$/L	Adult	54	196 (165, 224)	74.38-259.55 (168)
		Subadult	24	141 (105, 184)	11.43-278.87 (51)
Nucleated red blood cell count	$x10^{9}$/L	Adult	30	a	0-0.16 (45)
		Subadult	13	a	0-0.06 (26)
Albumin	g/L	Adult	27	28.8 (27.2, 30.2)	16.28-42.27 (68)
		Subadult	12	28.4 (26, 32)	22.32-41.53 (32)
Total protein	g/L	Adult	27	76.8 (73, 80.3)	59.14-80.99 (31)
		Subadult	12	69.8 (68.1, 76.8)	48.75-77.85 (26)
Globulin	g/L	Adult	27	47.3 (43.9, 50.9)	24.35-41.76 (30)
		Subadult	12	41.8 (35.4, 48)	19.63-40.24 (26)
Reticulocyte count	$x10^{9}$/L	Both	31	(2 counted in n=31)	1.0-1.0

[a]values too low

Rainfall had a significant effect on absolute lymphocyte (χ^2_1 = 6.59, P < 0.05), eosinophil (χ^2_1 = 5.32, P <0.05) and monocyte counts (χ^2_2=7.78, P < 0.05). There was a general trend for decreasing absolute lymphocyte and eosinophil count with increasing rainfall, however, the opposite was true for absolute monocyte count. There were no significant effects on any other blood parameters.

3.4.2.2 Blood smears and N:L ratio

Microcytosis (reduced RBC size), hypochromasia (pale cytoplasmic colour) and target cells (RBC surface disproportionate to volume) were noted in several blood smears. Two reticulocytes were found from examination of n = 31 reticulocyte smears from the sample group at LAMN (Table 3.2). N:L ratio differed significantly among sites (χ^2_4 = 33.43, P < 0.001) and maturity (χ^2_1 = 46.75, P < 0.001). NBGC had the greatest mean (± *SD*) N:L ratio (0.83 ± 0.42) followed by LAMN (0.82 ± 0.5), HP (0.77 ± 0.38), DP (0.71 ± 0.49) and AM (0.5 ± 0.54) (Figure 3.4). These differences were significant for AM and each of LAMN, HP and NBGC (P < 0.05 for all three) as determined by estimated marginal means. Across all sites, adults had significantly greater N:L ratios compared to subadults (0.84 ± 0.46, n = 225 and 0.5 ± 0.37, n = 61 respectively).

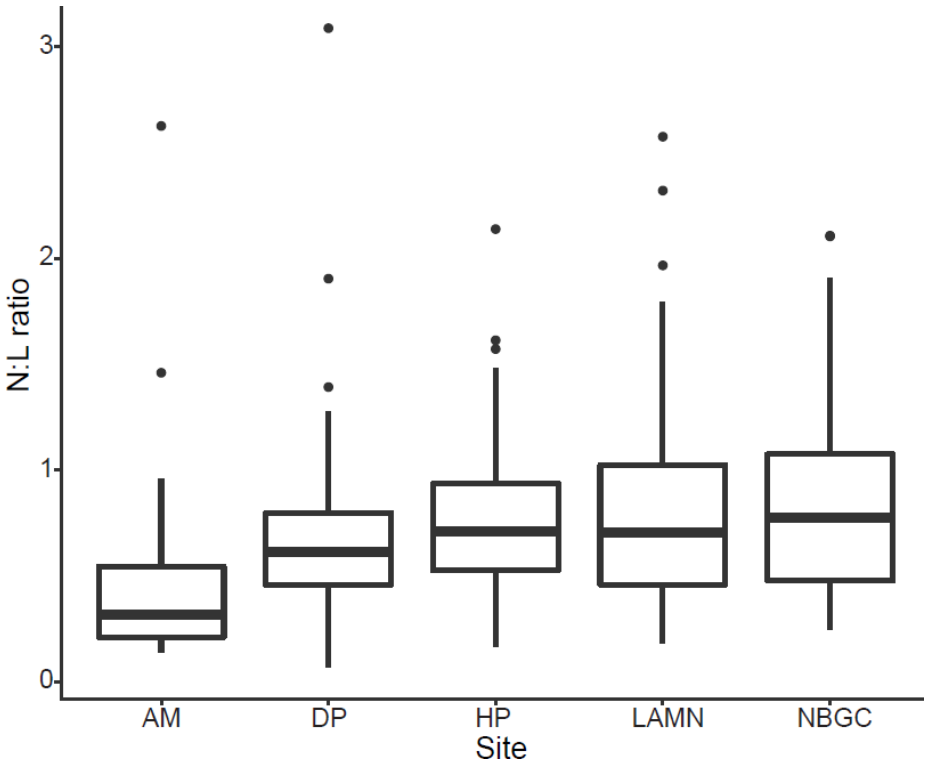

Figure 3.4 Box and whisker plot of the relative proportion of absolute neutrophils to absolute lymphocytes (N:L ratio) from eastern grey kangaroos (*Macropus giganteus*) sampled from Look At Me Now Headland (LAMN, n = 71) from 2017 to 2019, Nelson Bay Golf Course from 2015 to 2019, (NBGC, n= 81), Darlington Park (DP, n = 47) from 2017 to 2019, Heritage Park (HP, n = 62) in 2017 and Ainslie Majura Kangaroo Management Unit (AM, n = 25) in 2018. Box plots display the median, with the lower and upper limits of the box corresponding to the 25th and 75th percentiles. The upper whisker extends to the largest value no further than 1.5 times the interquartile range. The lower whisker extends to the smallest value at most 1.5 times the interquartile range. Data beyond the end of the whiskers are outliers and are plotted individually.

3.4.2.3 Trace element and heavy metals

Median (95% *CIs*) trace element and heavy metal concentrations at LAMN are compared to two other kangaroo populations (Table 3.3). Based on qualitative comparison of the data (due to the limited sample size at NBGC and WGA), there was a higher mean concentration of nickel, tin, mercury, cadmium and bismuth at LAMN compared to NBGC and WGA. LAMN had a lower iron, selenium, zinc and to a lesser extent magnesium concentration, compared to NBGC and WGA. WGA had the highest concentration of manganese, selenium and lead. Arsenic concentration was highly variable between all three sites; however it was highest at WGA and below the detection limit at NBGC.

Table 3.3 Median (95% confidence intervals, CIs) eastern grey kangaroo (*Macropus giganteus*) blood trace and heavy metal concentration (µg/L) from three different sites in 2019; Look At Me Now Headland (LAMN), Nelson Bay Golf Course (NBGC) and Woolgoolga (WGA), New South Wales (NSW), Australia.

Analyte (µg/L)	LAMN (n=33)	NBGC (n=5)	WGA (n=2)
Mg[a]	36314 (35519, 37189)	37806 (33328, 42283)	37920 (37608, 38230)
Mn[b]	25.3 (21.7, 30.2)	23.4 (11.2, 45.1)	40.8 (37.1, 44.6)
Co[c]	0.6 (0.5, 0.7)	0.5 (0.3, 0.7)	0.6 (0.4, 0.7)
Ni[d]	2.1 (1.8, 2.4)	1.3 (0.9, 2)	1.7 (1.3, 2.1)
Zn[e]	2275 (2216, 2332)	2553 (2239, 2866)	2462 (2401, 2523)
As[f]	120 (107, 131)	BDL[s]	541 (489, 594)
Al[g]	118 (97.4, 140.9)	132 (104, 168)	115 (102, 129)
Cr[h]	9.3 (9.1, 9.6)	9 (8.3, 12)	9.3 (9, 10)
Fe[i]	370991 (352749, 388513)	494037 (463713, 535697)	445786 (402065, 489507)
Cu[j]	344 (320, 370)	359 (346, 414)	362 (352, 372)
Se[k]	74.4 (54.2, 100)	91.6 (68.8, 114.4)	373 (352, 395)
Cd[l]	0.2 (0.1, 0.3)	0.1 (0, 0.5)	0.2 (0.1, 02)
Sn[m]	0.7 (0.6, 0.8)	0.4 (0.3, 0.6)	0.6 (0.5, 0.7)
Sb[n]	14.3 (12.3, 167.)	12.5 (10.2, 15.3)	12.7 (12, 13.5)
Hg[o]	8.6 (5.4, 14)	1.4 (1.1, 1.7)	2 (1.6, 2.5)
Tl[p]	0.1 (0.1, 0.2)	0.1 (0, 0.1)	0.1 (0.1, 0.1)
Pb[q]	16.6 (14.3, 18.8)	7.4 (5.1, 8.6)	24 (22.2, 25.8)
Bi[r]	0.2 (0.1, 0.2)	0.1 (0, 0.1)	0.1 (0.1, 0.2)

[a]Magnesium, [b]Manganese, [c]Cobalt, [d]Nickel, [e]Zinc, [f]Arsenic, [g]Aluminium, [h]Chromium, [i]Iron, [j]Copper, [k]Selenium, [l]Cadmium, [m]Tin, [n]Antimony, [o]Mercury, [p]Thallium, [q]Lead, [r]Bismuth, [s]below detection limit

3.4.2.4 Parasitological results

EPG differed significantly among sites (χ^2_3 = 22.97, P < 0.001). Kangaroos at LAMN had the highest EPG values (1660 ± 1444) followed by HP (1279 ± 1343) and NBGC (1173 ± 1644), all of which were significantly greater than DP (549 ± 834); as determined by estimated marginal means (Figure 3.5). Total tick counts differed significantly among sites (χ^2_4 = 24.34, P < 0.001) and sex (χ^2_1 = 10.66, P < 0.01). Across all sites, total tick counts were higher in males (5.03 ± 5.6, n = 115) compared to females (3.96 ± 5.26, n = 172). Kangaroos at NBGC had the greatest mean total tick count (6.48 ± 4.48) followed by LAMN (6.42 ± 6.63), DP (4.39 ± 4.95), HP (2.05 ± 3.91) and AM (0 ± 0) (Figure 3.6). Estimated marginal means showed that this difference was significant for LAMN and HP (P < 0.01) and DP and HP (P < 0.05). Mite presence-absence differed significantly among sites (χ^2_2 = 6.97, P < 0.05), seasons (χ^2_3 = 9.35, P < 0.05) and between sexes (χ^2_1 = 7.62, P < 0.01). Mites were seen more commonly in females (25%) compared to males (6%). Estimated marginal means showed a significant difference in occurrence of mites between two sites: HP and LAMN (P < 0.05). Mites were more commonly seen in kangaroos sampled at LAMN (38% of the sample population), followed by DP (14%) and HP (2%). Mite presence-absence was also significantly different among seasons, as determined by post hoc testing; spring accounted for 39% of mite observations, followed by summer (25%), autumn (11%) and winter (7%).

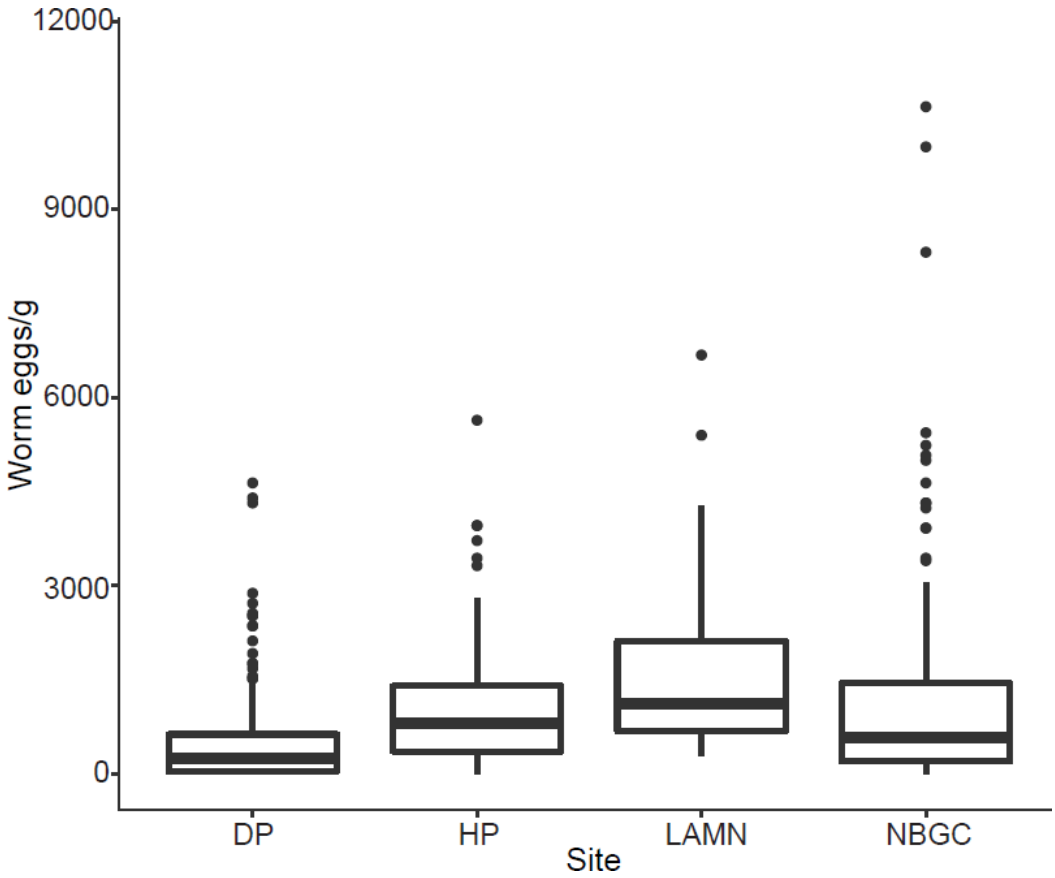

Figure 3.5 Box and whisker plot of worm eggs per gram of faeces (EPG) from eastern grey kangaroos (*Macropus giganteus*) sampled from Look At Me Now Headland (LAMN, n = 45) from 2017 to 2019, Nelson Bay Golf Course (NBGC, n = 172) from 2015 to 2019, Darlington Park (DP, n = 162) from 2017 to 2019 and Heritage Park (HP, n = 39) in 2017. Box plots display the median, with the lower and upper limits of the box corresponding to the 25th and 75th percentiles. The upper whisker extends to the largest value no further than 1.5 times the interquartile range. The lower whisker extends to the smallest value at most 1.5 times the interquartile range. Data beyond the end of the whiskers are outliers and are plotted individually.

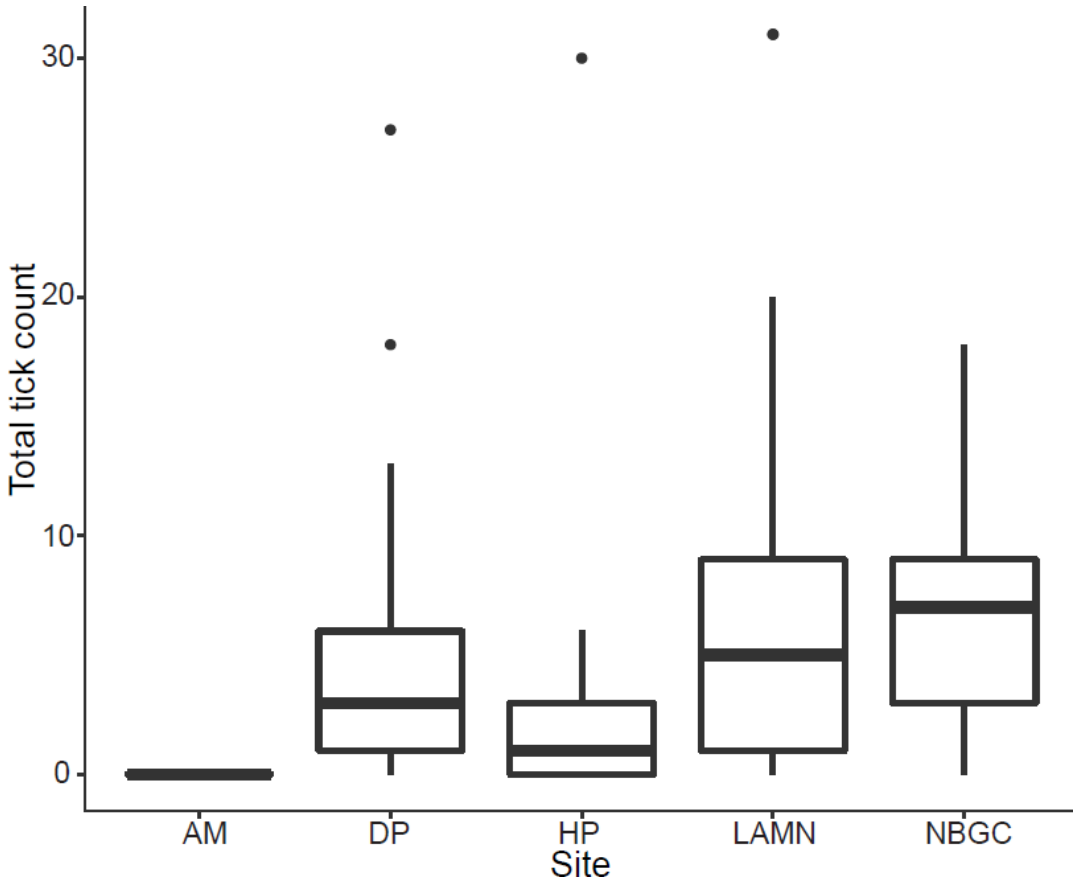

Figure 3.6 Box and whisker plot of total tick counts from eastern grey kangaroos (*Macropus giganteus*) sampled from Look At Me Now Headland (LAMN, n = 79) from 2017 to 2019, Nelson Bay Golf Course (NBGC, n = 54) from 2015 to 2019, Darlington Park (DP, n = 62) from 2017 to 2019, Heritage Park (HP, n = 64) in 2017 and Ainslie Majura Kangaroo Management Unit (AM, n = 28) in 2018. Box plots display the median, with the lower and upper limits of the box corresponding to the 25th and 75th percentiles. The upper whisker extends to the largest value no further than 1.5 times the interquartile range. The lower whisker extends to the smallest value at most 1.5 times the interquartile range. Data beyond the end of the whiskers are outliers and are plotted individually.

3.4.2.5 Body condition

A subjective body condition score was assigned to n = 30 animals from LAMN. 11 animals were in 'good'condition (n = 7 adults and n = 4 subadults), 12 animals were in 'thin' condition (n = 8 adults and n = 4 subadults) and seven animals were in 'poor' condition (n = 4 adults and n = 3 subadults). No animals were assessed as being in 'excellent' condition.

3.5 Discussion

This study highlights several health concerns in the LAMN kangaroo population which were not apparent in other populations at lower densities. Principle among these concerns is the significant number of animals with blood analytes outside of the species RI and high parasitism. Another concern is the number of animals in 'thin' and 'poor' body condition. We hypothesise that high animal density at LAMN is associated with nutritional stress and higher parasite prevalence, which in turn has a direct influence on haematological parameters. Densities of more than two kangaroos per ha^{-1} are considered high (Banks et al. 2000) and all of our comparative populations in which health concerns have not been identified, are below this figure. The population density at LAMN (5.4 individuals per ha^{-1}) is more than double that of many densities previously recorded for this species (Southwell 1984; Banks et al. 2000; Ramp and Coulson 2002; Brandimarti et al. 2020 [thesis Chapter 2]). Management of further population growth will be critical for the long-term improvement of health and welfare in this population. However, veterinary intervention on an 'as needed' basis is recommended in circumstances of animals showing obvious signs of disease, poor condition and compromised welfare.

A large number of adults and a lesser number of subadults sampled at LAMN were anaemic based on RI comparisons. Decreased HCT, RBC counts and HGB concentration were noted, consistent with clinical observations of mucous membrane pallor in sampled individuals. Anaemia can be characterised as either regenerative or non-regenerative based on the number and proportion of circulating immature RBCs (reticulocytes and NRBC) and manifests clinically as lethargy, reduced

exercise tolerance, impaired weight gain and growth (Canale and Lanzkowsky 1970; Thrall 2012). Anecdotal observations at LAMN noted that many single kangaroos appeared less active (lethargic) and had self-isolated from the larger group of kangaroos, which is unusual for a gregarious animal. Based on the absence of reticulocytes (except for two in total across all animals sampled), the anaemia observed is classified as non-regenerative, such that the HCT would not return to the normal RI if the disease impact is ongoing (Neiger et al. 2002; Thrall 2012). Blood smears revealed evidence for non-regenerative anaemia including hypochromasia, target cells and microcytosis. Microcytosis, however, was not reflected in the automated haematological results. The small size of microcytic RBCs can result in undercounting by automated cell counters, resulting in falsely elevated MCV calculations (Weiss 2010), which could explain this disparity. Non-regenerative anaemia is due to defective or reduced production of RBCs in the bone marrow (Thrall 2012) and can be caused by primary bone marrow disease, reduced availability of iron or secondary impacts on the bone marrow due to anaemia of chronic disease (Nemeth 2008). There are a number of chronic conditions that cause secondary anaemia including inflammation, renal disease, neoplasia and endocrine abnormalities; anaemia due to chronic parasitism (generally due to haemorrhage) and nutritional anaemias are also recognised (Weiss 2010). The effect of nutritional status on RBC counts in kangaroos has been partly addressed in Portas and Snape (2008), however investigation of the underlying cause of the anaemia in this population is critical.

Thrombocytosis (platelet number above the RI) was also seen in 28% of adults and 13% of subadults. Thrombocytosis is a common finding in acute blood loss and/or iron deficiency anaemia and has been observed in many animal species (Weiss 2010; Helmick and Milne 2012; Lee McMichael et al. 2015).

Age-related differences in blood parameters were also evident. Subadult kangaroos from LAMN had lower RBC indices (MCV, MCH and MCHC) compared to adults, although interestingly, most of these indices were either within or above the RI. The greater percentage of adults diagnosed with anaemia could reflect the chronic nutritional deficiencies at this site. Given the greater body size of adults and

the additional energy expenditure of lactating females, the nutritional requirements of adults is greater than subadults (Munn and Dawson 2001). Subadult kangaroos have an energy requirement approaching 70% of an adult female, therefore it is likely that the health of subadults will decline at weaning and there may be long-term impacts on growth with increased susceptibility to disease in adulthood (Munn and Dawson 2001; Huynh et al. 2015).

Rainfall has been shown to play a large role in determining erythrocyte values (Brandimarti et al. 2020 [thesis Chapter 2]). For example, the relationship between RBC count and rainfall is parabolic, with a decrease in RBC counts at 100 mm of rain (Brandimarti et al. 2020 [thesis Chapter 2]). In this study there was no significant effect of rainfall on any erythrocyte values, only differential WBCs. Nutritional stress caused by competition for food alongside prolonged drought may have masked the short-term effect of rainfall on erythrocytes, which was measured over less than six months.

N:L ratios have been used recently in wildlife populations as an indirect measure of stress (Davis et al. 2008). In response to stress, the number of neutrophils increase, while the number of lymphocytes decrease (Davis et al. 2008). Significantly higher N:L ratios found in kangaroos at the peri-urban sites LAMN, NBGC and HP compared to AM (a nature reserve) could indicate populations under stress. Previous research in Queensland established that kangaroos living within the peri-urban space had higher levels of stress hormone metabolites than their rural counterparts (Brunton et al. 2020). However, the opposite was true for populations in the ACT (Brunton et al. 2020). In our study, it was not possible to delineate the impacts of anthropogenic stress from other causes such as nutritional stress, high-density, and parasitism.

Many kangaroos at LAMN had TSP concentrations above the RI. Increased protein often results from haemoconcentration (Senay Jr and Christensen 1965), and prolonged skin ‘tenting’ was present in several individuals, indicating dehydration. Albumin concentrations, however, were within the normal RI, suggesting that a component of the elevated TSP concentration can be attributed to a true increase in globulins. In most animals, globulin concentrations were above the RI. Globulin

concentrations can increase in response to inflammation and infection (Serrano et al. 2018) as well as prolonged immunostimulation due to underlying disease. This increase could be attributed to chronic parasitism in this population, which is increased in adults who spend more time grazing in order to meet their energy requirements. Differentiation of the globulin fractions by serum protein electrophoresis would aid interpretation of globulin results.

Kangaroos (amongst others in the Macropodoidea superfamily) have been known to host the most diverse array of parasites of any known mammalian group (Beveridge and Chilton 2001), many of which have been previously documented (Beveridge and Arundel 1979; Arundel et al. 1990; Vogelnest and Portas 2008). The impact of parasitism can range from no effect to reduced growth and reproduction, clinical disease and mortality; depending on the species and parasite load (Gulland 1995). Nematode species such as *Rugopharynx rosemariae* have been known to cause severe hypertrophic gastritis (inflammation of stomach mucosa) in kangaroos, while large burdens of *Globocephaloides trifidospicularis* may cause haemorrhage, anaemia, and mortality in juvenile kangaroos (Arundel et al. 1990; Fazenda 2009).

Kangaroos at LAMN had a higher median EPG (range 280-6680) compared to any other site in this study, however the range overlaps with NBGC and HP. The high EPG at LAMN also exceeds those reported in studies of other kangaroo populations (Cripps et al. 2013, 2014, 2015). The high population density at LAMN is a likely driver of parasitism because the high contact rate between kangaroos promotes parasite transmission (Gortázar et al. 2006). High population density has been suggested to increase parasitism in other marsupial species, such as the Tasmanian devil (*Sarcophilus harrisii*) (Gregory et al. 1975; Arneberg et al. 1998). The mechanistic model for this pathway is that as host density increases, each parasite larvae or egg has an increased chance of contacting a host (May and Anderson 1978). Despite being a common and non-lethal practice for estimating nematode burdens, EPGs from LAMN should be interpreted with caution as they may not be representative of an animal's total worm burden, rather driven by a single species of nematode

present (Cripps et al. 2015).

Opportunistic necropsy of kangaroos from LAMN revealed large gastric nematode burdens (Figure 3.7A), accompanied by multi-focal haemorrhages of the gastric wall (Figure 3.7B). Scouring was also noted in some individuals. A limitation of our investigation was the lack of assessment of species and proportion of parasite species present. For this reason, the impact of gastric parasitism on the health status and blood parameters of this population is not able to be quantified. However, given the high occurrence of non-regenerative anaemia in the LAMN population, as well as increased eosinophil counts in some adult kangaroos, it is possible that pathogenic nematode species could be contributing to the poor health of kangaroos at LAMN. Identifying the parasites present is a recommended next step for future research.

Figure 3.7 (A) Large gastrointestinal worm burden in the stomach. (B) Multi-focal haemorrhages (indicated by arrows) of the gastric wall. (C) Tick and mites around the perimeter of the eye. (D) Orange patches associated with pruritis and inflammatory lesions of the axilla. (E) Mites and inflammation at the base of the teat in a female kangaroo (F) Emaciated male kangaroo dispatched by police due to reports by the public of an animal with severe emaciation and lethargy. All eastern grey kangaroos (*Macropus giganteus*) photographed were from Look At Me Now Headland (LAMN) during the study period (2017 to 2019).

External parasitism was also evident at LAMN, with significantly higher total tick counts compared to most other sites (Figure 3.7C), with a significant tick bias in males compared to females. This bias has been firmly established in many studies and is thought to occur because of the immunomodulatory effects of testosterone in males (Folstad and Karter 1992; Zuk et al. 1995; Tschirren et al. 2003; Pollocket al. 2012).

Kangaroos from LAMN had a higher incidence of mites, with 38% of the sample population having mite infestations. Larvae of the Trombiculidae family have been described in the agile wallaby (*Notamacropus agilis*) and bridled nailtail wallaby (*Onychogalea fraenataies*), and have been found to cause inflammatory lesions (Turni and Smales 2001; McLelland and Youl 2005; Old et al. 2009). Clinical examination of affected animals showed orange patches associated with pruritis and inflammatory lesions of the axilla (Figure 3.7D), inguinal region, perimeter of the eye and in the inner ear folds (Mullen and O'Connor 2019). There was a significantly higher incidence of mites in female kangaroos at LAMN, and often mites were found inside the pouch, attached to and around the base of the teats, which appeared reddened and swollen (Figure 3.7E). This sex difference could be attributed to the warm, humid and protected location of the pouch; which benefits the mite and allows close contact with the skin. There was also a higher incidence of mites in summer, when the climate at LAMN is hot and humid. Similar environmental conditions have been promoted as a likely cause of increased mite numbers in the bridled nailtail wallaby (Old et al. 2009). Kangaroos at LAMN were found to have the greatest incidence of mites compared to other local sites (DP and HP).

Body condition can indicate nutritional status and is intrinsically linked to parasite burden (Ndlovu et al. 2007; Sánchez et al. 2018). Most kangaroos sampled from LAMN were in 'thin' (40%) or 'poor' (23%) body condition and had a dull coat (Figure 3.7F) as well as pale oral and urogenital mucous membranes, a clinical finding consistent with anaemia. This study was unable to compare body condition with lower density sites, however, most sampled animals scored in the lowest two categories (Johnston et al. 1997).

A high intensity of grazing pressure has been reported at LAMN (Hunter and Hunter 2019). Given the high-density of kangaroos and their strong site fidelity (unpublished data), it is hypothesised that kangaroos are experiencing nutritional stress, and as a result, may not be able to mount an adequate immune response to their parasite burdens. It is well known that a lack of micronutrients, particularly zinc, selenium and iron, all of which were lower at LAMN compared to other sites studied, can lead to clinically significant immune deficiency (Cunningham-Rundles et al. 2005). An adequate immune response in kangaroos could also be inhibited by increased concentration of mercury and cadmium both of which are non-essential elements and can cause immunotoxicity (Moszczyński 1997; El-Boshy et al. 2015).

This study determined that the LAMN kangaroo population had one of the highest population densities recorded for this species, with evidence of chronic nutritional stress, parasitaemia and overall poor welfare (Figure 3.8).

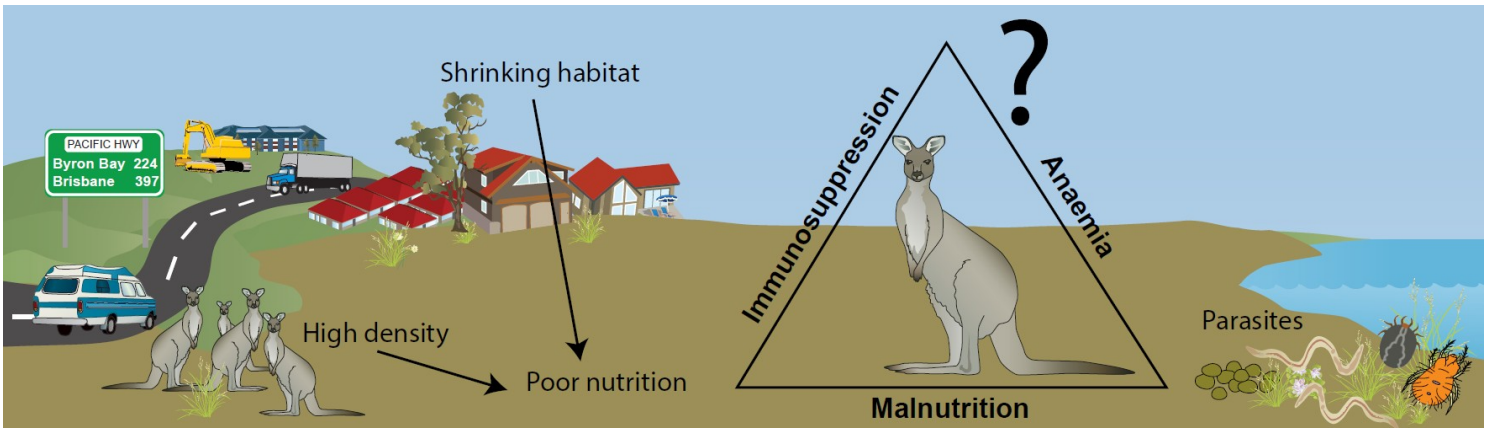

Figure 3.8 Hypothesised factors contributing to poor health and welfare of eastern grey kangaroos (*Macropus giganteus*) at Look At Me Now Headland (LAMN). Symbols courtesy of the Integration and Application Network, University of Maryland Center for Environmental Science (https://ian.umces.edu/symbols/).

These issues are a likely consequence of human-induced environmental modification and for this reason, management of this population is recommended. Short- and long-term management strategies, concurrent with ongoing health and disease monitoring, should be employed to ensure improved welfare outcomes for individuals. Short-term strategies could include lethal control (culling) which will reduce animal density but could be a controversial option, lacking local support. A targeted approach, whereby a veterinarian selects individuals with compromised welfare for humane euthanasia, based on signs of ill-thrift, poor body condition and associated symptoms (lethargy, reluctance to move), would achieve both improved animal welfare outcomes and reduce population density. Once the population size is reduced, reproductive management could be used as a longer-term solution to manage population growth. Reproductive management could also improve the health status of female kangaroos, reducing the metabolic demands of a pouch young and lactation (Gélin et al. 2015). In the long-term, local and regional planning must prioritise the maintenance of habitat connectivity for kangaroo populations, in order to prevent overabundance and enhance animal health and welfare. This study has demonstrated how local overabundance can result in concerning animal welfare outcomes. Future coastal developments must incorporate landscape ecology (Collinge 1996) such as wildlife movement corridors which may alleviate some pressures on urban kangaroos, however swift management interventions at LAMN are required to improve the health and welfare outcomes for this population.

The effect of testosterone suppression on health and parasite burden in male eastern grey kangaroos (*Macropus giganteus*)

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4.1 Abstract

Testosterone has a dualistic effect in males by promoting sexual ornamentation at the cost of immune defence. This trade-off has been demonstrated in several taxa; where males often host a greater parasite burden compared to female conspecifics. We suppressed testosterone in wild male eastern grey kangaroos (*Macropus giganteus*) for ten weeks using a novel gonadotropin-releasing hormone (GnRH) vaccine, Bopriva. We evaluated the impact of testosterone suppression on testes width, parameters of health, tick and worm burden in kangaroos using a Before-After-Control-Impact (BACI) experimental design. Given the potential impact of animal movement on parasite burden, core area use of a subset of males was also determined. Bopriva significantly reduced testosterone in male kangaroos as well as reducing the combined size of testes by 9.6% (P = 0.01). There was no detectable effect of testosterone suppression on parasite burden and core area use in *Treated* kangaroos compared to *Control* (placebo) and *Before* treatment animals. Our results suggest that a duration of suppression longer than ten weeks may be required to observe changes in parasite burden. Overall, this study provides a suitable framework for future studies to test whether reproductive hormones influence energy allocation, parasitism, and reproductive strategies in marsupials.

4.2 Introduction

Wildlife are home to a diverse community of parasites, many of which have the potential to harm their hosts (Pedersen and Fenton 2015). Parasitism can reduce host fitness, reproductive success and survival (Tompkins and Begon 1999; Tompkins et al. 2011). Parasites can also have sub-clinical effects by stimulating the energetically costly immune system and diverting resources away from other critical processes (Sheldon and Verhulst 1996). Parasites exist in a delicate balancing act with their host and the environment, in which parasite growth and replication is met by their host's immune defence (Sheldon and Verhulst 1996). This host-pathogen-environment interaction can be disturbed when animals are immunocompromised, have large energetic costs or when food is limited (Beldomenico and Begon 2010; Brandimarti et al. 2021 [thesis chapter 3]). Reproduction is another important energetic cost for hosts. For males, the reproductive hormone testosterone is costly to produce and can directly suppress immune function (Folstad and Karter 1992; Zuk et al. 1995; Pollock et al. 2012). When resources are limited, males can either allocate energy to secondary sexual signals (large antlers, body size or colourful plumage) which will improve their chance of finding a mate; or they can allocate energy to immunofunction, thereby potentially compromising their capacity to find a mate. This trade-off is referred to as the immunocompetence handicap hypothesis (ICHH, Folstad and Karter 1992).

The ICHH has been tested by multiple studies with mixed results. Findings are often ambiguous and sometimes contradictory (Roberts et al. 2004). On balance, however, the weight of evidence suggests that male animals have a higher parasite burden and greater susceptibility to disease compared to females (Folstad and Karter 1992; Zuk et al. 1995; Tschirren et al. 2003). For example, testosterone administration in female and castrated male mice increased their parasite burden, parasite egg production and infection duration, thereby influencing individual host survival (Alexander and Stimson 1988; Nakanishi et al. 1989). Other studies on the ICHH have shown opposite results. Male voles (*Microtus pennsylvanicus*, *Microtus ochrogaster*) mounted a stronger immune response compared to females when exposed to a novel antigen (Klein and Nelson 1998). In

addition to the often contradictory results among studies, evidence for the ICHH is especially lacking in wild populations that likely face resource shortages, compared to laboratory animals which always have an available food supply (De La Peña et al. 2020).

Host behaviour also influences levels of parasitism. Sex-specific behaviours, such as conspecific aggression and multiple mating partners can expose males to more parasites and aid their transmission (Seivwright et al. 2005; Grear et al. 2009; Shaw et al. 2018). Males also often have larger territories and move greater distances compared to females; however, this has a differential effect on parasite burden (Shaw et al. 2018). Increased movement can expose males to new and diverse parasite threats, but it can also reduce levels of parasitism as it allows hosts to evade contaminated pasture or infected conspecifics (Teitelbaum et al. 2018). The role that sex and behaviour plays in the acquisition of parasites remains largely unstudied in many vertebrates, including marsupials.

Eastern grey kangaroos (*Macropus giganteus*, hereafter kangaroos) are a sexually dimorphic, long-lived mammal, abundant throughout eastern Australia (Rioux-Paquette et al. 2015). Males are sexually dimorphic in weight (e.g. mature males 85 kg, females 42 kg) and forelimb size, with longer forearms, scapula bones and greater muscle development compared to females (Jarman 2000; Coulson 2008). These characteristics are used by males to gain mating rights to females, and also function to establish dominance by other display behaviours, such as grass-pulling, 'stiff-legged' walking and combat with rival males (Miller et al. 2010). Because testosterone increases skeletal muscle development (Emerson 2000), it is a likely driver of these sexual signals in male kangaroos. Male kangaroos are also known to occupy a larger home range than females (Fisher and Lara 1999; Brunton et al. 2019).

Kangaroo parasite communities are incredibly diverse and have a range of effects depending on the species present and the parasite load (Gulland 1995). Nematodes such as *Rugopharynx rosemariae* and *Globocephaloides trifidospicularis* are highly pathogenic in juvenile kangaroos (Arundel et al. 1990). However, some studies have found few impacts of these nematodes (at low levels) and other

parasite species on host growth or body condition (Cripps et al. 2014). Despite the importance of understanding the impacts of parasitism on wildlife health, there remains a paucity of evidence on the role of testosterone in this system. In other marsupials, the indirect effects of testosterone have been implicated as a causative agent in post-mating mortality of some dasyurid species (Bradley et al. 1980; Bradley 1987), however, the effect of testosterone on marsupials broadly has not been tested.

To address this knowledge gap, we experimentally manipulated testosterone and examined the relationship between testosterone, parasitism, core area use and health parameters in a large common marsupial, the kangaroo. Kangaroos were selected because males are sexually dimorphic and reproductive success is linked to testosterone concentration (Miller et al. 2010). We used a Before-After-Control-Impact (BACI) experiment, whereby testosterone was suppressed in wild adult males and their tick and worm burden, health parameters and core area use were compared *Before* and *After* treatment to a *Control* (placebo) group. The study was conducted over summer and autumn when the majority of worm species are acquired by kangaroos (Arundel et al. 1990), however ticks are consistently present in all months at the study site (unpublished data). At these times, haematological health parameters are likely to change in response to potential blood feeding or from limited nutrient absorption (Colditz 2008). For kangaroos in the *Control* group, we also predict that there will be a reduction in haematological values such as red blood cell counts, an increase in eosinophil counts, a systemic response to parasite contact, and a reduction in glucose concentration. We hypothesise that if testosterone is suppressed in males, they will have a lower parasite burden and occupy a smaller core area compared to before suppression (*Before*) and *Control* (placebo) animals. We predicted the core area use would contract in suppressed animals due to a reduction in energetic demand and the absence of mate-seeking behaviour.

4.3 Materials and methods

This study was conducted with animal ethics approval from The University of Sydney, 2015/917 and relevant scientific permits, SL102148.

4.3.1 Study site

The study was conducted at Nelson Bay Golf Course (NBGC), covering 70 ha of pastoral grassland and open dry sclerophyll vegetation in Port Stephens, New South Wales (NSW) (-32.728°S, 152.150°E). Southern and eastern sides of NBGC are surrounded by Tomaree National Park, however the northern side opens to Nelson Bay town centre and surrounding suburbs. Kangaroos at NBGC are free-ranging and are uniquely identifiable via ear tags and microchips (Herbert et al. 2020).

4.3.2 Manipulating testosterone

To suppress testosterone in male kangaroos, we used the commercially available drug Bopriva (400 µg/mL gonadotropin-releasing hormone (GnRH) protein conjugate, Zoetis, Australia), which stimulates antibodies to inhibit GnRH secretion (Pfizer 2020). GnRH regulates luteinising hormone and follicle stimulating hormone, which promote testosterone production (Randall et al. 1997). As antibodies neutralise GnRH, testicular function is suppressed (Janett et al. 2012a). Using a vaccination against GnRH (like Bopriva), is the only plausible means for transiently decreasing testosterone in kangaroos, as GnRH agonist implants are not effective in male macropodids (Herbert et al. 2004). Artificially elevating testosterone can lead to aggression, either towards conspecifics or people; for this reason, reducing testosterone in the *Treated* group rather than elevating testosterone was considered a safer option for achieving experimental manipulation.

Bopriva has not been previously tested for use in kangaroos, in which its administration is off-licence, providing the opportunity to evaluate its effectiveness in this species. Bopriva has been tested on cattle and results show it is safe and efficacious by reducing testosterone concentration, sexual and aggressive behaviour (Theubet et al. 2010; Janett et al. 2012a). In cattle it is administered

as a primary and booster injection, four weeks apart. Suppression typically peaks nine days after the booster and generally lasts for 12 weeks; however, the effect and duration of suppression varies between individuals (Pfizer 2020).

4.3.3 Administration of Bopriva and experimental design

4.3.3.1 Pilot study

From September 2018 to February 2019, a pilot study was undertaken in adult male kangaroos (n=2) to evaluate; 1) effectiveness of Bopriva in reducing testosterone concentration, 2) effectiveness of subcutaneous or intramuscular injection, and 3) whether either mode of administration caused an injection site reaction. Pilot animals were also fitted with global positioning system (GPS) collars to determine their core area use. After confirmation that both modes of administration caused a decline in serum testosterone with no unusual injection site reaction (Table 4.1), the primary study was initiated.

Table 4.1 Pilot study (n=2) serum testosterone concentration (nmol/L) and mean (± SEM) testis measurements (mm) *Before* and *After* treatment of adult male eastern grey kangaroos (*Macropus giganteus*) with Bopriva; a short-term testosterone suppressing vaccination. The impact of two types of injection on effectiveness of Bopriva in suppressing testosterone was trialled. The testosterone concentration of a female was 0.45 nmol/L. The pilot study was undertaken from September 2018 to February 2019 at Nelson Bay Golf Course, Port Stephens, New South Wales, Australia.

Animal identification		**013**	**144**
Injection type		Subcutaneous	Intramuscular
Injection site reaction	Primary vaccination	No	No
	Booster vaccination	No	No
Testosterone nmol/L	*Before*	44.03†	20.3
	After	1.74	0.45
Testis breadth (mm)	*Before* (n=2)	30.885 (0.92)	32.485 (0.12)
	After (n=2)	29.785 (0.99)	28.4*
Testis width (mm)	*Before* (n=2)	63.27 (0.93)	66.1 (0.5)
	After (n=2)	56.755 (1.75)	52.6*
Testis length (mm)	*Before* (n=2)	42.43 (0.03)	44.62 (1.42)
	After (n=2)	37.815 (3.92)	32.8*

†serum collected in 2017
*raw value presented as n=1

4.3.3.2 Primary study

The study was conducted from December 2018 to May 2019. Upon first capture, male kangaroos (n=23) were randomly assigned into two groups; *Treated* (Bopriva administered, n=13) and *Control* (placebo adults, n=7; placebo subadults, n=3) and GPS collars were attached to a subset of these animals (*Treated*, n=3; *Control*, n=2). *Treated* animals were given Bopriva (primary injection), whilst *Control* animals were injected with a placebo containing sterile medical grade water for injection (Sykes, Dandenong, Victoria (VIC) (Figure 4.1). Four weeks later, both *Control* and *Treated* animals were identified and remotely administered a second injection (Bopriva for *Treated* animals and a placebo for *Control* animals) (Figure 4.1). Some animals (n=4 *Treated* and n=3 *Control* [subadult n=1, adult n=2]) could not be found and did not receive a booster; these animals were subsequently excluded from the study.

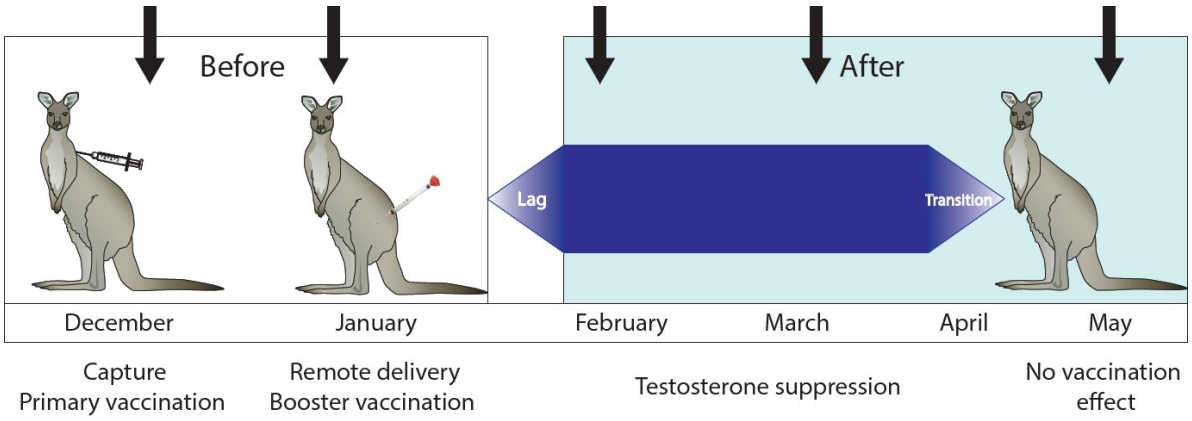


Figure 4.1 Bopriva vaccination timeline for adult male eastern grey kangaroos (*Macropus giganteus*) (n ≤23) from December 2018 to May 2019 at Nelson Bay Golf Course, NSW, Australia. The primary vaccination was hand-delivered using a syringe, and the booster vaccination was dart-delivered using a dart gun. The dark blue double-sided arrow indicates the period of testosterone suppression, with the 'lag' phase showing it takes nine days to achieve maximum suppression after the booster vaccination has been administered. The 'transition' phase indicates when suppression of testosterone starts to decline. The transition phase can be variable but usually starts 12 weeks after the booster vaccination. Data collected from the primary vaccination to the booster vaccination is considered *Before* treatment (white box), while data collected nine days post booster vaccination is considered *After* treatment (light blue box). Black arrows indicate when sampling occurred to determine parasite burden, health parameters and morphological measurements. Symbols courtesy of the Integration and Application Network, University of Maryland Center for Environmental Science (https://ian.umces.edu/symbols/).

4.3.3.3 Bopriva treatment protocol

A primary injection was given as a 1 mL subcutaneous injection of Bopriva in the dorsal interscapular region, with *Control* animals receiving 1 mL of sterile medical grade water for injection also in the dorsal interscapular region. A booster injection was administered by loading Bopriva (*Treated*) or water for injection (*Control*) into a disposable dart (Pneu-Dart type 'P' 1 cc 3/4 inch with gel collars Williamsport, Pennsylvania, United States) which was administered into the gluteal musculature using a CO_2-powered dart gun (X-Calibre, Pneu-Dart, Williamsport, Pennsylvania, United States), as a 1 mL intramuscular injection.

4.3.3.4 Animal capture and sample collection

Sampling of kangaroos to determine parasite burden, health parameters and morphological measurements occurred *Before* treatment, 'during' suppression and *After* treatment (no treatment effect) (Figure 4.1). Animal capture and sample collection were undertaken as described in Brandimarti et al. (2020) [thesis chapter 2]. Briefly, target male kangaroos were immobilised using Zoletil (Virbac, Milperra, NSW, Australia) at a fixed dose of 125 mg for subadults and 250 mg for adults. Whilst immobilised, the leg, pes, testes and weight of each animal were measured using standard protocols (Poole et al. 1982). Animals were classified as adult if leg length was > 52.3 cm (Poole 1973; Poole et al. 1982). The combined width of both testes (mm) were measured twice (by M. E. B) using digital Vernier calipers (Absolute, Mitutoyo, Japan) and the mean calculated. Whole blood was collected from the lateral caudal vein, and 1.3 mL was placed into a EDTA tube (Becton Dickinson and Co, North Ryde, Australia), and was subsequently preserved in a 1:1 ratio with Streck Cell Preservative (Streck, Omaha, United States) and stored at 4°C prior to haematological analyses. The remaining whole blood sample (≤5 mL) was placed into a plain serum tube, centrifuged, and serum collected and stored at – 20°C prior to protein analyses. Kangaroos were examined at multiple body sites for ticks, and total counts were performed using the methodology outlined in Brandimarti et al. (2021). Ticks were left in situ to minimise disturbance, and depending on the species present, they may either drop off or remain on the host to complete their life cycle (Veiga et al. 1998;

Beveridge and Emery 2015). All animals were visually examined for any injuries and abrasions, which were recorded. Animals were monitored inside capture bags for up to 2.5 h before release. If a faecal sample was not obtained during processing, a sample was usually collected from the capture bag or by opportunistically re-sighting animals <12 hours after capture. Faecal samples were then stored on ice bricks in a cooler bag prior to freezing (-20°C, for immunoassay) or processing (within 48 h) for quantification of gastrointestinal worm burden. Long-term investigations at NBGC facilitated the collection of any deceased study animals and necropsies were performed under the supervision of an experienced wildlife veterinarian to determine likely cause of death.

4.3.4 Quantification of worm burdens

Gastrointestinal worm burden were estimated by counting strongyle worm eggs in faecal samples. Faecal egg counts estimate the gastrointestinal worm burden of an individual in eggs per gram of faeces (EPG). Faecal egg counts were performed as previously described (Brandimarti et al. 2021 [thesis chapter 3]). This methodology was used because there is an assumed relationship between faecal egg output and nematode worm burden (Cabaret et al. 1998). This approach is non-destructive, repeatable and can be conducted in the field. Eggs can be detected in the faeces of kangaroos 12 weeks after ingestion of infective larvae (Cripps et al. 2014). Because worms are typically acquired during summer, we conducted *Before* sampling and initiated testosterone suppression during this time (Figure 4.1) providing opportunity for the host to invest in inhibition of infective larvae which will go on to produce eggs at the *After* timepoint.

4.3.5 Health parameters

Blood analyses was performed at the Veterinary Pathology Diagnostic Services (VPDS) at The University of Sydney (USYD), NSW within seven days of blood collection. The same methodology was used as outlined in Brandimarti et al. (2020) [thesis chapter 2]. Briefly, Streck preserved blood samples were analysed using the Sysmex XN1000i automated haematology analyser (Roche diagnostics, Australia) for the following parameters: haematocrit (HCT; L/L), haemoglobin (HGB; g/L),

total red blood cell count (RBC; $x10^{12}$/L), total white blood cell count (WBC; $x10^{9}$/L), platelet count (PLT; $x10^{9}$/L) and nucleated RBC count (NRBC; $x10^{9}$/L). Values were doubled to account for dilution with cell preservative and any haemolysed samples were excluded. Mean cell volume (MCV; fL; (HCT/1000)/RBC), mean cell haemoglobin (MCH; pg; (HGB/RBC)) and mean corpuscular haemoglobin concentration (MCHC; g/L; (HGB/HCT)) were calculated manually. Differential WBC counts were performed by differentiating 100 WBC cells (neutrophils, lymphocytes, eosinophils, monocytes and basophils) on a Diff Quik (Lab Aids Pty Ltd) stained blood smear. Albumin (g/L), total serum protein (TSP; g/L) and globulin (g/L) concentrations were analysed using the Konelab Prime 30i analyser (Thermo scientific, Australia). Blood glucose was measured immediately after blood collection as previously described (Brandimarti et al. 2021 [thesis chapter 3]), using a hand-held glucose monitoring device (Freestyle Optium Neo Blood Glucose Monitoring System, Abbott, Alameda, California).

4.3.6 GPS collar deployment and retrieval

Solar-powered GPS collars were fitted to the neck of a subset of kangaroos (*Treated*, n=5 (including n=2 pilot animals); *Control*, n=2). Two models of GPS unit were used; Radio tag-14 (primary study, Milsar, Gdansk, Poland) and GsmRadioTag-M9 collars (pilot study, Milsar, Gdansk, Poland). Counterweights were attached to the collars at 180° to the solar panels to optimise light exposure and solar charging efficiency. The final weight of GPS collars was approximately 131 g, or 0.2% of the average body weight of an adult male kangaroo. Collars were programmed to obtain GPS location fixes every 15 min after activation. In optimal conditions (open space), GPS accuracy was within 3-5 m, however, accounting for suboptimal conditions, 95% of fixes were within a 15-20 m radius. Data was successfully retrieved from n=4 *Treated* and n=1 *Control* animals; data from two (n= 1 *Treated* and n=1 *Control*) collars could not be retrieved for unknown reasons. The remaining collars logged GPS data for between 14 and 22 weeks. The GsmRadioTag-M9 collar on pilot animal 013 malfunctioned one month after deployment and was replaced with a Radio tag-14 unit for the

remainder of the study, however a significant period of GPS data was lost. To retrieve data, kangaroos were recaptured (methods previously described) 22 weeks post GPS collar deployment and collars were removed. Data was automatically downloaded to a base station upon return to USYD.

4.3.7 Measuring testosterone concentration

Serum testosterone (nmol/L) analyses were conducted on samples from the pilot study at the Royal North Shore Hospital, St Leonards, Sydney. Historic serum samples from animal's 013 and 144 in 2017 were analysed in addition to serum samples taken at four weeks (booster vaccination) and/or eight weeks after the primary vaccination (Figure 4.1). Thawed serum testosterone was analysed using the chemiluminescent microparticle immunoassay (ARCHITECT 2nd Generation Testosterone, Abbott Laboratories, Wiesbaden, Germany). The assay has been validated for use in kangaroos (Miller et al. 2010), however a serum sample from a female was included for comparison. Serum samples were initially diluted 1:3 with testosterone assay specific diluent (ARCHITECT 2nd Generation Testosterone kit). However, samples were reanalysed 'neat' when results were <0.45 nmol/L or at a 1:4 dilution when >35 nmol/L.

4.3.7.1 Faecal metabolite extraction

To analyse the testosterone concentrations for the primary study, the metabolite extraction protocol in Keeley et al. (2012) was modified for use in an herbivorous animal. Faecal samples were thawed, homogenised and dried overnight (14-18 h) at 70-80°C (Qualtex Thermstat, model: 0G24T, Watson Victor Ltd, Australia). Dried faeces were weighed (0.19 to 0.21 g), and steroid hormone metabolites extracted using 5 mL 80% methanol. Samples were rotated (Ratek Instruments, R.S.M. 6) overnight and centrifuged (Sorvall general-purpose RC-3 automated refrigerated centrifuge, Waltham, United States) at 1000g for 15 min and the decanted extract stored at -20°C prior to analysis.

4.3.7.2 Faecal testosterone enzyme immunoassay

Faecal testosterone and its metabolites were analysed using the ISWE testosterone EIA 'mini-kit' (Arbor Assays®, Ann Arbor, Michigan, United States). The EIA uses a polyclonal testosterone antibody (C196-6ML; 1:50), testosterone-peroxidase conjugate (C197-6ML; 1:50) diluted with phosphate buffer; and a testosterone standard (C198-1 mL; 1,000 ng mL-1) diluted with phosphate buffer to create a standard set (20 - 0.039 ng mL-1). Microtiter plates (Costar 96 well, high binding assay plate, Corning Inc. Kennebunk, Maine, United States) were pre-coated with goat anti-rabbit immunoglobulin (GARG) coating antibody (A009-25MG; 1:4000; Arbor Assays®) prior to use. Extract samples were diluted (1:10) with phosphate buffer, vortexed and loaded onto the plate at 50 µl/well along with standards and quality controls (1.25 and 0.156 ng/ml testosterone standards for high and low control) in duplicate. Prepared testosterone conjugate (50 µl) was added to each well followed by 50 µl of antibody solution (except to blank wells). The plate was mixed on a plate shaker (compact digital mini rotator, Thermo Fisher Scientific Co. Ltd, Waltham, United States) at 170 rpm in the dark at room temperature for 2 h. The plate was washed, 200 µl of tetramethylbenzidine (TMB) substrate added and incubated at room temperature for 30 min before the stop solution (50 µl/well of 4M sulfuric acid) was added and the absorbance read at 405 nm (BioTek 800 TS microplate reader, Vermont, United States) with a reference wavelength of 630 nm. Results were uploaded to MyAssays.com and calculated using four parameter logistic curve analysis.

4.3.7.3 Immunoassay validation

The immunoassay was validated for kangaroos by establishing a parallelism between the standard curve and a series of pooled faecal dilutions (adult and subadult males, and males during testosterone suppression). Comparing suppressed males with subadult males offers a biological benchmark to the magnitude of effect of Bopriva in adult male kangaroos. The inter-assay coefficient of variation (CV) measured from the high and low binding controls was 11.8% and 15.7% respectively. Intra-assay CV was 10.4% and 11.4% from 14 replicates of high and low controls run on the same plate. Assay sensitivity was 0.039 ng µL-1 (lowest readable standard). The known cross

reactivity of the testosterone antibody for testosterone is 100%; dihydrotestosterone 35.4%; progesterone 0.024%; corticosterone <0.004%; cortisol <0.004%; cortisone <0.004% and 17β-estradiol <0.004% (Arbor Assays 2020).

4.3.8 Statistical analyses

4.3.8.1 Testosterone concentration, health parameters and parasite burden

All data was analysed using R version 3.5.3 statistical software (R core team 2017) and significance was determined when $P < 0.05$. 'Spaghetti' line plots were produced for all observations from each individual during the study and visually examined to assess trends. Normality was also assessed for each explanatory variable using the Shapiro-Wilk normality test and by visual inspection of a histogram. A cube root transformation was used to transform non-normal native data to a normal distribution. Not all data could be normalised through transformation (Table 4.2), such that data distribution was factored into subsequent models. To determine the effect of treatment on testosterone concentration and health parameters for normally distributed variables (Table 4.2) a linear mixed effect model (lmm) using the 'lmer' function in the 'lme4' package was used. For non-normal variables with a Poisson distribution, a generalised linear mixed effect model (glmm) using the 'glmer' function in the 'lme4' package was used (Table 4.2). For non-normal variables with a variance greater than the mean, a negative binomial distribution was used using the function 'glmer.nb' in the 'lme4' package (Table 4.2). For both lmm and glmm models, the fixed and orthogonal effects were 'time' (*Before* and *After* treatment; [tr_date]), 'treatment group' (*Treated* or *Control* [treat]) and 'animal identification' [id], which was included as a random effect. Collinearity of models was assessed using the variance inflation factor and found to be below the cut-off level of < 4.3 (Craney and Surles 2002), suggesting low levels of collinearity. The ANOVA function in the 'car' package was then used to test (Type II Wald Chi-squared) analysis of deviance to determine significance. For significant interactions between [tr_date] and [treat], the 'emmeans' package was used to test post hoc pairwise combinations using Tukey's methods (Sokal and Rohlf 1995). To guard

against Type 1 error arising from multiple comparisons of health parameters, P values were corrected by applying the Bonferroni sequential probability correction for analyses of health parameters (Rice 1989).

Table 4.2 Explanatory variable distribution and model selection for determining the effect of treatmenton testosterone concentration and health parameters in adult male eastern grey kangaroos (*Macropus giganteus*) at Nelson Bay Golf Course, Port Stephens, New South Wales.

Variable	**Distribution**	**Model**
Testosterone[1]	Normal	LMM
Ticks	Neg binomial	GLMM
EPG	Neg binomial	GLMM
Weight	Neg binomial	GLMM
Teste width	Normal	LMM
Teste breadth	Normal	LMM
Teste length	Normal	LMM
Glucose[1]	Normal	LMM
White blood cell count	Normal	LMM
Neutrophil count	Poisson	GLMM
Lymphocyte count	Normal	LMM
Eosinophil count[1]	Normal	LMM
Monocyte count	Poisson	GLMM
Basophil count	Poisson	GLMM
Red blood cell count	Normal	LMM
Haemoglobin	Normal	LMM
Haematocrit	Normal	LMM
Mean corpuscular volume	Neg binomial	GLMM
Mean corpuscular haemoglobin	Neg binomial	GLMM
Mean corpuscular haemoglobin concentration	Neg binomial	GLMM
Platelet[1]	Normal	LMM
Nucleated red blood cell count	Poisson	GLMM
Albumin	Normal	LMM
Total protein	Normal	LMM
Globulin	Normal	LMM

EPG, worm eggs per gram faeces; [1]Cube root transformation applied

4.3.8.2 GPS data analyses using biased random bridge utilisation distributions

GPS fixes in the 24 h period post animal capture were removed to exclude any behavioural stress response following release (Herbert et al. 2020). GPS fixes from each collar were split into *Before* and *After* (nine days post booster, Figure 4.1) datasets for each animal, and even sampling was employed by matching the number of days *After* with the number of days *Before*. Raw data was cleaned and any erroneous GPS fixes were removed using a customised R function (Smith 2018). This included duplicate timestamps, missing recordings (likely signal dropouts) and fixes with a turn angle > 170° and distance > 200 m (movements unlikely to occur in kangaroos between the timescale of successive fixes) (Smith 2018). Core area size for each collared animal for both *Before* and *After* datasets was calculated using the 'adehabitatHR' package (Calenge 2015), which determines biased random bridge utilisation distributions (BRBs). We define an animal's core area, as the area in which most time is spent, as indicated by an aggregation of GPS locations (BRBs) across a defined period of time. Contour shapefiles (95% and 50%) were produced for visualisation of core areas. 'Realistic' area use can be modelled using BRBs because frequent reorientations towards preferred sites and space use intensity are accounted for (Benhamou 2011; Stark et al. 2017). Statistical comparisons of *Treated* and *Control* groups were not performed for the size of BRBs because data could only be downloaded from one *Control* animal. However, the raw and mean (± SEM) BRBs for *Control* and *Treated* animals respectively were described. A lmm was used to determine differences between the *Before* and *After* treatment on BRBs of the *Treated* group for both contours (normally distributed). The fixed and orthogonal factors were 'time' and 'animal identification' (random effect). The ANOVA function was used to determine significance.

Table 4.3 Biased random bridge utilisation distribution (ha) of each eastern grey kangaroo (*Macropus giganteus*) from Nelson Bay Golf Course, NSW, Australia *Before* and *After* (50% and 90% contours) treatment (*Control* and *Treated*). Average weight (kg) at first capture are presented alongside the number of days GPS data was logged for each animal. The *Treated* group includes n=2 animals from the pilot study conducted from September 2018 to February 2019. The primary study was conducted from December 2018 to May 2019.

ID	**Group**	**Weight**	**Days logged**		***Before***		***After***	
			Before	***After***	**50 (ha)**	**95 (ha)**	**50 (ha)**	**95 (ha)**
165	*Treated*	65.4	28	28	15	91	17	79
038	*Treated*	67.9	28	28	15	77	7	53
013	*Treated*	71.1	7	7	4	24	5	24
144	*Treated*	74.2	36	36	8	38	11	48
168	*Control*	59.2	17	17	3	19	7	47

ID, animal identification

4.4 Results

4.4.1 Pilot study

Serum testosterone concentration declined after treatment with Bopriva in both pilot animals regardless of the type of injection (Table 4.1). Testes measurements also declined after treatment (Table 4.1). There was no unusual injection site reaction on either intramuscular or subcutaneous administered animals.

4.4.2 Effect of treatment on testosterone concentration and testes measurements

Testosterone concentration was three times lower in *Treated* compared to *Control* animals during suppression (treat_date x treat interaction; $\chi^2_1 = 7.73$, P = 0.02, Figure 4.2), and was comparable to subadult males at this time (Figure 4.2). Suppression lasted at least 10 weeks, as the *Treated* mean at week 10 was 107.9 ng/g ± 29.5 (n=4), which is below *Control* (367.3 ng/g, n=1) and subadult (218.1 ng/g and 163.6 ng/g, n=2) concentrations. There was no treatment effect by the next sampling week (week 18, 281.2 ± 53, n=3). *Treated* animals had significantly smaller combined testes width *After* treatment compared to *Control* animals *Before* and *After* treatment (treat_date x treat interaction; $\chi^2_1 = 6.59$, P = 0.01) (Table 4.1, Figure 4.3).

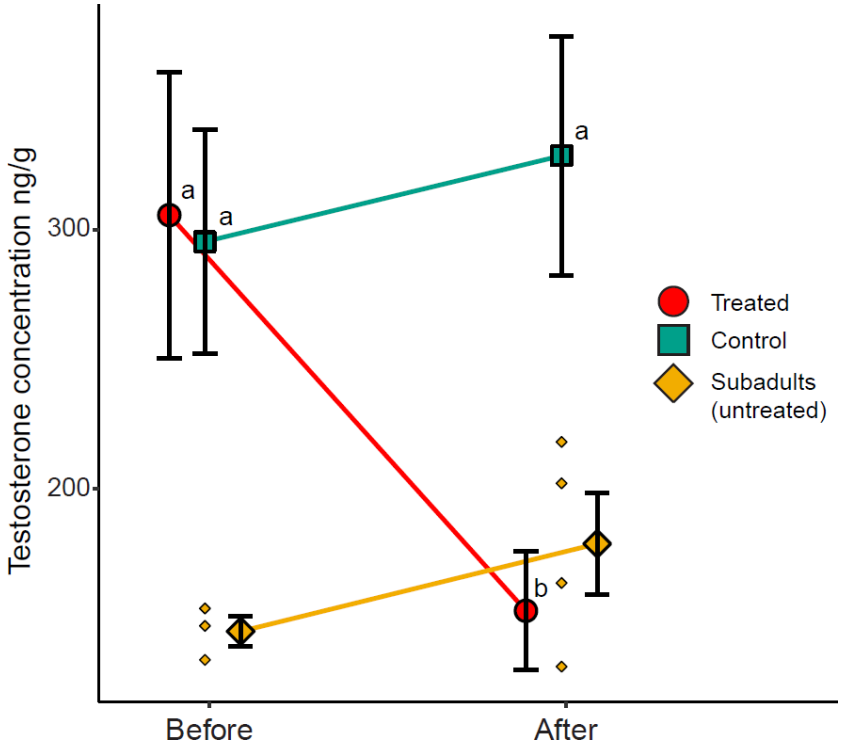


Figure 4.2 Mean faecal testosterone concentration (ng/g) in male eastern grey kangaroos (*Macropus giganteus*) sampled at Nelson Bay Golf Course, NSW, Australia in 2018–2019. Error bars indicate the standard error of the mean (SEM) for adult *Treated* (red circle; *Before* n=13, *After* n=15), adult *Control* (green square; *Before* n=12, *After* n=7) and subadult (placebo) (yellow diamond; *Before* n=3, *After* n=4)kangaroos. Small yellow diamonds depict raw data points for subadult animals as the sample size both *Before* and *After* treatment was ≤ 4. Latin letters (a-b) indicate the significant differences among levelsfor the 'time' × 'treatment group' interaction indicated by post hoc pairwise comparisons between *Treated* and *Control* groups.

Table 4.4 Mean (± SEM) testes width (mm) in *Control* and *Treated* male eastern grey kangaroos (*Macropus giganteus*) from Nelson Bay Golf Course, NSW, Australia *Before* and *After* treatment with Bopriva. The study was conducted from December 2018 to May 2019.

Measurement	***Before***		***After***	
	Control (n=7)	*Treated* (n=13)	*Control* (n=10)	*Treated* (n=12)
Width	59.6 (2.2)	55.9 (0.8)	59.2 (1.5)	50.8 (1.7)

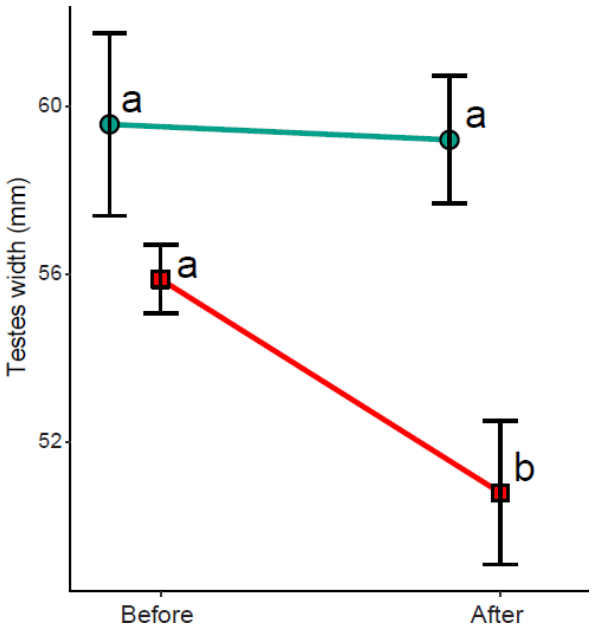


Figure 4.3 Mean combined testes width (mm) in male eastern grey kangaroos (*Macropus giganteus*) sampled at Nelson Bay Golf Course, NSW, Australia in 2018–2019. Error bars indicate standard error ofthe mean (SEM). *Control* (green circle) and *Treated* (red square) animals are shown *Before* (*Treated* n=13, *Control* n=7) and *After* treatment (*Treated* n=12, *Control* n=10). Latin letters (a-b) indicate the significant differences among levels for the 'time' × 'treatment group' interaction indicated by post hocpairwise comparisons.

4.4.3 Effect of treatment on parasite burdens

There was a trend for higher EPG in the *Control* group and lower EPG in the *Treated* group at the *After* timepoint, however this result was not statistically significant (treat_date x treat interaction; $\chi^2_1 = 2.71$, P = 0.1, Figure 4.4A). Total tick counts were lower in both *Control* and *Treated* groups at the *After* timepoint, however this result was not statistically significant ($\chi^2_1 = 3.69$, P = 0.054, Figure 4.4B).

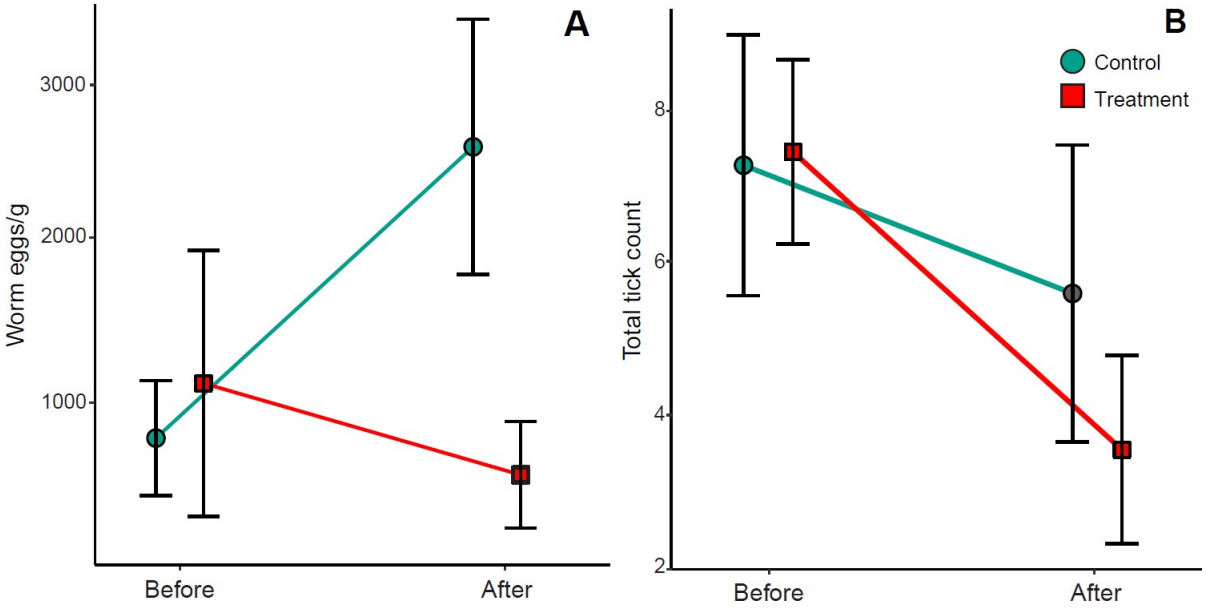


Figure 4.4 Mean worm eggs per gram of faeces (A) and total tick counts (B) *Before* and *After* treatment of adult male eastern grey kangaroos (*Macropus giganteus*) with Bopriva at Nelson Bay Golf Course, NSW, Australia in 2018–2019. The effect of 'treatment' and 'time' were not significant. Error bars indicate standard error of the mean (SEM). Faecal egg counts for *Control* (green circle) and *Treated* (red square) animals are shown *Before* (*Treated* n=13, *Control* n=8) and *After* (*Treated* n=15, *Control* n=9) treatment. Total tick counts for *Control* (green circle) and *Treated* (red square) animals are shown *Before* (*Treated* n=13, *Control* n=7) and *After* (*Treated* n=11, *Control* n=10) treatment.

4.4.4 Effect of treatment on health parameters

Total WBC count was significantly lower in both *Control* and *Treated* animals at the *After* timepoint (χ^2_1 = 23.31, P < 0.018 after Bonferroni correction, Figure 4.5B). MCHC was significantly higher in both *Control* and *Treated* groups at the *After* timepoint (χ^2_1 = 11.5, P = 0.01 after Bonferroni correction, Figure 4.5C). *Treated* animals had lower absolute lymphocyte counts at the *After* timepoint compared to the *Before* timepoint, although this interaction became non-significant after the application of the Bonferroni correction (treat_date x treat interaction; χ^2_1 = 6.231, P = 0.18, Figure 4.5A). There was no significant effect of treatment on absolute neutrophil, eosinophil, monocyte and basophil counts, or RBC, MCV, MCH, HGB, HCT, PLT, albumin, TP, globulin, glucose or NRBC concentration. There was no significant effect of treatment on body weight.

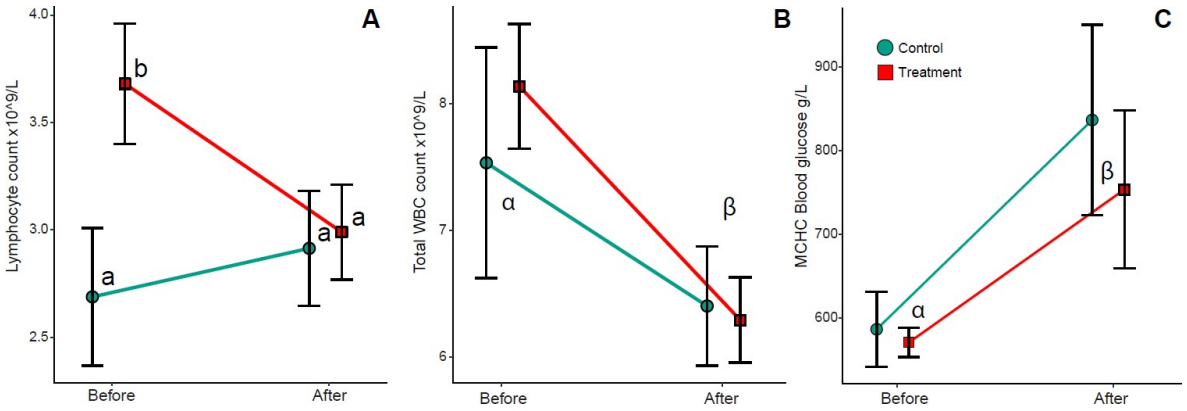


Figure 4.5 Absolute lymphocyte count (A), total white blood cell (WBC) count (B) and mean corpuscular haemoglobin concentration (MCHC) (C) of adult male eastern grey kangaroos (*Macropus giganteus*) treated with Bopriva at Nelson Bay Golf Course, NSW, Australia in 2018–2019. Error bars indicate standard error of the mean (SEM). *Control* (green circle) and *Treated* (red square) animals are shown *Before* (*Treated* n=12, *Control* n=6) and *After* (*Treated* n=12, *Control* n=8) treatment. Latin letters (a-b) indicate the significant differences among levels for the 'time' × 'treatment group' interaction indicated by post hoc pairwise comparisons. Greek letters (α-β) indicate significant differences between 'time'.

4.4.5 GPS data analyses using biased random bridge utilisation distribution

The raw BRB size of the *Control* animal was lower than the mean of the *Treated* group for both contours (95% and 50%) (Table 4.2, Figure 4.6, Figure 4.7). Within the *Treated* group, there was no significant effect of treatment on the size of BRBs in animals measured from the *After* dataset (Figure 4.6, Figure 4.7).

Table 4.5 Raw (*Control*) or mean (± SEM; *Treated*) biased random bridge utilisation distribution (ha) of *Control* and *Treated* eastern grey kangaroos (*Macropus giganteus*) from Nelson Bay Golf Course, NSW, Australia *Before* and *After* treatment with Bopriva. The *Treated* group includes n=2 animals from the pilot study conducted from September 2018 to February 2019. The primary study was conducted from December 2018 to May 2019. Two utilisation distribution contours (50% and 95%) were estimated.

Group	Sample size	*Before*		*After*	
		50% (ha)	95% (ha)	50% (ha)	95% (ha)
Treated	4	10.5 (2.7)	57.5 (15.8)	10 (2.6)	51 (11.3)
Control	1	3	19	7	47

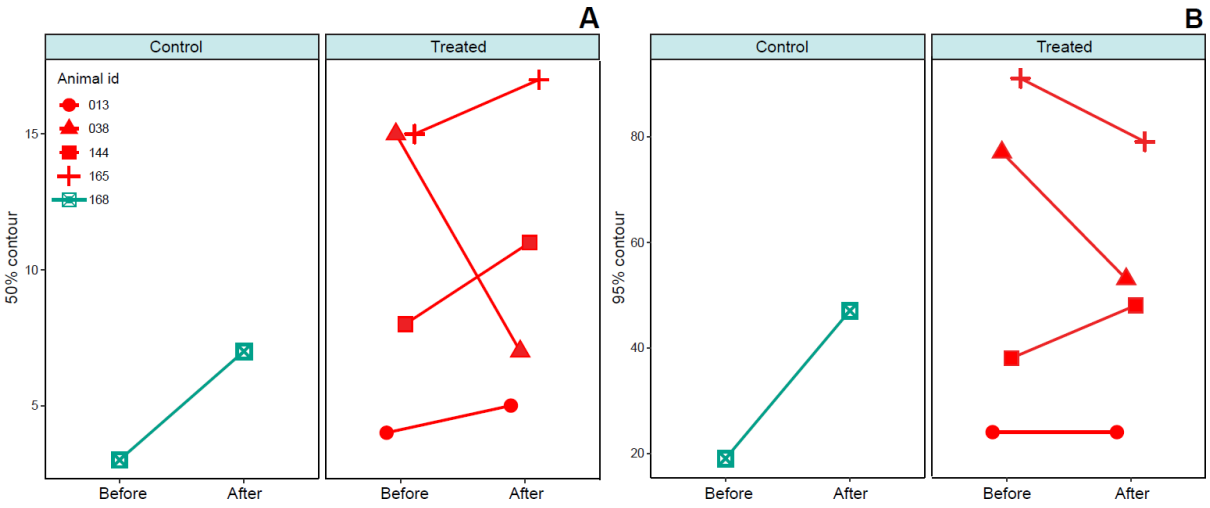
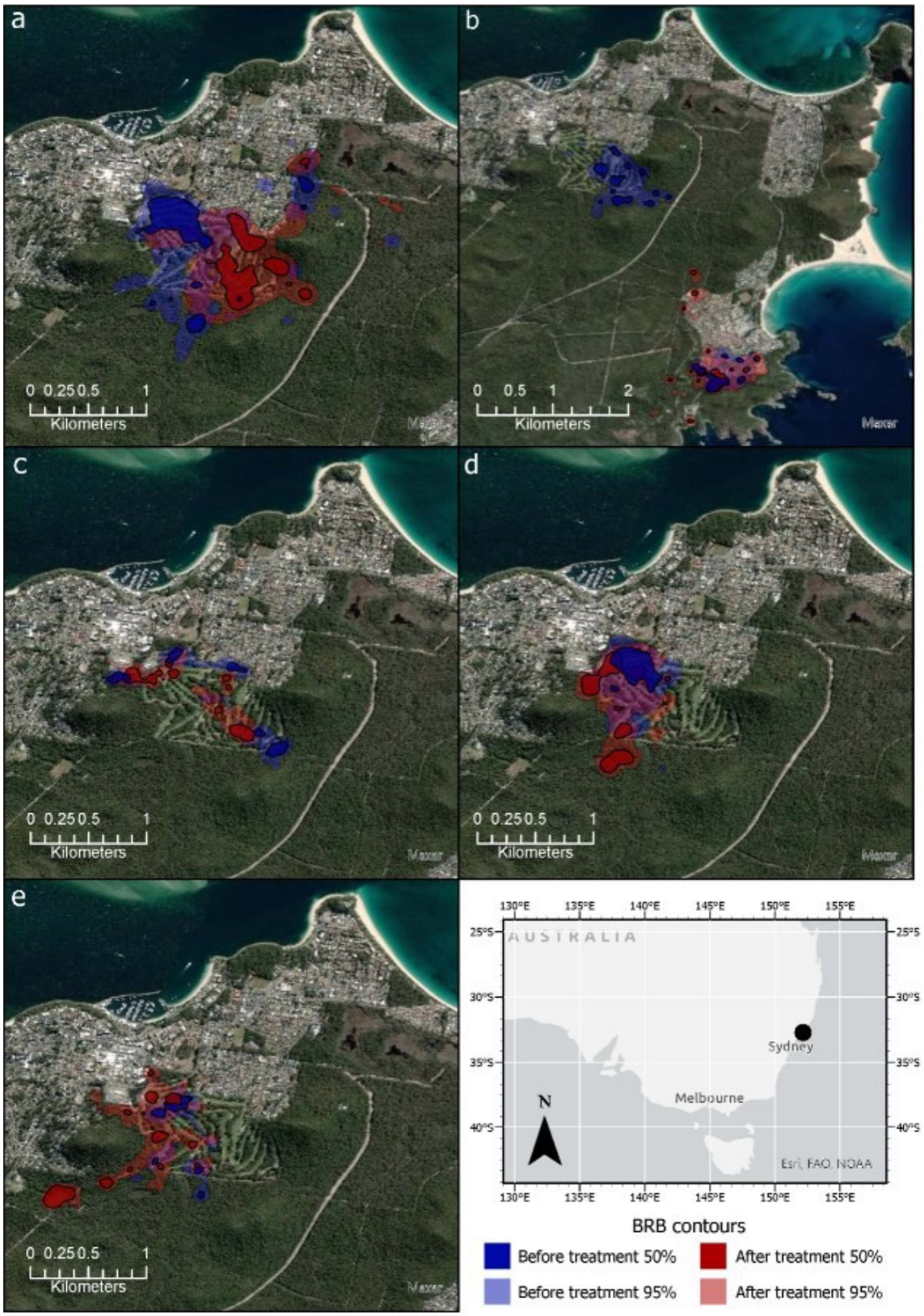


Figure 4.6 50% (A) and 95% (B) contour biased random bridge utilisation distributions (BRB) of *Control* (left box) and *Treated* (right box) eastern grey kangaroos (*Macropus giganteus*) from Nelson Bay Golf Course *Before* and *After* treatment. The *Treated* group includes n=2 animals (013, red circle; 144, red square) from the pilot study conducted from September 2018 to February 2019. The primary study was conducted from December 2018 to May 2019.


a
0 0.250.5 1
Kilometers
b
0 0.5 1 2
Kilometers
c
0 0.250.5 1
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e
0 0.250.5 1
Kilometers
130°E
135°E
140°E
145°E
150°E
155°E
25°S
30°S
35°S
40°S
AUSTRALIA
Sydney
Melbourne
N
Esri, FAO, NOAA
BRB contours
Before treatment 50%
After treatment 50%
Before treatment 95%
After treatment 95%

Figure 4.7 Biased random bridge utilisation distributions (BRB) of testosterone suppressed eastern grey kangaroos (*Macropus giganteus*) 165 (A), 038 (B), pilot 013 (C) and pilot 144 (D) *Before* and *After* treatment. The BRB of *Control* animal 168 (E) is also shown. Dark blue and dark red inner contours show the estimated 50% BRB contour *Before* and *After* treatment, respectively. Light blue and light red outer contours show the estimated 95% BRB contour *Before* and *After* treatment, respectively. The primary study was conducted from December 2018 to May 2019; the pilot study was conducted from September 2018 to February 2019 at Nelson Bay Golf Course, NSW, Australia.

4.4.6 Necropsy findings

Necropsies were conducted on two of the four deceased kangaroos (n=2 *Treated* (n=1 pilot) and n=2 *Control* (n=1 subadult)) recovered after the study. Both animals were emaciated. The *Treated* (pilot) animal had multiple, severe, necrotic hydatid cysts replacing the tissue of the right lung, fusing with the pericardial sac with extensive pyothorax and copious purulent effusion. Hydatid cysts have been observed previously in necropsies of other (untreated) animals at NBGC (unpublished data). There was no gross indication of abnormalities related to use of Bopriva. The *Contro*l (subadult) had localised but severe fibrinous peritonitis affecting the peritoneal surfaces of the liver, stomach, spleen, part of the small intestine and abdominal wall. There was no evidence of stomach or intestinal perforation or a large penetrating wound into the abdomen. Necropsies could not be performed on two animals due to storage limitations. However, the *Control* animal's death could be age-related as this animal had been a dominant male at NBGC for > 8 years and had been observed in thin body condition, isolating from the larger group prior to death.

4.5 Discussion

Bopriva effectively suppressed testosterone in male kangaroos for at least 10 weeks, facilitating comparison of parasite burden, health parameters and movement patterns to a *Control* (placebo) group. *Treated* kangaroos showed reduced testes size when compared with *Control* animals and there was a significant effect on WBC count and MCHC over time. There was no significant reduction in parasite burden or the size of the core area use of animals with testosterone suppression.

4.5.1 The effect of Bopriva in kangaroos

To our knowledge, the effectiveness of Bopriva has not been evaluated in a free-ranging wildlife species. As such, this study is the first to demonstrate that a short-term GnRH inhibitor is effective at achieving reversible testosterone suppression in a free-ranging marsupial. While the individual effect of Bopriva on testosterone concentrations varied, treatment longevity in this study was limited by sampling timepoints. At 10 weeks, *Treated* individuals were still suppressed; we would expect

reversal to have occurred between week 10 and week 18, but the exact duration of effect in each individual is unknown, as sampling between these timepoints was not undertaken. In addition to a reduction in testosterone *After* treatment, there was also a significant decline in testes width, which is consistent with findings from previous studies in cattle (Janett et al. 2012a; Janett et al. 2012b). The ability to associate quantifiable changes in combined testes width with treatment is useful for future studies, such that immunoassays may not be necessary to demonstrate treatment effectiveness.

4.5.2 The effect of testosterone on parasite burden

There was no significant effect of testosterone suppression on worm eggs or total tick counts *After* treatment or compared to *Control* animals. However, *Treated* animals did have reduced EPG and tick counts at the *After* timepoint. These trends are consistent with previous studies in wildlife models (Decristophoris et al. 2007; Negro et al. 2010; De La Peña et al. 2020), but due to the reduction in sample size throughout the course of this study, we are unable to support or reject the hypothesis that testosterone suppression lowers parasite burden. Surprisingly, *Control* animals who maintained their baseline testosterone concentration had an increase in EPG at the *After* timepoint. This could reflect a seasonal shift in egg output as the environmental conditions are warmer and wetter during autumn in the Port Stephens region compared to studies in Victoria (Arundel et al. 1990), benefiting parasitic persistence. However, it could also reflect a lack of correlation between worm burden and faecal egg counts. This could have occurred due to variation in the amount of faeces produced at the time of sampling, the species present and inherent host resistance (McKenna 1981).

Reduced testosterone may have also had an effect on parasite burden beyond the experimental timeframe (Martínez-Padilla et al. 2010). For example, parasite intensity in red grouse (*Lagopus lagopus scoticus*) treated with testosterone remained unchanged one- or six-months post-treatment (compared to *Controls*) but were significantly increased one-year post-treatment (Seivwright et al. 2005). Given the seasonality of most nematodes' lifecycle and infective patterns in kangaroos, it is

possible that the treatment and sampling length did not entirely capture changes in parasitism. To expand on the results from this study, a longer experiment should be completed across seasons, encompassing all phases of the nematodes lifecycle and across varying environmental conditions. To understand the dynamics of testosterone and parasitism, additional studies with a larger sample size that result in testosterone suppression over a longer timescale are needed. Longer testosterone suppression could be achieved by giving an additional booster shot of Bopriva before the effect reverses, and a larger sample size could be achieved by increasing the retention of animals in the study using more efficient, real-time tracking technology. Additional methods to quantify parasite burden and speciation of worm eggs could also be useful given the bias of EPG toward certain nematode species in kangaroos which may account for most of the egg output (Cripps et al. 2015).

4.5.3 The effect of testosterone on kangaroo health parameters

There was no effect of treatment on haematological parameters, with values within the normal adult reference interval range for the species (Brandimarti et al. 2020 [thesis chapter 2]). Using baseline haematological parameters as indicators of immune fitness is limited, as they reflect an individual's inflammatory demand and corticosteroid status at a single point in time, rather than their ability to mount an immune response (Demas et al. 2011). Measuring the immune response to a pathogenic challenge is considered a more accurate method for evaluating the effect of testosterone on the immune system (Demas et al. 2011) and further research should be undertaken by measuring immunoglobulin levels and/or antigenic stimulation (Demas et al. 2011). Although there was no effect of treatment on health parameters, there was an effect of 'time' on total WBC count and MCHC in both treatment groups, indicating seasonal variability in these parameters, as has been previously shown (Brandimarti et al. 2020 [thesis chapter 2]). Temporal variation of these parameters could also reflect an individual's response to parasitism and individual health status, both of which can fluctuate with season (Cripps et al. 2015).

Prior to Bonferroni corrections there was a significant decline in absolute lymphocyte counts of

Treated animals to a similar level in *Control* animals. This result should be considered within the context of the potential effects of vaccination on the immune system (that is, antibody production is artificially stimulated), however GnRH vaccinations have been shown to have minimal impact on haematological parameters (Massei et al. 2008, Turkstra et al. 2011). At the *Before* timepoint in *Treated* animals, absolute lymphocyte counts were significantly higher, followed by a decline. This likely reflects an immune response to Bopriva, as kangaroos had already received a primary injection during the *Before* sampling period. This is consistent with use of Bopriva in cattle, where immunoglobulin G antibodies were elevated at the time of the booster injection (Amatayakul-Chantler et al. 2012).

4.5.4 The effect of testosterone on movement patterns

There was no overall significant reduction in the size of the core area use by *Treated* animals when testosterone was suppressed. As such, this study shows that the size of the area occupied by individuals was not determined solely by testosterone. A range of factors determine an animal's core area, such as where resources, mates or escape routes are located and whether they are used frequently or rarely, and these factors can also change over time (Powell and Mitchell 2012). It was predicted that suppressed individuals would have a smaller core area due to a reduction in energetic requirements and reluctance to find a mate. However, this prediction was not reflected in the results of this study. This lack of change in core area size may have influenced parasite burden as mobility is a key predictor of infection risk (Shaw et al. 2018). By maintaining their core area with potentially infected conspecifics, kangaroos do not reduce their exposure rates to parasites. Further investigation using the methodology employed in this study on a greater sample size will allow a better understanding of the role of testosterone suppression on core area use.

The core area occupied by kangaroos was the golf course, which is typical of peri-urban kangaroos (Coulson et al. 2014). Kangaroos also occupied the surrounding Tomaree National Park and neighbouring suburbs to a lesser extent. *Before* and *After* contours overlapped often, regardless of

treatment group.

4.5.5 Looking to the future

For research purposes, Bopriva is a less invasive and reversible alternative to other methods of testosterone suppression in males (such as castration) and can be delivered remotely. These unique properties would be ideal for behavioural studies in kangaroos or other large mammals to explore social organisation and life history strategy. However, to test the ICHH, a GnRH vaccination with greater longevity (such as Gonacon) is recommended (Snape 2012), or repeated boosters of Bopriva are required.

4.6 Conclusion

This study used novel techniques that aimed to explore the life history strategy of a common, long-lived marsupial. No consistent change in parasite burden, most health parameters and the size of the core area use of kangaroos were observed. Understanding the role of testosterone for individual movement patterns is important, as male's commonly come into conflict with humans through aggressive behaviour, property damage and motor vehicle collisions (Brunton et al. 2019; Henderson et al. 2018a). Limitations of the study include the short sampling duration which likely confounded findings in relation to parasite burden, the small sample size and evaluation of baseline parameters over a relatively short period of time. Future correlative investigations could examine individuals with higher baseline testosterone versus males with lower testosterone to ascertain its impacts on the immune response and parasitism. Although further research on the duration of suppression will need to be established, there are a variety of applications and cross-disciplinary avenues for research which could capitalise on the use of Bopriva in kangaroos. We advise that the period of animal monitoring be extended, additional measures of parasite quantification be explored and that captive kangaroos could be used (allowing a greater sample size) to robustly test this hypothesis in future studies. The outcomes of this study have provided a platform for future research on the effects of testosterone on health and parasites in marsupials.

General discussion

Eastern grey kangaroos (hereafter kangaroos) are a large macropod, commonly found in peri-urban eastern Australia where they can reach high densities due to habitat fragmentation and the removal of natural predators (Herbert 2004). This thesis contributes knowledge of the impacts of anthropogenic changes on kangaroo health and welfare by developing an accurate tool to evaluate health. Integral to our understanding of health in kangaroo populations is the impact of parasitism. To date, there has been limited research on host-specific factors that impact parasite dynamics in marsupials. This thesis addressed knowledge gaps on the impact of parasites on haematological, glucose and serum protein values, and examined the influence of testosterone in determining fitness and parasite burdens in male kangaroos. While there is still much to learn, several outcomes of this thesis can be directly applied to kangaroo management in both general and specific scenarios.

As part of this thesis, a customised tool in the form of species-specific haematological reference intervals (RI) was developed which can be used to evaluate the health status of individuals and populations (Chapter 2). These RIs aid diagnosis of disease and may aid one part of understanding welfare in different populations of kangaroos. Additionally, climatic factors unique to the location of kangaroo populations were identified as a primary driving force of health parameters and welfare status.

A key finding of this work was that despite numerical abundance, the health and welfare of kangaroos in peri-urban areas can be at risk if habitat connectivity is not maintained. This outcome was clearly highlighted in Chapter 3, in which the health status of a high-density kangaroo population was evaluated utilising the developed RIs. It was determined that the population of kangaroos at Look At Me Now Headland (LAMN) were in poor health which could contribute to compromised welfare when compared to populations at lower density. Reduced habitat connectivity, concurrent with overgrazing and increased parasitism were likely drivers of reduced health in this population. These findings have direct management implications for this population

and highlight the utility of haematological RIs for evaluating health in a kangaroo population. Importantly, this work has been considered by local government, and a proposal for ongoing assessment of the health of kangaroos at the site has been accepted. The decision to support robust investigations of population health at LAMN, using RIs developed in this thesis, provides reassurance to the public that management decisions at this site are evidence-based and well founded.

Considering the impact of increasing kangaroo densities on individual and population health, the factors influencing male susceptibility to parasitism were tested on a small number of kangaroos. The experiment (Chapter 4) investigated the effect of testosterone suppression on health, including haematological values, parasite burden and core area use. This study demonstrated that a novel gonadotropin-releasing hormone (GnRH) vaccination (Bopriva) commercialised by Zoetis for temporary testosterone suppression in cattle, was effective at reducing testosterone concentrations in adult male kangaroos, and this was accompanied by a measurable reduction in testes size. However, these changes were not accompanied by statistically significant changes in parasite burden, most health parameters or the core area use of kangaroos. This outcome likely reflects the small number of animals that could be recaptured and the short experimental timeframe, as there were trends for lower parasite burdens at the *After* timepoint.

Taken together, these findings improve the current methodologies for measuring health in kangaroos, which is of relevance to applied management and fundamental research, and highlight the impacts of localised overabundance on one of Australia's most iconic mammals.

5.1 A tool to measure health in kangaroos

Decisions over kangaroo management in Australia can be divisive (McLeod and Hacker 2020). Tools for health evaluation must allow veterinarians and managers to rapidly assess health with accuracy, which should encompass a robust understanding of health parameter baseline for a given species. The haematological, glucose and serum protein RIs described in Chapter 2 will allow veterinarians and animal managers to better assess health and condition in kangaroos when compared to a healthy population, without destructive sampling. RIs are a robust and objective tool to measure kangaroo health, that will be useful for informing whether management interventions may be warranted to improve health and in some cases, welfare. Reference intervals also improve on the current methodologies used for assessing health in kangaroos by allowing repeatable sampling over time and by providing important details on a population's nutritional and disease status. Reference intervals provide stakeholders with the opportunity to measure effective management in a population by determining whether health parameters improved as a result of the management intervention. Due to the costs and labour associated with collecting and analysing blood samples, RIs are likely to be most useful in the management of kangaroo populations that are in semi-urban areas (Chapter 3). By developing this tool which facilitates longitudinal studies on health, managers can unambiguously justify the need for management and determine the subsequent effectiveness of their management intervention.

Reference intervals have already proved effective tools for assessing health in other marsupial species, such as the endangered bilby (*Macrotis lagotis*) resulting in ongoing health monitoring of bilby populations and contributing to management decisions for their recovery effort (Warren et al. 2015). Similar to bilby management, using RIs for the ongoing monitoring of kangaroo health will be vital for the welfare of populations existing in isolated peri-urban habitat pockets. Comprehensive health surveys should collect a suite of data, including the density of animals, site-specific attributes that might already be known (e.g. endemic disease status) as well as using RIs. Establishing

population density by performing direct observation counts or sweep counts (DTMS 2010) is an important first step in evaluating the necessity of health investigations in circumstances where the number of animals is not known, is of concern to land managers or has changed overtime. In circumstances where health investigations are performed to understand environmental exposure to pathogens (e.g. toxins), understanding animal density may not be required. However, understanding site-specific attributes such as the presence of disease or forage quality and climate can also indicate an animal's physical state (Harvey et al. 2020). When conducting surveys using RIs, clear aims of the sampling target, the sampling timeframe and utility of the most appropriate RI for the health evaluation should be described. Like many experimental investigations, sampling the greatest number of animals will provide the most accurate results (Sokal and Rohlf 1995), however, there are often constraints on time and resources. Sampling programs should, therefore, aim to sample the maximum number of animals (financially and logistically possible) in order to increase the chance of capturing a representative proportion of the population (e.g. 25%; Friedrich et al. 2012). When the investigation aims to examine the effect of co-factors (e.g. season, site, sex) a higher sampling target may be needed to meet the required number of individuals within each co-factor to ensure statistical robustness (see Friedrichs et al. 2012 for guidelines on sample sizes). For populations with a known clinical history of disease, or if the density is high, it would be appropriate to consult site-specific population means described in Chapter 2 (Figure 2.2 and Table 2.5).

With respect to the sampling target, it is imperative that a health survey aims to capture different demographic classes of the target population (e.g. equal proportions adult and subadult animals) using standardised sample collection, handling and processing of animals, consistent for the species. However, if resources were limited, it is possible that large male kangaroos could be targeted and act as sentinel animals for the population, based on the assumption that male kangaroos have high energetic costs and may be more immunocompromised than females or subadults (Folstad and Karter 1992). Alternatively, sampling subadult kangaroos could be beneficial for detecting early signs of poor health in a population given previous mass mortality events in kangaroos have

disproportionally affected this age category (Portas and Snape 2018). While these suggestions were not rigorously tested in this thesis, it was determined that standard analytical methodologies and preferably, the same laboratory is used to process all samples in order to minimise the introduction of laboratory and inter-laboratory error. However, using any animal pathology laboratory consistently throughout longitudinal sampling would suffice. If any specific abnormalities are detected or specific age cohorts are determined to have abnormal parameters of health when compared to the RI, further investigations are recommended.

As indicated in Chapter 2, several factors significantly influenced RIs, including site, rainfall, temperature and season. For this reason, it is recommended that populations be surveyed within a defined time period, ideally within one season of one year, to coincide with similar weather patterns so that a longitudinal health evaluation can be undertaken. Selecting a RI is also imperative. While maturity-specific RIs are recommended as the gold standard for the relevant age cohort (Chapter 2, Table 4.3), other factors could also be relevant to the specific population being evaluated and may impact on the interpretation of results. For example, for captive populations located within international zoos, season-specific RIs may be advantageous to use because of extreme climatic variability in some locations; however, for comparing blood results between different species of kangaroos, species-specific RIs may be appropriate. Importantly, an outcome of this work (Chapter 3) is the ability to quantify the number of individuals in a population that fall outside of the RI limits, providing a quantifiable measure of the impact of parasites, disease and physiological stressors within a population. However, only analytes with > 2.5% (non-parametric 95% confidence intervals, CIs) or > 5% (robust 90% CIs) of the population falling outside the central RI should be interpreted as notable, as less than these values are likely random error and are common in healthy populations (Friedrichs et al. 2012; Brandimarti et al. 2020 [thesis Chapter 2]).

This study has developed RIs derived from a wild population and provides an example of how they can be applied at a population-specific level. As such, this study provides a blueprint for the development of similar intervals for other kangaroo species. Similar management issues arise for

free-ranging, isolated and fragmented western grey (*M. fuliginosus*) and red kangaroo (*O. rufus*) populations, that may be at high-density or experiencing drought. Future studies should aim to develop species-specific RIs for these species.

Despite the relative ease of collecting a blood sample from kangaroos compared to destructive or less repeatable techniques like subjective body condition scoring, it remains an invasive procedure that requires access to specialised equipment such as cell preservatives or a centrifuge, and additional funding. Therefore, the next steps for assessing health in kangaroos will be incorporating non-invasive health and body condition assessments that are repeatable across populations of kangaroos. Currently, body condition indices using standardised residuals (King et al. 2011; Quesnel et al. 2017) or the 'scaled mass index' (Cripps et al. 2014; Gélin et al. 2015), derived from the ratio of body mass to skeletal length, are used (Moss and Croft 1999). However, their comparative application across sites has not been tested nor have they been validated against other measures of animal health.

5.2 Managing the health costs of high-density

Rapid urban development is fragmenting the landscape and altering the spatial structure of native habitats (Collinge 1996). The average home range size of mammals across the globe has reduced by 49% in response to urbanisation alone (Doherty et al. 2021). In addition to mammals; birds, reptiles, amphibians, fish and arthropods all demonstrate altered movement due to human disturbance. With adequate resource availability and dispersal opportunities, some populations of kangaroos will be able to persist, but for others in isolated habitat islands with limited connectivity, there are finite resources to sustain them. Chapter 3 provides an example of a peri-urban, isolated kangaroo population with poor health and welfare outcomes associated with high abundance. The results presented directly challenge the perception that abundant populations of kangaroos are thriving. The LAMN kangaroos are a signature example – an isolated kangaroo population which has attained high-density. This high-density, combined with prolonged drought and chronic nutritional stress, has

resulted in parasitism and poor health contributing to compromised welfare. Localised urbanisation, human activities on the headland and the construction of an extensive motorway could be the cause of reduced available habitat for the population, with reduced pathways for dispersal as the population grows.

Populations of wild animals often experience what would be considered 'poor welfare' under the Five Freedoms Paradigm as part of natural population regulation where they are exposed to disease, parasitism, predation, and starvation (Mellor 2017). This thesis used the Five Domains Model framework to guide and assess welfare in a wild population, focusing on the 3rd Domain, health. However, it should be recognised that welfare is not the sole determinant of whether management actions should be implemented (Kirkwood and Sainsbury 1996). Kirkwood and Sainsbury (1996) outline how welfare grounds are only one aspect of determining whether intervention and management of wildlife populations is warranted. These include: "1) the extent to which we are responsible for harm to them; 2) the extent to which the harmed animals are under our stewardship; 3) the severity of the problems that harm wildlife and 4) cultural and economic factors, including the popularity of the species involved" (Kirkwood and Sainsbury 1996; Stephen and Wade 2018, p.1). In the case of kangaroos at the LAMN headland in Chapter 3, human modifications to the environment increased the levels of human stewardship over the population, therefore, increasing the ethical license to intervene. If those same kangaroos were experiencing similar levels of poor welfare in a natural or undisturbed setting, it is less likely that intervention would be implemented.

This thesis did not test the effectiveness of management actions, however previous work has shown that decreased density could be achieved through lethal management, reproductive control or a combination of both (Herbert 2004; Descovich et al. 2016). Lethal control could be employed broadly to reduce the density of animals (culling), however given the high visibility of kangaroos at this site with locals and international visitors, a discretionary approach whereby a veterinarian selects individuals with compromised welfare for euthanasia may be more appropriate. Further research is needed to evaluate the most appropriate management options.

Where possible, mitigation strategies should occur pre-emptively and aim to reduce risk factors for poor health, such as maintaining habitat connectivity. This can be done by incorporating landscape ecology into coastal developments (Collinge 1996) such as wildlife movement corridors, which may alleviate some pressures on urban kangaroos and prevent the localised overabundance that can lead to poor health and welfare outcomes. In locations where previous developments have already isolated local kangaroo populations and there is limited capacity to provide movement corridors, kangaroo population density should be monitored long-term. Health assessments should be triggered if a population is found to be at high-density, or there is a significant increase in density, so that any change in health can be detected quickly and interventions can be made before the welfare of kangaroos is compromised to the extent described at LAMN. Future research needs to establish the density thresholds at which health assessments should be triggered in different ecosystems. Chapter 2 provides some baseline data on the RIs for a range of populations at different densities, but a comprehensive understanding of longer-term population trajectories is likely necessary to determine the relationship between density and health.

Chapter 3 provides evidence that any further ecosystem degradation or development at LAMN could further compromise animal health and contribute to compromised welfare. This includes the effect of a changing climate and alteration in seasonal patterns. Chapter 2 highlighted that climate can influence RIs in kangaroos. The effects of season on health have also been shown in dasyurids and echidnas (Andersen et al. 2000; Clark 2004) and in other macropods. In wallaroos (*O. robustus*), rainfall was shown to influence the nutrient content of plants (Ealey and Main 1967), which in turn contributes to animal health and body condition. These effects are likely to become more pronounced as Australia's climate changes. This study was conducted through an extended period of drought in eastern Australia (CSIRO 2018). Australia's climate is predicted to become warmer and drier over the next decade, with rainfall occurring in increasingly isolated and sporadic events (CSIRO 2020). Our results suggest that this changing climate will likely impact the population of kangaroos at LAMN. However, if the density of animals at LAMN can be reduced, vegetation communities could

recover, as has been shown in kangaroo populations at other sites (Gowans et al. 2010). The study hopes to incite swift management of kangaroos at the site which reduces the population density and alleviates welfare concerns.

5.3 The immunocompetence handicap hypothesis

Host parasite and disease susceptibility varies due to an individual's sex (McDonald et al. 2014). This variation can be partly explained by energy allocation, whereby males invest less in immune health in favour of increased sexual signalling and mating opportunities; compared to females who invest evenly across immune health and reproduction. These sex-specific life history strategies have flow on effects to disease transmission, infection duration/intensity and can even impact upon population sex ratios and future evolution, whereby older male animals are less represented in a population. The sex differences in disease dynamics of wildlife populations are often overlooked in epidemiological studies (Rosa et al. 2019), in part because individuals cannot be continuously monitored from the time they are infected until their time of death (McDonald et al. 2014). Chapter 4 describes a novel approach to understanding more about sex related disease susceptibility over a relatively short period of time. It aimed to understand if the male reproductive hormone – testosterone, plays a role in sex-related disease susceptibility shown in wildlife. To achieve this aim, testosterone was suppressed, and it was hypothesised that given the hormones role in determining immunocompetence, males would have improved blood parameters and less parasites compared to *Control* males. Despite a robust study design, the difficulty of understanding this complex system in wildlife persisted in this experiment.

The experiment began with a modest sample size (*Treated* n=13 and *Control* n=10), but the recapture rate of recruited animals declined throughout the study. This reduction in sample size, combined with the variability in parasite burden results meant that this study could not support or refute the ICHH in determining whether testosterone increased males' susceptibility to infection. Kangaroo recapture was limited as the targeted cohort spent a substantial amount of time off the

golf course in the surrounding national park, where capture and transportation of animals is more challenging. If target individuals were sighted, their repeat capture was also difficult because kangaroos became cautious around researchers, reducing the success rate of darting.

It is possible that the experimental approach was not able to detect changes in nematode burdens because most lifecycles are completed across multiple seasons (and our study was only conducted in summer and autumn). These shortcomings were likely exacerbated by the lack of site-specific parasitological research and the relatively short duration of testosterone suppression. Many studies have found greater success in assessing testosterone and parasite burdens by comparing *Control* groups with *Treated* groups that have elevated testosterone by the use of implants, or testosterone reduced by castration; both of which have either permanent or a longer duration of suppression, which may not be desirable in free-ranging animals.

To test the ICHH more rigorously in kangaroos, a 'beyond' Before-After-Control-Impact (BACI) approach could be adopted, whereby *Treated* and *Control* groups from one location are compared to *Control* groups from other locations (Underwood 1992). This study design would allow more accurate correlation of parasite trends, such as whether the increase in eggs per gram of faeces (EPG) in the *Control* group (determined in Chapter 4) is a result of seasonal fluctuations in parasite burden, sampling error, or an actual increase due to immunosuppression of *Control* animals. Additionally, future studies could consider using captive colonies of kangaroos to facilitate recapture of target individuals. However, conclusions regarding wild host-parasite-environment dynamics based on captive experiments must be interpreted with caution, as transmission of parasites in captivity could be higher than wild populations due to heavy faecal contamination; or alternatively, kangaroo nematode communities may be altered by anthelmintic treatment.

Male risk-taking behaviour and wider ranging movements both contribute to elevated male mortality and increased disease risk (McDonald et al. 2014). Therefore Chapter 4 examined whether core area use reduced when testosterone was suppressed. Home range size was not measured

because the collection of data on habitat, resources and other attributes of the environment (Powell and Mitchell 2012) was beyond the scope of the study. However, there was no overall reduction in the size of the core area use by *Treated* animals when testosterone was suppressed. Defining the boundaries around an animal's home range or core area is challenging (Powell and Mitchell 2012), and while the results of this study have limited applicability due to sampling limitations, the results suggest that the size of the area occupied by individuals was not determined solely by testosterone. If we had been able to accurately describe a kangaroo's home range, we may have seen different results. With further improvement to our experimental design, accurate testing of behavioural factors on disease susceptibility and the ICHH can be undertaken. While this study was unable to support or refute the ICHH in kangaroos, several key outcomes were achieved. These include using a novel approach to effectively suppress testosterone in kangaroos using a GnRH vaccination. This effect also resulted in a significant reduction in testes size; a readily observable trait that can be used to indicate suppression in future studies. Another key outcome described was the seasonal variability of white blood cell counts and mean corpuscular haemoglobin concentration, further highlighting the importance of season for animal health outcomes. However, further research is required to test whether testosterone influences energy allocation, parasitism and reproductive strategies in kangaroos.

5.4 Conclusions

Kangaroos have prospered on artificial grasslands created by land clearing for agriculture and suburban development, and are widespread throughout eastern and south-eastern Australia (Poole et al. 1982). The apparent success of this species within the built environment comes with a heightened risk of misadventure from overcrowding and density related disease, malnutrition, and motor vehicle collisions. Yet the management of large populations of kangaroos can be contentious amongst landowners, government bodies, and the general public, often making decisive action challenging.

This thesis sought to provide a tool to assess kangaroo health and establish whether population management to improve welfare is necessary on the grounds that animals are deemed unresponsive or incapable of appropriately responding to their environment and the stressors presented. This tool was successfully developed with the establishment of RIs and these were applied in two contexts: 1) on a high-density population of kangaroos undergoing localised habitat loss and geographic isolation (Chapter 3) and 2) to investigate the impacts of testosterone on fitness measures, with the aim of uncovering sex-specific variability in disease susceptibility and evolutionary mechanisms driving fitness trade-offs in male kangaroos (Chapter 4). It is anticipated that the haematological RIs will be broadly adopted as an effective tool for determining the health of kangaroos in a range of contexts, from the individual animal presented to a veterinary hospital to a wild population that may require management to improve population health.

Australian wildlife is currently at a crossroads where urgent action must be taken to stem the loss of endemic fauna. Land clearing is recognised as a contributing factor to Australian faunal species declines, and new land is constantly approved for clearing (Koskimäki et al. 2014; Woinarski et al. 2019). In addition, threats such as the warming and drying of Australia are only intensifying as a result of human-induced climate change (CSIRO 2020; Wintle et al. 2020). The latest independent review of Australia's Environment Protection and Biodiversity Conservation Act found that Australia's 'environmental trajectory is currently unsustainable' (Samuel 2020). Among the multiple recommendations is that a 'quantum shift' in the availability of environmental information be made to arm managers and decision makers with the information to guide Australia's future environment and biodiversity (Samuel 2020). This thesis investigated one of Australia's largest and iconic marsupials to establish and test a versatile tool for assessing kangaroo health, which was applied and tested in two contexts. By developing a validated and powerful health assessment tool, this thesis takes a step, albeit small, towards this 'quantum shift' of environmental information. It is also recognition that common species should not be forgotten when assessing the impacts of development on wildlife.

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