Stress, wildlife health and the conservation of a critically endangered marsupial, the woylie

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A thesis submitted to Murdoch University
to fulfil the requirements for the degree of
Doctor of Philosophy

December 2016

“It seems to me that the natural world is the greatest source of excitement,

the greatest source of visual beauty,

the greatest source of intellectual interest.

It is the greatest source of so much in life that makes life worth living.”

Sir David Attenborough

Author's Declaration

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

Stephanie Hing

Statement of Contributions

This thesis, composed of six first authored published papers, is my original research. My supervisors S.S.Godfrey, R.C.A.Thompson and E.J.Narayan are included as co-authors on all papers for their editorial input. S.S.Godfrey also advised on study design and provided fieldwork support. E.J.Narayan also provided laboratory training and support. The role of additional co-authors are outlined below.

Chapter 4 Immunologists A.Currie and S.Broomfield are included as co-authors as I collaborated with them to adapt an assay developed in human infants for woylies. Co-author S.Keatley provided technical laboratory assistance and K.L.Jones assisted with fieldwork and provided support for statistical analyses.

Chapter 5 This research was undertaken in collaboration with the Department of Parks and Wildlife (DPaW) and co-funded by DPaW and an Australian Research Council (ARC) grant. A.F.Wayne (DPaW) and A.Northover (ARC PhD student), were included as co-authors as they coordinated the translocation. A.Northover also conducted the faecal flotations and S.Keatley provided technical assistance with field and laboratory work.

Chapter 6 This research was undertaken in collaboration with co-authors, K.L.Jones and the conservation staff at Whiteman Park Reserve, which is headed by co-author C.Rafferty.

Abstract

Effective wildlife management requires an understanding of how animals cope physiologically with stress. When stressors are encountered, the hypothalamic pituitary adrenal (HPA) axis is activated releasing glucocorticoids, which can alter immune function and infectious disease dynamics. Investigations have suggested that stress associated immunosuppression and exacerbation of infection (particularly by *Trypanosoma* spp. of haemoparasite) may play a role in the decline of the woylie (syn. brush-tailed bettong, *Bettongia penicillata*), a critically endangered marsupial. This thesis aims to investigate the relationship between stress, immune function and parasite infection dynamics in woylies.

Woylies were trapped from wild populations, reserves and captivity with faecal and blood samples collected from over 300 individuals. Parallel parasitological and non-invasive endocrine analyses were performed to quantify endoparasites and faecal cortisol metabolites (FCM), end-products of HPA axis activation. I also adapted an assay developed in human infants to assess innate immunity in woylies. The novel results suggest that stress physiology and *Trypanosoma* infection status influence innate immunity.

Collecting longitudinal field data, I identified proximate factors that influenced woylie stress physiology, including season, sex, parasite status and body condition. I also explored woylies' response to translocation and a major bushfire that unexpectedly occurred at a field site. After translocation, FCM was significantly higher than before or at the time of translocation. However, the variation in FCM was not related to short-term changes in parasite infection dynamics. FCM was not significantly higher immediately after the fire, nor were there corresponding changes

in parasite load or body condition compared to the months preceding the fire. I suggest that woylies can maintain homeostasis at least in the period immediately after a fire provided they are managed appropriately. Thus this thesis provides new knowledge on woylie stress physiology and highlights the value of innovative tools to advance woylie conservation as they continue to face stressors in the future.

Acknowledgements

It takes a village to raise a PhD.

The vision for a PhD is able to be brought into reality thanks to funding bodies which generously support it. Thank you to Murdoch University, the Australian Academy of Science Margaret Middleton Foundation, Holsworth Wildlife Research Endowment, Foundation of National Parks and Wildlife, Australian Society for Parasitology and Australian Research Council.

A PhD is nurtured by supervisors. Thank you to S.S.Godfrey, R.C.A.Thompson and E.J.Narayan whose guidance was instrumental in the maturation, development and completion of the project.

A PhD needs to be nourished with support from generous colleagues particularly L.Pallant whose tireless logistical support was invaluable. Thank you also to my dedicated field volunteers for all the early mornings and late nights.

A PhD flourishes thanks to the support of collaborators: The Western Australian Department of Parks and Wildlife Science and Conservation Division Manjimup particularly A.F.Wayne, and C.Rafferty, K.Morley and the gang at Whiteman Park, and L.Aravidis and the team at Native Animal Rescue.

Most importantly, over its lifetime, a PhD needs to grow up in a happy environment like that provided by my super support crew: my family and 'Team Parasite' especially the indomitable K.L.Jones. Thanks also to all the rascally little woylies who participated in the study. To my colleagues, friends, family and loved ones (past and present), thank you all from the bottom of my heart for being my village and helping me raise this PhD.

Publications arising from this research

Hing S., Jones K.L., Rafferty C., Thompson R.C.A., Narayan E.J., Godfrey S.S. (2017) Wildlife in the line of fire: evaluation the stress physiology of a critically endangered Australian marsupial after bushfire. *Australian Journal of Zoology* doi: 10.1071/ZO16082

Hing S., Northover A., Narayan E.J., Wayne A.F., Jones K.L., Keatley S., Thompson R.C.A., Godfrey S.S. (2017) Evaluating stress physiology and parasite infection parameters in the translocation of critically endangered woylies (*Bettongia penicillata). EcoHealth* doi: 10.1007/s10393-017-1214-4

Hing S., Narayan E.J., Thompson R.C.A., Godfrey S.S. (2016) Identifying factors that influence faecal cortisol metabolites in critically endangered woylies (*Bettongia penicillata*) and implications for conservation management. *Journal of Zoology* doi: 10.1111/jzo.12428

Hing S., Currie A., Broomfield S., Keatley S., Jones K.L., Thompson R.C.A., Narayan E.J., Godfrey S.S. (2016) Host stress physiology and *Trypanosoma* haemoparasite infection influence innate immunity in the woylie (*Bettongia penicillata*). *Comparative Immunology, Microbiology and Infectious Diseases* 46, 32-39.

Hing S., Narayan E.J., Thompson R.C.A., Godfrey S.S (2016) The relationship between physiological stress and wildlife disease: consequences for health and conservation. *Wildlife Research* 43, 51-60.

Hing S., Narayan E.J., Thompson R.C.A., Godfrey S.S. (2014) A review of factors influencing the stress response in Australian marsupials. *Conservation Physiology* doi: 10.1093/conphys/cou027

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List of Abbreviations

BCI	body condition index
DPaW	Department of Parks and Wildlife
EDTA	ethylenediaminetetraacetic acid (anti-coagulant)
EIA	enzyme immunoassay
FCM	faecal cortisol metabolites
FGM	faecal glucocorticoid metabolites
GC	glucocorticoid
HPA	hypothalamic pituitary adrenal
IUCN	International Union for the Conservation of Nature
NAR	Native Animal Rescue
PCR	polymerase chain reaction
RIA	radioimmunoassay

Chapter 1 General introduction

Since the first study on links between stress and infectious disease was conducted by physician Dr Tohru Ishigami in the early 1900s (Ishigami 1918), the physiological stress response has been associated with changes in immune function (Sabioncello *et al.* 1997, Sapolsky *et al.* 2000) and altered infectious disease dynamics (Cohen *et al.* 1991, Cohen *et al.* 1997) in many studies of livestock, laboratory animals and humans (see reviews by Segerstrom and Miller, 2004, Sheridan *et al.*, 1994). Hormones including glucocorticoids, released as part of the physiological stress response, can influence susceptibility to and the outcomes of infectious disease via direct regulation of the immune system (Aapanius 1998, Sapolsky *et al.* 2000) and indirectly for example via modulation of behaviour (Glaser and Kiecolt-Glaser 2005). However, the relationship between stress, immunity and infection remains a neglected area in wildlife research (Martin *et al.* 2010). This is concerning because stress may influence the impact of disease on wildlife populations, especially endangered species that are often exposed to multiple threatening processes which may render them more vulnerable to the impact of stressors and infectious agents (Smith *et al.* 2008). In this thesis, I address the links between stress, immune function and infection dynamics in the context of endangered species conservation, focusing on a critically endangered Australian marsupial, the woylie (*Bettongia penicillata*).

Chapter 1 is presented in two parts. The first part, ‘Stress and disease in wildlife’, delineates significant knowledge gaps in our understanding of stress and disease in wildlife and proposes how a multi-disciplinary approach involving parallel investigations into stress physiology and infection parameters can be used for the benefit of wildlife conservation, population management and health. The second part, ‘A review of factors influencing the stress response in Australian marsupials’, identifies trends and future research directions for stress physiology research in Australian marsupial conservation. Following the two papers, I then explain how these over-arching themes relate to the specific context of conservation of a critically endangered Australian marsupial, the woylie.

1.1 The relationship between physiological stress and wildlife disease, consequences for health and conservation

The following is a published paper:

Hing S., Narayan E.J., Thompson R.C.A, Godfrey S.S. (2016) The relationship between physiological stress and wildlife disease: consequences for health and conservation. *Wildlife Research* 43, 51-60.

Introduction

Stress can be broadly defined as a change in the psychological, physiological and/or physical well-being of a living organism as a result of exposure to any biological and/or environmental factor that acts as a stressor (challenge to regulatory capacity). Wildlife encounter a range of stressors or threatening processes ranging from habitat loss to climate change, which may activate the hypothalamic pituitary adrenal (HPA) axis (stress response) (Madliger and Love 2014). The stress response is not inherently detrimental but rather is a complex and essential negative feedback system involving glucocorticoids among other neuro-endocrine mediators. Thus the HPA axis regulates the physiological, biochemical and behavioural processes required to maintain allostasis (homeostasis through change) (Sapolsky *et al.* 2000, McEwen 2005). Busch and Hayward (2009) proposed a log quadratic model for the relationship between glucocorticoids and fitness, which illustrated how the stress response (hereby referred to as 'stress') has a critical adaptive advantage up to a point, but when regulatory mechanisms are overcome, allostatic overload occurs and stress incurs fitness costs (McEwen 2005, Busch and Hayward 2009). For example, severe acute and unpredictable or chronic stressors may not allow the HPA axis to reach a recovery phase resulting in dysfunction of the negative feedback mechanism

and subsequent health impairment (Tsigos and Chrousos 1994).

In terms of infectious disease, cost of allostatic overload can include increased infection susceptibility, shedding of infectious agents, severity of clinical signs and poor prognosis (Biondi and Zannino 1997). As a result, shifts may occur in the host-parasite equilibrium, the healthy balance between host and parasites that have co-evolved over millennia (Aapanius, 1998, McLaren *et al.* 2007, Martin *et al.* 2009). Stress may affect the host-parasite equilibrium via complex interactions with and direct influence on the immune system (Reichlin, 1993, Munck and Náray-Fejes-Tóth, 1994, Daynes *et al.* 1995, Sapolsky *et al.* 2000). Mediators of the HPA axis for example glucocorticoids can have profound effects on different immunological processes (Sheridan *et al.* 1994, Aapanius 1998) via receptors on immune cells (Besedovsky and del Rey 1996) and changes in immune gene expression in target tissues (Padgett and Glaser 2003). Stress may also affect host parasite equilibrium indirectly for example by changing characteristics of the body's protective mucus membranes (Chester 1992) and via behavioural changes which may alter disease transmission pathways (Broom 2003). Studies in laboratory animals (Dhabhar and McEwen 2004), livestock (Salak-Johnson and McGlone 2007), fish (Maule *et al.* 1989) and humans (Biondi and Zannino 1997) indicate that stress can affect patterns of infectious disease (Sheridan *et al.* 1994). Stress has also been suggested as a factor influencing a wide range of diseases in wildlife including *Chlamydia* infection in koalas (Brearley *et al.* 2013), toxoplasmosis in various marsupials (Thompson *et al.* 2010), chytridimycosis in amphibians (Blaustein *et al.* 2012, Kindermann *et al.* 2012, Gabor *et al.* 2013), avian influenza in migratory birds (Weber and Stilianakis 2007) and white nose syndrome (Cryan *et al.* 2010) and zoonotic (spread from animals to humans) viruses in bats including Ebola and Hendra virus (HeV) (Groseth

et al. 2007, Plowright *et al.* 2014). However, associations between stress and disease in wildlife are not commonly empirically tested.

Stress has the potential to significantly alter wildlife immune function but the extent to which this translates into changes in patterns of infectious disease in wildlife populations remains unclear (Martin 2009, Blaustein *et al.* 2012). In order to assess this outstanding question, we evaluated the literature on physiological stress and infectious disease. We interrogated the ISI *Web of Science* (http://www.isiwebofknowledge.com) using keywords including: stress, glucocorticoids, wildlife, infection, disease, bacteria, virus, fungi, parasite and pathogen. We selected those studies which examined the influence of stress on infectious disease in wildlife using parallel measures of glucocorticoids and infection indices. The reference lists in these sources were also inspected to find further papers.

We concentrated on the relationship between extrinsic stressors and infectious disease in captive and free-ranging vertebrate wildlife including fish, amphibians, reptiles, birds and mammals. Previous reviews suggest stress can also affect immunity in other organisms across the evolutionary spectrum (Ottavani and Franceschi 1996) including invertebrates (Adamo 2012), molluscs (Saarinen and Taskinen 2005, Hooper *et al.* 2007) and corals (Peters 1984) but these were considered beyond the scope of this review which concentrates on stress and disease in vertebrate wildlife. Studies focusing on domesticated fauna in agriculture or aquaculture (Cohen and Kinney 2007) were excluded as they either examine selectively bred animals which may not react to stressors in the same way as free-ranging wildlife or wild caught animals or those with limited generations in captivity. This is due to vastly different stressor exposure and genetics as the stress response is

shaped by both previous exposure (Bejder *et al.* 2009) and heritable traits (Zhou *et al.* 2008).

We focused on glucocorticoids and their metabolites as stress physiology indicators for consistency and because glucocorticoids provide insights into the mechanisms underpinning the physiological stress response (Cooke *et al.* 2013). Glucocorticoids have been demonstrated to alter physiological, biochemical and behavioural processes to maintain allostasis during exposure to stressors (Sapolsky *et al.* 2000, McEwen 2005). They can also be measured in wildlife using minimally invasive methods (Sheriff *et al.* 2011) and are widely acknowledged as a practical tool in conservation physiology, wildlife research and management (Cooke *et al.* 2013, Narayan 2015).

The importance of increased attention to stress and wildlife disease

As the frequency and severity of stressors increase globally, the relationship between stress and disease in wildlife populations demands serious attention (Lafferty and Kuris 1999, Aguirre and Tabor 2008, Van Bressem *et al.* 2009, Blaustein *et al.* 2012). Infection can influence animal populations via sub-lethal effects reducing fitness as well as dramatic population level effects (Scott 1988, Aguirre and Tabor 2008). For example, Owen et al (2012) found that experimentally elevated plasma corticosterone can reduce the survival of Northern cardinals (*Cardinalis cardinalis*) infected with West Nile Virus (Owen *et al.* 2012). Similarly, Pedersen and Grieves (2007) demonstrated experimentally that stress, parasitism and malnutrition drive winter population crashes in white-footed mice (*Peromyscus leucopus*). In addition, improving our understanding of the relationship between stress and the incidence, prevalence, intensity, recrudescence and severity of disease in wildlife could benefit

biodiversity conservation and One Health (Zinsstag *et al.* 2011). One Health encompasses the collaborative goals of providing optimal health for people, animals (domestic and wild) and the environment by considering interactions between these three systems (Thompson 2013). Here we outline the importance of increased research, monitoring and management of stress and infectious disease in wildlife populations and discuss approaches for future investigation. We draw particular attention to three key anthropogenic stressors which human society can practically and directly manage: climate change, habitat disturbance and management interventions.

1. Climate change

Climate change can have a profound effect on disease dynamics in wildlife populations (Hoberg and Brooks 2015). In part, these effects may be mediated by stress because climate change involves a suite of proximate stressors such as thermal extremes and resource limitations (Geyer *et al.* 2011) which elicit a host stress response. For example chronic stress, such as that associated with resource limitations resulting from prolonged drought, is suspected as a major risk factor for chlamydiosis in koalas, a disease which threatens population survival (Davies *et al.* 2014).

Stress linked to extreme weather events has also been suggested as a contributing factor in mass mortality events in threatened wildlife species. For example, the normally benign bacteria *Pasteurella hamolytica*, which can be precipitated by stress, has been linked to catastrophic mass mortality events in the critically endangered saiga antelope (*Saiga tatarica*) following severe winters (Bekenov *et al.* 1998). Recent modelling of data from over 720 animal mass

mortality events in over 2400 populations indicates that mortality events associated with concurrent stressors and disease have increased in frequency from the 1940s to 2000s (Fey *et al.,* 2015). The imperative to investigate the relationship between stress and disease in wildlife is greater now than ever. Monitoring glucocorticoid parameters and infection indices in wildlife populations over time will allow comparisons to be made as threatening processes progress. In addition, if we can identify which host, pathogen and/or environmental factors (Figure 1) influence animals' responses to major threats such as climate change, we may be better equipped to make conservation decisions (for example designing selection criteria for managed relocation programs).

2. Habitat disturbance

Brearley *et al.*, (2013) reviewed the profound impact that habitat loss and fragmentation can have on the prevalence of wildlife disease and highlighted the role stress was likely to play as a mediator of this impact. Recent studies have examined the physiological stress response of wildlife to habitat loss and fragmentation (Davies *et al.,* 2013). There are also studies linking infection patterns in wildlife to habitat loss and fragmentation (Gillespie and Chapman 2006, Hing *et al.* 2013). However, there appears to be an absence of research which unites physiology and disease diagnoses, measuring glucocorticoid and infection indices in parallel in order to understand links between habitat loss, stress and infection patterns in wildlife. Investigating stress and disease in wildlife can also help us identify host factors particular to individuals, populations or species which are resilient (Jessop *et al.* 2013) in an increasingly urbanised world (Figure 1). For example, the Australian long nosed bandicoot (*Permales nasuta*) and southern brown bandicoot (*Isoodon*

obesulus) had similar faecal glucocorticoid metabolites in the suburbs of Sydney compared to National Parks, and metabolites did not covary with ectoparasite burden suggesting that as generalists able to flourish in a variety of environments, these species may possess traits which make them adaptable to stress and parasite infection in a changing landscape (Dowle *et al.* 2012). The question arises: what traits allow some individuals, populations or species to be more resilient to the synergistic effects of stress and infectious disease? Models have been developed in livestock for genetic selection and breeding for robustness based on the stress response (Mormède *et al.* 2011) and in the future, wildlife management too may employ similar strategies to build populations which are more resilient to the combined effects of stress and disease.

Climate change and habitat loss are amongst the key anthropogenic stressors which are significant in terms of The One Health paradigm which centres on inextricable links between environmental, animal and human health (Daszak *et al.* 2000) (Figure 2). These links are exemplified in modelling of global emerging infectious disease (EID) data from 1950 to 2008 which indicated a positive association between the number of threatened mammal and bird species and outbreaks of zoonoses in the Asia Pacific region (Morand *et al.* 2014). If environmental threats trigger a stress response in wildlife which precipitate infectious disease, there may be consequences for the transmission of zoonotic EID (Figure 2). This is particularly concerning because Jones *et al.* (2008) have highlighted that over 60% of global EID are zoonoses and wildlife represent the most common source (over 70%). Hence, improving our understanding of how stress affects patterns of disease in wildlife populations will benefit One Health.

Catastrophic contemporary disease outbreaks such as Ebola virus have raised

urgent questions about how environmental stressors and interactions between humans, livestock and wildlife influence the role of wildlife in disease emergence and transmission (Fenton *et al.* 2006, Myers *et al.* 2013, Plowright *et al.* 2014). However, the role of wildlife stress in EID epidemiology has yet to be elucidated. Studies have suggested that environmental stressors encountered by wildlife, including climate change and habitat loss, may precipitate zoonotic disease transmission (Hoberg and Brooks, 2015). Epidemiological studies have identified trends that suggest stress related immunosuppression in flying foxes (pteropid bats) (such as that associated with reproduction, poor nutrition, habitat loss, climate change) may have contributed to the emergence of zoonoses including HeV and Nipah, viruses spread from flying foxes to domestic animals and humans (see case study: physiological stress in bats and HeV control) (Plowright *et al.* 2008, Plowright *et al.* 2008, Plowright *et al.* 2011, Plowright *et al.* 2014)

3. Wildlife management interventions

An understanding of the relationship between physiological stress and disease in wildlife will help us improve the planning, design, execution and evaluation of wildlife management interventions. It is crucial that we characterise how wildlife respond to research and conservation interventions such as capture and handling (de Villiers *et al.* 1995, Narayan *et al.* 2012) and translocation (Kahn 2007) to assess risk, minimise harm and increase the efficacy of these activities. For example, it has been suggested that the stress of translocation is associated with increased risk of infectious disease in translocated wildlife (Teixeira *et al.* 2007, Dickens *et al.* 2010, Sainsbury and Vaughan-Higgins 2012) including recrudescence of latent and normally innocuous pathogens as well as increased vulnerability to diseases in the

release site to which the translocated animals may not have been previously exposed (Mihok *et al.,* 1992). However, parallel endocrine and infection investigations have rarely been carried out before, during and after wildlife translocations or indeed in instances of wild capture (moving of wild animals into captivity), to investigate the influence of stress and disease in translocation success and the health of translocated and resident populations. A study by Kahn *et al.* (2007) is a notable exception, finding that translocation of gopher tortoises (*Gopherus polyphemus*) did not appear to have a significant effect on corticosterone, immune parameters or infection with *Mycoplasma agassizii*, a common cause of upper respiratory disease in tortoises.

Stress and wildlife disease in management interventions also have relevance to One Health. For example, recent research suggests that stress and disease in wildlife is highly relevant to the management of bovine tuberculosis (*Mycobacterium bovis*), an economically significant livestock, wildlife and zoonotic disease with ramifications for agriculture and food security (Cross *et al.* 1998, George *et al.* 2014). Stress has been linked to increased shedding of *M. bovis* in badgers (*Meles meles*) in the United Kingdom (George *et al.,* 2014). These findings have direct relevance for *M. bovis* and badger management. They suggest that stressful interventions such as culling may contribute to increased shedding by wildlife hosts (Carter *et al.* 2007). Greater attention to stress and wildlife disease will help design effective wildlife policy and management plans with potential benefits to One Health, welfare, biosecurity, agriculture and associated financial consequences.

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Figure 1.

Factors which may influence the response of wildlife to stress and disease

Stressor, host, pathogen and environment factors which may influence the response of wildlife to stress and disease.

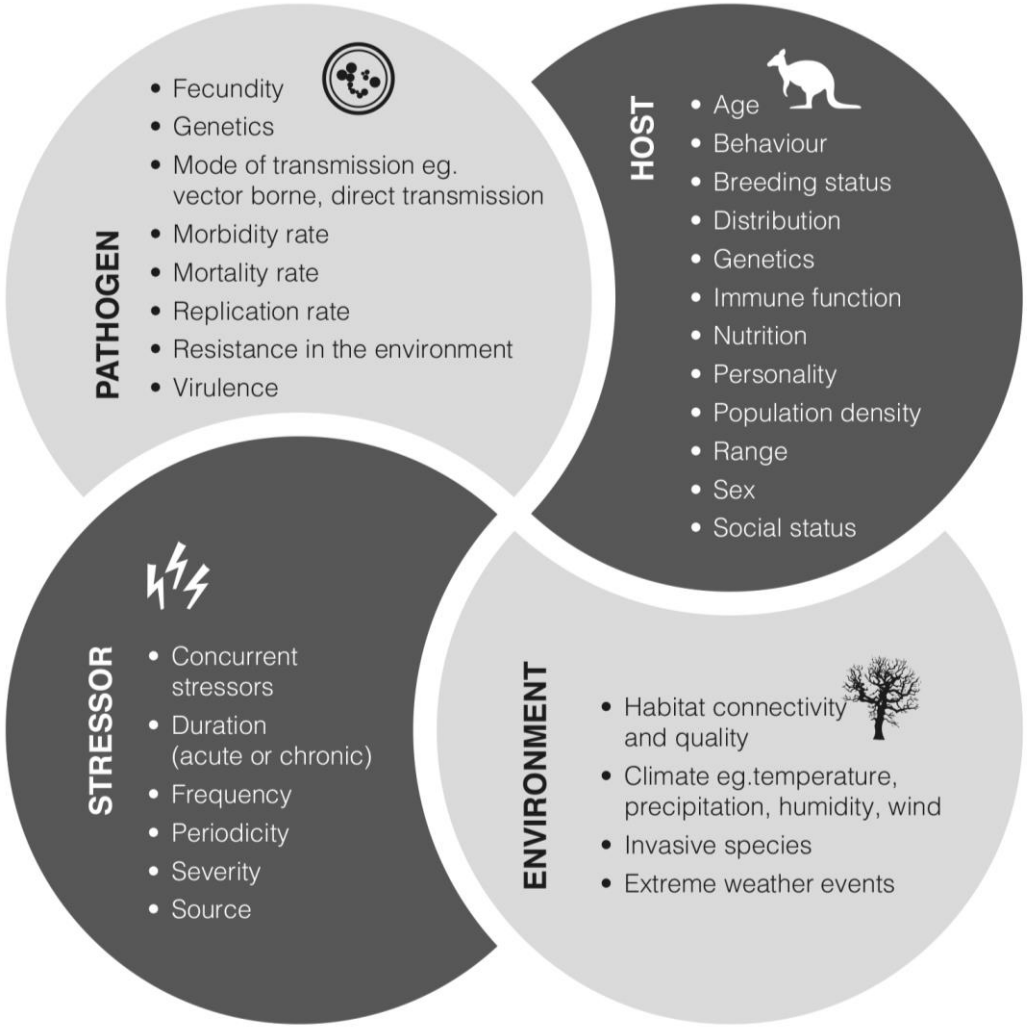
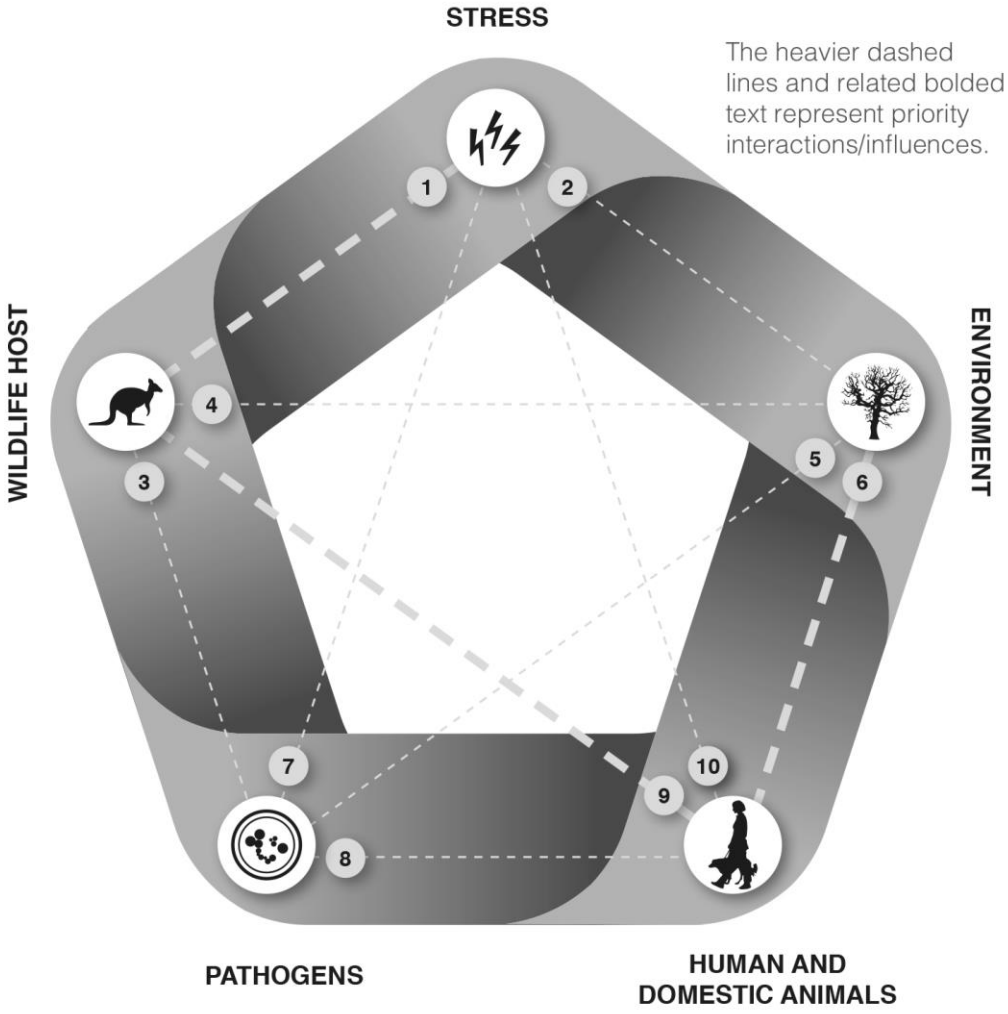


Figure 2.

Stress and disease in One Health

Conceptual flow diagram of the relationship between stress and disease and how it may influence animal and human health including emerging infectious disease. Links are bi-directional.

1. **Stress to wildlife can influence infection dynamics and wildlife may also be a source of stress eg. predators, invasive species**
2. The environment can be a source of stress and stress can also influence hosts' interaction with the environment
3. Pathogens can act as a stressor and stress can also affect infection dynamics
4. Environmental factors may influence the stress and disease response of wildlife and wildlife may also contribute to environmental change
5. Pathogens may be shed into the environment and environmental factors can facilitate persistence of pathogens
6. **Humans can be a source of environmental stress and environmental factors also influence human infection dynamics**
7. Pathogens may affect the wildlife host stress response and stress may exacerbate infection
8. Some pathogens can infect humans, domestic hosts and wildlife
9. **Interactions between humans, domestic hosts and wildlife can facilitate disease transmission**
10. Humans and domestic animals may be the source of stress for wildlife but may also be susceptible to many of the same stressors



Approaches to understand the relationship between stress and disease in wildlife

There are many different ways stress and disease can be investigated in wildlife but here we discuss the merits and challenges of an approach using glucocorticoids and infection indices measured in parallel. This approach has rarely been applied in wildlife but has been recommended as a means to investigate the impact of anthropogenic activities on wild animals (Muehlenbein 2009). Combining glucocorticoid and infection indices with assessments of host reproductive history (Narayan *et al.* 2012) and survivorship (de Villiers *et al.* 1995) has also been proposed as an approach to investigate the fitness costs of stress.

Glucocorticoid indices are advantageous for investigating stress and disease in wildlife because they provide a mechanistic understanding of the underlying physiological processes mediating the stress response and are being increasingly used in wildlife research (Cooke *et al.* 2013). We now have at our disposal minimally and non-invasive tools to investigate stress and disease in wildlife (Sheriff *et al.* 2011, Hunt *et al.* 2013, Narayan 2013). Using validated enzyme immunoassays and radioimmunoassays, glucocorticoids can be measured in the peripheral circulation (Palme *et al.* 2005). Alternatively, glucocorticoid metabolite concentration can be measured in faeces (Palme 2005), urine (McMichael *et al.* 2014), feathers (Bortolotti *et al.* 2008), saliva (Majchrzak *et al.* 2014), exhaled breath (Hunt *et al.* 2013) and water (Gabor *et al.* 2013). Research on glucocorticoid receptors may also prove useful to evaluate stress and disease in wildlife but these methods remain invasive (Liebl and Martin 2013).

To date, parallel glucocorticoid and infection parameters have been measured in few studies but these include species ranging from the seahorse (Anderson *et al.*

2011) to amphibians (Kindermann *et al.* 2012, Gabor *et al.* 2013), birds (Lindström *et al.* 2005, Kitaysky *et al.* 2010), lizards (Oppliger *et al.*, 1998) and non-human primates (Chapman *et al.* 2006, Clough *et al.* 2010), indicating its potential to be used in different taxa. This approach has been applied in a small number of stress and disease studies conducted on endangered species in challenging field conditions (Aguirre *et al.* 1995, Chapman *et al.* 2006). For example, plasma corticosterone positively correlated with fibropapilloma virus, an emerging disease threatening endangered green turtle (*Chelonia mydas*) (Aguirre *et al.* 1995). Stress and infection indices were also found to correlate in endangered red colobus monkeys (*Piliocolobus badius*) where a positive correlation was observed between poor nutrition, parasitism and faecal glucocorticoid metabolites (Chapman *et al.* 2006).

There are numerous challenges, theoretical and practical, to investigating stress and disease in wildlife using glucocorticoids and infection parameters in parallel, particularly methodological limitations and pitfalls in the interpretation of results. Firstly, researchers must keep in mind that the relationship between stress and infection is likely to vary depending on a variety of stressor, host, environment and pathogen factors (Figure 1). Secondly, caution must be exercised when interpreting the results of glucocorticoid assays (Lane 2006, Dantzer *et al.* 2014). For example, high glucocorticoid values are not necessarily detrimental and low values beneficial particularly if chronic stress leads to dysfunction of the HPA axis rendering an animal unable to mount an appropriate stress response (Busch and Hayward 2009, Narayan and Hero 2014). Caution must also be exercised when interpreting changes in glucocorticoids solely as physiological responses to stressors because variations in glucocorticoids can occur normally for example with diurnal rhythms, exercise, pain, season and reproduction (Lane 2006).

Another challenge in the interpretation of stress and wildlife disease studies is that infection itself can act as a stressor, elevating glucocorticoids as part of the host's efforts to mobilise energy and redistribute resources towards fighting infection whilst also preventing collateral damage to the body (Sapolsky *et al.* 2000, Dunn 2007, Laver *et al.* 2012). For example, wild captured rodents experimentally exposed to fleas had higher faecal glucocorticoid metabolite concentrations compared to controls (St Juliana *et al.* 2014) and male fence lizards (*Sceloporus occidentalis*) naturally infected with *Plasmodium mexicanum* mounted a greater corticosterone response to capture and handling than uninfected lizards (Dunlap and Schall 1995). The bi-directional relationship between stress and infection poses a challenge to establishing causality in observation studies. Experimental approaches can be used to investigate causality and the multi-dimensional nature of the stress disease relationship including: experimental stressors (Oppliger *et al.* 1998), experimental infections (Warne *et al.* 2011, Kindermann *et al.* 2012, Marino *et al.* 2014) and parasite/pathogen treatment experiments (Goldstein *et al.* 2005, Raouf *et al.* 2006, Pedersen and Greives 2008, Monello *et al.* 2010). However, experimental approaches have numerous logistical and ethical challenges, particularly when working with small populations of free-ranging endangered species. Captive populations may serve as useful surrogates for experimental studies of stress and wildlife disease provided that limitations are acknowledged, such as potential behavioural and physiological differences between captive and wild counterparts, and the stressors that may be alleviated or imposed by captivity itself (Narayan *et al.* 2012). There are also considerations when measuring infection indices in wildlife. For example, faecal egg counts for parasite oocysts and viral titres can naturally vary with demographic, temporal and spatial variables and potentially covary with the

same factors that influence the stress response (Plowright *et al.* 2014). Limitations of diagnostic tests (such as detection threshold, sensitivity and specificity) must also be considered, particularly if tests are being adapted for use in wildlife species for the first time. Continuous testing and validation of sampling methods, assay protocols and experimental designs will be necessary to build robust research programmes that can reliably and efficiently evaluate the relationship between stress and disease in wildlife species.

Case study: Physiological stress in bats and Hendra virus (HeV) control

Improving our understanding of the relationship between physiological stress and disease dynamics in bat populations may be a key strategy to help characterise the epidemiology of HeV, predict outbreaks and protect animal and human health (Plowright *et al.* 2008, Plowright *et al.* 2014, McMichael *et al.* 2014). HeV, a RNA virus of the family *Paramyxoviridae* and genus Henipavirus, was identified as an emerging zoonotic disease in Australia in 1994 (Field *et al.* 2007). *Pteropus* species of fruit bats including vulnerable species such as grey-headed flying fox (*Pteropus poliocephalus*), have been identified as the asymptomatic natural reservoir hosts (Young *et al.* 2000). Horses develop highly fatal respiratory or neurological disease after oronasal exposure to HeV virions in bat secretions including urine (Westbury 2000, Marsh *et al.* 2011). Following direct contact with sick horses, veterinary and stable staff have contracted severe meningoencephalitis, which in four cases proved fatal (Playford *et al.* 2010). East coast states of Australia continue to report sporadic equine cases (New South Wales Department of Primary Industries 2015).

The epidemiology of HeV remains incompletely understood, particularly the host and environment factors driving spill-over events (Hyatt *et al.* 2004, Smith *et al.* 2011). Changes in flying fox ecology associated with anthropogenic stressors including climate change, habitat fragmentation and urbanisation, have been proposed as drivers of HeV disease dynamics (Bradley and Altizer 2007, Plowright *et al.* 2011, Dietrich *et al.* 2015). These ultimate stressors are associated with proximate stressors such as nutritional stress which are in turn, overlaid on challenges such as the demands of reproduction (Plowright *et al.* 2008). Though experimental evidence is not available at this time, a leading hypothesis is that physiological stress alters bat immune function and increases the rate of virion shedding (Plowright *et al.* 2008, Plowright *et al.* 2008). Hence, physiological stress may be one of the important factors influencing HeV emergence and outbreaks.

Currently McMichael *et al.* (2014) are conducting parallel investigations into glucocorticoid metabolites and HeV dynamics. This valuable information on the relationship between physiological stress in bats and HeV will inform policy and management of flying fox populations. The potential effects of stress to flying foxes on HeV spill-over has been discussed at a policy and legislative level in Australia and on these grounds, highly disruptive methods employed overseas for ‘bat control’ such as culling (Florens 2012) are now banned in some Australian jurisdictions (Degeling and Kerridge 2013). However, stressful management interventions for ‘problem’ flying fox populations continue to be applied in Australia. For example, aversive stimuli such as noise cannons are used sporadically to drive bats away from urban centres where people complain about noise, smell and damage to gardens

(Degeling and Kerridge 2013). Information about significant stressors associated with HeV virion shedding would inform cost benefit evaluation of disruptive management approaches and would be a valuable resource upon which to formulate public health advice.

HeV provides a contemporary example of how the relationship between physiological stress and disease dynamics can inform responsible, evidence based One Health and wildlife conservation policy and management. More broadly, this case study may have implications for other emerging viral diseases for which bats act as reservoir hosts including Ebola (Calisher *et al.* 2006, Plowright *et al.* 2008, Smith and Wang 2013, Plowright *et al.* 2014).

Conclusion

Despite suggestions that stress plays an important role in disease dynamics, this remains a neglected area in wildlife research. Rhyan and Spraker (2010) described how mounting stressors may profoundly change disease transmission cycles using the analogy of 'adding bags of sand to a rowboat until it sinks'. Understanding the dynamics of stress and disease in wildlife will help us keep healthy biological systems 'afloat' by identifying novel management targets and assisting priority setting and policy decisions. Wider application of stress physiology and diagnostic tools to characterise links between stress and disease in wildlife will benefit One Health and biodiversity conservation. Greater collaboration between human and animal health experts, conservation researchers, comparative physiologists and disease ecologists will enable further progress in understanding the stress disease synergism to prevent the loss of iconic species and reduce the risk of contemporary and emerging infectious diseases.

We propose a defined approach using parallel glucocorticoid parameters and infection indices to investigate stress and disease in wildlife. This approach can be applied to examine the influence of physiological stress associated with key anthropogenic stressors including climate change, habitat disturbance and management interventions on disease dynamics. It is time to fully incorporate stress and wildlife disease in the overall management of environmental, animal and human health.

1.2 A review of factors influencing the stress response in Australian marsupials

The following is a published paper:

Hing S., Narayan E., Thompson R.C.A., Godfrey S.S. (2014) A review of factors influencing the stress response in Australian marsupials. *Conservation Physiology* doi:10.1093/conphys/cou027

Introduction

Characterisation of the stress response of animals, the physiological reaction to challenging stimuli, is essential to conservation because these processes underpin how wildlife responds to environmental change (Reeder and Kramer, 2005, Killen *et al.* 2013, Madliger and Love, 2014). For this reason, the field of wildlife stress physiology is increasingly being recognized as an integral component of conservation physiology (Cooke *et al.* 2013, Kersey and Dehnhard, 2014) and a key approach to improve wildlife welfare and management *in situ* and *ex situ* (Monfort, 2003, Bradshaw, 2010, Cooke *et al.* 2013). However, stress physiology remains an underused approach in Australian marsupial conservation.

An extensive body of literature highlights the complex roles of the stress endocrine system in the physiological responses of wildlife to stressors. Stress can be stimulatory (optimizing physiological systems for action), preparative (priming systems for action) or inhibitory especially chronic stress, which can cause permanent dysfunction of the endocrine stress response and lead to suppression of functions such as reproduction (Narayan and Hero, 2014a, 2014b). The stress response is a complex and vital physiological mechanism that enables individuals to

cope with stressful situations (Munck and Náray-Fejes-Tóth, 1994, Sapolsky *et al.* 2000). The complexities and importance of stress physiology have been recognized in many mammalian taxa (see reviews: marine mammals, Fair and Becker, 2000, carnivores, Young *et al.* 2004, felids, Brown, 2006, primates, Muehlenbein, 2009) as well as birds (Palme *et al.* 2005), amphibians (Gabor *et al.* 2013, Narayan, 2013) and fish (Iwama *et al.* 2011, Baker *et al.*,2013). However, in comparison to other taxa, relatively little is known about the response of Australian marsupials to conservation-relevant stressors and the conservation implications of the marsupial stress response.

Stress physiology in Australian marsupials is of particular interest owing to their conservation status, evolutionary importance and unique physiological characteristics. Over 40% of the 142 (60 of 142) extant Australian marsupial species are of conservation concern (Kennedy, 1992). Marsupials also occupy a unique evolutionary niche (Armati *et al.* 2006). Thus, investigation of how marsupials respond to stressors can provide further insights into the evolution of the physiological stress response, an area of ongoing scrutiny in evolutionary ecology (Boonstra, 2013). Australian marsupials are the most diverse extant marsupial radiation (Johnson, 2006) and possess many unique physiological characteristics, such as adaptations to specific climatic envelopes (Bradshaw, 1983), which may facilitate distinct physiological stress responses. Stress physiology is also a neglected area of research in marsupials from the Americas, however, for the purposes of identifying trends relevant to Australian marsupial conservation, this review focuses on the Australian context.

Marsupials are exposed to a suite of potential stress factors (McEwen, 2005, Bradshaw, 2010), such as habitat loss and increased predation pressure (Claridge *et al.* 2007). These factors have contributed to nationwide decline and extinction of

native species (Johnson, 2006). In order to manage and conserve marsupials into the future, it is crucial to characterize their physiological response to stress factors (Narayan *et al.* 2013). Understanding responses to extrinsic (environmental) stressors is particularly important, because these types of factors have been found largely to determine extinction risk among Australian marsupials (Fisher *et al.* 2003). Insights into marsupial stress physiology may also improve outcomes for injured and orphaned marsupials, because stress has been implicated as a major challenge to their successful care and survival (Jackson, 2007).

The aim of this review is to identify how stress physiology can be applied to the conservation of Australian marsupials. The stress physiology literature was examined to find studies on the stress response of marsupials to conservation-relevant stressors and those which highlighted the consequences of stress in marsupials. We surveyed the literature in view of three key questions. (i) What stress parameters and methods have been used for glucocorticoid quantification in marsupials? (ii) How have these tools been applied to identify significant stressors relevant to marsupial conservation? (iii) What are the significant knowledge gaps that require future research? We sought to evaluate whether stress physiology can be used to identify risk factors, aid management and improve conservation outcomes for threatened species of Australian marsupials.

The physiological stress response

The stress response involves the production of neuroendocrine mediators (adrenaline, noradrenaline and glucocorticoids) and is essential to maintain allostasis (homeostasis through change) during exposure to a stressor (McEwen, 2005, Wikelski and Cooke, 2006). The hypothalamic–pituitary–adrenal (HPA) axis, the neuroendocrine pathway underpinning the physiological stress response, is highly conserved across vertebrates, including eutherian mammals, marsupials and monotremes (McDonald, 1977, Nesse and Young, 2007). Several texts (Downs, 1980, Moberg and Mench, 2000) and reviews have outlined the HPA axis in detail (e.g. Reeder and Kramer, 2005, Sheriff *et al.* 2011). In summary, the HPA axis operates as a negative feedback system. Secretion of corticotrophin-releasing hormone from the hypothalamus stimulates release of adrenocorticotrophic hormone (ACTH) from the pituitary gland. Adrenocorticotrophic hormone promotes the release of many neuroendocrine mediators (Moberg and Mench, 2000), including the focus of this review, glucocorticoids (GCs) from the adrenal cortex (Möstl and Palme, 2002). The GCs (mainly cortisol and/or corticosterone) have complex interactions with virtually all biological processes (McLaren *et al.* 2007). As for eutherian mammals, GCs in marsupials are known to regulate a suite of biological processes from protein, carbohydrate and fat metabolism (Bradley and Stoddart, 1990) to essential renal (McDonald and Bradshaw, 1993), neurological (McAllan, 2006), cardiorespiratory and reproductive functions (Shaw *et al.* 1996). Ultimately, GCs influence wildlife health, fitness and survival (Jessop *et al.* 2013).

The HPA axis of marsupials shares many similarities with that of eutherian mammals (Hume *et al.* 1989, Booth *et al.* 1990). However, there are several unique characteristics which may constitute evolutionary adaptations to stressors that marsupials face in harsh habitats (McDonald, 1977). For example, in some macropod species, such as quokka (*Setonix brachyurus*), GCs appear to lack diabetogenic and nitrogen-mobilizing actions (Bradshaw, 1983, McDonald and Bradshaw, 1993). Early studies involving the removal of both adrenal glands (bilateral adrenalectomy) concluded that marsupials may be less dependent on the HPA axis for survival compared with eutherian mammals, because marsupials appeared to survive for longer periods post-adrenalectomy (McDonald, 1977). Early reports also mention that marsupials exhibit low levels of circulating GCs compared with eutherian mammals (Weiss *et al.* 1979) and have low sensitivity to an exogenous ACTH stimulation test (McDonald, 1977, Weiss *et al.* 1979). However, more recent studies have readily demonstrated a GC response to an ACTH stimulation test as part of the validation of non-invasive GC assays (Hogan *et al.* 2012, Narayan *et al.* 2013).

Research on the physiological stress response in marsupials

What stress parameters and methods have been used for glucocorticoid quantification in marsupials? There are several methods for measuring stress in wildlife (Sheriff *et al.* 2011). We have focused on GCs as physiological measures of stress because they provide a mechanistic understanding of the physiological stress response (Cooke *et al.* 2013). The other major group of hormones released in the stress response are catecholamines (adrenaline and noradrenaline). However, it is difficult to measure catecholamines in wildlife because they are released almost instantaneously when a stressor is encountered and have a very short half-life of <30 s in the circulation (Medeiros and Wildman, 2013). Little is known about the excretion of

catecholamines, and the known metabolites are unstable (Palme *et al.* 2005). Therefore, catecholamines are infrequently applied to evaluate the stress response in wildlife (Stoddart and Bradley, 1991, Dehnhard, 2007). Behavioural and haematological parameters are also used as measures of stress in wildlife. However, they can vary widely among individuals and lead to inconsistent results (McKenzie *et al.*, 2004, Young and Deane, 2006). Particularly in the case of behavioural parameters, the underlying neuroendocrine stress response can occur in the absence of significant behavioural changes (Hogan *et al.* 2011). In contrast, GCs mediate many of the consequences of stress for health, reproduction and survival (Cockrem, 2005, Reeder and Kramer, 2005). Glucocorticoids are also increasingly acknowledged as practical endocrine indicators of stress in many species and are currently being integrated into wildlife conservation worldwide (Möstl and Palme, 2002, Palme *et al.* 2005, Sheriff *et al.* 2011).

Endocrinology has undergone a revolution since Chester Jones *et al.* (1964) carried out the first study on GCs in Australian marsupials by collecting adrenal venous blood from brushtail possums (*Trichosurus vulpecula*). While invasive terminal sample collection was commonplace in the 1960s and 1970s and *post-mortem* sampling continues to be informative (e.g. Oates *et al.* 2007), these methods are not always practical or ethical, particularly if studying cryptic and endangered species. In the last 20 years, minimally invasive sampling has become more widely practised (Kersey and Dehnhard, 2014). Innovative minimally invasive techniques to measure GCs and their metabolites have been broadly applied to identify stressors to wildlife (Sheriff *et al.*, 2011). Assays have been validated for use in various wildlife species to allow GCs to be quantified in blood, faeces, hair (Sheriff *et al.*, 2011), urine (e.g. Narayan, 2013) and even water (Gabor *et al.*, 2013).

Assays in marsupials initially relied on chromatographic techniques (Chester Jones *et al.* 1964, Weiss and McDonald, 1966), but now radioimmunoassays (RIAs) and enzyme immunoassays (EIAs) are used, with EIAs having the advantage of lower resource costs, versatile equipment and lack of radioactive materials (Sheriff *et al.* 2011). These assays have been employed in studies of Australian marsupials, including EIA (e.g. Narayan *et al.* 2013) and RIA for faecal glucocorticoid metabolites (FGM, Oates *et al.* 2007), EIA for plasma GC (e.g. Baker and Gemmell, 1999) and EIA for GC in hair (Brearley *et al.* 2012). The FGM EIAs are the most common method used in studies of Australian marsupials (Figure 3).

Key factors to consider when integrating stress physiology into marsupial conservation include considering what is to be measured, standardized protocols and validation (Möstl and Palme, 2002, Sheriff *et al.* 2011). Firstly, if measuring GC in the circulation, it is important to consider that blood should be collected within 2–3 min of capture (Romero and Reed, 2005). This is rarely possible in marsupial field studies, because trapping and handling cryptic, sparsely distributed, often nocturnal marsupials with minimal resources in challenging terrain is not conducive to immediate collection. Hence, measuring FGM has proved to be a popular method. Recent research has explored the relative value of FGM analysis on fresh vs. older samples (by quantifying decay rates of FGMs, Evans *et al.* 2013). Metabolites of GCs appear to be relatively stable in bilby faeces for up to 19 days (Evans *et al.* 2013). However, as a precaution this validation needs to be done for each species owing to species specificity in decay rates of FGMs (Evans *et al.* 2013).

It is critical that assays have been validated in the laboratory as well as physiologically (see reviews: Möstl and Palme, 2002, Millspaugh and Washburn, 2004, Touma and Palme, 2005, Sheriff *et al.* 2011). For example, the ACTH stimula-

tion test is a widely accepted method of biological validation because it demonstrates the cause-and-effect relationship between HPA axis activation and minimally invasive GC measurements. The ACTH stimulation test has been carried out to validate chromatography for plasma GC in Tasmanian devils (Weiss and Richards, 1971), quolls (Weiss and Richards, 1971), phascogales (Bradley, 1990) and quokkas (Bradshaw, 1983) and also to validate FGM EIAs in bandicoots (Dowle *et al.* 2012), numbats (Hogan *et al.* 2012), koalas (Narayan *et al.* 2013b, Davies *et al.* 2013a), bilbies (Narayan *et al.* 2012) and wombats (Hogan *et al.* 2011).

Interpretation of different parameters and assays should also be considered carefully. A range of potential methodological [e.g. sample collection, storage and extraction procedures, fraction of glucocorticoid measured, i.e. total, free, plasma corticosteroid-binding globulin (CBG) bound or albumin bound] and biological confounders (e.g. diurnal or seasonal fluctuations, reproduction, diet, pain and exercise) need also be considered when interpreting results and management implications (Millspaugh and Washburn, 2004, Palme, 2005, Lane, 2006, Evans *et al.* 2013). The GC fraction measured is also noteworthy. For example, if the assay measures total cortisol, an increase in free cortisol may occur without an increase in total cortisol, depending on the rate of steroid metabolism (Hajduk *et al.* 1992). Assays may measure non-protein-bound or free levels of the steroid. In circulation, GC can be bound to CBG, and CBG levels have been measured in marsupials to elucidate the amount of free vs. bound cortisol (Schmidt *et al.* 2006). Free GCs are biologically active, but bound GCs may also exert their effect on cells via receptors for bound GCs (Sheriff *et al.* 2011). The identity of the primary GC is important to consider because it too can differ between marsupial species (Oddie *et al.* 1976).

As GC play a vital role in regulating metabolism, activity, reproduction and response to environmental challenges (Boonstra, 2013), fluctuations in GCs occur normally. Glucocorticoids can vary with diurnal rhythms (Than and McDonald, 1973, Allen and Bradshaw, 1980), reproductive status (Narayan *et al.* 2013b) and season (Humphreys *et al.* 1984, Bradley and Stoddart, 1992). For example, diurnal variations in GC have been documented in brushtail possums (Than and McDonald, 1973) and koala (Johnston *et al.* 2013), and significant differences in FGM were noted between lactating and non-lactating female koalas (Narayan *et al.* 2013). Seasonal variations in GCs have also been recorded in sugar gliders (Bradley and Stoddart, 1992) and scaly-tailed possums (*Wyulda squamicaudata*, Humphreys *et al.* 1984). Such naturally occurring variation in GCs should be considered in order to avoid misinterpreting a normal GC fluctuation as a significant physiological response to a stressor. Study design and analyses must also acknowledge and try to account for these variables (Lane, 2006). Considering methodological and biological factors when designing minimally invasive GC methods will aid the incorporation of stress physiology into marsupial conservation.

Identification of significant stressors to marsupials

A range of broad categories of stress factors have been identified in the marsupial stress physiology literature, including habitat loss and fragmentation, captivity, translocation and climate. It must be noted that each of these ultimate stress factors (root causes) may entail a number of potential proximate (immediate) factors. For example, habitat loss and fragmentation are associated with proximate factors including nutritional stress (Chapman *et al.* 2007), altered social contact rates (Gillespie *et al.* 2005) and changing patterns of parasite infection (Gillespie *et al.* 2005, Figure. 4). Assessing the stress response of marsupials to these factors will

allow mitigation of stressors which may have a detrimental effect on health, welfare and fitness and thereby increase conservation success.

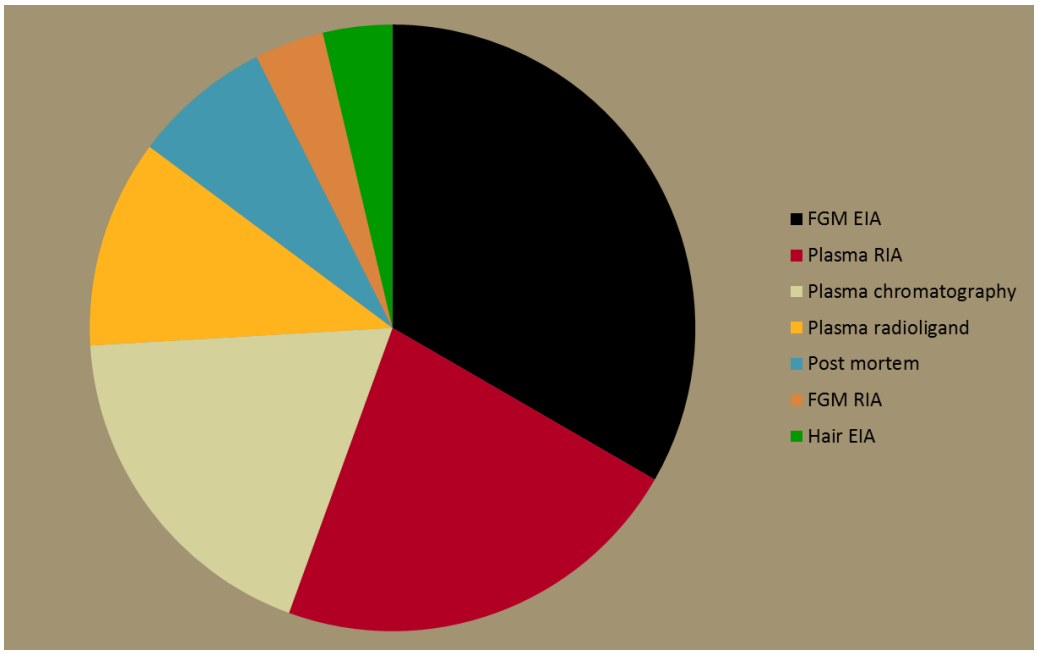


Figure 3. Stress parameters and methods used in marsupials. The most commonly measured stress parameters in marsupials are faecal glucocorticoid metabolites (FGM) using enzyme-immunoassay (EIAs, 33%). Radio-immunoassays (RIAs) and chromatography were also popular methods to measure plasma glucocorticoids (total n=29 publications)

Habitat loss and fragmentation

Habitat loss and fragmentation have a severe impact on wildlife population viability and the persistence of endangered species (McCallum and Dobson, 2002, Acevedo-Whitehouse and Duffus, 2009). Stress could be a consequence and could contribute to species decline in fragmented habitat (Brearley *et al.* 2012, Johnstone *et al.*, 2012). Habitat loss and fragmentation may act as stressors through proximate mechanisms, such as reduced resource availability, increased competition, altered behaviour and social and disease stressors associated with changes in population density (Mbora and McPeek, 2009, Brearley *et al.* 2013). Marsupials tend to experience greater physiological stress at higher densities compared with lower densities as measured by plasma GC (Thomas, 1990, Johnstone *et al.* 2012). Northern quolls may be an

exception, with no association found between plasma GC and the population density of quolls in the Kimberley region of Western Australia (Schmitt *et al.* 1989).

Habitat fragmentation may also increase stress through the creation of more habitat edges. Squirrel gliders (*Petaurus norfolcensis*) living in edge habitats exhibited significantly higher cortisol compared with counterparts in interior habitats, as measured by GC metabolites in hair (Brearley *et al.*, 2012, 2013). Long-nosed bandicoots (*Perameles nasuta*), in contrast, had similar FGM concentrations in National Parks (continuous habitat) and suburban backyards (fragmented habitat, Dowle *et al.*, 2012), although other concurrent stressors pertaining to urban and wild locations may also explain these differences. Differences in physiological stress between marsupials in fragmented compared with continuous habitat may also underlie differences in immunocompetence and infection patterns (Johnstone *et al.*, 2012).

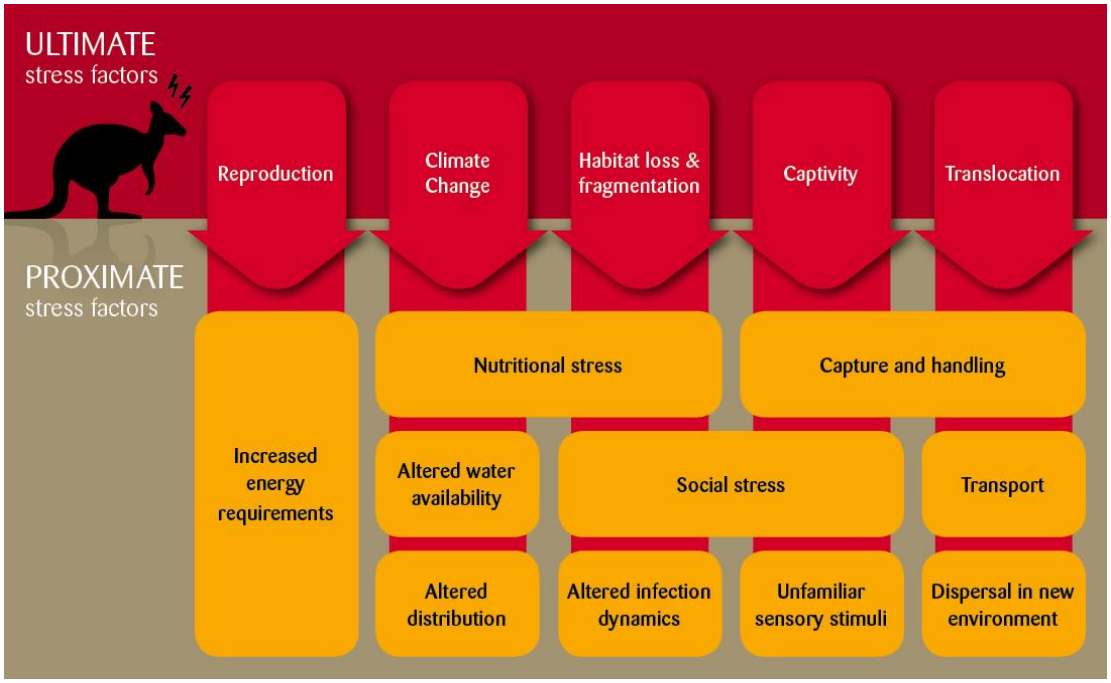


Figure 4. Ultimate and proximate stressors to marsupials

Captivity

Many species of marsupial are dependent on human care in zoos, wildlife parks and rehabilitation facilities (Kennedy, 1992). Numerous conservation programmes rely on captive breeding to supplement wild populations (Fa *et al.* 2011). In order to maximize conservation outcomes and animal welfare, it is imperative to understand how marsupials respond to potential stressors in captivity. The captive environment may entail a range of potential biotic and abiotic stressors that they would not otherwise face in their native habitat, such as frequent human contact, abnormal social grouping, confinement, inability to exhibit natural behaviours, artificial light, alien odours and artificial substrates (Morgan and Tromborg, 2007). To an extent, captivity can also alleviate potential stressors, such as resource limitations, exposure to climatic extremes, disease and predators (Wielebnowski, 2003). Few studies have investigated the stress physiology of marsupials in relationship to captivity, but stressors identified to date include social isolation (McKenzie and Deane, 2005), social status (Stoddart and Bradley, 1991) and capture and handling (Narayan *et al.* 2013). Health-related concerns, such as arthritis, dental disease, upper respiratory tract infections and associated veterinary procedures have also been found to be associated with increased mean FGM concentrations in captive bilbies (*Macrotis lagotis*, Narayan *et al.* 2012).

Using FGM, Hogan *et al.* (2012) investigated the response of captive numbats (*Myrmecobius fasciatus*) to 20 key stressors classified in several categories: conspecific interactions (such as introduction or separation of animals), environment (such as rain and extreme temperatures), handling and health (including injuries, veterinary procedures and dietary changes) and anthropogenic disturbances (such as events and maintenance activities). Handling, conspecific interactions and health-related factors were found to elicit a significant physiological stress response, but

interestingly, anthropogenic disturbances less so (Hogan *et al.* 2012). The lack of a statistically significant response to anthropogenic disturbances does not necessarily indicate that these factors did not affect captive numbats (Hogan *et al.* 2012). Indeed, with regular exposure, the animals may have habituated to anthropogenic disturbances (Bejder *et al.* 2009).

Captivity can also alter or provide abnormal social groupings, leading to social stress. Stoddart and Bradley (1991) investigated social status and stress in captive male sugar gliders (*Petaurus breviceps*). The odour of a dominant male from another group elicited an increase in plasma GCs, whereas no changes in these stress parameters occurred with exposure to odour from a castrated male or a female (Stoddart and Bradley, 1991). These insights provide valuable information for the management of social groups in captivity.

While non-gregarious marsupials, such as numbats and sugar gliders, may experience stress associated with interaction with conspecifics, social marsupials may be stressed by social isolation. When tammar wallabies (*Macropus eugenii*), which are social marsupials that live in groups, were moved into captivity and isolated they exhibited a physiological stress response measured as an increase in FGM (McKenzie and Deane, 2005). As it was not possible to identify faecal samples individually from group-housed wallabies, comparisons between isolated and group-housed animals could not be made (McKenzie and Deane, 2005). Stress associated with social isolation is well characterized in other social mammals, including laboratory rodents (Weiss *et al.* 2004), livestock (Carbajal and Orihuela, 2001) and primates (Sapolsky *et al.* 1997). However, there appears to be a lack of studies on the impact of psychological stressors, including social stressors, on marsupials in

captivity despite many marsupials (including many social macropod species) commonly being kept in zoos and wildlife parks.

Wildlife in captivity provide surrogate study specimens due to the logistical difficulties of working with free-ranging populations. Hence, a greater understanding of how Australian marsupials respond to captivity will aid the interpretation of results from experiments which use captive animals. Furthermore, measuring and monitoring stress in captive marsupials will improve conservation outcomes, particularly as the future of many endangered Australian marsupials relies on captive insurance populations.

Capture and handling

Capture and handling are relatively well-characterized stressors in aquaculture species (Baker *et al.* 2013) and eutherian mammals, particularly laboratory animals and livestock (Grandin, 2007). A small number of studies have suggested that handling also elicits a stress response in both captive and wild-caught marsupials. Physiological indices, plasma GC and FGM indicate an acute stress response to handling in some species, including koalas (*Phascolarctos cinereus*, Hajduk *et al.*, 1992, Narayan *et al.* 2013), numbats (Hogan *et al.* 2012) and wombats (*Lasiorhinus latifrons*, Hogan *et al.* 2011). Behavioural reactions to capture and handling cannot always be accurate indicators of physiological stress. The behavioural reactions of captive wombats wane with regular handling, but increasing levels of FGM indicate that their physiological stress response does not diminish (Hogan *et al.* 2011). This suggests learned helplessness, where the animal is in a state of stress but stops displaying associated behaviours because the stressor has continued (Hogan *et al.* 2011). These results highlight the need for physiological measures of stress in

addition to behavioural measures. Capture and handling are common in marsupial management, and the physiological stress response to these procedures has been shown to have consequences for health and survival in wildlife (Baker *et al.* 2013). Faecal glucocorticoid metabolites can be used to investigate the impact of capture and handling stress in marsupial conservation and develop ways in which this stress could be managed.

Climate

Physiological stress associated with climatic factors such as extremes of temperature have been well documented in other mammalian species since the birth of the concept of stress (Selye, 1936). However, perhaps because it has been assumed that marsupials possess adaptations to harsh conditions (McDonald, 1977, Claridge *et al.* 2007), there is little research into the adrenocortical stress response of marsupials to climatic extremes. Evaluating the response of marsupials to climate change using GCs will provide important information about how climate change may affect a wide range of biological functions. This approach will allow key stressors associated with climate change to be identified and predictions to be made about the impact of these stressors under different climate change scenarios. King and Bradshaw (2010) characterized the physiological response of the critically endangered Barrow Island Euro (*Macropus robustus isabellinus*) to prolonged drought and associated factors, such as nutritional stress and lack of water, spanning 8 months during 1993–94. Euros exhibited increased plasma GCs and haematological changes, namely decreased eosinophils (Bradshaw, 2010, King and Bradshaw, 2010). These results suggested down-regulation or redistribution of the T-helper 2 arm of the immune system, which is responsible for defence against macroparasites (Ezenwa *et al.* 2010, King and Bradshaw, 2010). The study suggested that immune function of the

Euro might have been suppressed as a result of elevated corticosteroids. Thus, although marsupials possess many unique physiological adaptations, they are not immune to the impact of climatic stressors.

The lack of attention given to the adrenocortical response of marsupials to climatic factors is of concern, because stressors related to climate change are a significant threat to global biodiversity (Geyer *et al.* 2011). Climate can affect marsupial health, for example, seasonal debility has been recorded in quokka in association with elevated plasma cortisol, weight loss, dehydration, parasite and bacterial infection (Miller and Bradshaw, 1979). Furthermore, climate change can influence the occurrence and distribution of wildlife populations (Loyola *et al.* 2012, Haby *et al.* 2013). Indeed, climate change has been suggested as a contributory factor in past, present and future range contractions and local extinctions of Australian marsupials, including northern bettongs (*Bettongia tropica,* Abell *et al.* 2006, Bateman *et al.* 2012), koalas (Adams-Hosking *et al.* 2011) and quokkas (Gibson, 2001). Recent studies by Davies *et al.* (2013b, 2014) in koalas in south-west Queensland have found higher FGM in koalas at the arid edge of their range, particularly during drought and during winter. Their results suggested that koalas may be struggling to cope at the periphery of their range, and this may be exacerbated by variations in dietary composition with ongoing climate change (Davies *et al.* 2013b, 2014). Investigations into the effects of climate change on wildlife, such as range contractions or (in some cases) expansions, are often approached from the standpoint of how climate change alters the ecology of the system (Thomas *et al.* 2004), but associations between climate change and wildlife stress physiology remain an important area for further investigation (Wikelski and Cooke, 2006).

What are the significant research gaps that require attention to strengthen Australian marsupial conservation?

Stress physiology is rarely applied to Australian marsupial conservation despite being long recognized as an important area of research in mammology and comparative endocrinology (McDonald, 1977), veterinary and human medicine (as models for disease and the evolution of physiological systems, Stein-Behrens and Sapolsky, 1992, Baudinette and Skinner, 2001). Major knowledge gaps remain, including a lack of studies focusing on marsupials of conservation concern and the need for investigations into the consequences of stress in marsupials (Figure 5). In addition, reports spanning decades suggest that stress is also a major challenge to the rearing and rehabilitation of sick, injured and orphaned marsupials (Jackson, 2007). However, there appears to be a lack of published systematic investigations of stress physiology of marsupials in wildlife care. Young macropods are reported to be particularly vulnerable to mortality associated with dysfunction of the stress response (Hopwood and King, 1972). Hence, future insights into stress physiology in marsupials can facilitate improvements to *in situ* and *ex situ* management of marsupials.

In our view, based on the synthesis of current knowledge available on marsupial conservation, priority areas for stress physiology research in endangered marsupials include assay validation (particularly of minimally invasive methods, such as FGM) and quantifying the response of Australian marsupials to conservation-relevant stressors, such as introduced predators, invasive species, diseases, anthropogenic activities, habitat loss and fragmentation and climate change. We acknowledge the significant logistical challenges associated with studying the stress

response in Australian marsupials, many of which are cryptic, nocturnal and threatened, including resource limitations (for example, time- and labour-intensive trapping and sample collection) and the only recent development of validated assays. Captive populations may provide feasible candidates for assay validation (Hogan *et al.* 2011, Narayan *et al.* 2012). Once key stressors can be identified, measures to manage stress can be trialled, and if successful, incorporated into management plans.

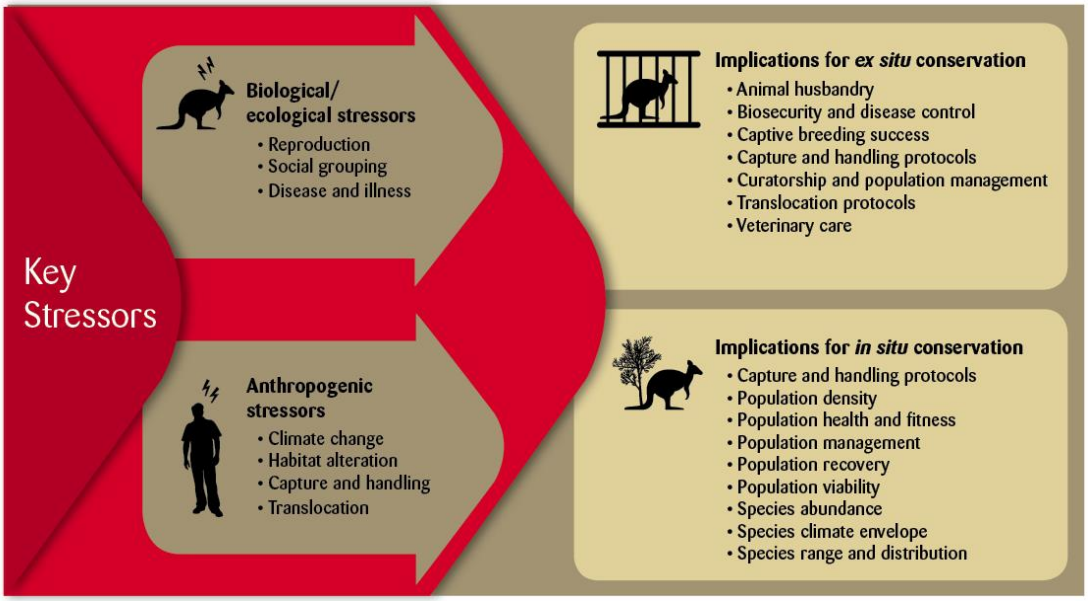


Figure 5. Key stressors to Australian marsupials documented in the literature and potential implications for is situ and ex situ conservation efforts.

Physiological stress assessments in natural populations using conservation physiology tools are becoming immensely useful for understanding conservation challenges for other threatened wildlife in Australia (Kindermann *et al.* 2012, Graham *et al.* 2013). Marsupial conservation can benefit in a similar manner through the integration of stress physiology assessments into ecological monitoring programmes. Thus, networking and collaborations between field managers and stress physiologists will boost Australian marsupial conservation programmes.

Taxonomic coverage

Glucocorticoids have been used in 29 studies to understand the stress response to conservation-relevant stressors in 21 of the 142 extant species of Australian marsupials (15%, Table 1). The majority of species (60%) examined in these studies were listed as of least concern by the International Union for the Conservation of Nature (IUCN). There are an estimated 60 Australian marsupials of conservation concern (Kennedy, 1992), but glucocorticoids have been used to investigate the stress response of only one critically endangered (Stead-Richardson *et al.* 2010) and two endangered species of Australian marsupial (Weiss and Richards, 1971, Hogan *et al.* 2012, Table 1). Characterization of the physiological stress response of marsupials of conservation concern is needed because threatened species such as these are likely to be the most vulnerable to stressors, hence, these species may benefit most from the application of stress physiology to characterize the stress response and identify optimal management in the presence of threatening processes.

Other neglected stressors

Many factors which have been found to elicit a significant stress response in other wildlife species have yet to be investigated in Australian marsupials. Challenges for marsupials, such as predators (Ramp *et al.* 2005, Parsons and Blumstein, 2010) and invasive species (Dickman, 1996), have been investigated in terms of their behavioural and ecological effects, but the physiological response of marsupials to these threats has yet to be scrutinized fully. In these instances, stress physiology would allow more complete evaluation of the impact of these threats and thus assist in the design of more effective threat-management strategies. It is perhaps not surprising that these stressors remain unexplored given that experimental -

Table 1. Summary of studies using glucocorticoids to understand the stress response of marsupials

Species	IUCN	Captive/ wild	Sample	Assay	Molecule	Keywords	Reference(s)
Antechinus flavipes Yellow-footed antechinus	LC	Wild-caught	Plasma	Radioligand assay	Total CORT	Semelparity	McDonald *et al.* 1981,
A. stuartii^ Brown antechinus	LC	Wild-caught	Plasma	Chromatography	Total CORT, free, CBG bound	Semelparity	Bradley *et al.*1980
A.swainsonii Dusky antechinus	LC	Wild-caught	Plasma	Radioligand assay	Total CORT	Semelparity	McDonald *et al.* 1981, McDonald *et al.*1986
Dasyurus hallucatus Northern quoll	EN	Wild	Plasma	Radioligand Gel electrophoresis	Total, free, CBG, albumin bound CORT	Species differences, reproduction, population density	Schmitt, 1989
Isoodon macrourus Northern brown bandicoot	LC	Wild	Plasma	Equilibrium dialysis	Total, free, albumin bound CORT, corticosterone	Season, species differences	Kemper *et al.* 1990
Lasiorhinus latifrons Wombat	LC	Captive	Faeces	EIA	FGM (CORT)	Validation, handling	Hogan *et al.*2011
Macropus robustus isabellinus Barrow island euro	LC	Wild	Plasma Whole blood	RIA Haematology	Total CORT	Climate	King and Bradshaw, 2010
Macrotis lagotis Bilby	VU	Captive	Faeces	EIA	FGM (CORT)	Validation, acute and chronic stress	Narayan *et al.*, 2012
Perameles nasuta Long-nosed bandicoot	LC	Wild	Faeces	EIA	FGM (corticosterone)	Habitat, population density	Dowle *et al.* 2012
Phascogale tapoatafa Brush-tailed phascogale	NT	Captive	Plasma	RIA	Total CORT, free, CBG bound	Semelparity	Schmidt *et al.* 2006
Phascolarctos cinereus Koala	LC	Captive and wild	Faeces	EIA	FGM (CORT)	Validation, handling	Narayan *et al.* 2013
		Wild caught	Plasma Whole blood	RIA Haematology	Total CORT	Capture	Hajduk *et al.*, 1992
		Wild	Faeces	EIA	FGM (CORT)	Validation, climate	Davies *et al.*, 2013a,b Davies *et al.*, 2014

Species	IUCN	Captive/ wild	Sample	Assay	Molecule	Keywords	Reference(s)
Potorous gilbertii Gilbert's potoroo	CR	Captive, Wild-caught	Faeces	EIA	FGM (CORT)	Reproduction, captivity	Stead-Richardson *et al.*, 2012
Myrmecobius fasciatus Numbat	EN	Captive	Faeces	EIA	FGM (CORT)	Validation, multiple stressors	Hogan *et al.*, 2012
P.calura Red-tailed phascogale	NT	Wild-caught	Plasma	Chromatography Gel electrophoresis RIA	Total CORT, free CORT, CBG bound, albumin bound, total corticosterone	Semelparity	Bradley, 1990
Qyulda squamicaudata Scaly tailed possum	DD	Wild	Plasma	Radioligand	Total CORT free CORT, albumin bound	Season	Humphreys *et al.* 1984
Isoodon obesulus Southern brown bandicoot	LC	Wild	Plasma	Radioligand Haematology	Total CORT	Population density	Thomas,1990
Petaurus norfolcensis Squirrel gliders	LC	Wild	Hair	EIA	CORT	Habitat loss	Brearley *et al*,2011 Brearley *et al.*2012
Petaurus breviceps Sugar gliders	LC	Wild	Plasma	RIA	Total CORT, free CORT, CBG bound, albumin bound	Reproduction	Bradley and Stoddart 1991
		Wild	Whole blood via chronically placed catheter	Equilibrium dialysis, gel electrophoresis	Total CORT CBG bound, albumin bound, Catecholamines	Social status	Stoddart and Bradley 1991
Macropus eugenii Tammar wallaby	LC	Captive	Serum	RIA	Total CORT	Season	McKenzie and Deane 2003
			Serum	EIA	Total CORT Total corticosterone	Validation, Social isolation, Feeding	McKenzie *et al.* 2004
			Faeces	EIA	FGM (corticosterone)	Social isolation	McKenzie and Deane, 2005
Tarsipes rostratus Honey possum	LC	Wild-caught*	Faeces	RIA	FGM (CORT)	Captivity	Oates *et al.*, 2007
Trichosurus vulpecular Brush-tail possum[#]	LC	Wild	Plasma	RIA	Total CORT	Translocation	Baker and Gemmell, 1999

Cortisol (CORT), plasma corticosteroid binding globulin (CBG), IUCN status: DD= data deficient, LC = Least Concern, NT = Near Threatened, VU = Vulnerable, EN = Endangered, CR = Critically Endangered, ^ Select papers, which reflect the complex links between stress and reproduction have been included from the body of literature on semelparity in antechinus as this has been previously reviewed eg. Naylor *et al* 2008, Bradley 2003, #Select papers have been included from the literature on glucocorticoids in brush-tail possums as the foundations of this research dating back to 1960s has previously been reviewed eg. McDonald 1977

- manipulation of these factors in marsupials is difficult and opportunities to do so are rare. An exception may be translocation stress, because translocations are widely used as a conservation strategy for Australian marsupials (Sheean *et al.* 2012). Translocation entails a combination of many potential stressors, including capture and handling, transport and dispersal in a new environment (Teixeira *et al.* 2007, Figure 4), but few studies have explored the response of wildlife to these events during translocation (Franceschini, 2007) and how these can be designed to manage stress. Baker and Gemmell (1999) demonstrated that brushtail possums experience stress following translocation, as indicated by elevated levels of plasma cortisol following capture and translocation. Given that marsupial translocations are an important and widely used conservation tool, a key question is: what aspects of translocation are most stressful, and consequently, how can translocation success be optimized? Most importantly, stress physiology tools can also be used to gauge the adaptation of animals post-translocation.

Understanding individual and species differences

The reasons underlying individual and species differences in the stress response are rarely examined in marsupials or wildlife in general (Morgan and Tromborg, 2007, Mason, 2010). Physiological differences between individuals and species are not random, in that differences can be determined by a myriad of factors, such as animal temperament, prior experience, environmental requirements and characteristics of biology (Wielebnowski, 2003, Bejder *et al.* 2009, Mason, 2010). A key question is: what characteristics make some animals more resilient than others? The reasons for individual and species differences may indicate variation in vulnerability to decline and thus may shed light on desirable or undesirable traits and inform conservation priority setting and resource allocation. For example, plasma cortisol-

- (free, CBG bound and albumin bound) in northern brown bandicoot (*Isoodon macrourus*) indicated that this species appeared to be less stressed by seasonal changes compared with sympatric marsupials, such as the northern quoll (*Dasyurus hallucatus*, Kemper *et al.* 1989). *Isoodon macrourus* may be more resilient to climatic changes because they are generalists in terms of diet and habitat and do not experience the same breeding stress as other marsupials (Kemper *et al.*1989). Hence, in the case of extremes of weather, if resources for conservation management were limited, they could be prioritized for more specialized species, which may require more support, or in a triage situation, prioritized for generalists who are more likely to survive. Marsupial species also differ in their response to factors associated with reproduction, such as higher energy expenditure and intraspecific aggression. Antechinus species undergo semelparity while others, such as northern quoll (*D. hallucatus*), avoid fatal stress-related pathology associated with breeding (Schmitt *et al.* 1989). The appropriate design of population-monitoring programmes may depend heavily upon this knowledge of species-specific stressors.

Individual differences in the stress response of Australian marsupial species have been explored by Narayan *et al.* (2012). Individual bilbies reacted differently to short-term stressors depending on the degree to which they had habituated to long-term stressors and their health condition (Narayan *et al.* 2012). In contrast, Hogan *et al.* (2012) showed no significant variation among individual responses of captive numbats to the same stressors. An individual animal's stress response to characteristics of a captive environment may be influenced by a variety of factors, for example, past experience, time in captivity and nature of the contact (Morgan and Tromborg, 2007, Narayan *et al.* 2013a). Potentially, with the advancement of molecular technologies, genetic markers associated with the variation in glucocorti-

coid data could be identified in marsupials to breed more resilient populations selectively. Human research has revealed that the stress response is moderately to highly heritable, and the neuropeptide Y gene has been identified to regulate individual variation in the effects of stress (Zhou *et al.* 2008).

The consequences of stress

Marsupial stress physiology studies have largely concentrated on the essential first steps, such as biological and laboratory validation of assays and comparing individuals and populations of marsupials (Table 1). As small fluctuations in GCs are essential for survival, demonstrating changes in GC in itself does not necessarily indicate a potentially detrimental stress response. However, it is clear that prolonged high plasma cortisol (chronic stress) can reduce survival of marsupials (Bradley *et al.* 1975). Incorporating GC monitoring into routine fauna monitoring of wild populations may provide insights into fluctuations between GC levels and population growth and fitness.

Acute vs. chronic stress can have different effects on fitness (Sapolsky *et al.* 2000), hence, it is important to understand the consequences of both acute stressors, such as capture and handling, and chronic stressors, such as confinement (Stead-Richardson *et al.* 2010). Indeed, there may be an interaction between responses to acute and chronic stressors, as observed in bilbies where individuals experiencing long-term stress associated with injuries and health issues demonstrated a greater FGM response to acute stressors (Johnstone *et al.* 2012, Narayan *et al.* 2012).

In order to determine whether a stressor poses a significant threat to an animal, further investigations are needed into associations between GC and biological functions (Madliger and Love, 2014), including immune defences, disease dynamics, reproduction and fitness (Moberg and Mench, 2000). Measuring these parameters in parallel to GC and comparing baseline GC with changes in response to specific stressors will help discern between normal vs. potentially detrimental variations in GC.

It is crucial that links between GC, biological function and fitness are fully characterized to understand the impact of stressors on Australian marsupials, particularly species of conservation concern. For example, the health consequences of stress are many and varied (Sapolsky *et al.* 2000), with serious implications for a variety of infectious diseases (Kriek, 1994, Narayan, 2013). In marsupials, stress has been associated with changes in body condition, bacterial and parasitic infection (Kemper *et al.*, 1989).

Stress may play a role in driving disease dynamics in Australian marsupials. For example, stress is implicated as a potential trigger for clinical disease in koalas infected by Chlamydia (Canfield *et al.*1991). Stress has also been suggested as a factor exacerbating *Toxoplasma gondii* infection, potentially contributing to the decline of Australian native fauna (Thompson *et al.* 2010). In addition, *Trypanosoma* species of haemoparasites, which may play a role in the decline of the critically endangered woylie (*Bettongia penicillata*, Thompson *et al.*, 2010, Botero *et al.* 2013), may be exacerbated by stress (Brown *et al.* 2003). A small number of studies have been conducted in marsupials to investigate associations between stress and disease (Dowle *et al.* 2012, Robert and Schwanz, 2013). However, we are yet to

investigate and characterize fully the health and conservation implications of physiological stress in marsupials. By doing so, disease risk factors may be identified and mitigated to protect healthy populations for the future

Reproduction

The relationship between stress and reproduction is relevant to marsupial conservation because chronic stress can limit breeding (Bradley *et al.* 1980, Oates *et al.* 2007). Hence, it is important to consider associations between stress and reproduction for the sustainability of free-ranging populations and captive-breeding programmes. Chronic stress associated with breeding can be associated with fatal pathology (Dickman and Braithwaite, 1992, Bradley, 2003). For example, in Antechinus species and the red-tailed phascogale (*Phascogale calura*), the negative feedback system that regulates GC release is abolished during the breeding season, resulting in dysregulated and increased GC release and mass mortality (Bradley *et al.* 1980, McDonald *et al.* 1986, Bradley, 1997). A decrease in plasma CBG results in an increase in total free (biologically active) cortisol to levels which appear to be immunosuppressive, decreasing serum immunoglobulins (Bradley *et al.* 1980). Consequently, gross post-mortem and histopathology reveal severe haemorrhagic gastrointestinal tract pathology, including ulceration (Bradley and Monamy, 1991), increased invasiveness of micro-organisms and parasites (Bradley *et al.* 1980, Dickman and Braithwaite, 1992), liver pathology associated with Listeria monocytogenes (Barker *et al.*, 1978) and recrudescence of haemoparasites, such as Babesia spp. (Cheal *et al.*, 1976, Barker *et al.*, 1978).

The association between chronic stress and animal reproduction has also been determined experimentally. To determine the pattern of adrenal activity that

was responsible for semelparity, Bradley *et al.* (1975) administered 10 mg/kg/day exogenous corticosterone to wild-caught male antechinus over 2 weeks to simulate chronic stress during the breeding season. The mortality rate of the treatment group was significantly higher than that of the control group (Bradley *et al.* 1975). These insights indicate that conservation success for semelparous marsupial species may rely on mitigation of proximate stressors associated with reproduction, such as energy deficits.

Other dasyurid species, such as northern quoll (*D. hallucatus*), do not experience fatal stress-related pathology associated with breeding (Schmitt *et al.* 1989). However, even in marsupial species that do not undergo semelparity, stress may influence breeding success, and stress physiology can be a highly informative tool for troubleshooting reproductive failure. For example, honey possums (*Tarsipes rostratus*) are vulnerable to chronic stress in captivity, and stress appears to be an important factor impeding reproductive success (Oates *et al.*, 2007). Few Gilbert's potoroo (*Potorous gilbertii*), Australia's most endangered marsupial, have been successfully bred in captivity, and Stead-Richardson *et al.* (2010) investigated whether stress may diminish reproduction in captive *P. gilbertii*. If this were the case, Stead-Richardson *et al.* (2010) surmised that captive potoroos would demonstrate significantly higher FGM compared with their wild counterparts, but their results suggested the reverse.

There are several possible explanations for differences in the relationship between stress and reproduction in different studies (captive and wild) and species, including the following: differences in protocols, methods and techniques, species differences in GC responses, length of time in captivity, characteristics of the captive environment, and the nature, frequency, duration and severity of stressors (Wielebnowski, 2003). Interpreting stress physiology and reproduction can also be problematic given the complexity of the interactions between GCs and reproductive hormones (oestrogens, progesterone and testosterone, Lane, 2006). Nevertheless, there is an extensive body of literature on stress and reproduction in other species indicating that stress can have a significant impact on reproductive success depending on a variety of individual, species, stressor and environmental factors (Tilbrook *et al.* 2000, Karsch and Breen, 2009). For example, it has been consistently demonstrated that chronic stress reduces the secretion of gonadotrophins and inhibits reproduction in non-rodent mammals (Tilbrook *et al.* 2000), hence, it is likely to be beneficial to marsupial conservation to consider stress physiology when evaluating the reproductive performance marsupials in situ and ex situ.

Conclusions

Studies on the stress physiology of Australian marsupials have direct significance for the management of these unique species because they highlight significant factors that may influence conservation outcomes, i.e. reproduction, habitat loss, captivity, climate, capture, handling and translocation. There remain many other potential stressors that are yet to be investigated. There is a broad range of situations where knowledge about the stress response could inform marsupial management plans. In captive populations, understanding what social groupings, housing conditions,

environmental provisions and husbandry techniques incur more or less stress would help to improve welfare, health and fitness. For example, Hogan *et al.* (2011) demonstrated learned helplessness in captive wombats in response to handling and suggested that 'gentling', originally an equine training technique involving gradual, patient husbandry, may improve wombat management. Demonstration of the stress response of social marsupials to isolation (McKenzie and Deane, 2005) suggests that isolation should be avoided if possible, for instance if an individual requires off-exhibit treatment, the patient should be accompanied by conspecifics. The design of conservation protocols would also benefit from an understanding of the stress response, for example, insights into which stages of translocation are most stressful for marsupials and how provisions such as lighting, substrate and sound barriers might be used during transport to decrease the stress inherent in translocation. Stress physiology would also aid in evaluation of the efficacy of such measures.

In wild populations, understanding the stress response to major threats, such as habitat loss and climate change, is critical for conservation planning. Stress physiology research has indicated that marsupials such as koalas (Davies *et al.* 2014) and squirrel gliders (Brearley *et al.* 2012, 2013) are vulnerable at habitat edges, so resource allocation may prioritize these populations. Data can also be applied to the design of Australia's protected area network to strengthen the case for increased connectivity (Soulé *et al.* 2004). The response of marsupials to stress factors may have a myriad of implications for in situ and ex situ conservation, but further studies are required to establish whether stress has a significant impact on the health and fitness of Australian marsupials, or indeed, if it contributes significantly to extinction risk and species survival.

While there is a body of literature on marsupial stress physiology spanning over 50 years, Australian conservation research and management programmes are yet to realize the potential benefits of stress physiology fully. Significant knowledge gaps remain in identifying how marsupials respond to stressors, the factors which modulate individual and population stress responses and the conservation implications of stress in marsupials. However, the rapid recent development of several non-invasive assays to measure stress parameters holds much promise for furthering this field of research into Australian marsupial conservation. Given urgent concerns about the future of endangered Australian marsupials, understanding how they respond to the stressors is critical for predicting how populations will cope and how best to manage them into the future.

1.3 Aims of thesis

Here, I introduce the specific context of this thesis and outline the proceeding chapters. This research focuses on stress and wildlife health in the context of the conservation of a critically endangered native Australian marsupial, the woylie (syn. brush-tailed bettong, *Bettongia penicillata*). Once abundant and widely distributed across much of the Australian mainland, the number of woylies has decreased by over 90% since 1999 and remnant populations are restricted to 1% of their former range (Thompson 2014, Yeatman *et al.* 2016). Despite exhaustive investigations, the causes of the woylies' decline remain unclear (Wayne *et al.* 2013). In **Chapter 2**, I introduce the woylie and study sites.

Stress, compromised immune function and infectious disease are particularly relevant to the conservation of the woylie as these have been suggested as potential drivers of the species' recent population decline together with introduced predators especially feral cats (Wayne *et al*. 2008). Evidence comes from existing population biology, haematology, disease ecology and parasitology studies in woylies. Longitudinal population biology studies have revealed distinct spatio-temporal and density-dependent patterns in the woylie declines which are suggestive of infectious disease agent(s) (Wayne *et al.* 2015). High population densities, together with associated potential stressors such as increased competition and nutritional stress, has preceded population declines (Wayne *et al.* 2015). In addition, haematology studies have found differences in leukocyte (white blood cell) profiles between declining and stable populations which are also suggestive of 'immunological stressors' (Pacioni et al., 2013). In particular, parasitological studies have identified that *Trypanosoma* species of protozoan haemoparasites (blood parasites) especially *T.copemani*, are

thought to be linked to the woylies' decline (Botero *et al.* 2013, Thompson *et al.* 2014). Virulent trypanosomes have been found more commonly in declining woylie populations (Botero *et al*. 2013, Thompson *et al*. 2014). Together, this evidence led to the hypothesis that stress-induced immunosuppression has exacerbated the impact of parasite infections including trypanosomiasis, contributing to the decline of the woylie (Botero et al., 2013). In this thesis, I explore the basis for this hypothesis.

The overall aim of this thesis was to investigate the relationship between physiological stress, immune function and parasite infection dynamics in the woylie, in the context of their conservation management. As a multidisciplinary study, specific aims span the fields of stress physiology, immunology, parasitology and conservation science.

In terms of stress physiology, I aimed to develop the field and laboratory protocols necessary to measure the concentration of faecal cortisol metabolites (FCM) in woylies in a real-world conservation context. **Chapter 3,** a longitudinal study, details the development of these protocols and provides baseline data for our understanding of woylie stress physiology. This data aids interpretation of results in the following chapters.

To investigate the premise of the hypothesis that stress may influence immune function and haemoparasite infection dynamics in woylies, I aimed to develop a technique to measure key immune function parameters in woylies and use this technique to investigate the relationship between physiological stress and immune function. In **Chapter 4,** I detail the first immune function study in woylies.

I also aimed to identify endoparasites of woylies using molecular and traditional techniques in order to compare infection patterns with the aforementioned stress physiology and immune function parameters. In terms of conservation science, a key aim was to assess the stress physiology of woylies in the context of real-world conservation relevant stressors (translocation and bushfire) and these are evaluated in **Chapter 5** and **Chapter 6**.

Chapter 2 General methods

2.1 Woylies

Woylies (syn. brush-tailed bettongs, *Bettongia penicillata*) are Australian marsupial rat kangaroos (Superfamily Macropodoidae, Family Potoroidae) (Figure 6). Adults measure approximately 330mm in body length, 300mm in tail length and range in weight from approximately 1200 to 1600g. They are continuous breeders with females able to undertake embryonic diapause and simultaneously nurture weaned young, pouch young and a developing embryo (Smith 1989). Woylies are nocturnal, resting in shallow nests under grass trees (*Xanthorrhoea* spp.) during the day and foraging at night for a diet consisting mainly of underground (hypogeous) fungi as well as bulbs and seeds (Claridge *et al.* 2007). They play a vital role as ecosystem engineers, maintaining native forests by digging and turning over soil and facilitating the sporulation of hypogeous fungi upon which native flora rely (Fleming *et al.* 2014).

Once abundant and widely distributed across much of the Australian mainland, woylies have undergone dramatic population declines and can now only be found in south-west Western Australia (Figure 7) (Wayne *et al.* 2013). Today they are listed as endangered under the Western Australian *Wildlife Conservation Act 1950* and National *Environment Protection and Biodiversity Conservation Act 1999* and critically endangered on the International Union for the Conservation of Nature Red List of Threatened Species (Wayne *et al.* 2008). Woylies are also recognised as an Evolutionarily Distinct and Globally Endangered (EDGE) species, "*a unique and irreplaceable part of the world's natural heritage…currently sliding silently towards extinction*" (Isaac *et al.* 2007, Zoological Society of London 2014).

Figure 6. An adult woylie at Native Animal Rescue, Perth, Western Australia

2.2 Study sites

This study was carried out across four sites in south-west Western Australia, a globally significant biodiversity hotspot home to a rich diversity of endemic flora and fauna (Brooks *et al.* 2002).

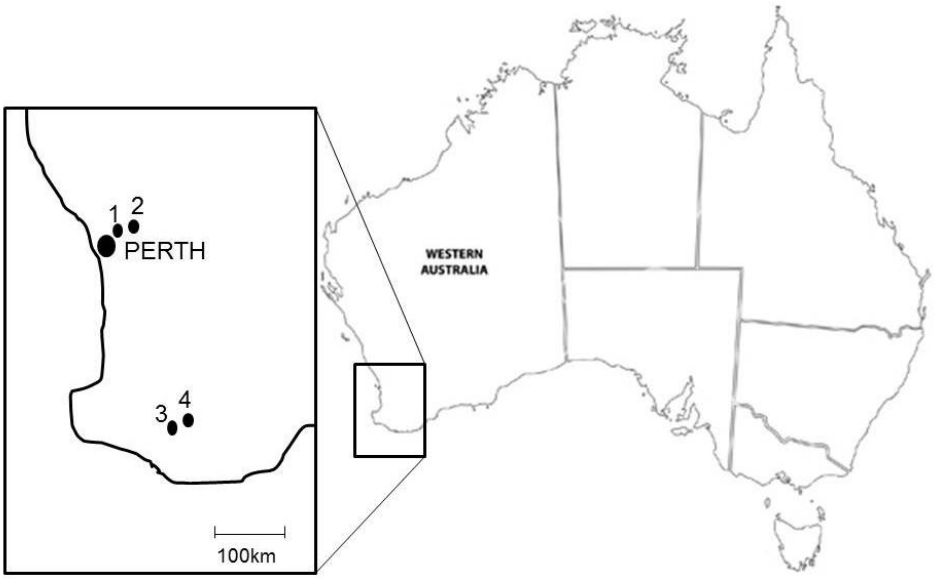


Figure 7. Location of study sites in south-west Western Australia:
1. Native Animal Rescue, 2. Whiteman Park Woodland Reserve, 3. Perup Sanctuary, 4. Greater Kingston National Park

Native Animal Rescue

At monthly intervals from late 2013 to early 2015, we trapped a captive population of woylies (15 adults, 6 male, 9 female) (Figure 8). This population was housed in a naturalistic enclosure divided into four adjacent pens (3 to 4 adult woylies per 35m x 55m pen) at Native Animal Rescue (NAR), a wildlife facility managed by the Fauna Rehabilitation Foundation in Malaga, Perth, Western Australia (Figure 9). The woylies were brought to NAR in late 2010 from the Kingston and Perup indigenous populations and supplemented in early 2011 with individuals from a private collection in Roleystone, a predominantly rural area approximately one hour's drive away from NAR (Thompson 2014). The pens were enclosed by 2 m high anti-predator electrified fences. Traps at NAR were set at sunset and trap checking commenced just before sunrise the following day.

Figure 8.Trap setup at Native Animal Rescue

Figure 9. Outside the predator proof enclosures at Native Animal Rescue

Figure 10. Inside the predator proof enclosures at Native Animal Rescue

Whiteman Park Woodland Reserve

From early to late 2014, we trapped woylies at Whiteman Park Woodland Park Reserve, a 150ha nature park (expanded to 200 hectares in August 2014) surrounded by a predator proof fence in Perth, Western Australia. This population was established in 2010 with rehabilitated and captive raised woylies and it has been supplemented with further individuals of varied origin (Rafferty and Pacioni 2012).The Whiteman Park trapping protocol was to set traps before sunset and check traps throughout the night.

Perup Sanctuary

Prior to a planned translocation by the Western Australian Department of Parks and Wildlife (DPaW), we trapped woylies in the source population at Perup Sanctuary, a 423ha predator-proof enclosure located approximately 300km south east of Perth. The Sanctuary was established in 2010 to protect an insurance population of woylies sourced from the Perup and Kingston indigenous populations. The DPaW trapping protocol at Perup was to set traps before sunset and check them at sunrise the following morning.

Greater Kingston National Park

Located in south-west Western Australia, in the Greater Kingston National Park and Dryandra Woodland, there are now only two remnant indigenous populations of woylie remaining. To supplement woylie populations at Warrup and Walcott within the Greater Kingston National Park, woylies were translocated from Perup Sanctuary in June 2014. We trapped and sampled woylies at Warrup and Walcott before and after the translocation (Figure 11). At the time of translocation, traps were set before sunset and woylies were processed, transported and released during the night.

Figure 11. Juvenile woylie during fieldwork in the Greater Kingston National Park

Chapter 3 Identifying factors that influence stress physiology of the woylie

The following is a published paper:

Hing S., Narayan E.J., Thompson R.C.A., Godfrey S.S. (2016). Identifying factors that influence stress physiology of the woylie, a critically endangered marsupial. *Journal of Zoology* doi: 10.1111/jzo.12428

Interpreting and applying stress physiology indicators in wildlife conservation requires an understanding of natural fluctuation in these indicators and the factors which influence those fluctuations. Faecal cortisol metabolites (FCM) are commonly used and minimally invasive stress physiology indicators in wildlife research and management but the factors influencing natural fluctuation in FCM have not been previously investigated in woylies. In this chapter, I present a longitudinal study which provides baseline data on FCM in woylies and identifies factors influencing FCM fluctuations in a captive population over time.

Introduction

Global biodiversity is threatened by a growing intensity and range of challenges or stressors. Stress physiology can provide insights into how animals respond to stressors (Cooke *et al.* 2013). An essential part of the stress response involves the hypothalamic pituitary adrenal (HPA) axis, which aims to maintain homeostasis by modulating physiological and behavioural responses to stressors (Landys *et al.* 2006, Busch and Hayward, 2009, Parry-Jones, Webster and Divljan, 2016). Measuring glucocorticoids and their metabolites provide a means to monitor the HPA axis, the underlying neuroendocrine mechanisms that determine how an organism functions under changing conditions (Wikelski and Cooke, 2006). Faecal glucocorticoid metabolites (of either cortisol or corticosterone), measured using minimally invasive methods, are commonly used in wildlife (Keay *et al.* 2006) and are particularly practical when blood sample collection immediately following capture is not possible (Romero and Reed, 2005).

Interpretation of faecal glucocorticoid metabolite results is aided by knowledge of factors that influence baseline values. A variety of factors have been shown to correlate with faecal glucocorticoid metabolite values in wildlife species, ranging from sex to season (Millspaugh and Washburn, 2004). Parasites, including endoparasites (Clough, Heistermann and Kappeler, 2010) and ectoparasites (St Juliana *et al.* 2014), have also been associated with alterations in faecal glucocorticoid metabolite concentration in wildlife hosts. In part, links between infection patterns and host stress physiology may be due to the effects of glucocorticoids on immune function (Biondi and Zannino, 1997, Sapolsky, Romero and Munck, 2000). Stress associated immunosuppression and exacerbation of infectious disease could be a significant threat to wildlife (Beldomenico and Begon,

2010) and understanding the relationships between host stress physiology and parasitism is important for wildlife research and conservation.

Stress exacerbating the impact of parasitic infections has been suggested to contribute to the ongoing decline of the woylie (syn. brush-tailed bettong, *Bettongia penicillata*), a critically endangered Australian marsupial (Botero *et al.* 2013, Thompson *et al.* 2014). Woylies were once abundant and widespread across much of mainland Australia but became locally extinct across most of their range by the 1970s. Remnant populations are now confined to Western Australia (Wayne *et al.* 2013a). Recent studies suggest that the distribution and abundance of woylies is related to stressors including habitat fragmentation, proximity to agriculture and invasive predators (Wayne *et al.* 2013b, Yeatman *et al.* 2016). In addition, more virulent trypanosomes (protozoan hemoparasites) have been found more commonly in declining woylie populations (Botero *et al.* 2013, Thompson *et al.* 2014), and white blood cell counts in declining populations are suggestive of "immunological stressors" (Pacioni *et al.* 2013). Hence, stress-induced immunosuppression has been hypothesised to exacerbate the impacts of parasite (especially trypanosome) infections, contributing to declines (Botero *et al.* 2013). Associations between faecal cortisol metabolites (FCM), immune cell (phagocyte) function and trypanosomes have since been found, which support the stress-induced immunosuppression hypothesis (Hing *et al.* 2016). Thus, understanding how parasite infection may influence long-term variation in FCM in woylies is pertinent. In this study, we asked what factors such as season, sex, body condition index, female reproductive status and the presence of parasites influenced variation in FCM in woylies, in a longitudinal study of a captive population.

Materials and methods

Trapping and sample collection

We studied a captive population of woylies (15 adults, 6 male, 9 female), housed in four adjacent outdoor naturalistic enclosures (3 to 4 adult woylies per 35m x 55m pen) at Native Animal Rescue in Malaga, Western Australia. Woylies had access to underground fungi, native forage (bulbs, seeds etc.), insects and a year round supplementary ration of fruit and vegetables. We trapped all individuals monthly from September (austral-spring) in 2013 to June (austral-winter) in 2015. Galvanized wire Sheffield traps (220 x 220 x 550mm) (Sheffield Wire Products, Western Australia), baited with a mixture of peanut butter and oats, were set just prior to sunset and checked before sunrise (maximum total duration in the trap was 8 to 10 hours). We pooled the faeces deposited by each individual woylie at the bottom of their trap but time since defaecation could not be determined. We acknowledge that changes in FCM concentration can occur over time, and this could have influenced our results (Laver *et al.* 2012). Woylies were individually identified by a unique microchip code. Animals were weighed, females were checked for the presence or absence of pouch young (pouch status), and the size of pouch young (mm) was estimated by palpation of the pouch.

Faecal samples were collected from underneath the trap, and stored frozen at -20°C until they were prepared for FCM assays. All faecal samples were extracted within sixteen months of collection. A blood sample (400 to 1000µl) was collected from the lateral caudal vein into an EDTA MiniCollect tube (Greiner Bio-One, Germany) to enable DNA extraction and trypanosome PCR. EDTA blood samples were stored at -20°C and processed within six months of collection. In some cases, all assays could not be completed for every sample (e.g. due to insufficient sample

volume) but a total of 269 faecal samples and 208 blood samples were analysed. This work was carried out under a Western Australian Department of Parks and Wildlife Regulation 17 License to Take Fauna for Scientific Purposes (SF009623) and Murdoch University Animal Ethics Permit (RW2611/13).

Faecal cortisol metabolite (FCM) enzyme immunoassay (EIA)

FCM were analysed by an enzyme immunoassay (EIA) previously used for woylies (Hing *et al.* 2016). In summary, faecal samples (0.2g dry weight) were lyophilised (freeze-dried) and extraction carried out using 90% ethanol and heat treatment (80^{o}C for 10min). Extracts were assayed for FCM by EIA using a polyclonal anti-cortisol antiserum R4866 protocol (Narayan *et al.* 2012, Hing *et al.* 2016). The R4866 anti-cortisol antiserum has been reported to cross react 100% with cortisol metabolites and less than 10% with other steroids (Webster, Narayan and de Vos, In Press). Results were expressed as FCM concentration (pg/g) on a dry weight basis.

Parasitology analyses

We performed microscopic and molecular parasitology analyses to determine what endoparasites were present. To detect gastrointestinal parasites (nematodes and protozoans), one gram wet weight of faeces was floated for 10 minutes using a concentrated sodium nitrate ($NaNO_3$) solution with centrifugation (Dryden *et al.* 2005). The area under the coverslip was observed systematically under a BX51 microscope (Olympus, Japan) at 20x objective and eggs were classified as strongyle or oxyurid types (as these were the two major groups observed).

To detect trypanosome blood parasites, DNA extraction and *Trypanosoma* PCR amplification from blood samples were carried out using previously described

protocols validated for woylies (Botero *et al.* 2013, Hing *et al.* 2016). Presence or absence of *Trypanosoma* species in peripheral circulation as indicated by PCR positive or negative results was recorded. The ears of woylies were also visually inspected for the presence or absence of ticks.

Statistical analyses

We used linear mixed effect models to investigate which factors influenced FCM fluctuations in woylies. To fulfil model assumptions of data conforming to a normal distribution, FCM (the dependent variable) was log-transformed. Fixed effects included in our model were: season (summer/autumn/winter/spring), sex (male/female), body condition index, presence or absence of oxyurid eggs on faecal flotation, presence or absence of strongyle eggs on faecal flotation, PCR positive or negative for trypanosomes and the presence or absence of ticks. Two-way interactions between these effects were also included. Woylie ID nested within pen was included as a random effect in all models to account for repeated measures from the same individuals. Body condition index was derived from the residuals of a regression of hindfoot (pes) length to weight, calculated separately for males ($p<0.001$, co-efficient=28.5, $R^2=0.2114$) and females ($p<0.001$, co-efficient=21.6, $R^2=0.0806$) and adjusted for pouch young size in females by including pouch young size as a covariate. We were also interested in the effects of female reproductive activity on FCM, so we re-ran the models described above for females only (n=169) and included pouch status (0 = empty or 1 = pouch young present) as a fixed effect.

To ensure there was no strong multicollinearity between explanatory variables, we calculated the variance inflation factor (VIF) for all explanatory variables included in the maximal model, and ensured no variables had a VIF higher

than 3 prior to modelling. To determine the minimal adequate models, we undertook model simplification by stepwise reduction, removing non-significant terms from the maximal model until further model reductions resulted in significant changes in model deviances ($p<0.05$) (Crawley, 2007). Significance ($p\leq0.05$) was tested in a likelihood ratio test (χ^2). Models were run using R 3.1.0 and the packages 'lme4' (Bates *et al.* 2015) and 'car' (Fox and Weisberg, 2011).

Results

Overall, FCM concentration ranged widely from 0.03 to 457.40 pg/g. Host and environmental factors that significantly affected FCM in woylies included season, body condition index and sex ($p<0.05$) (Table 2). Modelling revealed that mean log-transformed FCM was lowest in summer (2.5 ± 0.2 SE, n=108 samples), moderate in spring (2.9 ± 0.1, n=50) and highest in the cooler months of autumn (3.3 ± 0.1 SE, n=71) and winter (3.3 ± 0.2 SE, n=40) (Figure 12a). Overall, there was a significant but weak relationship between body condition index and FCM (Table 2).

The relationship between body condition index and FCM differed between the sexes with a more marked negative relationship in males compared to females (Figure 12b). Females (3.1±0.1 SE) had a higher mean FCM compared to males (2.7±0.1 SE) (Figure 12c). In female woylies, the presence or absence of pouch young overall did not have a significant effect on FCM (coefficient = 0.03, SE=0.40, df=1, χ^2=1.7, p=0.193). However, there was a significant interaction between pouch status and season (coefficient = -0.79, SE = 0.72, df=1, χ^2=8.15, p=0.04), with the greatest difference noted in winter when mean FCM in females with pouch young was higher (3.86 ± 0.20, n=119) than females without pouch young (2.46 ± 0.59, n=52).

Oxyurid pinworm eggs were present in 17% of 269 faecal samples, and were significantly associated with FCM (Table 2). When woylies were shedding oxyurid eggs, they had higher mean FCM than when oxyurid eggs were not detected (Figure 13a). An interaction between body condition and oxyurids also influenced FCM. When woylies were shedding oxyurid eggs, there was a weak positive relationship between body condition index and FCM (Table 2). A weak negative relationship between body condition and FCM was observed when oxyurid eggs were not detected (Figure 13b). Woylies were also infected by trypanosomes (57% of 208 blood samples positive), strongyles (25% of 269 faecal samples positive) and ticks (44% infested of 260 inspections) in this study. Interactions between trypanosome and strongyle status, and between strongyle and tick status also influenced FCM. FCM was lowest when neither trypanosomes nor strongyles were detected (Figure 13c) and when neither ticks nor strongyles were detected) (Figure 13d).

Table 2. Coefficients for the minimum adequate linear mixed effect model of factors affecting faecal cortisol metabolites (FCM log-transformed). Significant factors in bold (n=202 readings from 15 individuals).

Fixed effects	Coefficient	SE	Df	χ^2	p-value
Body condition index	**-0.001**	**0.001**	**1**	**3.905**	**0.048**
Season	**1.047**	**0.205**	**1**	**30.561**	**<0.001**
Sex	**-0.447**	**0.168**	**1**	**6.577**	**0.010**
Trypanosomes	0.418	0.190	1	0.987	0.321
Oxyurid pinworm eggs	**0.522**	**0.221**	**1**	**5.134**	**0.024**
Strongyle nematode eggs	1.166	0.375	1	0.059	0.808
Ticks	0.288	0.213	1	0.129	0.720
Body condition index:sex	**-0.447**	**0.002**	**1**	**4.522**	**0.034**
Body condition index:pinworm eggs	**0.005**	**0.003**	**1**	**3.970**	**0.046**
Trypanosomes:strongyle eggs	**-1.166**	**0.416**	**1**	**7.838**	**0.005**
Strongyle eggs:ticks	**-0.835**	**0.391**	**1**	**4.563**	**0.033**

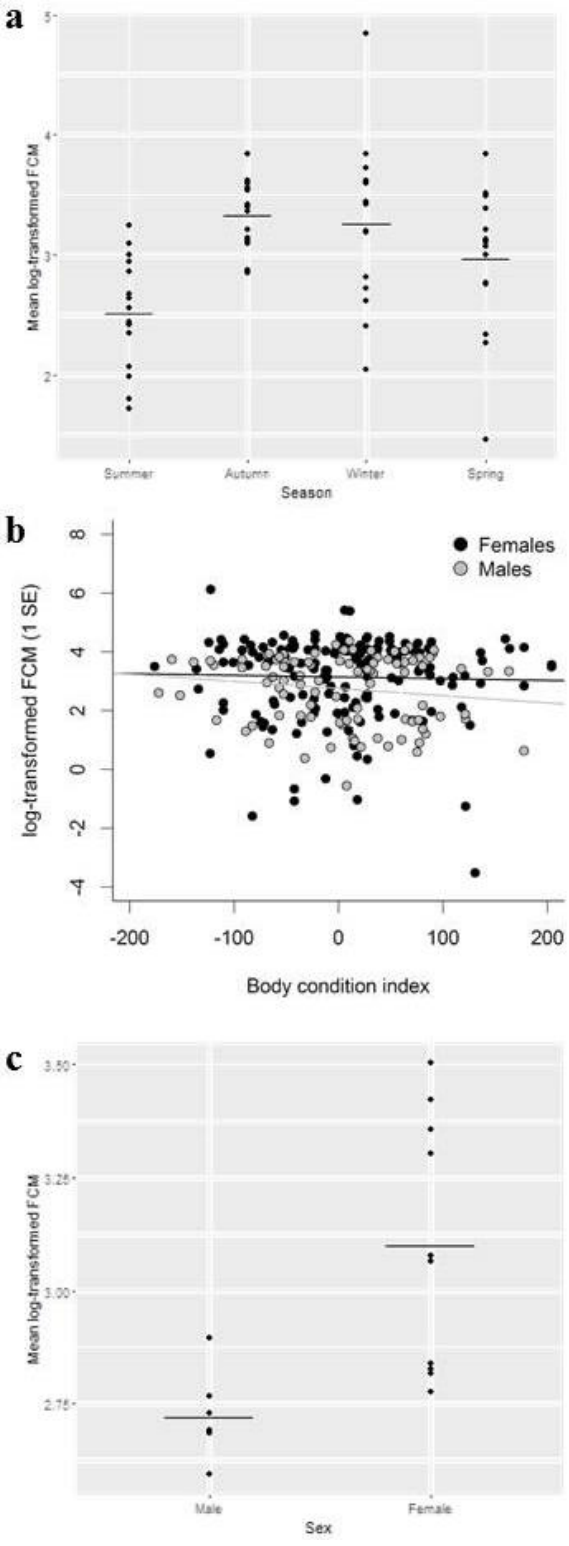


Figure 12. Host and environment factors which influence faecal cortisol metabolites in woylies. In (a) and (c), dots represent (averaged) FCM concentration of individual woylies and horizontal lines mark the overall mean.(a) Season, (b) Interactive effect of body condition and sex, (c) Sex.

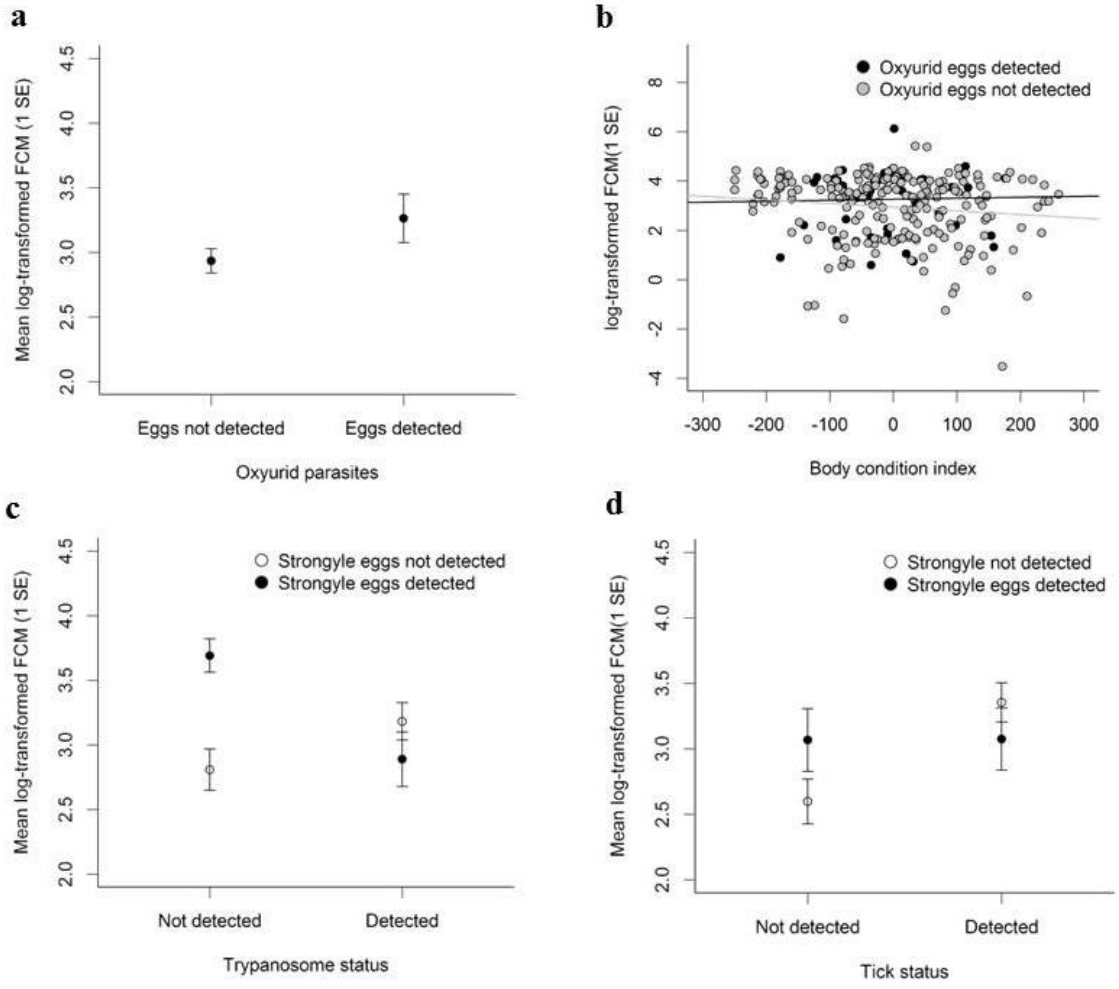


Figure 13. Parasite factors which influence faecal cortisol metabolites in woylies: (a) Oxyurid pinworm status (b) Interactive effect of body condition index and oxyurid status, (c) Interactive effect of trypanosome and strongyle status, (c) Interactive effect of strongyle and tick status.

Discussion

We identified several host, environmental and parasitological factors that influenced baseline FCM levels of captive woylies housed in semi-natural enclosures. These fluctuations in FCM may represent natural variations in HPA axis activity essential to survival (Crespi *et al.* 2013) but they may also represent the physiological response of woylies to environmental and biological stressors (such as parasites and climate) which are of importance to conservation science.

Glucocorticoids are essential to the physiological and behavioural responses that allow animals to adapt to changing conditions (Jessop, Woodford and Symonds, 2013). Therefore it is not unexpected that we noted significant seasonal variation in FCM in woylies as in other wildlife studies (including marsupials) of faecal glucocorticoid metabolites (Romero, 2002). For example, winter peaks in FCM in koala (*Phascolarctos cinereus*) were hypothesised to be due to low winter temperatures and rainfall and associated resource limitations and metabolic demands (Davies *et al.* 2014).

The important question in understanding seasonal variation is what proximate stressors are driving ultimate seasonal changes? Seasonal reproduction and changing resource quality and quantity are often cited as proximate causes of seasonal fluctuations in faecal glucocorticoid metabolites (Romero, 2002). However, our study provides a rather unique perspective as these woylies are continuous breeders and were supplementary fed throughout the study. Levels of anthropogenic noise and human interaction (including handling), which have been reported to constitute stressors in other marsupials (Narayan *et al.* 2013) are also unlikely explanations as these factors remained relatively constant across the study period. Alternatively, peak FCM in woylies during the cooler seasons may be associated with circadian rhythms (Lane, 2006), shortened day length (Steinmetz *et al.* 2006), or increased metabolic demands such as energy mobilisation for thermoregulation (Steffen and Musacchia, 1985). Seasonal variation in woylie stress physiology should be considered in the timing of management interventions as it may influence their response to interventions.

Woylie body condition remained within a healthy range throughout the study and body condition was only weakly negatively associated with FCM. This is consistent with other wildlife studies that show broader relationships between stress and body condition (Mumby *et al.* 2015) that may be due to regulation of metabolism by glucocorticoids (Sapolsky *et al.* 2000). The presence of only a weak effect in our study may be due to the protection of the captive study population from acute stressors such as limited food resources. In a study of free-ranging woylies during a translocation program, a significant and more pronounced negative association between body condition index and FCM after translocation was found (Hing *et al.* 2017). The negative relationship between body condition index and FCM in our current study was more marked in males compared to females. This may be associated with sex differences in endocrine regulation of metabolism and body weight (Shi and Clegg, 2009), but these interactions are currently uncharacterised in woylies. Longer-term monitoring of FCM and body condition in free-ranging populations that are exposed to other potential stressors is required to investigate the potential ramifications for woylie health and conservation.

Females had higher mean FCM than males, a pattern also reported in other Australian marsupials including the bilby (*Macrotis lagotis*) (Narayan *et al.*, 2012), koala (Narayan *et al.* 2013) and southern brown bandicoot (*Isoodon obesulus*) (Dowle, Webster and Deane, 2012). These results may be a reflection of sex differences in glucocorticoid metabolism (Lane, 2006) and could suggest that male and female woylies have different physiological sensitivities to stressors, a pattern that has been observed in other species (Handa *et al.* 1994).

Females woylies are capable of caring for young 96% of the time (Thompson *et al.* 2015) as they are continuous breeders and can undertake embryonic diapause (Smith, 1989). Consequently, the majority of samples collected from female woylies during this study (n=171 in total) were collected when they were carrying pouch young (n=119). We found no association between female pouch status and FCM, which is consistent with previous suggestions that stress-induced reproductive inhibition is not a major concern for woylie conservation (Wayne *et al.* 2013a). Nevertheless, the greatest difference between females with versus without pouch young was noted in winter which may suggest greater physiological demands on mothers during this time.

When woylies were shedding oxyurid pinworm eggs, they had significantly higher FCM concentration compared to when oxyurid eggs were not detected. This may reflect parasite induced stress or exacerbation of infection by stress. Itching associated with the perianal deposition of oxyurid eggs has been found to cause mild chronic stress in rats (Silveira *et al.* 2003). In addition, experiments have shown that stressor exposure increases the shedding of oxyurid eggs in captive ground squirrels (*Citellus armatus*) (Noble, 1966). We also found that the relationship between body condition index and FCM was influenced by the presence or absence of oxyurid eggs. However the relationship was weak, so the biological significance of this effect is unclear.

We sought to investigate the impact of parasite co-infection on host stress physiology because animal hosts are commonly infected by multiple endoparasite and ectoparasite types with potential effects on host immunity and health (Ezenwa *et al.* 2010). While we found FCM was lowest when neither trypanosomes nor strongyles were detected and when neither ticks nor strongyles were detected, other results found in this study suggest that the relationship between stress physiology and parasite co-infection in woylies is complex. For example, in trypanosome positive woylies mean FCM was higher when strongyle eggs were not detected compared to when strongyle eggs were detected. This is consistent with findings from studies of free-ranging woylies. When free-ranging woylies were trypanosome positive, strongyle egg counts decreased as FCM increased (potentially making eggs less likely to be detected if counts dropped below the detectable threshold) (Hing *et al.* 2016). It is possible that these interactions reflect the influence of stress physiology on different arms of the immune system (such as T helper 1 and T helper 2 responses) responsible for defence against micro-parasites (such as trypanosomes) and macro-parasites (such as strongyles) (Padgett and Glaser, 2003, Hing *et al.* 2016). The immune response to parasites and the potential coordinating role of glucocorticoids, while widely explored in other species (Sapolsky *et al.* 2000), remain areas for further exploration in woylies.

Future projects that use FCM in woylies should be aware of the strengths and limitations of our approach. In general, given how woylies must be trapped and handled, FCM are more practical physiology metrics in this species compared to other measures like plasma glucocorticoids. However, as the application of FCM in woylie research and management remains in its infancy, further optimisation would strengthen our ability to interpret results of the EIA used in our study. High

performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS), though costly and resource intensive, would allow identification of hormone metabolite constituents in woylie faeces, which would be valuable for example to pinpoint sex-related differences in glucocorticoid metabolism (Monfort, 2003). Of most importance is the biological relevance of the hormonal titre, that is, does the FCM data relate to biological events of interest? Adrenocorticotropic hormone (ACTH) challenge for assay validation is not feasible for woylies housed in large enclosures because it is not practical nor ethically permissible to trap and collect faecal samples three times a day for several days as previously performed in a woylie (n=1) in a zoo study (Fanson *et al.* 2015). However, we have demonstrated that the R4866 FGM EIA can detect variation in FCM concentration related to translocation (Hing *et al.* 2017) and in association with differences in immune function (Hing *et al.* 2016). Thus the protocol we employed provides physiologically relevant information by monitoring FCM in relation to environmental and management factors.

Conclusions

This longitudinal study revealed insights into factors influencing stress physiology indices in a captive population of woylies. Sex and seasonal factors had the greatest influence on FCM fluctuations and parasite parameters showed some interesting and complex interactions that are yet to be fully understood. This study provides a baseline for understanding what factors should be considered when carrying out potentially stressful conservation interventions or other anthropogenic activities that may influence the future survival of this unique and critically endangered species.

Chapter 4 Host stress physiology and *Trypanosoma* haemoparasite infection influence innate immunity in the woylie

The following is a published paper:

Hing S., Currie A., Broomfield S., Keatley S., Jones K.L., Thompson R.C.A, Narayan E.J., Godfrey S.S. (2016) Host stress physiology and *Trypanosoma* haemoparasite infection influence innate immunity in the woylie. *Comparative Immunology, Microbiology and Infectious Diseases* 46, 32-39.

In collaboration with immunologists working in human medicine, I conducted the first immune function study in woylies. I adapted a phagocytosis assay developed in pre-term human infants for use in woylies. Phagocytosis, the engulfment of foreign particles by white blood cells (leukocytes) is an integral component of front line immune defences including against *Trypanosoma* species of haemoparasites. The adapted phagocytosis assay was applied in parallel with analyses of FCM and parasites (including trypanosomes) to investigate the premise of the hypothesis that stress, immune function and *Trypanosoma* infection dynamics are linked in woylies.

Introduction

Globally, wildlife populations are under increasing pressure from infectious diseases (Altizer *et al.* 2003). Host immunity is a key factor determining the impact of disease on animal populations (Klein 1993). Therefore, investigating immune function in wildlife species can inform population management and conservation (Downs and Stewart 2014). However, significant knowledge gaps remain in wildlife immunology including lack of basic species-specific data and outstanding questions surrounding factors that influence immune responses and infection patterns (Klein 1993, Martin *et al.* 2010). The relationship between immune function and infection has rarely been examined in wildlife despite suggestions that these processes play a part in health, population decline and extinction (Plowright, Sokolow, *et al.* 2008, Aguirre and Tabor 2008, Acevedo-Whitehouse and Duffus 2009). The need to address these outstanding questions in wildlife immunology is especially urgent for small populations of endangered species because they may be particularly vulnerable to the effects of infectious disease due to concurrent stressors and their influence on immune function (Blaustein *et al.* 2012). Addressing knowledge gaps in wildlife immunology in part depends on translating progress made in research focusing on health in human populations to wildlife (Pedersen and Babayan 2011).

Marsupials, including many of Australia's most threatened species, have long been singled out as having an "extra-ordinary susceptibility to certain infections" (Rowlands 1976), which may influence population health and survival (Peacock and Abbott 2014). The woylie (or brush-tailed bettong, *Bettongia penicillata*) is an ecologically important, and critically endangered marsupial, which has undergone over 90% declines in population size since 1999 (Wayne *et al.* 2013). There are now only two remnant populations of woylie in south-west Western

Australia. Although the cause of the recent woylie decline is unknown, it has been suggested that stress, immunosuppression and infectious disease may have exacerbated the impact of introduced predators (Wayne 2008, Yeatman and Groom 2012). Trypanosomes (protozoan haemoparasites, *Trypanosoma spp.*) have been identified as possible disease candidates due to their association with the decline of the woylie (Botero *et al.* 2013, Thompson, Wayne, *et al.* 2014). *Trypanosoma* species are endemic in Australian mammals and occur in free-ranging woylie populations (Thompson *et al.* 2014). Woylie are host to multiple species of trypanosomes, which fall into several clades (Botero *et al.* 2013, Thompson *et al.* 2013). Botero *et al.* (2013) found that Clade A, including *Trypanosoma copemani*, appears more virulent and prevalent in a declining woylie population (Upper Warren, Western Australia) compared to a higher prevalence of less virulent clades in a stable population (Karakamia Wildlife Sanctuary, Western Australia). This observation has been further supported by a temporal association between decline patterns in the Upper Warren and *T. copemani* prevalence (Thompson, Wayne, *et al.* 2014). It has been suggested that woylies in the declining population may be less efficient at responding to more virulent genotypes of this endemic parasite (Botero *et al.* 2013). In addition, a haematological study found variation in differential leukocyte counts between stable woylie populations and those sampled during or soon after decline (Pacioni *et al.* 2013). In the majority of populations sampled (including stable populations in sanctuaries), neutrophils were the predominant leukocyte type (Pacioni *et al.* 2013). In the declining populations of the Upper Warren region of Western Australia, lymphocyte and neutrophil counts were similar, which the authors suggested was consistent with involvement of immunologic challenges in the declining population (Pacioni *et al.* 2013). Hence, understanding leukocyte function

and *Trypanosoma* infection in woylies addresses outstanding questions in the conservation of this species.

A key component of innate cell-mediated immunity and the host immune response to *Trypanosoma* haemoparasites is phagocytosis, the engulfment and destruction of foreign particles by phagocytic leukocytes (Jones and Hancock 1983). Deviations from normal phagocytic function such as that which might occur with stress (Baccan *et al.* 2004) can render animals more susceptible to disease (Muñoz *et al.* 2014). Phagocytosis has been described as a particularly difficult immunological process to measure (Gil and Culver 2011). However, different methods have been used to assess phagocyte function ranging from microscopy (Muniz-Junqueira *et al.* 2003) to flow cytometry (Bassøe 2002, Lockwood *et al.* 2016). Phagocytosis flow cytometry is a high throughput, quantitative assay, which assesses leukocytes' capacity to phagocytose fluorescently labelled bacteria (Bassøe 2002, Neaga *et al.* 2011). It has numerous advantages for use in comparative immunology and has proven to be a rapid, reliable tool to evaluate phagocytosis in different species ranging from teleosts (*Oncorhynchus mykiss*) (Li *et al.* 2006), mice and macaques (*Macaca fascicularis*) (Neaga *et al.* 2011) to salamanders (*Ambystoma tigrinum*), (Froese *et al.* 2005), seals (*Phoca vitulina, P. groenlandica, Halichoerus grypus*) (Frouin *et al.* 2010), camels (*Camelus bactrianus)* (Abdurahman and Saad 1996), sea turtles (*Chelonia mydas*) (Rossi *et al.* 2009, Muñoz *et al.* 2014), beluga whales *(Delphinapterus leucas*) (de Guise *et al.* 1995) and Tasmanian devils (*Sarcophilus harrisii*) (Kreiss *et al.* 2008). The *ex vivo* nature of the phagocytosis flow cytometry assay also has advantages over *in vivo* immunologic tests such as allograft rejection, which may be ethically challenging to carry out in critically endangered species. The phagocytosis flow cytometry protocol adapted for this study (Prosser *et al.* 2013) was

particularly well suited for use in the woylie as it requires small blood volumes (<25µl) and, with some modifications, did not require species-specific markers or reagents.

We aimed to identify the factors that affect phagocytosis in the woylie, including host stress physiology and investigated the influence of phagocyte function on *Trypanosoma* haemoparasite infection status. We adapted a small blood volume phagocytosis flow cytometry protocol developed for very preterm human infants (Prosser *et al.* 2013) and validated in newborn lambs (Lockwood *et al.* 2016), to perform the first study of immune function of the woylie. We then investigated factors which influence host immunity and host-parasite interactions to determine their impact, if any, on phagocytosis. We hypothesised that woylies exhibit deficits in phagocyte function, these deficits are associated with parasite status and together, these factors may in part have contributed to the species' dramatic decline.

Materials and methods

Animal trapping and sample collection

We trapped woylies three times during the winter and spring of 2014 (June, July and November) in four adjacent naturalistic enclosures (35m x 55m each) at Native Animal Rescue in Western Australia. Fifteen adult woylies (9 females, 6 males) were housed (3 to 4 adult woylies per pen). In each trapping session, 32 galvanized wire Sheffield traps (220 x 220 x 550mm) (Sheffield Wire Products, Western Australia) were set just prior to sunset and checked before sunrise. All 15 woylies were re-captured each month. At each capture, woylies were individually identified by a unique microchip code, examined for ticks and females were inspected for the presence or absence of pouch young (pouch status).

Faecal samples (deposited overnight) were collected (from underneath the trap), and a blood sample was collected from the lateral caudal vein (400 to 1000µl). At each time point, half the blood volume from each animal was collected in an EDTA MiniCollect tube (Greiner Bio-One, Germany) for DNA extraction and *Trypanosoma* PCR. The other half of the blood sample was collected in a lithium heparin MiniCollect tube (Greiner Bio-One, Germany) for phagocytosis flow cytometry. EDTA and faecal samples were stored at -20°C and processed within ten months of collection. Lithium heparin samples were kept at room temperature and processed within 24 hours of collection, as the phagocytosis flow cytometry assay required live viable cells. In some cases, all assays could not be completed for every sample (e.g., due to insufficient sample volume) but a total of 41 faecal samples, 40 lithium heparin samples and 39 EDTA samples were analysed. Valid samples were collected for each individual for at least 2 time points.

This research was carried out under a Western Australian Department of Parks and Wildlife Regulation 17 License to Take Fauna for Scientific Purposes (SF009623) and Murdoch University Animal Ethics Permit (RW2611/13).

Phagocytosis flow cytometry assay

We investigated phagocytosis in woylie by adapting a flow cytometry protocol developed for very pre-term human infants (Prosser *et al.* 2013). As this was the first time that flow cytometry has been carried out to assess phagocytosis in the woylie, the amount of fluorescently labelled bacteria added to whole blood was first titrated. Titration was performed by adding phRodo® Red *Staphylococcus aureus* Bioparticles® Conjugate for Phagocytosis prepared as per the manufacturer's instructions (hereby referred to as phRodo®) (Gibco Life Technologies, Mulgrave

Australia), to whole blood samples from two individuals opportunistically sampled as part of a pilot study. Aliquots of phRodo® ranging from 0 to 50ul were added and plotted against the percent of phagocytosing leukocytes. The proportion of phagocytosing leukocytes was maximal above 10ul of phRodo labelled *S. aureus* and a clear separation in fluorescence of positive cells above that of negative cells was evident at 10 ul. Hence, 10ul of phRodo® was added to treated samples for the assay.

Samples were prepared for flow cytometry as described by Prosser *et al.* (2013) with some modifications. In brief, as per the woylie titration, 10ul of phRodo® was added. Heparinsed whole blood (25µl) was incubated at 37°C, 5% CO_2 in the dark for 60 minutes with 5% foetal calf serum media (80µl). Cells were washed twice with cold PBS (1ml) (Gibco Life Technologies, Mulgrave Australia) and the supernatant was removed by vacuum suction. Red blood cells were lysed with FACSLyse (1ml) (BD, North Ryde, Australia) and the remaining cell pellet resuspended in Stabilising Fixative (200ml) (BD). Analysis was performed on a BD FACSCanto flow cytometer (BD). Detection of phRodo® fluorescence was by a 450nm violet long-pass filter. No compensation was required for this single colour panel. We aimed to record twenty thousand events for each sample. Data was processed in FlowJo v.10. Single cells were gated and doublets excluded based on side-scatter height (SSC-H) and side-scatter width (SSC-W). Phagocytes were subsequently identified during FACS analysis as those with intermediate to high forward-scatter (FSC) and side-scatter (SSC) properties, whereas lymphocytes have low SSC and FSC. Untreated whole blood samples served to determine the cut-off for fluorescence for phagocytosis in each donor.

DNA extraction and PCR amplification for Trypanosoma species

We used an established and validated PCR protocol to detect *Trypanosoma* parasites in the blood stream of woylies (Botero *et al.* 2013, Thompson *et al.* 2014, Dunlop *et al.* 2014). DNA extraction and *Trypanosoma* PCR amplification were carried out as per previously described protocols (Botero *et al.* 2013, Thompson *et al.* 2014, Dunlop *et al.* 2014). Whole genomic DNA was extracted from frozen whole blood samples stored in EDTA using a DNA Blood Kit following the manufacturer's guidelines (Qiagen, Hilden, Germany). Species-specific primers for *Trypanosoma* species were used in a nested PCR: *T. vegrandis* (TVEF, TVER, TVIF and TVIR), *T. copemani* (S825F, SLIR, WoF and WoR) and H25 (H25EF, H25ER, H25IF and H25IR) (Botero *et al.* 2013). Negative and positive controls were used for each PCR, with the positive control derived from a known stock and the negative control containing neither blood nor tissue. Presence or absence of *Trypanosoma* species in peripheral circulation as indicated by PCR positive or negative results was recorded as trypanosome status (+ or -). We did not quantify the number of parasites in the blood stream because qPCR has not been developed for this host-parasite system.

Faecal cortisol metabolite (FCM) enzyme immunoassay (EIA)

Faecal samples were prepared for faecal cortisol metabolite (FCM) enzyme immunoassay (EIA) by lyophilising (freeze-drying) to remove water while inhibiting glucocorticoid metabolite breakdown by micro-organisms (Wasser *et al.* 2000, Palme and Chadi 2013). Extraction was carried out according to protocols previously described using 90% ethanol and heat treatment (Wasser *et al.* 2000, Palme and Chadi 2013).

Extracts were assayed for FCM by enzyme immunoassay (EIA) using a polyclonal anti-cortisol antibody R4866 protocol (Narayan *et al.* 2012). We demonstrated parallelism (between dilutions of pooled faecal extracts and the cortisol standard curve), recovery of exogenous cortisol added to extracts ($y=1.1026x - 0.8897$, $R^2 = 0.9929$), sensitivity of the assay (2.04 ± 0.39pg/well, n=15) and degree of intra-assay variation (CV 4% for high binding internal control and CV 6% for low binding internal control) and inter-assay variation (CV 2% for high binding internal control, CV 12% for low binding internal control). Dilution factors were 1:8 for male woylie and 1:16 for female woylie based on the 50% binding point on the parallelism curve. Results were expressed as FCM concentration (pg/g) on a dry weight basis.

Statistical analysis

We used linear mixed effect models (lmer) in R 3.1.0 (Bates *et al.* 2015) using the packages 'lme4' (Bolker 2015) and 'car' (Fox and Weisberg 2011) to investigate the host and environmental factors that may influence percent of phagocytosing leukocytes and phagocytosis index. The study had a nested design with repeated samples from individual woylies in their respective pens. Percent of phagocytosing leukocytes and phagocytosis index of each woylie were the dependent variables. To fulfil model assumptions of data conforming to a normal distribution, the percent of phagocytosing leukocytes was arc-sine square root transformed, phagocytosis index was scaled by z-transformation (using the function 'scale' in R) (Becker *et al.* 1988) and FCM was log-transformed. Sex (male/female), season (winter/spring) and FCM were included as fixed effects. Two-way interactions between these effects were also included. Woylie ID nested within pen was included as a random effect in all models to account for repeated measures from the same individuals in their respective pens.

To determine the minimal adequate models, we undertook model simplification by stepwise reduction, removing non-significant terms from the maximal model until further model reductions resulted in significant changes in model deviance (Crawley 2007). Significance ($p \leq 0.05$) was tested in a likelihood ratio test (χ^2).

We were also interested in the effects of female reproductive activity on immune function, so models were re-run for females only (n=26 samples from 9 females). In the same way described above, we used lmer to investigate the effect of season, pouch status (0 = empty or 1 = pouch young present) and FCM on percent of phagocytosing leukocytes and phagocytosis index.

To investigate if immune function influenced the likelihood of trypanosome infection, we used a generalised linear mixed effect model (glmer) (Bates *et al.* 2015) to explore the influence of FCM, percent of phagocytosing leukocytes, phagocytosis index, season, and tick status (0=absent, 1=present) on trypanosome status. Trypanosome status (0 = PCR negative or 1 = PCR positive) was the dependent variable with a binomial error distribution. Model simplification was carried out in the same way as the lmer models, described above.

Results

We demonstrated phagocytosis in woylie leukocytes by flow cytometry (Figure 14). As expected, phagocytosis (higher mean fluorescence in the presence compared to in the absence of phRodo®) was negligible in lymphocytes (Figure 14c), but was detected in phagocytes (Figure 14d). The median fluorescence (*x*-axis: PE-A pHRodo) was an indirect indicator of phagocyte functional efficiency or *Staphylococcus aureus* particle uptake (phagocytic index). The ratio of phRodo® positive cells indicated the percentage of the phagocyte population that phagocytosed the phRodo® labelled *S.aureus* particles (percent

phagocytosing leukocytes). Woylies had competent phagocytes with a mean percent phagocytosis of 91% ± 2.6 and mean phagocytosis index of 4730 ± 388.

Factors influencing phagocytosis parameters

There was a significant positive association between phagocytosis index and the percent of phagocytosing leukocytes (Table 3 and 4, Figure 15). We also found a significant interaction between sex and FCM on the percent of phagocytosing leukocytes (Table 3).

In females, lower FCM was associated with higher percent of phagocytosing leukocytes, but the opposite relationship was detected in males (Figure 16). In female woylies, the presence or absence of pouch young did not significantly affect phagocytosis index or the percent of phagocytosing leukocytes ($p>0.05$).

Table 3. Summary of the minimal adequate linear mixed effect model of factors influencing the percent of phagocytosing leukocytes (arc-sine square root transformed).

Fixed effects	Coefficient	Df	χ^2	p-value
FCM	-0.0853	1	2.382	0.123
Phagocytosis index	**-0.064**	**1**	**21.878**	**<0.001**
Sex	-0.482	1	0.027	0.870
Season	-0.120	1	0.000	0.983
FCM:phagocytosis index	0.054	1	3.048	0.081
FCM*sex	**0.0156**	**1**	**5.090**	**0.024**
Season:trypanosome status	0.2896	1	3.284	0.070

Table 4. Summary of the minimal adequate linear mixed effect model of factors influencing phagocytosis index (z-transformed).

Fixed effects	Coefficient	Df	χ^2	p-value
FCM	1.386	1	0.363	0.547
Percent of phagocytosing leukocytes	**5.354**	**1**	**15.843**	**<0.001**
Trypanosome status	1.570	1	0.991	0.320
FCM:percent of phagocytosing leukocytes	-0.679	1	1.806	0.179
FCM*trypanosome status	**-0.591**	**1**	**4.191**	**0.041**

FCM was log transformed, phagocytosis index was z-transformed and percent of phagocytosing leukocytes was arc-sine square root transformed.

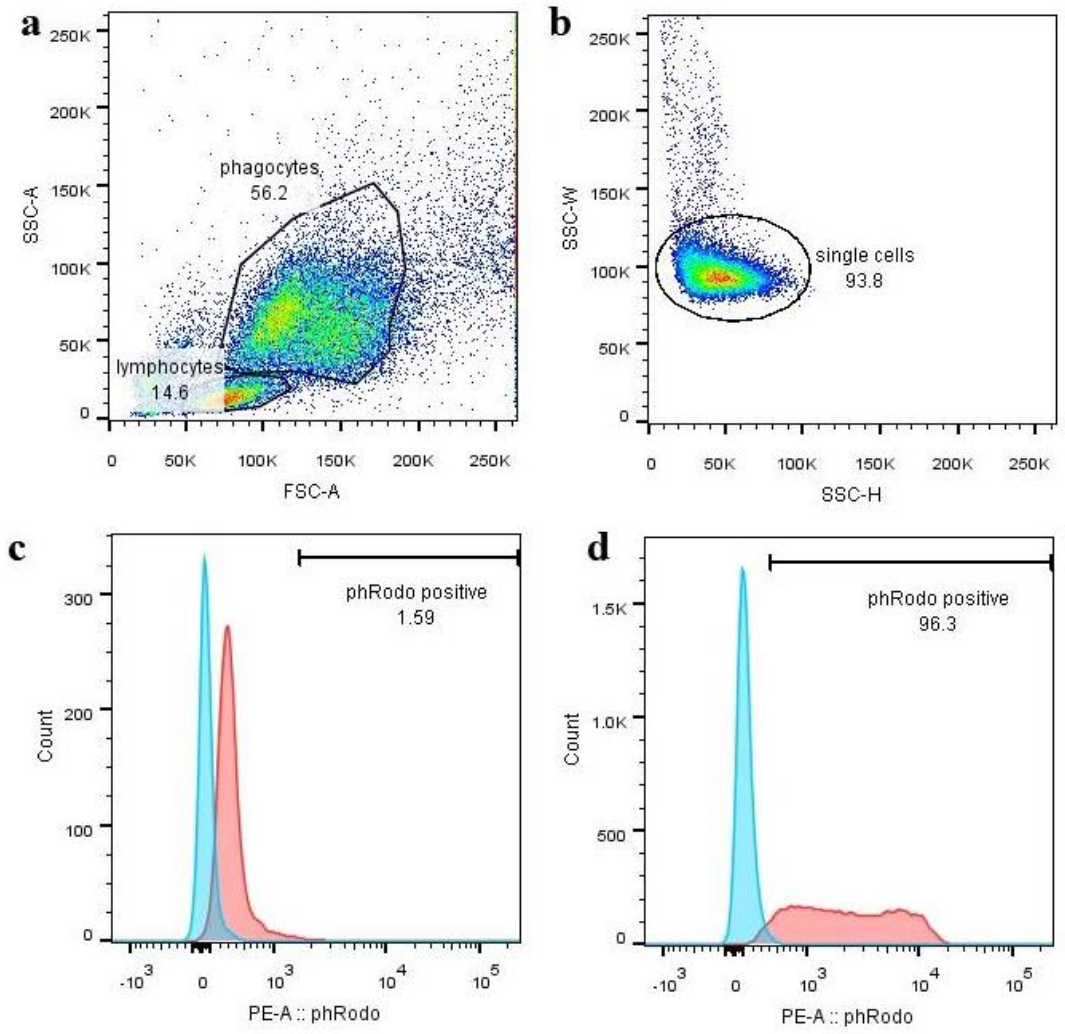


Figure 14. Analysis of phagocytosis using flow cytometry with pHrodo-labelled bacteria. The top two panels indicate inclusion gates for: a) phagocytes and lymphocytes, based on forward-scatter area (FSC-A) and side-scatter area (SSC-A), and b) single cells, based on side-scatter height (SSC-H) and side-scatter width (SSC-W). The bottom two panels show histograms of pHrodo fluorescence (PE-A:phRodo) and cell count of c) lymphocytes and d) phagocytes in the presence (pink) and absence (blue) of pHRodo® labelled bacteria.

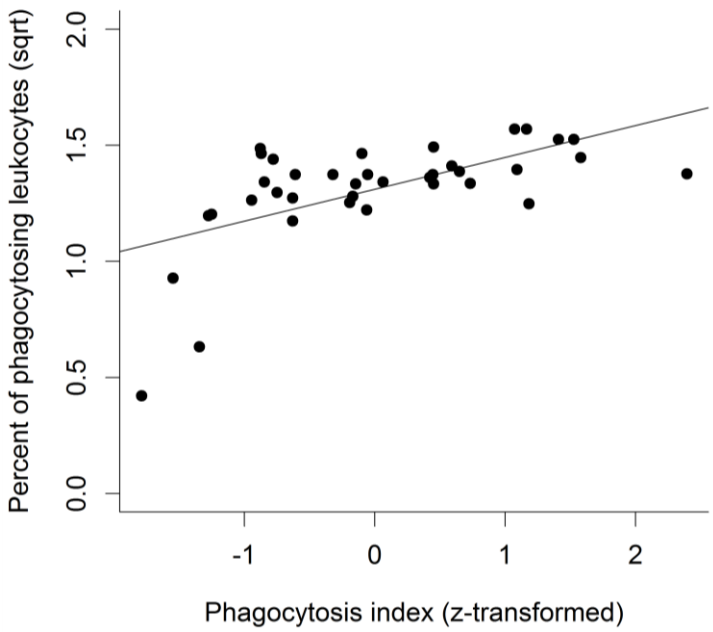


Figure 15. The relationship between the percent of phagocytosing leukocytes (arc-sine square-root transformed) and phagocytosis index.

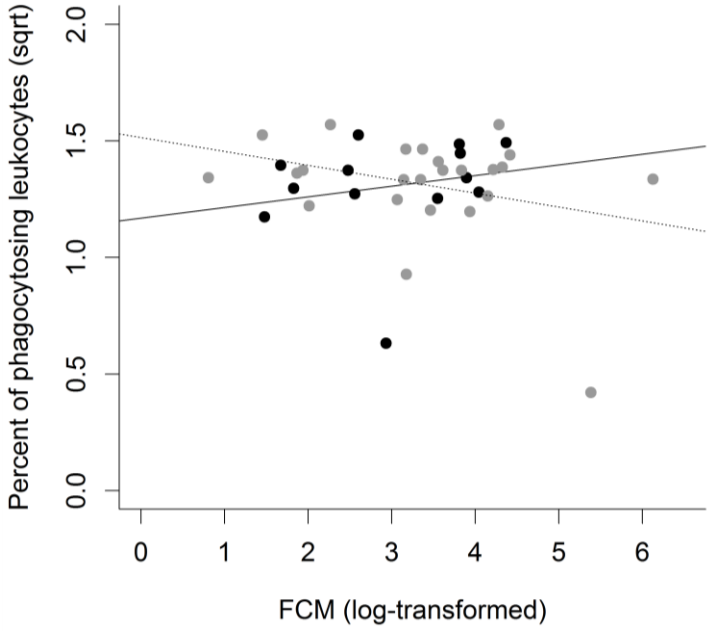


Figure 16. Percent of phagocytosing leukocytes and FCM (log-transformed) by sex.The relationship between the percent of phagocytosing leukocytes (arc-sine square-root transformed) and faecal cortisol metabolite concentration (log-transformed) for males (black symbols, solid line, percent= 0.05FCM+1.16) and females (grey symbols and dotted line, y= -0.06FCM + 1.51).

Table 5. Fluctuating trypanosome parasitaemia detected by PCR

Animal ID	June	July	August
WC2842	-	+	-
WC2920	+	+	+
K1642-1680	+	+	+
YH-W2930-18	+	+	+
K1305-1268	+	+	+
K1282-1289	-	+	-
WC2807	-	-	-
WC2830	+	+	-
K1483-1610	+	+	NA
K1294-1291	+	+	-
WC2844	+	-	+
WC2930	+	+	NA
WC2841	NA	+	+
K1639-1265	-	+	NA
K1677-1635	-	NA	-

Relationships between phagocytosis parameters and trypanosome infection

Overall Trypanosoma prevalence was 52% over the course of the study, and all cases were identified as *T. copemani*. The trypanosome status of five of the fifteen individuals changed from month to month during this study (Table 5). We found a significant interaction between Trypanosoma status and FCM on phagocytosis index (Table 4). When woylies were trypanosome positive, higher FCM was associated with lower phagocytosis index, but when woylies were trypanosome negative, higher FCM was associated with higher phagocytosis index (Figure 15). Sex, season, FCM, the presence or absence of ticks and phagocytosis parameters did not have a significant association with *Trypanosoma* status ($p>0.05$).

Discussion

In this first investigation into woylie immune function, we adapted a phagocytosis flow cytometry protocol developed in very pre-term human infants (Prosser *et al.* 2013) to characterise factors that affect phagocytosis in the woylie. Using this protocol, uniquely combined with minimally invasive physiology and parasitology techniques in individually-identified woylies sampled repeatedly over time, we determined that host stress physiology and *Trypanosoma* infection status influence phagocytosis.

We found a significant positive relationship between the percent of phagocytosing leukocytes and phagocytosis index. This differed from the findings of Prosser *et al.* (2013), where preterm infants were found to have fewer phagocytes capable of engulfing bacteria than term infants, but overall had a similar phagocytic index due an increased compensatory uptake of the amount of bacteria in the cells that were phagocytosing. When woylies had a lower percent of phagocytosing leukocytes, they also had a lower phagocytosis index and in these instances, they may be vulnerable to disease. Though none of the animals in this study showed observable signs of clinical disease, these were captive animals that were protected from some of the stressors (eg. nutritional stress) and the full spectrum of pathogens that might be found in the wild. Deficiencies in phagocytosis have also been shown to be associated with a range of infectious conditions in rodent (Jones and Hancock 1983) and human subjects (Lekstrom-Himes and Gallin 2000). Alternatively, woylies may employ alternative immunological mechanisms to compensate when phagocytosis index and percent of phagocytosing leukocytes are both low in comparison to the population mean.

The effect of an interaction between sex and FCM on percent of phagocytosing leukocytes is likely to reflect the three-way relationship between sex hormones, glucocorticoids and immune function (Da Silva 1995, Da Silva 1999). Our results appear to be consistent with comprehensive reviews of this relationship in laboratory rodents and humans, which highlight that oestrogens exert their effect on immune function in part due to an oestrogen augmented glucocorticoid response (Da Silva 1995). In general, females are considered more 'immunoreactive' than males to the immunosuppressive effects of glucocorticoids because oestrogens potentiate glucocorticoids (Da Silva 1995), this leaves females more vulnerable to inflammatory stimuli and autoimmune diseases (Da Silva 1995). Hence, our results may suggest that female woylies, similar to females of other species, may also be at higher risk of these conditions. In males, testosterone is described as immunosuppressive but the relationship between testosterone hormones, glucocorticoids and immune function is not well understood (Duffy *et al.* 2000) and the positive relationship between FCM and percent of phagocytosing leukocytes in male woylie requires further exploration. Development of sex hormone assays for woylies would allow for further investigation of the relationship between sex hormones, immunity and infection in the woylie.

Given the influence of sex hormones on immunity (Bouman *et al.* 2005), we expected female woylies phagocytosis parameters would vary with reproductive state. In studies on humans, aspects of innate immunity such as phagocytosis have been found to be upregulated in pregnant women to compensate for weakened adaptive immunity (Barriga *et al.* 1994). However, pouch status did not significantly affect phagocytosis index or percent of phagocytosing leukocytes in female woylies. As continuous breeders with no defined breeding season, woylies may have

developed mechanisms to maintain a steady state system through different phases of the female reproductive cycle to protect dam and young from erratic fluctuations in maternal immune function. However, generalisations are drawn with caution given the small number of data points where females did not have pouch young (n=6). Indeed, female woylies are rarely without young and this may be another factor contributing to unique endocrine and immunological patterns throughout the reproductive cycle. Similar to other marsupials, embryonic diapause enables female woylies to simultaneously gestate a foetus, lactate to support pouch young and nurture young at heel (Renfree 1980), which may translate to a distinct relationship between female reproductive status and immune function.

The only relationship we found between infection state and phagocytosis parameters involved an interaction with stress hormones (FCM). The negative relationship between FCM levels and phagocytosis index in trypanosome positive woylie may indicate that during periods of *Trypanosoma* parasitaemia, woylies are more vulnerable to the immunosuppressive effects of glucocorticoids (Munck and Guyre 1991). Alternatively, a combination of host stress physiology and infection status may affect the efficiency of leukocyte function (Davis *et al.* 2008). Laboratory rodent studies focused on elucidating the pathogenesis of Chagas disease (*Trypanosoma cruzi*) in humans have highlighted the important role of stress-related immunocompromise in the course of trypanosomiasis (Santos *et al.* 2005). Our results are consistent with the hypothesis that stress-related immunosuppression may also be associated with the course of trypanosomiasis in the woylie. These findings may have relevance for woylie health and conservation, as they suggest that woylies experiencing concurrent stressors and active infection may be more likely to have compromised immune function. However, we acknowledge that PCR, while

indicative of the presence or absence of the parasites in peripheral circulation at a given point in time, may not be an indicator of true infection prevalence, as parasitaemia fluctuates, trypanosome concentration can drop below detectable levels, particularly in chronic infections (Dunlop *et al.* 2014). Consequently, trypanosome status can change from month to month (Table 3) and intermittent low grade parasitaemia makes simple light microscopy of woylie peripheral blood smears an unreliable means to quantify trypomastigotes (life cycle stage in mammalian host bloodstream). If logistically feasible, cytometry could be used to quantify trypomastigotes in fresh blood immediately following collection. However, higher volumes (several millilitres) of blood and species specific antibodies are required (Carneiro *et al.* 2007), neither of which were available at the time of this study.

We did not detect single factor associations between phagocytosis parameters and FCM or trypanosome status (Table 1). The study population, which was well-provisioned and protected from terrestrial predators, may not have been stressed to an extent (allostatic overload) where immune function and infection dynamics were affected by host stress physiology. Physiological stress and glucocorticoids have been associated with changes in phagocytosis in laboratory rodents (Sesti-Costa *et al.* 2010) but our ability to detect a relationship between stress and phagocytosis may be less than in controlled laboratory studies where relatively severe experimental stressors are imposed. In the future, using a bench top flow cytometer in the field, it would be valuable to assess phagocytosis of the last two remaining wild woylie populations who may encounter additional stressors, such as introduced predators, from which the animals in our study were protected.

Phagocytosis parameters did not appear to have a significant effect on trypanosome presence. However, the immune response to trypanosomes is complex

(Stenberg 2012), and we were only able to test a single component of immune defense. Nevertheless, the phagocytosis flow cytometry protocol used in this study may be applied to further our understanding of the pathogenesis of trypanosomiasis in varied host species. While *Trypanosoma* infection status was not found to be affected by FCM or phagocytosis parameters, the degree of parasitaemia or pathology could be affected. Future research could consider quantitatively assessing degree of parasitaemia by, for example, using concentration techniques (Bettiol *et al.* 1998) or real-time quantitative PCR (de Freitas *et al.* 2011). We also found no association between the presence or absence of ticks and trypanosomes despite suggestions that ticks act as vectors for trypanosomes in woylie (Thompson *et al.* 2014). Previous studies indicate that the woylies included in the present study are chronically infected with trypanosomes (Thompson *et al.* 2013). Hence, even if ticks are vectors for trypanosomes in woylie, detecting ticks on an individual is not a pre-requisite for these individuals to be PCR positive for trypanosomes.

Seasonal changes in host immunity, including phagocytosis, have been noted in animals including humans (Møller *et al.* 2003). However, we did not find an effect of season on phagocytosis parameters in woylie. This may indicate that phagocytosis index and percent of phagocytosing leukocytes in woylie are not subject to seasonal fluctuations, which may otherwise render woylie particularly vulnerable to infectious disease at certain times of the year. However, the study only considered samples collected over two 'seasons' (winter and spring), so it is possible that an effect was not detected looking only at these two seasons. Seasonal changes in immune function are thought to be associated with resource quality and availability or according to the 'seasonal stimulus hypothesis', with UV exposure and vitamin D (Cannell *et al.* 2006). Hence alternative explanations for absence of detected seasonal differences in

phagocytosis parameters in these woylie may be associated with their protection from seasonal fluctuations such as year round supplementary feeding in this population and the species' nocturnal habits.

Here, we focused on only one aspect of the innate immune system, so studies into other aspects of the woylie immune system may reveal other patterns. We have avoided making generalisations about overall immune function in woylie because immunocompetence encompasses a complex multivariate response to many different kinds of parasite and pathogens. A variety of other tests such as lymphocyte proliferation with mitogens, mixed lymphocyte reactions and genomics could be used in the future to assess different components of the woylie immune system. There are limitations to the interpretation of our flow cytometry results. For example, we were unable to determine specific phagocyte populations and absolute numbers of monocytes or neutrophils. Further improvement of the method will be possible with the development of species-specific reagents, such as those being developed for other marsupial species, which allow for more precise identification of cell types (Morris *et al.* 2014). An in vitro bacterial killing assay and microscopy would also provide complementary measurements of immune function (Millet *et al.* 2007, Kreiss *et al.* 2008).

We have conducted the first study on immune function in the critically endangered woylie. Key results indicate that host immunity is likely to have ramifications for woylie conservation. If indeed infectious disease played a role in the species' decline, it may have occurred when percent of phagocytosing leukocytes and phagocytosis index were both low leaving woylies more vulnerable to infectious disease. Most notably, our findings indicate that an interaction between host stress physiology and *Trypanosoma* haemoparasites are associated with the functional

efficiency of woylie phagocytes. This is consistent with the hypothesis that stress-related immunosuppression may also be associated with infection status in the woylie. To manage wildlife species that may be threatened by multiple stressors and infectious disease challenges, we need to consider how these processes influence immune function because these interactions have the potential to influence health and conservation outcomes.

Chapter 5 Evaluating stress physiology and parasite infection parameters in the translocation of woylies

The following is a published paper:

Hing S., Northover A., Narayan E.J., Wayne A.F., Jones K.L., Keatley S., Thompson R.C.A., Godfrey S.S. (2017) Evaluating stress physiology and parasite infection parameters in the translocation of critically endangered woylies (*Bettongia penicillata*). *EcoHealth*.doi: 10.1007/s10393-017-1214-4

In this chapter, I assess the potential impact of a real-world management intervention, translocation, on woylie physiology and health. Translocation is a commonly applied strategy in the conservation of endangered species including the woylie. Stress may be important in fauna translocation because it has been suggested that it can exacerbate the impact of infectious disease on translocated wildlife. However, few studies explore this hypothesis by measuring stress physiology and infection indices in parallel during wildlife translocations. In collaboration with the Western Australian Department of Parks and Wildlife, I was able to compare FCM and parasite analyses in woylies before, during and after a translocation.

Introduction

Translocation involves the deliberate transport and release of animals from one site to another and is a widely applied strategy in wildlife management (Dickens *et al.* 2010). Identifying factors that influence the health and welfare of translocated wildlife is important to achieve positive conservation outcomes, including establishing new populations, supplementing wild populations and mitigating the impact of anthropogenic activities (Harrington *et al.* 2013). Physiological stress is a key factor that has been suggested to influence animal health and welfare and translocation success (Teixeira *et al.* 2007). It is increasingly recognised that translocation involves potential cumulative physical, physiological and psychological stressors (challenging stimuli), including capture, handling, confinement, transport and release into an alien environment (Teixeira *et al.* 2007, Dickens *et al.* 2010).

When animals encounter stressors, this activates the hypothalamic pituitary adrenal (HPA) axis, the essential neuroendocrine response that endeavours to maintain homeostasis through change (allostasis) (McEwen 2005). When stressors are unusual, unpredictable, severe, prolonged or cumulative, as they may be during translocation (Teixeira *et al.* 2007), the capacity for the HPA axis to maintain homeostasis may be overwhelmed (allostatic overload) (McEwen 2005, Busch and Hayward 2009). HPA axis dysregulation, failure of the negative feedback system, may result in either hypo- or hyper-activity of the HPA axis when stressors are encountered (Dickens *et al.* 2010). Dickens *et al.* (2010) outline the theoretical basis for chronic stress in translocated wildlife, including a prolonged increase in glucocorticoids and HPA axis dysregulation. For example, evidence of HPA axis dysregulation was found in chukar partridge (*Alectoris chukar*) five days after translocation and Capiro *et al.* (2014) found that faecal cortisol metabolites were

elevated in three Indian rhinoceros (*Rhinoceros unicornis*) up to nine weeks after they were translocated between zoos.

Chronic stress can compromise immune function and increase susceptibility to infectious disease (Martin 2009). Hence, recommendations have been made for studies that measure stress physiology and infection parameters in wildlife translocation (Letty *et al.* 2000, Dickens *et al.* 2010). However, a review of 199 mammalian and avian reintroduction projects found that only three projects measured stress indicators and only one examined parasite parameters (Harrington *et al.* 2013). To date, translocation stress studies suggest that chronic stress, days to months after translocation, is potentially a major factor in translocation failure (Teixeira *et al.* 2007, Dickens *et al.* 2009). Infectious disease is also acknowledged as a significant threat to wildlife involved in translocations (Aiello *et al.* 2014). However, the relationship between stress and infection patterns in wildlife translocation remains a neglected area in wildlife research.

Translocation is a mainstay of conservation management of threatened Australian fauna, including critically endangered species such as the woylie (*Bettongia penicillata*) (Morris *et al.* 2015). Woylies are small (1 to 1.8 kg) nocturnal marsupials and important ecosystem engineers (Fleming *et al.* 2014). They were once abundant across much of Australia but today are listed as critically endangered, their current decline, which began in 1999, is ongoing (Wayne *et al.* 2013). Only two remnant populations remain in south-west Western Australia (within the Greater Kingston National Park and Dryandra Woodland) (Wayne *et al.* 2013). To ensure the species' survival, several thousand woylies have been translocated to over fifty different sites across Australia (including sanctuaries, reserves and predator-free off-

shore islands), making it perhaps one of the most abundantly translocated Australian native animal species (Groom 2010, Morris *et al.* 2015)

A parallel study, focusing on the same translocation event as the current study, suggested that increases in some parasite parameters (prevalence of coccidian parasites and strongyle nematode egg counts) at one of the destination sites after translocation may be associated with translocation stress (Northover *et al.* 2015). Stress has also been suggested as a factor that may influence the impact of infectious disease on translocated eastern bettongs (*Bettongia gaimardi*), a species closely related to woylies (Portas *et al.* 2014). Others assert that stress and parasites are not a concern in the translocation of related species such as burrowing bettongs (*B. lesueur)* (Bannister *et al.* 2016). However, there is a paucity of studies measuring stress physiology and infection indices to test these hypotheses, particularly chronic stress beyond the immediate period after translocation. In this current study, we aimed to test these outstanding hypotheses.

We investigated the influence of host, environmental and parasitological factors on the stress physiology of both translocated and resident woylies (residing in the destination sites) during a translocation to supplement a wild population. Faecal cortisol metabolites (FCM) were measured as indicators of host stress physiology (von der Ohe and Servheen 2002). This minimally invasive approach is particularly useful for stress physiology evaluation in wildlife, including woylies (Hing *et al.* 2014). We analysed FCM and parameters of helminth and protozoan parasites before, at and after translocation in light of two key questions: 1) did translocation influence woylie stress physiology? and 2) among translocated woylies, what factors (including parasite parameters) were associated with host stress physiology?

Materials and methods

Trapping and sample collection

This study was carried out during an established translocation program. In June 2014, over three consecutive nights, woylies (n=182) were trapped from Perup Sanctuary (a 423ha predator-proof enclosure) in south-west Western Australia. After capture and processing, translocated woylies were transported 20km (approximately thirty minutes by vehicle) to one of two unfenced destination sites within the Greater Kingston National Park, Warrup (n=90) and Walcott (n=92). At the time of translocation, half of the translocated woylies included in this study (n=18/39 translocated woylies) were treated with a subcutaneous injection (0.2mg/kg) of the anti-parasitic drug ivermectin (Ivomec©) as part of a parallel study on the same translocation event (Northover *et al.* 2015). Trapping was also undertaken at Perup Sanctuary three months before the translocation (March 2014). Resident woylies at the destination sites, Warrup and Walcott, were trapped one to two months before the translocation (April/May 2014) and six months after the translocation (December 2014) (Table 6).

All trapping was carried out using 22 cm x 22 cm x 58 cm wire cage traps (Sheffield Wire Products, Welshpool, WA) baited with universal bait (oats, peanut butter and sardines). Traps were set before sunset and checked the following morning, except during translocation when all trapping, capture and handling was carried out overnight. Woylies were individually identified by unique ear tag codes in both ears. Animals were weighed and head length was measured (mm). Sex and age were recorded, and females were checked for the presence or absence of pouch young. Pouch young size was also measured (crown-rump length, mm) where applicable.

Table 6. Number of faecal samples collected before, at and after translocation by site, month and residence status

Site	Residence status	Before (March-May)	At (June)	After (December)	Total
Source					43
Perup	**Translocated**	4	39	n/a	41
Sanctuary	**Resident**	41	n/a	n/a	
Destinations					
Warrup	**Translocated**	n/a	n/a	18	18
	Resident	15	n/a	48	63
Walcott	**Translocated**	n/a	n/a	5	5
	Resident	11	n/a	42	53
Total		71	39	113	223

Blood (400 to 1000 µl) was collected from the lateral caudal vein into an EDTA MiniCollect tube (Greiner Bio-One, Germany) and stored at -20°C for *Trypanosoma* haemoparasite DNA extraction and PCR. Faeces were collected from newspaper laid underneath the trap. Immediately following collection, faeces were temporarily stored in a cooler for transportation. Within six hours of collection, two grams of each sample was placed in 10% buffered formalin at 4°C for faecal flotation, and the remainder was frozen at -20°C for FCM assays. A randomly selected subset of faecal samples was included in this study for FCM assays, sample size is detailed in Table 6.

Faecal cortisol metabolites (FCM) enzyme immunoassay (EIA)

Using methods previously described (Hing *et al.* 2016), faecal samples were freeze-dried, cortisol metabolites extracted (0.2g dry weight) using 90% ethanol and heat treatment, and the concentration of FCM (picograms per gram of faeces dry weight) was measured by polyclonal anti-cortisol antibody (R4866) enzyme immunoassay (EIA). This assay has been laboratory-validated by demonstrating: parallelism (based

on the 50% binding point on the parallelism curve), percentage recovery (88%, y=1.1026x – 0.8897, R^2 = 0.9929), sensitivity (2.04 ± 0.39 pg/well, n=15), intra-assay (CV 4% for high-binding internal control, and CV 6% for low-binding internal control) and inter-assay variation (CV 2% for high-binding internal control, CV 12% for low-binding internal control). Dilution factors were 1:8 for male woylies and 1:16 for female woylies (Hing *et al.* 2016). It should be noted that cortisol metabolism, chiefly by the liver and accumulation of FCM in faeces, occurs over time (von der Ohe and Servheen 2002). Hence, FCM measured at the point of translocation were not a reflection of acute translocation stress but a reflection of host stress physiology in the period immediately preceding the translocation.

Parasitology analyses

We performed simple faecal flotation as described by Northover *et al.* (2015) to count helminth parasite eggs and detect coccidian protozoan parasite oocysts. Briefly, 1 gram of each faecal sample was suspended in super-saturated sodium nitrate (NaNO3) solution (SG 1.37) for 15 minutes using Fecalyzer® containers (EVSCO Pharmaceuticals, USA, modified from Zajac and Conboy 2012). To gain a semi-quantitative estimate of parasite eggs per gram, we systematically scanned the area under the coverslip (22 mm x 22 mm) under a BX51 microscope (Olympus, Japan) at 10x objective using white light. We counted only strongyle and *Strongyloides*-like eggs as these were the major groups observed. The presence of coccidian oocysts (0 or 1) was also noted, but they were not counted due to often innumerable numbers.

An established, validated PCR protocol was used to detect *Trypanosoma* protozoan haemoparasites in peripheral blood samples (Botero *et al.* 2013, Dunlop *et*

al. 2014, Hing *et al.* 2016). Extraction and PCR amplification of whole genomic DNA of *Trypanosoma* was carried out using a DNA Blood Kit (Qiagen, Hilden, Germany). The positive control was derived from a known stock, and the negative control contained neither blood nor tissue.

Statistical analyses

We used a linear mixed effects model to investigate the question: Did translocation influence woylie stress physiology? The response variable was FCM concentration (pg/g), which was log-transformed to fulfil the model assumptions of data conforming to a normal distribution. Fixed effects included translocation and host factors that may also influence FCM: stage of translocation (before, at or after), site (Perup Sanctuary, Warrup or Walcott), residence status (resident or translocated), sex (male or female) and body condition index. As in previous studies, body condition index was derived as the residuals from a regression of head length to weight, it was calculated separately for males ($p=<0.001$, co-efficient=20.41, $R^2=0.17$) and females ($p=<0.001$, co-efficient=23.42, $R^2=0.23$) and adjusted for pouch young size in females (as pouch young size contributes to weight) (Northover *et al.* 2015). Woylie identification was included as a random effect in all models, as some individuals (n=20) were caught in more than one trapping session.

We also used linear mixed-effect models to investigate whether parasite infection patterns influenced the stress physiology of translocated woylies at translocation and after translocation (as this compares results immediately prior to the translocation and after the translocation). Fixed effects were: stage of translocation, ivermectin treatment, strongyle egg count, *Strongyloides*-like egg count, the presence or absence of coccidian oocysts and trypanosome status. *Strongyloides*-like and strongyle egg counts were scaled prior to analysis using 'rescale' in the R package 'MASS' (Ripley *et al.* 2015). The model was re-run with and without outlier results for *Strongyloides*-like egg counts.

The maximal models for both sets of analyses included all fixed effects and their two-way interactions. To determine the minimal adequate models, we undertook model simplification by step-wise reduction, removing non-significant terms from the maximal model until further model reductions resulted in significant changes in model deviances (Crawley 2007). Significance ($p \leq 0.05$) was tested in a likelihood ratio test (χ^2). Models were run in R 3.1.0 (Bates *et al.* 2015) using the packages 'lme4' (Bolker 2015) and 'car' (Fox and Weisberg 2011).

Results

Host and translocation factors influencing FCM

In both translocated and resident woylies, FCM was significantly higher after translocation (December 2014) compared to before (March-May 2014) or at translocation (June 2014) (Table 7, Figure 17a). Specifically, after translocation there was no significant difference in mean log-transformed FCM between resident (3.30 ± 0.06 pg/g, n=90) and translocated woylies (3.37 ± 0.10 pg/g, n=23). An

interaction between site and stage also had a significant influence on FCM, before translocation, the mean FCM at each site varied with the highest mean FCM at Walcott, moderate at Warrup and the lowest at Perup Sanctuary.

Host factors also influenced FCM in both resident and translocated woylies (Table 7). After translocation, there was a significant negative relationship between FCM and body condition index (BCI), with higher FCM associated with lower BCI (Figure 17b), while at other time-points there was no significant relationship between BCI and FCM. Sex had a significant influence on FCM, both on its own and in interactions with site and stage (Figure 17c, d). At translocation, males and females had similar mean FCM, whereas before and after translocation females had higher mean FCM. Across all sites, FCM was higher in females than in males (Figure 17d).

Parasite factors influencing FCM

One or more *Trypanosoma* species was detected in 46% (103/223) of the blood samples included in this study. Strongyle and *Strongyloides*-like eggs were ubiquitous in woylie faecal samples, with egg counts up to 393 and 154 eggs per gram of faeces respectively. Coccida oocysts were detected in 16% (35/223) of faecal samples.

Parasite factors were related to FCM in translocated woylies (Table 8). At the time of translocation, *Strongyloides*-like egg count increased as FCM decreased, but there was no relationship between *Strongyloides*-like eggs and FCM after translocation (Figure 18a). Including or omitting outlier values for *Strongyloides*-like egg counts did not alter which variables were identified as significant.

Interactions between parasite types also influenced FCM in translocated woylies. Mean FCM was lowest when both trypanosomes and coccidia were detected, compared to when either or

neither were detected (Figure 18b). When trypanosomes were detected, there was a significant negative relationship between strongyle egg count and FCM (Figure 18c). Anti-parasitic drug treatment did not significantly affect FCM. After translocation, there was no significant differences between the mean log-transformed FCM of woylies treated with the anti-parasitic (3.15±0.14 pg/g, n=12) and untreated woylies (3.56±0.13pg/g, n=11).

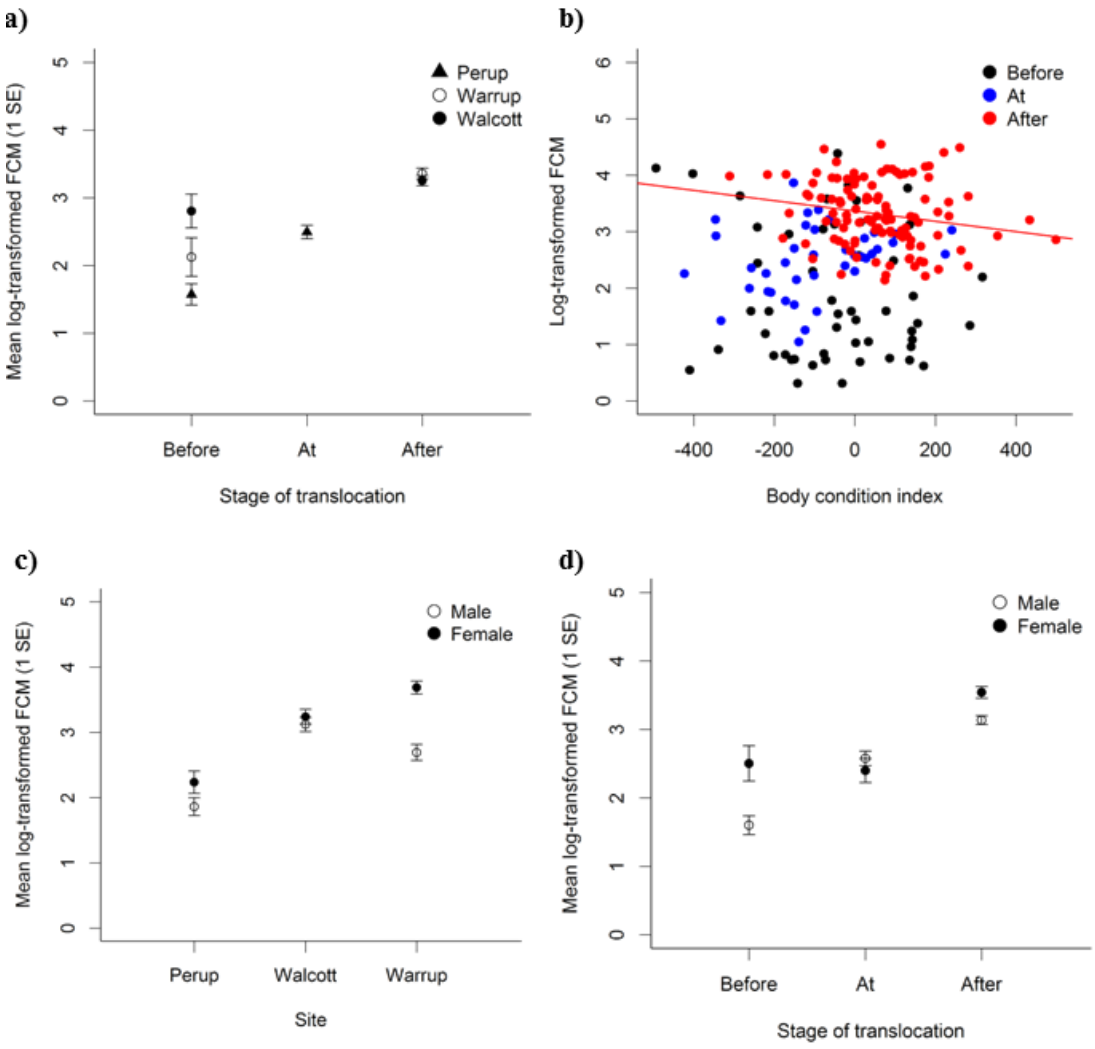


Figure 17. Host and translocation factors influencing FCM (log-transformed) in woylies.Note: resident and translocated individuals are pooled together because residence (translocated versus resident) had no significant effect on FCM.

Table 7.Summary of the minimal adequate linear mixed effects model of host and translocation factors influencing FCM (log-transformed). Translocated and resident woylie combined (there being no significant difference between them). Asterisks (*) indicate significant terms.

Fixed effects		**Coefficient**	**SE**	**Df**	χ^2	**p-value**
Site*	- Perup Sanctuary	-1.187	0.296	2	38.892	<0.001
	- Walcott	2.027	0.422			
	- Warrup	1.775	0.379			
Stage*	- Before	-0.770	0.292	2	60.074	<0.001
	- At translocation	0.675	0.369			
	- After	0.233	0.305			
Sex*	- Male	-0.674	0.253	1	20.379	<0.001
	- Female	0.234	0.196			
Body condition index		-0.002	0.001	1	3.512	0.061
Site:stage*		-0.752	0.353	1	4.541	0.033
Site:sex*		-0.583	0.442	2	13.189	0.001
Stage:sex*		1.076	0.319	2	23.354	<0.001
Stage:BCI*		0.003	0.001	2	12.393	0.002
Sex:BCI		0.001	0.001	1	3.157	0.076

Table 8. Summary of the minimal adequate linear mixed effects model of parasite factors influencing FCM (log-transformed) in translocated woylies

Fixed effects	**Coefficient**	**SE**	**Df**	χ^2	**p-value**
Strongyle egg count	-0.108	0.145	1	1.827	0.176
Strongyloides-like egg count*	-0.440	0.159	1	10.922	0.001
Trypanosome status	-0.519	0.158	1	2.612	0.106
Coccidia status	-0.429	0.237	1	0.051	0.821
Strongyle egg count: Trypanosome status*	-0.904	0.363	1	6.194	0.013
Strongyloides-like egg count:Trypanosome status	-0.419	0.320	1	1.712	0.191
Strongyloides-like egg count:Stage*	4.506	1.350	1	11.134	0.001
Trypanosome status: Coccidia status*	0.998	0.331	1	9.064	0.003

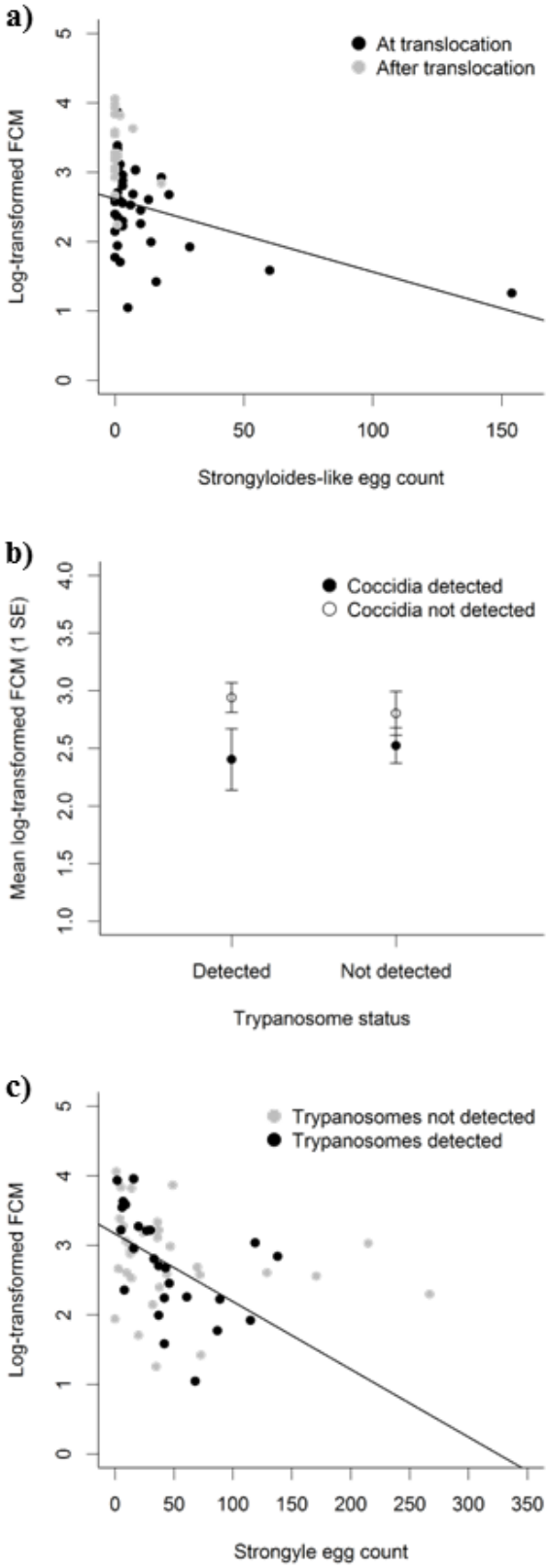


Figure 18. Parasite factors influencing FCM (log-transformed) in translocated woylies

Discussion

We measured FCM and parasite infection parameters in woylies during translocation to help address the paucity of studies measuring stress physiology and infection indices in relation to fauna translocations. We found that FCM in woylies varied significantly during the translocation. In addition, we identified host and parasite factors, such as body condition index and an interaction between micro- and macro-parasites, that were associated with variations in the stress physiology of woylies during translocation.

The influence of translocation on woylie stress physiology

FCM was highest six months after translocation, but there was no significant difference between residents and translocated woylies. These results may indicate that translocation was a stressful experience for both translocated and resident woylies or, alternatively, that they were responding to stressors independent of the translocation. With respect to the first proposition, studies comparing the stress physiology of translocated and resident free-ranging wildlife before and after translocation are rare (Pinter-Wollman *et al.* 2009), however, a transient (days to weeks) stress response to the introduction of new animals has been noted in newly introduced (Patt *et al.* 2012) and resident (Schmid *et al.* 2001) animals. In a study of translocated zebra, following an initial increase, FCM returned to before translocation levels within 11 to 18 weeks, suggesting zebra had acclimated to their new environment (Franceschini *et al.* 2008). In contrast, we did not find evidence for a return to before translocation FCM levels in translocated or resident woylies six months after translocation. It would be concerning if translocation entailed prolonged elevations in glucocorticoids, which can have a negative impact on fitness (Bonier *et al.* 2009)

Alternatively, resident and translocated woylies may have been responding similarly to stressors independent of the translocation, such as seasonal changes (Romero 2002) or exposure to predators (Scheuerlein *et al.* 2001). FCM in captive woylies is significantly influenced by season, although the seasonal peak in captive woylies occurs in the cooler seasons (Hing *et al.* 2016). The peak in FCM observed six months after translocation (December, austral-summer) may also have coincided with increased stress associated with feral cat abundance, which reaches a summer maxima in some parts of Australia (Jones and Coman 1982). The predator stress hypothesis is supported by our finding that before the translocation, mean FCM was lowest in Perup Sanctuary, a feral-predator free fenced area and higher at the destination sites where foxes and cats are present (Yeatman and Groom 2012).

Future study design elements that would improve our understanding of stress physiology in woylie translocations include: sampling of comparative control reference population(s) not influenced by translocations, more complete sampling before translocation to ascertain baseline FCM over time and high frequency serial sampling after translocation. For example, more frequent sampling after translocation would allow assessment of the short-term physiological response to translocation or definition of the time period over which populations return to before translocation FCM levels. Information about woylies' physiological response to population density, intraspecific and inter-specific competition for resources, predation and seasonal changes would also improve our understanding of the relative significance of potential stressors. To determine whether higher FCM after translocation is indicative of biologically significant HPA axis dysfunction, translocated and resident animals could be exposed to experimental stressors, such as a standardised capture-restraint 'hold'(Anderson *et al.* 2015) or administration of exogenous agents to

assess HPA axis negative feedback (Dickens *et al.* 2010). However, this may be logistically and ethically challenging in woylies and other free-ranging wildlife.

The influence of host factors on the stress physiology of woylies during translocation

Body condition

After translocation, not only was mean FCM at its peak, higher FCM was also associated with lower BCI. Links between host stress physiology and body condition are unsurprising given that glucocorticoids, including cortisol, are known to regulate a number of factors that influence body condition, such as energy metabolism, exercise and feeding behaviour (Moberg and Mench 2000). Higher faecal corticosterone metabolites were similarly associated with lower body mass index in translocated European wild rabbits (Cabezas *et al.* 2007). The significant negative relationship between BCI and FCM after translocation (and not before or at the point of translocation), may be indicative of translocation stress (Teixeira *et al.* 2007) or seasonal changes in stress physiology and condition (Mumby *et al.* 2015). For example, seasonal variation in resource availability may influence both BCI and FCM. Ninety percent of the woylies' diet is mycorrhizal fungi (underground truffles). The abundance of these fungi have been shown to diminish in summer (Meney *et al.* 1993) which may have contribute to nutritional stress and diminished body condition six months after translocation (December 2014, austral summer).

Sex

Across all sites, females had higher FCM than males. This pattern is consistent with previous findings in woylies (Hing *et al.* 2016) and other marsupials (Kemper *et al.* 1989, Dowle *et al.* 2012, Narayan *et al.* 2013). It is likely that sex differences are associated with inherent endocrine differences between male and females (Handa *et al.* 1994) as well as sex differences in response to varying population (eg. density) or site attributes (e.g., predators, resources). Given that these patterns in host stress physiology could influence how male and female woylies respond to translocation, it may be pertinent to explore the implications of sex differences for woylie translocation protocols. Indeed, in a comprehensive review of translocation stress, Teixeira *et al.* (2007) suggested that sex differences in stress response to translocation was a neglected area requiring further exploration.

The influence of parasite infection on the stress physiology of translocated woylies

Parasite infection levels were related to FCM in translocated woylies but not as predicted. Parasite richness, or the number of parasite species, has been found to correlate with FCM in other wildlife species (Muehlenbein 2006). Hence, we expected that FCM would be highest when concurrent infections by more than one parasite type were found. Unexpectedly, FCM was lowest when both trypanosomes and coccidia were detected in translocated woylies, compared to when either or neither were detected. We may infer from this that during the present study, woylies were physiologically able to tolerate concurrent infection by likely endemic parasites such as *Trypanosome* and coccidians due to a long shared evolutionary history (St Juliana *et al.* 2014).

There was a significant negative relationship between strongyle egg count and FCM when trypanosomes were detected in translocated woylies. These results may reflect the complex interactions between host stress physiology, immune function and parasite infection (Ezenwa and Jolles 2011). A vital component of immunity to parasites is where T-helper cells coordinate key arms of the immune system (T-helper response) (Spellberg and Edwards 2001). The T-helper 1 (Th1) response is important for defense against micro-parasites (intracellular life stages, such as trypanosomes) and the T-helper 2 (Th2) response is key to defense against macro-parasites (extra-cellular, such as strongyle nematodes) (Spellberg and Edwards 2001). Cortisol and other neuroendocrine HPA axis mediators can shift the immune response from Th1 to Th2 (Padgett and Glaser 2003). Higher FCM in translocated woylies may have favoured a Th2 response, leading to decreased strongyle egg count and a diminished Th1 response, leading to detection of *Trypanosoma.* However, it should be noted that little is known about woylie immune response to parasites (Hing *et al.* 2016), and, in other species, the response is complex and influenced by more than simply the balance between Th1 and Th2 responses (Gossner *et al.* 2012). For instance, further investigation into the complex host, environmental and parasite factors which influence *Strongyloides*-like egg counts in woylies is needed to understand the relationship between and host stress physiology and these parasite parameters.

Lastly, we found no evidence that anti-parasitic treatment alleviated physiological stress associated with parasite infection. This follows from the parallel study on the same translocation event that questioned the benefits of treating woylies during translocation given the lack of apparent benefit to host health (Northover *et al.* 2015).

Conclusion

This study provides rare insight into variations in host physiology and parasite infection parameters during translocation of a critically endangered species. After translocation, we found that FCM was highest and body condition decreased with increasing FCM, which may be indicative of translocation stress or stress associated with factors independent of the translocation. In addition, interactions between parasite types were related to FCM in translocated woylies, which may underscore important relationships between stress physiology, immune function and parasite infection. Our results confirm outstanding hypotheses that stress physiology and infection patterns can change significantly during translocation but further investigation is required to determine how these patterns influence translocation success. An improved understanding of how host stress physiology may change over space, time, and according to factors such as population density and predators would help elucidate how woylies respond to translocation and aid the design of future translocation programs.

Chapter 6 Wildlife in the line of fire: evaluating the stress physiology of a critically endangered Australian marsupial after bushfire

The following is a published paper:

Hing S., Jones K.L., Rafferty C., Thompson R.C.A., Narayan E.J., Godfrey S.S. (2017) Wildlife in the line of fire: evaluating the stress physiology of a critically endangered Australian marsupial after bushfire. *Australian Journal of Zoology* doi: 10.1071/ZO16082

In this chapter, I assess the potential impact of a real-world natural disaster, a bushfire, on woylie physiology and health. Australian native fauna are thought to be well-adapted to fire-prone landscapes but fire may still pose considerable challenges or stressors to wildlife. Unexpectedly a bushfire broke out at one of our study sites, Whiteman Park Reserve and this provided the opportunity to investigate an 'experiment in nature'. In collaboration with the reserve staff, I was able to compare FCM and parasite analyses in woylies before and after the fire.

Introduction

Bushfires are a globally significant abiotic factor influencing wildlife populations (Payne *et al.*, 2014) and are ubiquitous in the Australian landscape where they have influenced the evolutionary history of native fauna and flora (Doherty *et al.* 2015). In addition to direct mortality, fire may entail several proximate stressors including extreme heat and smoke, the necessity to flee (Christensen, 1980), changes to food availability, diet quality and composition (Vernes, Castellano and Johnson, 2001), disruption of social networks (Banks *et al.* 2012), destruction of shelter, and an influx of predators (Torre and Díaz, 2004). Stressors such as these may increase activation of the hypothalamic pituitary adrenal (HPA) axis (Moberg and Mench, 2000). The stress response to fire may vary among taxa, species or populations (Santos *et al.* 2014). In humans, encountering a single fire emergency can result in a considerable stress response (Proulx, 1993) and post-traumatic stress disorder with related neuropsychological and physiological changes (Chen *et al.* 2006). Adult female African elephants (*Loxodonta africana*) have also been found to have higher faecal glucocorticoid metabolites (downstream end-products of HPA axis activation) after a major fire event compared to three months before the fire occurred (Woolley *et al.* 2008). However, there are no studies evaluating the physiological stress response of Australian wildlife to bushfire.

As unexpected and severe stressors can cause immunosuppression and exacerbate infectious disease (Biondi and Zannino, 1997), it is important to understand the physiological stress response of wildlife to fire and the potential ramifications for their health. This is especially relevant for small populations of endangered species that are vulnerable to stochastic events, particularly when they are confined to limited areas (Kaplan Smith, 2000, Legge *et al.* 2008) where the

impact of fire is predicted to intensify with climate change (Lunney, Lunney and Recher, 2008).

Woylies (syn. brush-tailed bettong, *Bettongia penicillata*), a critically endangered native Australian marsupial, have undergone a dramatic and continuing population decline (Wayne *et al.* 2013, Yeatman *et al.* 2016). Remnant native populations are now only found in the south-west of Western Australia, and insurance populations are housed in several predator-proof reserves. While Australian marsupials are thought to be well adapted to fire-prone landscapes, woylies have been observed after fires to demonstrate behavioural responses consistent with shock (Christensen, 1980). Severe and unusual fires have also had devastating effects on small, isolated populations of marsupials including quokka (*Setonix brachysurus*) in south-west Western Australia (Bain, 2016), ringtail possums (*Pseudocheirus peregrinus*) in Sydney, New South Wales (Russell, Smith and Augee, 2003) and koalas (*Phascolarctos cinereus*) in south-west Victoria (Wallis, 2013). Since remaining populations of woylies are small and isolated, and thus vulnerable to stochastic events within a fire-prone landscape (Bryant, 2008), it is pertinent to investigate their response to fire. In addition, it has been suggested that, among other potential drivers, stress-related changes in immune function and exacerbation of infectious disease may play a role in the woylie's decline (Botero *et al.* 2013, Hing *et al.* 2016). Hence, understanding the impacts of fire on woylie stress physiology and population health will aid future conservation management.

An insurance population of woylies encountered a bushfire in December 2014. The fire broke out on December 14th, 2014 in Woodland Park Reserve, a 200ha nature park surrounded by a predator-proof fence in Whiteman Park, Perth, Western Australia. The fire burned out-of-control for up to thirty six hours. Due to

concerns that the fauna within the enclosure would be trapped, emergency gates were opened allowing escape from the fire. By the time the fire was extinguished on December 16th 2014, it had burned approximately 90% of the total area of the reserve (Figure 19).

We aimed to investigate the stress hormone response of woylies to the fire by measuring faecal cortisol metabolites (FCM) before and immediately after the event. Faecal glucocorticoid metabolites (of either cortisol or corticosterone) are commonly used as stress physiology metrics in wildlife and can be measured using minimally invasive methods (Keay *et al.* 2006). We hypothesised that there would be a significant increase in FCM immediately following the fire compared to the months preceding the fire. Recommendations have been made to assess fitness indices (body condition and parasite load) in the context of bushfires (Sutherland and Dickman, 1999). Thus, we also assessed whether health parameters including body condition and parasite load influenced stress physiology before and after the fire. We predicted an overall increase in FCM and an increased impact of body condition and parasite load on host stress physiology after the fire.

Figure 19. a) unburnt and b) burnt areas of Whiteman Park Woodland Reserve. Photographs were taken immediately after the fire (December 16th, 2014).

Materials and methods

Trapping and sample collection

We studied a population of woylies at Whiteman Park Woodland Park Reserve in Perth, Western Australia. Woylies were trapped in June 2014 (n=35) and October 2014 (n=23) as part of an existing study. On December 16th 2014, two days after the bushfire had started and after it had been extinguished, we trapped 19 woylies. Galvanized wire Sheffield traps (220 x 220 x 550mm, Sheffield Wire Products, Western Australia), baited with whole peanuts, were set and checked during the evening (maximum total duration in the trap was not more than two hours). Woylies were individually identified by unique ear tag and microchip code. Animals were weighed, females were checked for pouch young (pouch status), and the size of pouch young (mm) was estimated by palpation of the pouch.

Faecal cortisol metabolite (FCM) enzyme immunoassay (EIA)

Faecal samples were collected from newspaper laid underneath the trap, and (sample volume permitting) two grams was preserved in 10 mL of formalin for faecal flotation, with the remainder stored frozen at -20 °C for FCM assays. All assays could not be completed for every sample (e.g., due to insufficient sample volume), but a total of 77 faecal samples were assayed for FCM, of which 51 were also analysed for parasites.

FCM concentration was measured using an enzyme immunoassay (EIA) previously used for woylies (Hing *et al.*, 2016). In summary, faecal samples (0.2 g dry weight) were lyophilised (freeze-dried), and extraction was carried out using 90% ethanol and heat treatment (80 ^{o}C for 10 min). Extracts were assayed for FCM by EIA using a polyclonal anti-cortisol antiserum R4866 protocol (Narayan *et al.*

2012, Hing *et al.*, 2016). Results were expressed as FCM concentration (pg/g) on a dry weight basis.

Faecal flotation for parasite egg counts

To detect gastrointestinal parasites (nematodes and protozoans), one gram wet weight of faeces was floated for 10 minutes using a concentrated sodium nitrate ($NaNO_3$) solution with centrifugation, after washing (Dryden *et al.* 2005). The area under the coverslip was observed systematically under a BX51 microscope (Olympus, Japan) at 10x objective and eggs were classified as oxyurid, strongyle or *Strongyloides*-like nematodes (as these were the three major groups observed).

Statistical analyses

We used linear mixed effect models to make population level comparisons of FCM, comparing a total of 58 samples before the fire to a total of 19 immediately after the fire. Fitness indices (body condition and parasite load) were also included to investigate how a potential stress response to fire may relate to these variables. To fulfil model assumptions of data conforming to a normal distribution, FCM (the dependent variable) was log-transformed.

Fixed effects included in our model were: month (June/October/December), sex (male/female), body condition index, and oxyurid, strongyle and *Strongyloides*-like egg counts. All two-way interactions between these effects were also included. We checked for collinearity between covariates and all Variance Inflation Factors (VIF) were below 2.5. Body condition index was derived from the residuals of a regression of hindfoot (pes) length to weight, calculated separately for males ($p=0.06$, co-efficient=18.15, $R^2=0.10$) and females ($p<0.0001$, co-efficient=17.55,

R^2=0.37). In the calculation of body condition index, we adjusted for pouch young size in females by including it as a covariate. Woylie ID was included as a random effect in all models to account for repeated measures from the same individuals.

To determine the minimal adequate models, we undertook model simplification by stepwise reduction, removing non-significant terms from the maximal model until further model reductions resulted in significant changes in model deviances (Crawley, 2007). Significance ($p \leq 0.05$) was tested in a likelihood ratio test (χ^2). Models were run using R 3.1.0 with the packages 'lme4' (Bates *et al.*, 2015) and 'car' (Fox & Weisberg, 2011).

Results

Month was the only fixed effect remaining in the minimum model. The month of collection had a significant effect on FCM (co-efficient = -0.40, SE=0.25, df=1, χ^2=6.93. *P*=0.03). However, mean FCM was not higher two days after the fire compared to the preceding months of October and June (Figure 20). Mean FCM concentration before the fire was 6.58±1.17 pg/g in June and 14.60±2.78 pg/g in October. After the fire, the mean FCM concentration was 9.75±1.95 pg/g. There were no significant interactions between the fixed effects considered. Differences in FCM did not relate significantly to sex or body condition (*P*>0.05).

Month was not found to be a significant factor influencing strongyle, *Strongyloides*-like and oxyurid egg counts (*P*>0.05). Mean strongyle egg counts before the fire were 2.4±0.5 in June (n=28) and 4.0±2 in October (n=12) and 0.5±0.3 after the fire in December (n=10). Mean *Strongyloides*-like egg counts before the fire were 5.4±0.9 in June and 10.3±3.9 in October and 0.7±0.3 in December. Mean oxyurid egg counts before the fire were 0.7±0.3 in June, no samples were positive in

October and 13 eggs were found in one faecal sample in December. We did not find evidence for the predicted population-level increase in FCM after the fire nor a relationship between FCM and parasite load.

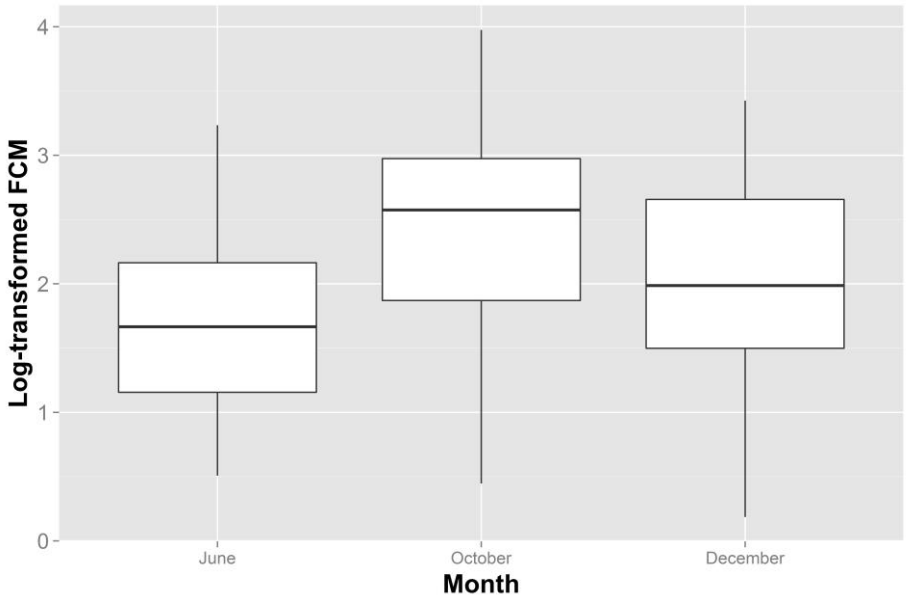


Figure 20. Faecal cortisol metabolite (FCM) concentration (log-transformed) before and after the fire. Bars represent the median, lower (25%) and upper (75%) quartiles and minimum and maximum values). (June n=35, October n=23, December n=19).

Discussion

An acute stress response was predicted given that the woylies would have experienced significant stressors during the bushfire, including extreme temperatures, loss of habitat, and possibly increased exposure to predators. However, we did not find a temporal association between the fire and an increase in FCM. Given the severity of the fire, within two days of the event woylies may have been stressed to a point of allostatic overload, that is, a state where the HPA axis can no longer maintain homeostasis through change (allostasis) (McEwen, 2005). Allostatic overload is associated with HPA axis dysregulation resulting in either hypo- or

hyper-activity of the HPA axis in response to stressors (Dickens, Delehanty and Romero, 2010). However, the absence of peak FCM after the fire is less likely to indicate allostatic overload and HPA axis dysfunction in woylies after fire, as we found no significant relationship between FCM and fitness indices (body condition and parasite load) that may be aberrant in allostatic overload. For example, a permanent reduction in body weight was observed in rats exposed to an experimental stressor (forced restraint) for three days (Harris *et al.* 1998). The woylies were also examined by veterinarians after the fire and found to be in good general health (S. Hing and K. Jones, personal observation, December 16th, 2014).

In this study, the lack of apparent acute effects of fire on FCM and fitness indices is more likely to be associated with adaptations of woylies to fire-prone landscapes (Christensen, 1980). Studies in woylies (Christensen, 1980) and northern bettongs (*B. tropica*), a closely related species, have suggested that bettongs are flexible in their response to fire events (Vernes *et al.* 2001). Woylies in our study displayed previously identified behavioural responses to fire including seeking out unburnt refugia (K. Jones, observation, December 16th 2014, Christensen, 1980). Bettongs are also known to increase foraging activity for a short period immediately following a fire due to increased productivity of fire-attenuated species of mycorrhizal fungi, a major food source (Johnson, 1995, Vernes, Johnson and Castellano, 2004). Johnson (1995) noted that fresh eastern bettong (*B. gaimardi*) diggings appeared as early as two days after an experimental fire, suggesting that bettongs commence increased foraging activity "almost as soon as the fires had gone out". These behaviours may have helped woylies respond to altered conditions after the fire.

In addition, efforts by reserve staff to manage woylies in the period immediately following the fire may have prevented stress from reaching levels where it may compromise their health. Woylies were provided with supplementary feed of herbivore pellets, fruits, hay and vegetables immediately after the fire was extinguished. Invasive predator control was also instigated almost immediately after the fire to minimise the possible impact of predation by cats and foxes on surviving woylies, due to the gates having been opened during the fire (G. Deegan, *pers.comm.*). Indeed, the potential impact of fire may be greater on remnant wild woylie populations exposed to the ongoing threat of feral predators because native Australian fauna facing multiple potential stressors are likely to be more vulnerable to population decline (Narayan, 2015, Narayan and Williams, 2016).

Another possible explanation for an absence of peak FCM after the fire is that a single session of opportunistic sampling was insufficient to capture an acute stress response. This explanation may be less likely given the severity of the fire and the immediacy of the sampling afterwards. However, delays of up to three days between the adminstration of exogenous adrenocorticotropic hormone (ACTH) and a peak in faecal glucocorticoid metabolite concentration have been noted in other marsupial species (Narayan, Evans and Hero, 2014). In a zoo study, samples were collected three times a day for several days in an enclosure in order to detect peak faecal glucocorticoid metabolite concentration in a woylie within four days after ACTH administration (Fanson *et al.* 2015). While such intense sampling is not feasible in free-ranging woylie populations, sampling woylies later than two days after fire may improve our ability to detect a bush-fire response if it is present. A final alternate explanation is that the animals sampled may not have been those individuals most severely affected by the fire. However, this is less likely considering the

pervasiveness of the heat, smoke and influx of vehicles and firefighters (K. Jones, observation, December 16th 2014).

Our study was not able to gauge chronic effects of the fire on woylies and we would recommend long-term FCM monitoring after fire events to shed light on these prolonged effects. A closely related species, the Tasmanian bettong (*B. gaimardi*), has been found to return to pre-fire behavioural activity by four months after fire (Johnson, 1995), and other marsupials such as koalas are also known to recover rapidly after fire (Matthews *et al.* 2016).

Bushfires are an ever present concern to wildlife researchers and managers in Australia, and managing fire is an important consideration in the conservation of woylies and their habitat (Taylor, 1991). However, we found little evidence to suggest that stress-related changes had a negative impact on the health of an insurance population of woylies two days after a major bushfire in their reserve. Our results suggest that woylies may be able to maintain allostasis, at least in the period immediately after a fire, provided that they display the appropriate behavioural responses and are well protected from concurrent stressors during this time.

Chapter 7 General Discussion

7.1 Summary

This thesis offers the first insights into the relationship between host stress physiology, immune function and parasite infection in a critically endangered Australian marsupial, the woylie. These factors have been suggested as potential drivers of the species' decline. Blood and faecal samples were collected from over 300 individual woylies during intensive nocturnal fieldwork (across captive, reserve and wild woylie populations) to provide unprecedented information about the role of stress in wildlife health and conservation. Exhaustive laboratory work including parallel endocrinology, immunology and parasitology analyses found evidence that host stress physiology in woylies is associated with important health parameters including body condition, innate immune function and parasite infection (nematodes and *Trypanosoma* haemoparasites).

The main results of this thesis are briefly summarised here. In **Chapter 1,** I introduced the literature and theories about the relationship between physiological stress and wildlife disease. I highlighted the urgent need to address gaps in our knowledge about how anthropogenic factors (such as management interventions) may influence disease dynamics. I also conducted a comprehensive review to identify key stressors to Australian marsupials because effective management of these species, including the woylie, requires an understanding of their response to ongoing stressors. After introducing the study species and sites in **Chapter 2**, I then moved to the specific context of woylie stress physiology in **Chapter 3**. Factors including season, sex, body condition and parasite status were associated with FCM concentration. Overall, mean FCM was lowest in summer and highest in the -

- cooler months of autumn and winter. Females had higher mean FCM than males. There was a significant but weak negative association between body condition and FCM which is consistent with broader patterns identified in other wildlife studies (Mumby *et al.* 2015). When woylies were shedding oxyurid nematode eggs they had higher mean FCM compared to when they were not shedding.

In **Chapter 4**, I tested the premise of the hypothesis that there are links between stress, immunity and infection in woylies. This study represented a unique One Health collaboration between veterinarians, immunologists and ecologists. I successfully adapted an *ex vivo* assay developed in pre-term human infants to assess a key immunological process, phagocytosis (the engulfment of foreign particles), in the woylie.

In **Chapter 5** and **Chapter 6**, I evaluated how real-world conservation relevant events may influence stress physiology and health parameters in woylies. In **Chapter 5** I focused on woylies' response to translocation, a mainstay of woylie conservation management. Stress physiology and body condition measures after translocation suggested a stress response in translocated and resident woylies at the destination sites, or changes due to factors independent of the translocation for example encounters with introduced predators.

In **Chapter 6**, I assessed the potential impact of bushfire on woylie stress physiology, health and conservation. Unexpectedly, FCM was not found to be higher immediately after the fire compared to the months previous nor were there changes in the woylies' condition or parasite load. I hypothesise that this may be associated with woylies' adaptations to fire prone landscapes. It is very unusual to be able to opportunistically sample wildlife, particularly critically endangered species, immediately following a major bushfire.

Thus, overall this thesis provides an in depth examination of the stress physiology of woylies in a range of conservation relevant contexts.

7.2 Stress physiology

Stress physiology is widely purported to be a useful tool in wildlife research and management, helping us understand how animals respond to challenges (Cockrem 2005, Jessop *et al.* 2013). In this thesis, I have demonstrated how measurement of FCM can be applied in a real-world conservation context to provide valuable insights for future conservation management. In this way, the project contributes to the development of minimally invasive tools in the emerging and rapidly developing field of conservation physiology (Cooke *et al.*, 2013). However, this research also emphasises that glucocorticoid metrics do not always change with potentially stressful events as we would predict (Busch and Hayward 2009) and stress may not always be immunosuppressive (Martin 2009). In this way, this research raises key outstanding questions about wildlife stress physiology.

What is the ideal time to sample woylies after a stressor in order to detect peak FCM? Findings support suggestions that woylies, similarly to other marsupials, may have a prolonged delay of longer than two days between encountering a stressor and peak FCM (Fanson *et al.* 2015). What constitutes a 'normal' versus potentially detrimental (allostatic overload) (McEwen 2005) stress response in a novel species? It is probable that conducting a longitudinal study and measuring variables of individual fitness including body condition and parasite load helped us interpret FCM results. In future, routine FCM monitoring of a single free-ranging population at regular intervals (and as they encounter various stressors) would help define the limits beyond which stress stops having an adaptive advantage and begins to incur fitness costs (Busch and Hayward 2009). This would also address another outstanding question: whether the same long-term patterns in FCM are seen in free-ranging and wild woylie populations.

Further limitations such as the high costs of the FCM EIA and the logistics of frozen sample transport, could be addressed if a wildlife endocrinology laboratory were to be established in Western Australia. Such facilities could also cater for numerous ongoing wildlife endocrinology studies at various universities in Perth and service government and non-government organisations interested in assessing the stress physiology of the state's unique and threatened fauna.

7.3 Immunology

Effective host immune function is essential to organisms' defenses against parasites and pathogens. However, despite their vulnerability to the impact of infectious disease, few studies test the immune competency of wildlife (Pedersen and Babayan 2011), particularly endangered species including Australian marsupials (Kreiss *et al.* 2008). This thesis provides the first immune function study in the woylie and supports previous studies that suggest an immunological challenge may have played a role in the woylies' decline (Pacioni *et al.* 2013). Overall, it was encouraging to find that woylies have competent phagocytes which challenged assumptions about marsupials 'rudimentary' immune system (Jurd 1994). Nonetheless, in terms of woylie conservation, this study raises several important future questions.

We are yet to investigate the health consequences for woylies when both the percent of phagocytosing leukocytes and phagocytosis index (a measure of the functional efficiency of phagocytes) are low. In addition, the functional efficiency of the woylies' phagocytes may be influenced by host stress physiology and *Trypanosoma* haemoparasite infection. As it is feared that woylies in declining populations may be particularly susceptible to pathogenic *Trypanosoma* spp. (Botero *et al.* 2013), applying the methods used in this thesis on blood samples from a free-

-ranging population is recommended.

As practical and ethical limitations may preclude *in vivo* experiments, further development of *ex vivo* techniques (for example the development of species specific reagents) may allow for further investigation. The logistics and timing constraints inherent in live cell assays make broad-scale application of the assay we adapted difficult in woylie field conservation. However, if the necessary equipment and human resources were available then these and other immunoassays in future could answer outstanding questions about woylie immunology including: what factors influence baseline immune function parameters and does immune function in declining populations vary in comparison to stable populations?

7.4 Parasitology

I identified evidence of infection by a variety of endoparasite in woylies including *Trypanosoma* haemoparasites and a range of nematodes. Many of these parasites remain to be identified to a species level, which would help us further our understanding of the relationship between stress and polyparasitism, a phenomena which may influence individual fitness (Ezenwa *et al.* 2010). A central question raised by this research pertains to cause versus effect, is the relationship between FCM and parasite status the result of the stress of infection or vulnerability of stressed individuals to infection? Practical and ethical considerations limit the opportunities for experimental studies in critically endangered species such as the woylie but this thesis demonstrates how conservation interventions and unexpected events, as well as parasite treatment experiments (Hing *et al.* 2017, Northover *et al.* 2015), may serve as opportunities to test cause versus effect.

Further experimental studies or causal component models are required to understand interactive effects among different parasite types and host stress physiology. While it is possible that the interactive effects of different parasite types on immune function and FCM identified in **Chapter 3** and **Chapter 5** reflect the influence of stress physiology on different arms of the immune system responsible for defence against micro-parasites and macro-parasites, reconciling these findings will require further investigations into causal pathways. In addition, quantitative values for parasite load could be used to investigate whether host stress physiology influences parasite load or vice versa. Means to quantitate trypanosomes in woylies are not available at this time but may become available for use in future studies.

7.5 Woylie conservation

My research supports existing studies which found associations between declining woylie populations and immunological challenges (Pacioni *et al.* 2013) and infection with potentially pathogenic *Trypanosoma* haemoparasites (Botero *et al.* 2013, Thompson *et al.* 2014). During periods of *Trypanosoma* parasitaemia, woylies displayed immune function variables that indicate they may be more vulnerable to the immunosuppressive effects of glucocorticoids or, alternatively, that stress physiology and *Trypanosome* infection may affect the efficiency of leukocyte function. This, together with broader relationships found between FCM and fitness variables (including body condition and some parasite variables), suggests that stress, immunosuppression and infection are relevant to woylie conservation. Hence, woylies' stress response to planned interventions as well as unexpected events should be considered carefully in future species management.

Consideration of factors which influence woylie stress physiology may help improve the outcomes of conservation management and research, for example to aid decisions about the timing of interventions and the handling of certain cohorts such as female woylies which may be more immunoreactive to the effects of glucocorticoids. Management interventions such as translocation may influence woylie stress physiology while they may be more resilient in response to other stressors to which they have a long evolutionary history, such as bushfire. Understanding the relationship between stress and health parameters including infection dynamics, is of importance to woylie conservation because it will help us understand the potential consequences of ongoing stressors and conserve species even under challenging conditions.

7.6 Conclusion

This thesis highlights the importance of the relationship between stress, immune function and infection in wildlife health and conservation. In human medicine stress is now well accepted as a key epidemiological driver and it is deemed insufficient "to deal with stress-related illness and ignore the conditions in which people live and work that gave rise to it" (Marmot 2007). This paradigm also applies to wildlife health. It is important that wildlife management takes consideration of how the conditions in which animal live influences their physiology, immune function and health. For worldwide, wildlife are under threat and the unique native fauna of Australia, in particular, is besieged by multiple stressors (Woinarski *et al.* 2015) which may influence their immune function and the impact of infectious disease (Aguirre and Tabor 2008). It is our duty as the ultimate cause of many of these stressors and also as custodians of the environment, to ensure healthy wildlife populations persist in the face of ongoing stressors.

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