Understanding the physiological impacts of stress on the Australian marsupial species, the Koala (*Phascolarctos cinereus*), within New South Wales and South Australia

By: Miss Renae Charalambous (17724337)
Principal Supervisor: Dr. Edward J Narayan

Western Sydney University
Master of Research
Submitted June 2019

Photograph by Miss Renae Charalambous
The work presented in this thesis, to the best of my knowledge and belief, is original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

Miss Renae Charalambous
Dedications

This thesis is wholeheartedly dedicated to all the koalas across Australia who desperately require our help to safeguard their survival.
Acknowledgments

I would like to take this opportunity to express recognition to the people, where if not for them, this project would not have been possible. To my supervisor Edward, thank you for encouraging me to pursue this project. Thank you for providing guidance when I was lost, support when I was defeated, and most of all, for all you have taught me; because of you, I am now rich. I look forward to continuing our research with the aim of helping those who need us. To everybody at Port Stephens Koalas, Port Macquarie Koala Hospital, Friends of the Koala and the Adelaide Koala and Wildlife Hospital, thank you for everything you have done, and continue to do for koalas. If not for your generosity, open mindedness and amazing wealth of records collected over the years, this project would have been impossible. Your continued dedication to wildlife astonishes me. Furthermore, your willingness for me to visit your facilities and your disposition to share all you know has completely exceeded all expectations. I look forward to hopefully collaborating again in the future. To my partner Neil Doszpot, thank you for absolutely everything you do. It would not have been possible for me to complete this thesis if not for your endless support, whether you are encouraging me to keep going when I lose focus, or to take a break when I am burnt out, you always know how to make a bad situation good again. I love you. To both the Charalambous and Doszpot families, thank you for all of your support throughout the last two years of this degree. It has not been easy, but I thank you all for showing me love and support when I needed it most. I love you all. To Jack Nakhoul, thank you for all your help in the laboratory. To Russell Thompson, thank you for all of your help with statistics. To Matthew Van Leeuwen, thank you for all your help with proof reading, for answering all the questions I have thrown at you, and for always supporting me when I need it most. To my dogs, Chester and Rhuben, thank you for always keeping me sane. To my two best friends Alannah Cain and Jadan Hutchings, thank you for always, always being there for me when I need anything at all. Lastly, thank you to all of my friends for making this journey worthwhile, you are all amazing.
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Chapter 1: Literature Review

Koalas in Australia

Koalas (*Phascolarctos cinereus*) are arguably one of Australia’s most iconic species and attract many tourists to Australia every year, with tourism both directly and indirectly contributing to conservation programs (Green and Higginbottom, 2000). Despite their popularity, koalas have experienced steady population declines over the last 18-24 years (Woinarski and Burbidge, 2016). In 2018, over their 1.5 million square kilometre geographic range, it was estimated that between 47,860 and 85,695 koalas remained in the wild (Figure 1) (Australian Koala Foundation 2018). Koalas are found in a range of habitats such as coastal islands, tall eucalypt forests, and low inland woodlands across Queensland, New South Wales, South Australia and Victoria (Figure 2) (Ellis et al., 2002, Seabrook et al., 2011). To be suitable koala habitat, there needs to be A) the presence of Eucalyptus tree species preferred by koalas B) the presence of other koalas (Ellis et al., 2002, Seabrook et al., 2011).

![Figure 1: 'Koala numbers by Federal Electorate with Potential Habitat' detailing the number of koalas remaining within their geographic range throughout Australia (Australian Koala Foundation 2018).]
Figure 2: ‘Koala Habitat Atlas’ detailing the geographic range of koalas throughout Australia (Australian Koala Foundation 2018).

Despite relying on a very dominant and widespread species of gum tree for survival, koalas are currently listed as “vulnerable to extinction” by the IUCN Red List of Threatened Species in their resident Australian states (New South Wales, Queensland, South Australia and Victoria) (Figure 3) (Woinarski and Burbidge, 2016).

Figure 3: IUCN Red List of Threatened Species Scale (least concerned, near threatened, vulnerable to extinction, endangered, critically endangered, extinct in the wild, extinct).
The vulnerable conservation status of the koala has been highly contested in the past due to the uncertainty of relevant population parameters and the marked variation in population trends across Australia (Melzer et al., 2000). In 2019, “The Australian Koala Foundation” claimed that the species was “functionally extinct” (AKF, 2019). The term “functionally extinct” can refer to a species whose population has declined to the point where it can no longer play a significant role in their ecosystem (Adams-Hosking, 2019). Additional definitions of “functionally extinct” includes a population that is no longer viable; where there are not enough individuals left to reproduce, or a population that is so small they suffer from inbreeding (Adams-Hosking, 2019). There is no scientific evidence to claim whether koalas are actually functionally extinct, however Australian biodiversity remains subject to change due to the progression of climate change and an increasing human population, and it is expected that due to this, koala populations will continue to decline over the next 20-30 years (Melzer et al., 2000, Woinarski and Burbidge, 2016).

Population Ecology and Conservation

There is clear evidence that recent global climate change trends have a significant impact on ecosystems and the species that live within them (Hughes, 2003). Understanding the biological and physiological implications of these impacts is important when considering the extent of a species ability to survive the stress of climate change (Narayan and Williams, 2016). To put this in perspective, Australia has warmed ~0.8°C over the last century, with minimum temperatures rising faster than maximum temperatures (Hughes, 2003). Global climate predictions suggest that the bioclimates of some species of plants and vertebrates may disappear entirely with as little as 0.5-1.0°C of warming (Hughes, 2003). An examination of 5 greenhouse climate scenarios was performed on the distribution of 42 vertebrate species with a threatened conservation status within the south-east of Australia (Brereton et al., 1995). The 5 greenhouse climate scenarios included the following: A) a temperature increase of 1.0°C, a summer rainfall increase of 5% and a winter rainfall decrease of 5%; B) a temperature increase of 2.0°C, a summer rainfall increase of 7.5% and a winter rainfall decrease of 7.5%; C) a temperature increase of 3.0°C, a summer rainfall increase of 10% and a winter rainfall decrease of 10%; D) a temperature increase of 3.0°C, and an overall rainfall increase of 10% in all months; E) a temperature increase of 3.0°C, and an overall rainfall decrease of 10% in all months.
The result of this examination predicted that under all 5 scenarios, there would be range reductions for 41 of the 42 vertebrate species, with 15 vertebrate species predicted to have no suitable bioclimatic available in south-east Australia (Brereton et al., 1995). Furthermore, under enhanced greenhouse scenarios, a temperature rise of 3.0°C with less than 10% increased rainfall is predicted to have the most damaging impact on the south-east Australian bioclimate and the species within it (Brereton et al., 1995). The stress of global climate change is having profound consequences on the ability of ecosystems and the species within them to adapt fast enough to ensure their survival (Brereton et al., 1995, Hughes, 2003, Narayan and Williams, 2016). A recent example of ecosystems and endemic species not having the ability to adapt quickly enough to the impacts of climate change can be seen when looking at the Australian Great Barrier Reef. Under conditions expected in the 21st century, global warming and its influence on ocean acidification is expected to compromise carbonate accretion which will reduce coral cover and survivorship on reef systems (Hoegh-Guldberg et al., 2007). The stress of climate change, declining water quality and overexploitation of key species has, and will continue to result in the loss of diversity in reef communities with systems moving increasingly towards the point of functional collapse (Hoegh-Guldberg et al., 2007).

Often, the species which are struggling to adapt and survive in the wild as a result of climate change are housed in captivity for breeding and recovery purposes (Morgan and Tromborg, 2007). There are many challenges associated with keeping wildlife in captivity before releasing them back to the wild that are known to influence stress (Morgan and Tromborg, 2007). The primary challenge of housing animals in captivity for rehabilitation involves the prevention of an animals engagement with species-typical behaviours (Morgan and Tromborg, 2007). In more detail, complications of captivity that prevent these behaviours can be abiotic (artificial lighting, loud or aversive sounds, arousing odours, uncomfortable temperatures and substrates), where others are confinement specific (restricted movement, reduced retreat space, forced proximity to humans, reduced feeding opportunities, abnormal social groups) (Morgan and Tromborg, 2007). Once an animal has undergone rehabilitation, translocation is used to return that animal to the wild (Molony et al., 2006). There are many challenges associated with translocation including the fact that rehabilitated individuals can have a low chance of survival.
following their release (Molony et al., 2006). Factors that affect post-release survival include 1) stress associated with handling and moving the animal to a new location, 2) pre-release conditioning to avoid predators and identify suitable food sources, 3) suitability of the release location (i.e. the initial site may still contain the direct threat that lead to the animal being held in captivity) (Molony et al., 2006).

There is a very delicate balance to account for when assisting with wildlife exposed to climate change (Morgan and Tromborg, 2007, Molony et al., 2006). If an ecosystem cannot sustain an animal species, they are removed and housed in captivity until they are able to be released. However, there are stressors associated with both remaining in a climate change affected ecosystem, and being held in captivity pending release (Morgan and Tromborg, 2007, Molony et al., 2006). Wild koalas are overly sensitive to human handling, especially when already subject to sub-clinical stress of environmental trauma and disease (Obendorf, 1983). A study was performed to measure stress in koalas in response to frequent photography and handling by tourists (Webster et al., 2017). Elevated levels of stress were identified in male, but not female koalas in response to frequent photography (Webster et al., 2017). In response to handling by tourists, most koalas across a range of life-history stages had exhibited elevated stress levels, with joeys, pregnant or lactating females, and some males particularly affected (Webster et al., 2017). In order to achieve successful rehabilitation, it is integral that stress is not exacerbated for koalas in captivity, and that they are not exposed to additional pressures from humans.

A study was performed on Asiatic black bears (Ursus thibetanus) who were rescued from a bear bile farm to investigate whether stress in animals contained in poor captive environments could be reduced over time through rehabilitation (Narayan et al., 2018). Stress hormones were measured at the time of the bears' rescue and then compared after 22 weeks of living in a sanctuary where environmental enrichment and veterinary care were provided to improve animal welfare (Narayan et al., 2018). Results concluded that most of the rescued bears still exhibited stress responses after they were rescued, but they were able to modulate this response to lower their stress in ideal conditions through humane care (Hing et al., 2016, Narayan et al., 2018). Koalas also have the capability to
be rehabilitated through appropriate clinical care however it can be a very complex and long-term process. This will be discussed throughout the upcoming chapters.

It is known that glucocorticoids play a large role in the neuroendocrine stress system, allowing animals to respond adaptively to a diverse range of acute, physical, social and environmental stressors (Delehanty and Boonstra, 2009). However, since the system responsible for responding to stress is closely tied to the physiological controls of reproduction and immunity, an animal's ability to respond adaptively is compromised under conditions of chronic stress such as a changing climate or being held in captivity (Delehanty and Boonstra, 2009). Being able to understand stress is imperative so that the risk that vulnerable wild and captive animals face is known, and from there we can help them in their quest for survival.

What is Stress?

There is no current definition of stress, both acute and chronic, that is not contentious. Acute stress can be described as a change in the psychological, physiological and/or physical wellbeing of an animal (Hing et al., 2016). These changes are often brought about by capture and handling, limited food resources, or the presence of an imminent threat such as a predator (Hing, 2016). Exposure to an acute stressor is not necessarily fatal, and is not known to directly jeopardise an animal's survival (Adamo et al., 2013). Acute stress is managed by executing a response known as 'fight or flight', which induces an animal's metabolic rate in order to manage the immediate threat (Adamo et al., 2013).

On the contrary, chronic stress can be described as an exceptional event that acts as a challenge to an animal's regular capacity (Hing et al., 2016). Chronic stress distorts an animal's allostasis (process of achieving homeostasis), moving it substantially away from an optimal point of homeostasis (ability to adjust to maintain internal constancy) (McEwen and Wingfield, 2010). Chronic stress can become fatal in the long term, and more recently has been known to jeopardise an animal's survival through limited growth and reproductive abilities, and additionally, the onset of disease (Johnstone et al., 2012, Narayan and Williams, 2016). Without intervention, the consequences of chronic stress can become irreversible. The decision of whether or not a perceived situation is stressful is determined by that animals sensory input (the things they see and hear) as well as their
memories (what happened last time they encountered a similar situation) (Hing et al., 2016). For the purpose of this literature review, acute stress can be defined as a sensory input stemming from a change in wellbeing, whereas chronic stress is when stressors persist to the point of inhibiting the body from regulating homeostasis. The most common stressors effecting wild animals in Australia are primarily involved with changes to the environment (Narayan and Williams, 2016). The most dominant environmental changes effecting koalas include the introduction of non-native and invasive species to an area such as dogs, fragmenting native habitats through the implementation of roads, agriculture and housing, and climate related issues such as fire and drought (Narayan and Williams, 2016).

The introduction of non-native and introduced species is well-recognised as a challenge to biodiversity conservation (Butchart et al., 2010). Among the non-native and introduced species, those who are domesticated (such as dogs and cats) pose the greatest conservation challenges of all, due to their close association with humans (Home et al., 2018). Dogs are ubiquitous in most terrestrial landscapes with global populations estimated to be close to a billion (Home et al., 2018). Additionally, the biological traits and broad morphological adaptability of dogs allow them to occur at densities higher than any other similar sized wild carnivore (Home et al., 2018). Due to their close relationship with humans, dogs engage in free-ranging behaviour which allows them to interact with wildlife in a number of potentially dangerous ways including direct predation, fear-mediated behavioural changes, competition and disease transmission (Home et al., 2018). For koalas, dogs present as a stressor as they continually appear in native koala habitat, and act as a risk to their population through unnatural predation.

Fragmenting native habitats is occurring at an unprecedented speed and scale, but is a direct response to several human-induced changes in the global environment including the implementation of roads, agriculture and housing (Said et al., 2016). Habitat fragmentation is a landscape level process in which a specific habitat is progressively subdivided into smaller, isolated fragments with altered adjacency patterns and spatial characteristics (Said et al., 2016). Essentially, when habitat is reduced through fragmentation, the amount and connectivity of suitable wildlife habitat is limited (Brearley et al., 2013). This has been shown to affect population viability, lower genetic
diversity, and cause inbreeding depression which can make individuals more susceptible to existing or novel diseases and stochastic environmental events (Brearley et al., 2013). As the human population continues to grow exponentially, natural ecosystems will be increasingly fragmented as they are converted into roads, space for agriculture and houses (Cheptou et al., 2017). For koalas, habitat fragmentation presents as a stressor as this species is continually forced to adapt and change around an increasing human population. This means that the survival of koala populations will be further jeopardised as their habitat is continually fragmented.

Climate related issues such as fire and drought pose as a particularly lethal stressor for koalas (Lunney et al., 2014). Analyses of climate trends over the last five decades (1960-2009) show that the mean Australian temperature has increased by ~0.7°C and is projected to rise by 0.6-1.5°C by 2030 (Lunney et al., 2014). An understanding of how animal and plant species alike will respond to this temperature rise, and if they will be able to adapt to ongoing climate change is important (Adams-Hosking et al., 2011, Adams-Hosking et al., 2012). A term called ‘refugia’ refers to a core region where animals and plants are able to persist during a time of change and when their wider geographic range will become uninhabitable (Adams-Hosking et al., 2011). However, if climate change persists, refugia may not be a viable possibility for some species, especially those who rely on a single tree species for their survival (such as koalas). It is believed that animals and plants who belong in cooler locations are less susceptible to climate change than animals and plants who belong in warmer locations (Ashcroft, 2010). Occupying the east-coast of Australia, koalas live in both cooler and warmer locations of the country, meaning climate change is a potentially lethal stressor for local populations with limited refugia (Ellis et al., 2002, Seabrook et al., 2011).

In some situations, stressors can be managed so that they are merely acute, and the animal can manage them quick enough so as not to cause serious damage to their body. However, it is when these stressors continually persist that they prevent the body from regulating homeostasis, and this can be referred to as chronic stress (Narayan and Williams, 2016).
The Stress Response

The stress response is a reaction to an unpredictable, uncontrollable and/or aversive stimulus (also known as a stressor) (Beehner and Bergman, 2017). When the stress response is activated, the hypothalamic-pituitary-adrenal (HPA) axis begins releasing a cascade of hormones tasked with the job to manage said stressor (Narayan and Williams, 2016) (Figure 4). This process begins with a small neuroendocrine structure called the hypothalamus, which is situated above the brainstem, releasing corticotrophin-releasing hormone (CRH) (Tasker and Herman, 2011, Narayan and Williams, 2016). The release of CRH signals the anterior pituitary, a small hormone-secreting gland that sits below the hypothalamus, to release adrenocorticotrophic hormone (ACTH) (Tasker and Herman, 2011, Narayan and Williams, 2016). When ACTH is released, it begins circulating in the blood, and the result is an increased output of glucocorticoids from the adrenal cortices (also known as the adrenal glands) (Narayan and Williams, 2016). Glucocorticoids then act to assist metabolically and behaviourally by diverting the storage of glucose to fat so as to instead partition energy by supplying it to all parts of the body for the upcoming challenge (Narayan and Williams, 2016). Additionally, the production of glucocorticoids will assist in balancing pH after the challenge, and also acts a chemical blocker within the negative feedback process to CRH secretion and HPA axis synergy (Narayan and Williams, 2016).

The function of the HPA axis in response to stress comes at the cost of diverting energy away from other corporal bodily functions (Narayan and Williams, 2016). If the stressor experienced is acute (short lived), then the body can return to homeostasis through a negative feedback mechanism (Beehner and Bergman, 2017, Narayan and Williams, 2016). On the contrary, if the stressor experienced is chronic (persists for an extended period of time), then the body is stuck in a state where resources that are integral for survival are continually diverted in an attempt to keep the HPA axis activated (Beehner and Bergman, 2017, Narayan and Williams, 2016). Ongoing stress can diminish the feedback loop and lead to over expression of glucocorticoids (Narayan and Williams, 2016). As a result, chronic stress has been found to inhibit growth, reproduction and function of the immune system (Narayan and Williams, 2016).
Figure 4: A diagram outlining how the hypothalamic-pituitary-adrenal axis functions in response to stress. Once a stressor has been sensed, the hypothalamus produces corticotrophin-releasing hormone, which then signals the anterior pituitary to produce adrenocorticotropic hormone, which then signals the adrenal cortices to produce glucocorticoids. The negative feedback loops assists in this process by signalling the anterior pituitary and hypothalamus to reduce their own secretion.
The Physiological Impact of Stress

The stress response operates like a classic life history trade-off: aim, to prioritise present energy usage over future energy storage (Wingfield and Sapolsky, 2003). For this reason, the stress response is adaptive over the short-term but unsustainable over the long-term (Beehler and Bergman, 2017). Generally speaking, activation of the HPA axis and exposure to chronic stress has been proven to subsequently decrease an animal’s health and longevity (Juster et al., 2010). This is because chronic stress has shown to be connected with a number of adverse health effects and disorders, most likely the result of the reallocation of resources during the stress response, as the body gives higher priority to survival over maintenance (Sapolsky, 2004, Whirledge and Cidlowski, 2010). Some of the major negative health implications associated with chronic stress include the suppression of growth, reproduction and function of the immune system (Lattin and Romero, 2014).

When stress is encountered and the HPA axis has been activated, the function of genes relating to growth often become suppressed through the repression of hormones (Donatti et al., 2011). Lack of one particular hormone called “Insulin-Like Growth Factor-1” (IGF-1) has proven to reduce offspring growth both during and after gestation (Donatti et al., 2011). Chronic stress can suppress the production of IGF-1, the hormone responsible for bone related growth, and this hormone is important in the formation of skeletons, in particular promoting longitudinal femur growth (Yan et al., 2016). Chronic stress has also been shown to reduce the growth of in utero offspring and their body weight after birth through the suppression of IGF-1 (Emack et al., 2008). Reduced growth is consistently obvious in in utero males compared to females with chronically stressed mothers (Emack et al., 2008). It is presumed that this is due to males exhibiting an overall higher growth rate than females, making it possible that males are more vulnerable to insult during gestation (Emack et al., 2008). Furthermore, reduced body weight after gestation as a result of maternal stress can be critical to the survival of offspring through increased evolutionary disadvantages such as a decrease in species specific biological fitness (Emack et al., 2008). The negative consequences of excessive glucocorticoid production on growth in koalas can cause stunted development through the production of IGF-1 which is required for survival (Emack et al., 2008).
Activation of the HPA axis in response to stress has been shown to influence immune function through increased morbidity and mortality (Brearley et al., 2013). This is concerning as stress exacerbates the impact of disease on animals who may already be struggling for survival (Hing et al., 2016). Disease in response to stress can be explained through the production of glucocorticoids in response to activation of the HPA axis (Hing et al., 2016). Glucocorticoid production can have profound physiological effects on an animals immunological process via the receptors on immune cells and changes in immune gene expression in target tissues (Hing et al., 2016). Glucocorticoids influence the trafficking of leukocytes (white blood cells responsible for fighting infection) and suppresses the secretion of proinflammatory cytokines (regulators of inflammation as a response to infection to heal and repair) (Chrousos, 2009). The pathogenesis of chronic stress related disease can be summarised through the sustained, excessive secretion of multiple homeostatic systems (Chrousos, 2009). These diseases represent the effects of two physiological processes whose mediators are supposed to be secreted in a quantity-limited and time-limited fashion, but have instead gone awry (Chrousos, 2009). The negative consequences of excessive glucocorticoid production on disease in koalas include inflamed tissues or systemic infection, anti-chlamydial antibodies as a sign of infection, organ dysfunction and clinical signs of chlamydiosis (fatal if left untreated) (Grogan et al., 2018).

Additional to increased morbidity and mortality, and decreased growth, activation of the HPA axis in response to stress has been shown to inhibit reproduction (Chrousos, 2009). Precise levels of glucocorticoids are required for proper reproductive function, and if this balance is disrupted, so is fertility (Whirledge and Cidlowski, 2010). When stress is encountered in females, the production of glucocorticoids inhibits the release of gonadotropin-releasing hormone (GnRH) (Whirledge and Cidlowski, 2010, Wingfield and Sapolsky, 2003). GnRH is the hormone responsible for the release of follicle-stimulating hormone (FSH) (FSH stimulates the ovarian follicle, causing an egg to grow), and luteinizing hormone (LH) (LH initiates ovulation by releasing the egg), both key parts of reproduction (Whirledge and Cidlowski, 2010, Wingfield and Sapolsky, 2003). The impact of glucocorticoid production on reproduction results in an extended follicular stage, making the overall reproductive cycle length longer and/or more irregular (Wingfield and Sapolsky, 2003). Furthermore, uterine maturation can become impaired
during stress by at least two mechanisms (Wingfield and Sapolsky, 2003). Firstly, stress in some species can be associated with a decline in levels of progesterone (progesterone mediates preparation of the uterine wall for implantation during the luteal phase) (Wingfield and Sapolsky, 2003). Secondly, stress in most species decreases proactive female behaviours designed to increase the likelihood of sex (i.e. proceptivity), as well as responsiveness to proceptive behaviours on the part of a male (i.e. receptivity) (Wingfield and Sapolsky, 2003). When stress is encountered in males, the production of glucocorticoids precedes a decline in testosterone concentration (Whirledge and Cidlowski, 2010). Leydig cells are responsible for the production of testosterone, the hormone required to regulate male fertility. Elevated glucocorticoids are known to decrease testosterone biosynthesis by Leydig cells, as well as induce Leydig cell apoptosis, and spermatagonia apoptosis within the seminiferous tubules (Whirledge and Cidlowski, 2010). Arguably, far more suppressive than a decrease in testosterone is erectile function (Wingfield and Sapolsky, 2003). Erectile dysfunction stems from the complex interplay between parasympathetic and sympathetic activation of the autonomic nervous system, with parasympathetic tone being a prerequisite for an erection in most species, and the transition to sympathetic tone mediating ejaculation (Wingfield and Sapolsky, 2003). As a result, the production of glucocorticoids during stress can block the capacity for an erection (inability to establish parasympathetic tone), or can cause premature ejaculation (accelerating the transition to sympathetic tone) (Wingfield and Sapolsky, 2003). The negative consequences of excessive glucocorticoid production on reproduction in koalas can cause infertility through the imbalance and inhibition of important hormones required for reproduction in both males and females (Chrousos, 2009).

**How to Measure Stress**

Measuring glucocorticoids is important for determining the lethal and sublethal effects of stress (Narayan et al., 2013). When measured, glucocorticoids have the ability to represent chronic stress and measuring chronic stress can provide insight into an animals well-being such as any permanent negative changes relating to inhibition of growth, reproduction and immune function (Sheriff et al., 2011). The four main biological samples used to measure chronic stress include blood, saliva, excreta (faeces and urine) and integumentary structures (hair and feathers) (Sheriff et al., 2011).
Blood has typically been the substance of choice from which to obtain glucocorticoids from in many species (Sheriff et al., 2011). This is because blood is able to give a snapshot of the stress that an animal is experiencing in that exact instant (Sheriff et al., 2011). However, the primary issue surrounding this method is that the capture and handling of the animal associated with blood collection is argued to be a stressor in itself (Hajduk et al., 1992). One particular study found that the stress of capture and handling promoted glucocorticoid production, and a substantial decrease in these levels was not observed until 6 hours after the capture and handling occurred (Hajduk et al., 1992). Although invasive, collecting blood from animals to measure stress is possible. The act requires a sophisticated skill set that presupposes a detailed knowledge of the ecology, evolution and behaviour of the proposed animal (Sheriff et al., 2011). Additionally, the handler is required to capture the animal in its natural habitat, restrain them (possibly anesthetise them), and collect the sample with a minimum of stress (Sheriff et al., 2011).

Saliva is another biological fluid used to measure glucocorticoids and has gained popularity due to the fact that it is less invasive to obtain than blood (Wood, 2009). The use of saliva is gaining traction and has been used as a successful measure of stress in many clinical and captive studies (Lutz et al., 2000, Anisman et al., 2001, Yaneva et al., 2004, Pearson et al., 2008). The primary issue surrounding this method involves setting up a saliva collection site such as a lick or chew site to ensure it remains non-invasive (Sheriff et al., 2011). When setting up a collection site, there needs to be a method that ensures only a single, known animal deposited its saliva (Sheriff et al., 2011). Without a collection site, saliva can be collected by hand (swab or syringe) but this can be dangerous for the handler, time consuming and can encourage stress from capture and handling (Sheriff et al., 2011). If these obstacles can be overcome, salivary glucocorticoids are a very reliable indication of stress with the benefit of being less invasive than blood (Sheriff et al., 2011).

Much like saliva, excreta such as faeces and urine have been increasingly used to measure glucocorticoids in laboratory, domestic, zoo and free-ranging animals (Sheriff et al., 2011). There have been multiple studies performed that have successfully evaluated glucocorticoids in excreta to determine its effectiveness in measuring stress (Millsapugh and Washburn, 2004, Webster et al., 2017, Ziegler and Wittwer, 2005). Compared to
blood and saliva, measuring glucocorticoids in excreta is almost completely non-invasive and can be performed by untrained personnel (Sheriff et al., 2011). For example, faecal samples can easily be collected from animals in the field after an observer notes defecation (Ganswindt et al., 2010). For smaller animals, live-trapping is often required to collect their faecal samples, however this is less invasive than the capture and handling associated with blood and saliva collection (Sheriff et al., 2011). The primary issue associated with this method is that faecal samples cannot provide as many physiological indices of stress as blood does, however, many studies have shown the adequacy on excreta in measuring basic indices of stress (Sheriff et al., 2011).

Finally, integumentary structures such as hair and feathers are the most ideal biological sample to be used in glucocorticoid collection for the purpose of measuring stress, in particular, chronic stress (Sheriff et al., 2011) (Figure 5). This is because the slow growth rate of hair and feathers mean that the time scale in which glucocorticoid levels can be measured is in weeks and months rather than in hours or days, as is the case for blood, saliva and excreta (Sheriff et al., 2011). The primary issue associated with this method is that it is a very new technique and thus, there is not empirical evidence to definitely conclude that integumentary structures yield 100% accurate results (Sheriff et al., 2011). However, there have been multiple studies performed since 2011 on a variety of species that successfully use hair or feathers to measure glucocorticoids as an indicator of stress such as on brown bears (Cattet et al., 2014), chimpanzees (Carlitz et al., 2016), lemurs (Rakotoniaina et al., 2017), and muskoxen (Di Francesca et al., 2017).

Once samples from one of the aforementioned biological samples are collected, an analyses called an immunoassay should be performed (Sheriff et al., 2011). The most common types of immunoassays performed are either a radioimmunoassay (RIA) or an enzyme immunoassay (EIA) (Sheriff et al., 2011). Both RIA and EIA are competitive binding assays and are highly sensitive, which is important as they necessitate an antibody directed against certain parts of the steroid molecule of interest (Sheriff et al., 2011). Once the immunoassay is performed, validating glucocorticoid measurements is the next critical step, as it is important to understand what could compromise validity in the research and how to attend to any errors (Sheriff et al., 2011). The assay must be validated for the species of interest, and even though such validations are routinely done
and implicitly understood, it is done to ensure proper quantification of glucocorticoids and validity of results (Sheriff et al., 2011). The four general validation requirements applied to measure glucocorticoids are specificity, parallelism, accuracy and limitations (Buchanan and Goldsmith, 2004, Mostl et al., 2005). Specificity is important in an immunoassay to ensure a false result is not reached. It ensures that other steroids known to be present in the matrix do not interfere significantly with the immunoassay and therefore skew the results (Sheriff et al., 2011). Parallelism helps to demonstrate that serial dilutions of the sample result in a linear decrease in immunoassay values that are parallel to the standard curve (Sheriff et al., 2011). Accuracy ensures that added glucocorticoids over a range of concentrations correlate directly to the amount recovered by extraction (Sheriff et al., 2011). And finally, limitations of the immunoassay include nonspecific binding, absence of false positives or negatives, sensitivity and precision (Sheriff et al., 2011).

Figure 5: A diagram showing the proposed mechanism of cortisol incorporation into integumentary structures such as hair and feathers. Cortisol is incorporated via passive diffusion from (A) blood capillaries, (B) sweat, (C) sebum, and (D) external sources (Lee et al., 2015).
Aims and Hypothesis

The aim of this research thesis is to understand the physiological impacts of stress on the Australian marsupial species, the koala. This will be achieved through 3 different research projects.

Chapter 2 aims to perform a retrospective analysis whereby admission records for 12,543 wild, rescued koalas admitted into clinical care within New South Wales will be studied in order to determine trends in clinical admissions and diagnosis over a period of 29 years. It is hypothesised that 1) there will be a difference between the prognosis and outcome of koalas based on year admitted into care (1989-2018) 2) there will be a difference between the prognosis and outcome of koalas based on location admitted into care (Port Stephens, Port Macquarie & Lismore) 3) there will be a difference between the prognosis and outcome of koalas based on their gender (male & female) 4) there will be a difference between the prognosis and outcome of koalas based on their age (adult, joey, juvenile & mature).

Chapter 3 aims to analyse haematology in 30 wild, rescued koalas admitted into clinical care within South Australia. Measuring haematology is a traditional biomarker of immune function, whereby blood samples consisting of leukocyte counts, neutrophil to lymphocyte ratios, and urea levels are tested to detect trends between stress and disease. It is hypothesised that the three haematological biomarkers will be able to assist in diagnosing disease influenced by stress therefore strengthening the outcomes of clinical intervention.

Chapter 4 aims to perform an innovative pilot study whereby glucocorticoids will be measured in hair samples for 45 wild, rescued koalas admitted into clinical care within New South Wales. The extraction of glucocorticoids, in particular cortisol, has been seen as an effective biomarker of chronic stress as seen in species including amphibians, marsupials and mammals, however no such research has been performed to date where cortisol is extracted from koala hair in order to assess chronic stress. It is hypothesised that there will be cortisol detected in all koala patients admitted into care, with no significant difference between each prognosis.
Chapter 2: Retrospective analysis of environmental stressors impacting koalas in NSW

Abstract

The koala (*Phascolactos cinereus*) is currently listed by the IUCN as vulnerable to extinction with a decreasing population trend. This listing can be attributed to both the recent climate trends impacting ecosystems, and human induced environmental change from extensive land clearing and habitat fragmentation. These have both been proven to induce stress, which in turn influences the onset of disease. This study performed a retrospective analysis whereby admission records for 12,543 wild, rescued koalas admitted into clinical care within New South Wales were studied in order to determine trends in clinical admissions and diagnosis over a period of 29 years. Results indicated that between all three locations (Port Stephens, Port Macquarie and Lismore), the most common prognosis for koalas admitted into care was disease, the most common disease for koalas admitted into care was signs of chlamydia, and the most common outcome for koalas admitted into care was released. Within Port Stephens, mature aged and female koalas were found to have more disease than any other age or gender, while juvenile aged and male koalas were found to be released more than any other age or gender. Additionally, there were fewer koalas with disease and fewer koalas released in Port Stephens as each year progressed. Within Port Macquarie, mature aged and male koalas were found to have more disease than any other age or gender, while juvenile aged and female koalas were found to be released more than any other age or gender. Additionally, there were more koalas with disease and fewer koalas released in Port Macquarie as each year progressed. Within Lismore, adult aged and female koalas were found to have more disease than any other age or gender, while joey aged and male koalas were found to be released more than any other age or gender. Additionally, there were more koalas with disease and fewer koalas released in Lismore as each year progressed. Determining trends in clinical admissions and diagnosis over such a substantial period of time is an important determinant for the continuing decline of koalas throughout Australia, and in particular New South Wales. It is integral that any further decline of koala populations is prevented, however this can only be achieved through informed recommendations through research projects such as these.
Introduction

In theory, Australia should have relatively few conservation concerns; it’s population density is low (~3km$^{-2}$) by global standards (~50km$^{-2}$), most of the continent remains sparsely settled and little modified, and the nation is relatively affluent (Sanderson et al., 2002). However, since 1788, 30 mammal species endemic to Australia have become extinct, with 55 others experiencing a worsened conservation status (Woinarski et al., 2015). Koalas are a folivorous marsupial whose distribution is tied to its food source (Woinarski et al., 2015). Koalas living in the North-East of Australia have experienced a 40% clearance of ideal habitat and a population decline of 80% since 1990 (Rhodes et al., 2015). It is unknown how many koalas remain in the wild throughout all of Australia, however the Australian Koala Foundation estimate that there could be less than 80,000 wild koalas remaining (AKF, 2019). Despite being listed as “vulnerable to extinction” by the IUCN in 2014, the conservation status of koalas remains highly contentious primarily due to the uncertainty of population parameters (Woinarski and Burbidge, 2016). Furthermore, with respect to the eligibility of koalas against IUCN criteria, drought was listed as the predominant threat associated with this species (Woinarski and Burbidge, 2016). Contrary to this, there is now outstanding evidence to suggest that drought alone is not the biggest threat affecting populations of koalas in Australia (Narayan and Williams, 2016). Climate change not only results in koalas being affected by drought, but increasing global temperatures make it hard for koalas to thrive within their natural habitat (Narayan and Williams, 2016). Furthermore, human population growth is highest throughout the East of Australia, which amplifies competition for suitable habitat between koalas and humans (Narayan and Williams, 2016). Therefore, it is absolutely necessary that the conservation status for this species be re-evaluated as koala populations throughout Australia increasingly dwindle and their suitable habitat is minimised.

As outlined in a study of koalas living in the south of Australia, the biggest threats affecting koala populations derive from human population growth and global climate change (Narayan and Williams, 2016). Due to significant infrastructural change across suitable koala habitat, there is a high proportion of koalas who present to clinical care with injuries consistent with motor vehicle trauma and trauma stemming from animal attacks (Gonzalez-Astudillo et al., 2017). Between 1997 and 2013 in the South-East of
Australia, 15.5% of koalas admitted into clinical care were diagnosed with motor vehicle trauma, and 5.2% of koalas admitted into clinical care were diagnosed with trauma stemming from animal attacks (Gonzalez-Astudillo et al., 2017). Of particular concern is that these patients had no presence of underlying disease, suggesting potential for impact on local populations by healthy, breeding koalas being prematurely removed (Gonzalez-Astudillo et al., 2017). Due to increased stress from global climate change and the amplified presence of humans across suitable koala habitat, there is a high proportion of koalas who present to clinical care with symptoms of chlamydia disease (Gonzalez-Astudillo et al., 2017). Between 1997 and 2013 in the South-East of Australia, 66.3% of koalas admitted into clinical care were diagnosed with chlamydia disease or presented signs consistent with chlamydia disease (Gonzalez-Astudillo et al., 2017). Pathogens that rely on frequency-dependent transmission, such as chlamydia disease, can influence population dynamics by increasing mortality from wasting and blindness, and decreasing population recruitment through impairment of reproduction (Gonzalez-Astudillo et al., 2017). However, studies have found that diseases such as chlamydia only play a detrimental role within the population when other extrinsic factors inducing physiological stress are present, such as human population growth and global climate change (Gonzalez-Astudillo et al., 2017, Narayan and Williams, 2016).

With increased exposure to stress due to human population growth and global climate change, it is integral to understand the population dynamics of koalas such as how stress affects gender and age. It has shown that HPA axis response patterns differ markedly between variables such as gender (male & female), and age (adult, joey, juvenile & mature) (Verma et al., 2011, Maniam et al., 2014). This is because individuals have varying biological capabilities to respond to the stressors which elicit a responses from the release of glucocorticoids (Maniam et al., 2014). Both human and animal studies have shown that males and females react differently from a physiological perspective in response to stress (Verma et al., 2011). Studies show that females produce a higher level of cortisol in response to stress when compared to males (Verma et al., 2011). This finding reflects an increased sensitivity of the adrenal cortex in females compared to males (Verma et al., 2011). Similarly to gender, both human and animal studies have shown that age impacts the ability to respond to stress (Maniam et al., 2014). Those who fall into the younger age brackets are likely to experience a decreased glucose disposal
rate and increased susceptibility to disease in response to stress when compared to older, developed individuals (Maniam et al., 2014).

This study aims to perform a retrospective analysis whereby admission records for 12,543 wild, rescued koalas admitted into clinical care within New South Wales will be studied in order to determine trends in clinical admissions and diagnosis over a period of 29 years. It is essential that this study is carried out, particularly for populations of koalas in New South Wales as there is a significant lack of published data for this species in that region. Furthermore, this study will contribute valuable information relating to the current conservation status of koalas as there is a vast amount of uncertainty relating to population parameters. Performing a retrospective analysis of admissions into clinical care will provide an educated recommendation to update the conservation status to one which is more representative of the current state of koalas in Australia. It is hypothesised that 1) there will be a difference between the prognosis and outcome of koalas based on year admitted into care (1989-2018) 2) there will be a difference between the prognosis and outcome of koalas based on location admitted into care (Port Stephens, Port Macquarie & Lismore) 3) there will be a difference between the prognosis and outcome of koalas based on their gender (male & female) 4) there will be a difference between the prognosis and outcome of koalas based on their age (adult, joey, juvenile & mature).

Methods
This project was performed under strict animal and human care guidelines. Animal ethics was granted by Western Sydney University (A12373). Admission forms and hospital records for 12,543 wild koalas admitted to one of three locations (Port Stephens Koalas, Port Macquarie Koala Hospital and Friends of the Koala) over a 29-year period were collected and analysed. The information recorded included each patients gender, age, suburb found, prognosis, prognosis sub-category, outcome, and year admitted. Once the information for all 12,543 koala patients were recorded in Microsoft® Excel, they were analysed in IBM SPSS Statistics®. Two tests were run for each hypothesis, with the aim of summarising the data to determine patterns, and these tests included a descriptive statistical analysis and a generalised linear model. Age was plotted against both the admission outcome and prognosis and the results were displayed in separate column graphs. Gender was plotted against both the admission outcome and prognosis and the
results were displayed in separate column graphs. Place was plotted against both the admission outcome and prognosis and the results were displayed in separate column graphs. And finally, year was plotted against both the admission outcome and prognosis and the results were displayed in separate column graphs. Raw data for descriptive statistics were transformed into proportions to allow for comparisons to be drawn, and this made it possible to successfully plot age, gender, place and year against outcome and prognosis.

For each koala patient admitted into clinical care, the suburb that they were found in was recorded to determine distribution trends. ArcGIS was used to map the distribution of koalas admitted to all three koala hospitals (Port Stephens Koalas, Port Macquarie Koala Hospital and Friends of the Koala). Postcodes within the three regions (Port Stephens, Port Macquarie and Lismore) and the number of koalas admitted within each postcode were transcribed into comma-separated documents (CSV) on Microsoft® Excel. The CSV sheets were then uploaded separately as a base layer on ArcGIS. Once uploaded, a dot distribution map was generated and koala “hot zones” were attained.

Finally, using Microsoft® Excel, information from admission forms and hospital records for all 12,543 koala patients was used to graph the frequency of both prognosis and disease. Percentages of total koalas admitted to clinical care based on both prognosis and disease was calculated and the percentages were then used to create three column graphs, with the data displayed in descending order.
Results

Distribution Maps

The suburbs where koalas were most frequently found prior to being admitted into clinical care at Port Stephens Koalas was Salamander Bay (335 koalas), Anna Bay (276 koalas) and One Mile (195 koalas) (figure 1).

Figure 1: Distribution trends displayed as hot zones for koalas admitted into care at Port Stephens Koalas between 1989 and 2018.
The suburbs where koalas were most frequently found prior to being admitted into clinical care at the Port Macquarie Koala Hospital was Port Macquarie (430 koalas), Armidale (14 koalas) and Limeburners Creek (13 koalas) (figure 2).

**Figure 2:** Distribution trends displayed as hot zones for koalas admitted into care at the Port Macquarie Koala Hospital between 1989 and 2018.
The suburbs where koalas were most frequently found prior to being admitted into clinical care at Friends of the Koala was Goonellabah (1,219 koalas), Lismore (1,004 koalas) and Wyrallah (444 koalas) (figure 3).

**Figure 3:** Distribution trends displayed as hot zones for koalas admitted into care at Friends of the Koala between 1989 and 2018.
Prognosis and Outcome

A total of 12,543 wild koalas were admitted to one of three locations (Port Stephens Koalas, Port Macquarie Koala Hospital and Friends of the Koala) over a 29-year period (1989-2018). Each koala was given a prognosis on entering care, and these included appearing healthy on assessment, being attacked by cattle, being collared for tracking, being diagnosed with a disease, being dead on arrival, being attacked by a dog, being caught in a fire, being harassed by humans, being hit by a car, being an orphan, being attacked by a snake, having an unknown prognosis, or being displaced in an unsuitable environment. Of all 12,543 koalas over the three locations, 34.49% were given the prognosis disease within their admission to clinical care (figure 4). Following disease, 24.36% of koalas were given an unknown prognosis, and 16.18% were given an appeared healthy prognosis within their admission to clinical care (figure 4).

![Figure 4: The prognosis that was given to each wild rescued koala patient (n=12,543), displayed as a percentage, when they were admitted to one of three locations (Port Stephens Koalas, Port Macquarie Koala Hospital and Friends of the Koala) between 1989 and 2018.](image-url)
A total of 12,543 wild koalas were admitted to one of three locations (Port Stephens Koalas, Port Macquarie Koala Hospital and Friends of the Koala) over a 29-year period (1989-2018). Of all 12,543 koalas over the three locations, 3,941 (34.49%) were given the prognosis disease. Diseases included having signs of chlamydia, having an infection, having poor body condition, having damage to one or many organs, having injuries to one or both eyes, being very old, experiencing trauma to the head, being dehydrated, having a tick infestation, having koala retrovirus, having injuries to one or many claws, and having an injury to one or both legs. The patients that were given the prognosis disease were then provided with a diagnosis. Over the three locations, the most common diagnosis of disease was signs of chlamydia (51.46%), followed by infection (24.18%) and poor body condition (10.13%) (figure 4.5).

**Figure 4.5**: The disease diagnosis that was given to each wild rescued koala patient (n=3,941), displayed as a percentage, when they were admitted to one of three locations (Port Stephens Koalas, Port Macquarie Koala Hospital and Friends of the Koala) between 1989 and 2018.
A total of 12,543 wild koalas were admitted to one of three locations (Port Stephens Koalas, Port Macquarie Koala Hospital and Friends of the Koala) over a 29-year period (1989-2018). Each koala’s outcome was recorded after their time, whether long or short in care, and these included being released into the wild, being euthanised, providing advice to the member of public, recording a sighting of a koala, being dead on arrival, dying while in care, being trapped and relocated to a new area, unknown, transferred to a different captive facility, still in care, unable to capture, koala escaped from care/self-release, and member of the public would not hand the koala over to the carer. Of all 12,543 koalas over the three locations, 20.7% had an outcome of released after their admission to clinical care (figure 5). Following released 17.44% of koalas had an outcome of euthanised, and 17.28% had an outcome of advice only after their admission to clinical care (figure 5).

![Figure 5](https://example.com/koala-outcomes.png)

**Figure 5**: The outcome that was given to each wild rescued koala patient (n=12,543), displayed as a percentage, after they were admitted to one of three locations (Port Stephens Koalas, Port Macquarie Koala Hospital and Friends of the Koala) between 1989 and 2018.
Of the 12,543 wild koalas admitted to one of three locations (Port Stephens Koalas, Port Macquarie Koala Hospital and Friends of the Koala) over a 29-year period (1989-2018), an analysis was performed to compare their prognosis with their age. Age categories for koalas include joey, juvenile, adult, mature and unknown, and prognosis categories for koalas include appearing healthy on assessment, being attacked by cattle, being collared for tracking, being diagnosed with a disease, being dead on arrival, being attacked by a dog, being caught in a fire, being harassed by humans, being hit by a car, being an orphan, being attacked by a snake, having an unknown prognosis, or being displaced in an unsuitable environment (some prognosis categories were excluded in figures as they weren’t statistically significant). The prognosis that occurred the most between all three locations was disease (34.4%), and the age category that was affected the most by this prognosis was mature koalas (42.2%), followed by adult koalas (37.4%) (figure 6).

Of the 12,543 wild koalas admitted to one of three locations (Port Stephens Koalas, Port Macquarie Koala Hospital and Friends of the Koala) over a 29-year period (1989-2018), an analysis was performed to compare their outcome with their age. Age categories for koalas include joey, juvenile, adult, mature and unknown, and outcome categories for koalas include being absent on arrival, providing advice to the member of public, died in care, dead on arrival, euthanised, still in care, member of the public refusing to admit the koala to the hospital, record of sighting, released into the wild, relocated to a new location, released themselves/escaped from clinical care, transferred to another caring facility or organisation, unable to capture, and unknown (some outcome categories were excluded in figures as they weren’t statistically significant). The outcome that occurred the most between all three locations was euthanised (17.4%) and the age category that was affected the most by this outcome was mature koalas (26.9%), followed by adult koalas (19.6%) (figure 7).

Of the 12,543 wild koalas admitted to one of three locations (Port Stephens Koalas, Port Macquarie Koala Hospital and Friends of the Koala) over a 29-year period (1989-2018), an analysis was performed to compare their prognosis with their gender. Gender categories for koalas include male, female and unknown, and prognosis categories for koalas include appearing healthy on assessment, being attacked by cattle, being collared for tracking, being diagnosed with a disease, being dead on arrival, being attacked by a
dog, being caught in a fire, being harassed by humans, being hit by a car, being an orphan, being attacked by a snake, having an unknown prognosis, or being displaced in an unsuitable environment (some prognosis categories were excluded in figures as they weren’t statistically significant). The prognosis that occurred the most between all three locations was disease (34.4%), and the gender category that was affected the most by this prognosis was considerably even with female koalas (38.9%), and male koalas (38.3%) (figure 8).

Of the 12,543 wild koalas admitted to one of three locations (Port Stephens Koalas, Port Macquarie Koala Hospital and Friends of the Koala) over a 29-year period (1989-2018), an analysis was performed to compare their outcome with their gender. Gender categories for koalas include male, female and unknown, and outcome categories for koalas include being absent on arrival, providing advice to the member of public, died in care, dead on arrival, euthanised, still in care, member of the public refusing to admit the koala to the hospital, record of sighting, released into the wild, relocated to a new location, released themselves/escaped from clinical care, transferred to another caring facility or organisation, unable to capture, and unknown (some outcome categories were excluded in figures as they weren’t statistically significant). The outcome that occurred the most between all three locations was euthanised (17.4%) and the gender category that was affected the most by this outcome was female koalas (21.7%), followed by male koalas (20.1%) (figure 9).

Of the 12,543 wild koalas admitted to one of three locations (Port Stephens Koalas, Port Macquarie Koala Hospital and Friends of the Koala) over a 29-year period (1989-2018), an analysis was performed to compare their prognosis with their location. Location categories for koalas include Port Stephens, Port Macquarie and Lismore, and prognosis categories for koalas include appearing healthy on assessment, being attacked by cattle, being collared for tracking, being diagnosed with a disease, being dead on arrival, being attacked by a dog, being caught in a fire, being harassed by humans, being hit by a car, being an orphan, being attacked by a snake, having an unknown prognosis, or being displaced in an unsuitable environment (some prognosis categories were excluded in figures as they weren’t statistically significant). The prognosis that occurred the most between all three locations was disease (34.4%), and the location category that was
affected the most by this prognosis was *Lismore* (37.1%), followed by *Port Macquarie* (27.5%) (figure 10).

Of the 12,543 wild koalas admitted to one of three locations (Port Stephens Koalas, Port Macquarie Koala Hospital and Friends of the Koala) over a 29-year period (1989-2018), an analysis was performed to compare their outcome with their location. Location categories for koalas include Port Stephens, Port Macquarie and Lismore, and outcome categories for koalas include being absent on arrival, providing advice to the member of public, died in care, dead on arrival, euthanised, still in care, member of the public refusing to admit the koala to the hospital, record of sighting, released into the wild, relocated to a new location, released themselves/escaped from clinical care, transferred to another caring facility or organisation, unable to capture, and unknown (some outcome categories were excluded in figures as they weren't statistically significant). The outcome that occurred the most between all three locations was *released* (20.7%) and the location category that was affected the most by this outcome was *Port Macquarie* (71.4%), followed by *male* koalas (49.3%) (figure 11).

Of the 12,543 wild koalas admitted to one of three locations (Port Stephens Koalas, Port Macquarie Koala Hospital and Friends of the Koala) over a 29-year period (1989-2018), an analysis was performed to compare their prognosis with the year they were admitted. Year categories ranged from 1989-2018, and prognosis categories for koalas include appearing healthy on assessment, being attacked by cattle, being collared for tracking, being diagnosed with a disease, being dead on arrival, being attacked by a dog, being caught in a fire, being harassed by humans, being hit by a car, being an orphan, being attacked by a snake, having an unknown prognosis, or being displaced in an unsuitable environment (some prognosis categories were excluded in figures as they weren't statistically significant). The prognosis that occurred the most between 1989-2018 was *disease* (34.5%), and the year that was affected the most by this outcome was 1992 (56.0%), followed evenly by 1990 and 1991 (52.7%) (figure 12).

Of the 12,543 wild koalas admitted to one of three locations (Port Stephens Koalas, Port Macquarie Koala Hospital and Friends of the Koala) over a 29-year period (1989-2018), an analysis was performed to compare their outcome with the year they were admitted.
Year categories for ranged from 1989-2018, and outcome categories for koalas include being absent on arrival, providing advice to the member of public, died in care, dead on arrival, euthanised, still in care, member of the public refusing to admit the koala to the hospital, record of sighting, released into the wild, relocated to a new location, released themselves/escaped from clinical care, transferred to another caring facility or organisation, unable to capture, and unknown (some outcome categories were excluded in figures as they weren’t statistically significant). The outcome that occurred the most between 1989-2018 was released (20.7%) and the year that was affected the most by this outcome was 1989 (50.7%), followed by 1999 (47.7%) and 1990 (45.9%) (figure 13).
Figure 6: The prognosis that was given to each wild rescued koala patient (n=12,543), displayed according to their age category when they were admitted to one of three locations (Port Stephens Koalas, Port Macquarie Koala Hospital and Friends of the Koala) between 1989 and 2018.
**Figure 7:** The outcome that was given to each wild rescued koala patient (n=12,543), displayed according to their age category when they were admitted to one of three locations (Port Stephens Koalas, Port Macquarie Koala Hospital and Friends of the Koala) between 1989 and 2018.
Figure 8: The prognosis that was given to each wild rescued koala patient (n=12,543), displayed according to their gender category when they were admitted to one of three locations (Port Stephens Koalas, Port Macquarie Koala Hospital and Friends of the Koala) between 1989 and 2018.
Figure 9: The outcome that was given to each wild rescued koala patient (n=12,543), displayed according to their gender category when they were admitted to one of three locations (Port Stephens Koalas, Port Macquarie Koala Hospital and Friends of the Koala) between 1989 and 2018.
Figure 10: The prognosis that was given to each wild rescued koala patient (n=12,543), displayed according to their location category when they were admitted to one of three locations (Port Stephens Koalas, Port Macquarie Koala Hospital and Friends of the Koala) between 1989 and 2018.
Figure 11: The outcome that was given to each wild rescued koala patient (n=12,543), displayed according to their location category when they were admitted to one of three locations (Port Stephens Koalas, Port Macquarie Koala Hospital and Friends of the Koala) between 1989 and 2018.
Figure 12: The prognosis that was given to each wild rescued koala patient (n=12,543), displayed according to the year they were admitted into one of three locations (Port Stephens Koalas, Port Macquarie Koala Hospital and Friends of the Koala) between 1989 and 2018.
Figure 13: The outcome that was given to each wild rescued koala patient (n=12,543), displayed according to the year they were admitted to one of three locations (Port Stephens Koalas, Port Macquarie Koala Hospital and Friends of the Koala) between 1989 and 2018.
Statistics

Of the 12,543 wild koalas admitted to one of three locations (Port Stephens Koalas, Port Macquarie Koala Hospital and Friends of the Koala) over a 29-year period (1989-2018), the most common prognosis was disease. Results show that within Port Stephens, there was no significant trend between gender and disease (p=0.117) and year admitted and disease (p=0.316), but there was a significant trend between age and disease (p=0.000) (table 1). Within Port Stephens, results show that for age, mature koalas are 2.267 times as likely to be affected by disease than adult koalas, juvenile koalas are 1.091 times as likely to be affected by disease than adult koalas, and joey koalas are 0.345 times as likely to be affected by disease than adult koalas (table 2). Furthermore, results show that for gender, male koalas are 0.658 times as likely to be affected by disease than female koalas (table 2). Finally, results show that for each year, koalas are 0.959 times as likely to be affected by disease than the previous year (table 2).

Table 1: A model effects test for koalas admitted to Port Stephens Koalas between 1989 and 2018.

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Table 2: A parameter estimate test for koalas admitted to Port Stephens Koalas between 1989 and 2018.

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</table>
Of the 12,543 wild koalas admitted to one of three locations (Port Stephens Koalas, Port Macquarie Koala Hospital and Friends of the Koala) over a 29-year period (1989-2018), the most common prognosis was disease. Results show that within Port Macquarie, there was no significant trend between gender and disease (p=0.527), however, there was a significant trend between age and disease (p=0.000) and year admitted and disease (p=0.000) (table 3). Within Port Macquarie, results show that for age, mature koalas are 2.378 times as likely to be affected by disease than adult koalas, juvenile koalas are 0.156 times as likely to be affected by disease than adult koalas, and joey koalas are 0.276 times as likely to be affected by disease than adult koalas (table 4). Furthermore, results show that for gender, male koalas are 1.125 times as likely to be affected by disease than female koalas (table 4). Finally, results show that for each year, koalas are 1.064 times as likely to be affected by disease than the previous year (table 4).

**Table 3**: A model effects test for koalas admitted to Port Macquarie Koala Hospital between 1989 and 2018.

<table>
<thead>
<tr>
<th>Tests of Model Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Age</td>
</tr>
<tr>
<td>Gender</td>
</tr>
<tr>
<td>Year Admitted</td>
</tr>
</tbody>
</table>

**Table 4**: A parameter estimate test for koalas admitted to Port Macquarie Koala Hospital between 1989 and 2018.

<table>
<thead>
<tr>
<th>Parameter Estimates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameter</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Age Mature</td>
</tr>
<tr>
<td>Age Juvenile</td>
</tr>
<tr>
<td>Age Joey</td>
</tr>
<tr>
<td>Age Adult</td>
</tr>
<tr>
<td>Gender Male</td>
</tr>
<tr>
<td>Gender Female</td>
</tr>
<tr>
<td>Year Admitted</td>
</tr>
</tbody>
</table>
Of the 12,543 wild koalas admitted to one of three locations (Port Stephens Koalas, Port Macquarie Koala Hospital and Friends of the Koala) over a 29-year period (1989-2018), the most common prognosis was disease. Results show that within Lismore, there was no significant trend between gender and disease (p=0.219), and year admitted and disease (p=0.068), however there was a significant trend between age and disease (p=0.000) (table 5). Within Lismore, results show that for age, mature koalas are 0.985 times as likely to be affected by disease than adult koalas, juvenile koalas are 0.433 times as likely to be affected by disease than adult koalas, and joey koalas are 0.411 times as likely to be affected by disease than adult koalas (table 6). Furthermore, results show that for gender, male koalas are 0.925 times as likely to be affected by disease than female koalas (table 6). Finally, results show that for each year, koalas are 1.008 times as likely to be affected by disease than the previous year (table 6).

**Table 5:** A model effects test for koalas admitted to Friends of the Koala between 1989 and 2018.

<table>
<thead>
<tr>
<th>Source</th>
<th>Type III</th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wald Chi-Square</td>
<td>df</td>
<td>Sig.</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>102.316</td>
<td>4</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>1.510</td>
<td>1</td>
<td>0.219</td>
<td></td>
</tr>
<tr>
<td>Year Admitted</td>
<td>3.337</td>
<td>1</td>
<td>0.068</td>
<td></td>
</tr>
</tbody>
</table>

**Table 6:** A parameter estimate test for koalas admitted to Friends of the Koala between 1989 and 2018.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Exp(B)</th>
<th>95% Wald Confidence Interval for Exp(B)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lower</td>
</tr>
<tr>
<td>Age Mature</td>
<td>.988</td>
<td>.062</td>
</tr>
<tr>
<td>Age Juvenile</td>
<td>.433</td>
<td>.345</td>
</tr>
<tr>
<td>Age Joey</td>
<td>.411</td>
<td>.327</td>
</tr>
<tr>
<td>Age Adult</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Gender Male</td>
<td>.925</td>
<td>.817</td>
</tr>
<tr>
<td>Gender Female</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Year Admitted</td>
<td>1.008</td>
<td>.999</td>
</tr>
</tbody>
</table>
Of the 12,543 wild koalas admitted to one of three locations (Port Stephens Koalas, Port Macquarie Koala Hospital and Friends of the Koala) over a 29-year period (1989-2018), the most common outcome was released. Results show that within Port Stephens, there was no significant trend between gender and release (p=0.683), however there was a significant trend between age and release (p=0.000) and year admitted and release (p=0.000) (table 7). Within Port Stephens, results show that for age, mature koalas are 0.762 times as likely to be released than adult koalas, juvenile koalas are 2.097 times as likely to be released than adult koalas, and joey koalas are 0.345 times as likely to be released than adult koalas (table 8). Furthermore, results show that for gender, male koalas are 1.042 times as likely to be released than female koalas (table 8). Finally, results show that for each year, koalas are 0.957 times as likely to be released than the previous year (table 8).

**Table 7:** A model effects test for koalas admitted to Port Stephens Koalas between 1989 and 2018.

<table>
<thead>
<tr>
<th>Source</th>
<th>Type III</th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wald Chi-Square</td>
<td>df</td>
<td>Sig.</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>20.514</td>
<td>3</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>.167</td>
<td>1</td>
<td>0.683</td>
<td></td>
</tr>
<tr>
<td>Year Admitted</td>
<td>28.403</td>
<td>1</td>
<td>0.000</td>
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</tr>
</tbody>
</table>

**Table 8:** A parameter estimate test for koalas admitted to Port Stephens Koalas between 1989 and 2018.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Exp(B)</th>
<th>95% Wald Confidence Interval for Exp(B)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lower</td>
<td>Upper</td>
<td></td>
</tr>
<tr>
<td>Age Mature</td>
<td>.762</td>
<td>.566</td>
<td>1.026</td>
<td></td>
</tr>
<tr>
<td>Age Juvenile</td>
<td>2.097</td>
<td>1.274</td>
<td>3.452</td>
<td></td>
</tr>
<tr>
<td>Age Joey</td>
<td>.345</td>
<td>.165</td>
<td>.723</td>
<td></td>
</tr>
<tr>
<td>Age Adult</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender Male</td>
<td>1.042</td>
<td>.855</td>
<td>1.270</td>
<td></td>
</tr>
<tr>
<td>Gender Female</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Year Admitted</td>
<td>.957</td>
<td>.941</td>
<td>.972</td>
<td></td>
</tr>
</tbody>
</table>
Of the 12,543 wild koalas admitted to one of three locations (Port Stephens Koalas, Port Macquarie Koala Hospital and Friends of the Koala) over a 29-year period (1989-2018), the most common outcome was released. Results show that within Port Macquarie, there was no significant trend between age and release (p=0.211) and gender and release (p=0.865), however there was a significant trend between year admitted and release (p=0.000) (table 9). Within Port Macquarie, results show that for age, mature koalas are 0.650 times as likely to be released than adult koalas, juvenile koalas are 1.325 times as likely to be released than adult koalas, and joey koalas are 0.691 times as likely to be released than adult koalas (table 10). Furthermore, results show that for gender, male koalas are 0.966 times as likely to be released than female koalas (table 10). Finally, results show that for each year, koalas are 0.780 times as likely to be released than the previous year (table 10).

**Table 9:** A model effects test for koalas admitted to Port Macquarie Koala Hospital between 1989 and 2018.

<table>
<thead>
<tr>
<th>Source</th>
<th>Type III</th>
<th>Wald Chi-Square</th>
<th>df</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td>4.510</td>
<td>3</td>
<td>0.211</td>
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<tr>
<td>Gender</td>
<td></td>
<td>.029</td>
<td>1</td>
<td>0.865</td>
</tr>
<tr>
<td>Year Admitted</td>
<td></td>
<td>97.709</td>
<td>1</td>
<td>0.000</td>
</tr>
</tbody>
</table>

**Table 10:** A parameter estimate test for koalas admitted to Port Macquarie Koala Hospital between 1989 and 2018.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Exp(B)</th>
<th>95% Wald Confidence Interval for Exp(B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age Mature</td>
<td>0.650</td>
<td>.349 - 1.209</td>
</tr>
<tr>
<td>Age Juvenile</td>
<td>1.325</td>
<td>.766 - 2.290</td>
</tr>
<tr>
<td>Age Joey</td>
<td>0.691</td>
<td>.335 - 1.428</td>
</tr>
<tr>
<td>Age Adult</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Gender Male</td>
<td>0.966</td>
<td>.650 - 1.436</td>
</tr>
<tr>
<td>Gender Female</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Year Admitted</td>
<td>0.780</td>
<td>.742 - .819</td>
</tr>
</tbody>
</table>
Of the 12,543 wild koalas admitted to one of three locations (Port Stephens Koalas, Port Macquarie Koala Hospital and Friends of the Koala) over a 29-year period (1989-2018), the most common outcome was released. Results show that within Lismore, there was a significant trend between age and release (p=0.000), gender and release (p=0.001) and year admitted and release (p=0.000) (table 11). Within Lismore, results show that for age, mature koalas are 0.000 times as likely to be released than adult koalas, and joey koalas are 2.850 times as likely to be released than adult koalas (table 12). Furthermore, results show that for gender, male koalas are 1.297 times as likely to be released than female koalas (table 12). Finally, results show that for each year, koalas are 0.939 times as likely to be released than the previous year (table 12).

**Table 11:** A model effects test for koalas admitted to Friends of the Koala between 1989 and 2018.

<table>
<thead>
<tr>
<th>Source</th>
<th>Type III</th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wald Chi-Square</td>
<td>df</td>
<td>Sig.</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>95.736</td>
<td>4</td>
<td>0.000</td>
<td></td>
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<tr>
<td>Gender</td>
<td>10.968</td>
<td>1</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Year Admitted</td>
<td>159.195</td>
<td>1</td>
<td>0.000</td>
<td></td>
</tr>
</tbody>
</table>

**Table 12:** A parameter estimate test for koalas admitted to Friends of the Koala between 1989 and 2018.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Exp(B)</th>
<th>95% Wald Confidence Interval for Exp(B)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lower</td>
</tr>
<tr>
<td>Age Mature</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Age Juvenile</td>
<td>1.549</td>
<td>1.215</td>
</tr>
<tr>
<td>Age Joey</td>
<td>2.850</td>
<td>2.264</td>
</tr>
<tr>
<td>Age Adult</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Gender Male</td>
<td>1.297</td>
<td>1.105</td>
</tr>
<tr>
<td>Gender Female</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Year Admitted</td>
<td>0.939</td>
<td>0.929</td>
</tr>
</tbody>
</table>
Discussion

The aim of this study was to perform a retrospective analysis of wild, rescued koalas admitted into clinical care within New South Wales in order to determine trends in clinical admissions and diagnosis over a period of 29 years. Three major koala hospitals within New South Wales were visited whereby a total of 12,543 admission records for koalas admitted into clinical care between 1989 and 2018 were collected. The hospitals visited included Port Stephens Koalas, located in Port Stephens, Port Macquarie Koala Hospital, located in Port Macquarie, and Friends of the Koala, located in Lismore. It is important to note that due to the stochastic nature of admissions into clinical care based on altered population density, the number of koalas admitted into each hospital and the available temporal data was not uniform at each location. As a result of this, proportional data was used when comparing locations while the majority of analyses focused on trends within individual sites.

Of the 12,543 koalas admitted into care, majority came from populations located on the East Coast of Australia (figure 1, 2 & 3). Identifying habitat preference is central to conservation, and it is important to understand why koalas are located on the East Coast of Australia, and what are the threats that lead to their admission into clinical care. Habitat is a species-specific concept defined as “the resources and conditions present in an area that produce occupancy” (Callaghan et al., 2011). For koalas, their home-range should include habitat resources that provide the necessary food, shelter and space requirements for reproduction, survival and movement between trees (Callaghan et al., 2011). Survival and population growth is heavily dependent on suitable home-ranges, and lack thereof has direct implications for the risk of extinction (Callaghan et al., 2011). The major factor defining a suitable home-range for a koala population depends on their preference for a relatively small number of eucalypt species that are able to provide a nutrient-rich foliage (Smith, 2004). Evidence suggests that foliar chemistry can explain differences in nutritional value and subsequent food selection by koalas between and among eucalyptus species (Moore et al., 2004). Studies have shown that there is a higher-frequency of visitation by koalas compared to other species to eucalypts such as the Tasmanian blue gum (Eucalyptus globulus) and manna gum (Eucalyptus viminalis) (Moore and Foley, 2005). This is likely due to these eucalypt species containing higher concentrations of nitrogen and lower concentrations of plant secondary metabolites, and
this suggests that foliar chemistry may influence koala distribution and abundance (Moore and Foley, 2005). Despite koalas existing in their suitable home ranges along the East Coast of Australia, their populations are experiencing a steep decrease and many are admitted into clinical care (Woinarski and Burbidge, 2016). This can be attributed to increased spatial competition between koalas and humans with anthropogenic activity resulting in reduction and fragmentation of suitable koala habitat (Goldingay and Dobner, 2014).

One response to decreasing suitable habitat for koalas is translocation. Translocation is a wildlife management tool employed to establish, re-establish or augment wildlife populations, particularly in response to intentional habitat destruction, to minimise human-wildlife conflicts, and to reduce densities of over-abundant species (Whisson et al., 2012). Previously, translocation has been used in the management of koala populations to reduce the effect of high-density populations on forest habitats, and lessen the risk of density-related animal welfare issues (Whisson et al., 2012). One study aimed to research the success of translocation on koala populations, as it is a possible conservation option for koala populations currently experiencing a steep home range decline. Between 1997 and 2007, over 3000 koalas were captured, sterilised and translocated to other home-ranges in mainland Australia (Whisson et al., 2012). Results of the translocation revealed densities of <0.4 koalas/hectare, despite release densities of 1.0 koalas/hectare (Whisson et al., 2012). Radiotracking indicated that the low densities could be attributed to a 37.5% mortality of translocated individuals within the first 12 months, post release (Whisson et al., 2012). With such high mortality rates associated with translocation, it could be argued that a more appropriate response would be the augmentation of human behaviour, selecting alternate areas for investment and development.

It is a clear commonality that literature suggests that koala populations are declining as a direct result of human activity, most notably habitat destruction and fragmentation (figure 1, 2 & 3). We are also aware that koalas do not exhibit high survival rates in response to translocation, and therefore, these animals remain in their compromised home ranges. Any disturbance to an animals home range activates the stress response (discussed in chapter 1), and if the stressor/s is not mitigated, the excessive production
of glucocorticoids can leave the animal with a compromised immune system (Narayan and Williams, 2016). It is therefore not surprising that of the 12,543 koalas admitted into care through 1989-2018, 34.49% presented with a disease (figure 4). Of the 34.49% of koalas that presented with a disease, 51.46% of them were diagnosed with chlamydia or displayed signs of chlamydial infection (figure 4.5). *Chlamydia pecorum* is an infectious bacterial pathogen that operates as a significant threat to koala conservation (Waugh et al., 2016). Chlamydia is primarily a sexually transmitted infection in koalas, however there is anecdotal evidence for vertical transmission (Waugh et al., 2016). Efforts to understand this disease have found ocular infections of chlamydia can lead to debilitating blindness, while urogenital tract infections of chlamydia can lead to cystitis and/or ascending infections of the reproductive tract and sterility (Wan et al., 2011). In the wild, chlamydia in koalas can be identified through red, swollen eyes and a brown, wet bottom (Kollipara et al., 2013). Koalas infected with this disease often starve to death as their eyes become so swollen, they are unable to find food or shelter. Depending on the stage of chlamydia, koalas admitted into clinical care can be administered with antibiotics as a treatment for the disease, although keeping in mind that this can adversely affect the intestinal microflora and health of the animal (Kollipara et al., 2013). A late diagnosis of chlamydia may require euthanasia on the grounds of welfare, as the disease is incredibly painful for the infected koala (Kollipara et al., 2013).

This retrospective analysis identifies some important trends in admissions of koalas into clinical care (figure 6-13) (table 1-12). In most instances, adult and mature aged koalas were more susceptible than joey and juvenile aged koalas to disease, and female koalas were more susceptible than male koalas to disease (figure 6-13) (table 1-12). The variation in sex and age specific selection pressures can have a fundamental influence on animal survival (Gaillard et al., 2000). This is due to the fact that fitness requirements differ between sexes and among age classes in distinctive ways (Gaillard et al., 2000).

When koalas are born, they are roughly 2cm long and remain blind and hairless in their mothers pouch feeding off milk from a teat; at this stage, they are referred to as a joey (AKF, 2019). At about 6 months of age, the koala exits the mothers pouch and weans off of milk and on to leaves; at this stage, they are referred to as a juvenile (AKF, 2019). To assist this transition, the mother will produce a substance called “pap”, which is a micro-
organisms necessary for making digestion for her juvenile koala possible (AKF, 2019). At about 12 months of age, koalas leave their mothers in search of their own home range; at this stage, they are referred to as an adult (AKF, 2019). From 7 years and above, koalas transition from an adult to a mature koala (AKF, 2019). At both the adult and mature stage of life, survival becomes harder for koalas as they no longer have their mother to protect them from predators, and to provide food and shelter. As an adult, koalas are on the move with the potential to cross roads with cars and enter landscapes with dogs and other predators. These reasons likely contribute to our results indicating that adult and mature aged koalas were more likely than joey and juvenile aged koalas to experience disease among other identified stressors.

Female koalas begin breeding at 3 years of age, and generally produce one offspring per year (AKF, 2019). During and after gestation are both a nutritionally demanding time for a female koala, as they are required to not only feed themselves, but also their young (AKF, 2019). This can become extremely stressful if resources become scarce, and this could easily take a toll on the female koala during not only a 35-day gestation period, but the remaining 365 days that they are responsible for their young. Unlike female koalas, at no stage in their lives are male koalas responsible for another koala, young or old. The higher expectations on females over males are likely why our results indicated that female koalas were more likely than male koalas to experience disease among other identified stressors.

There was a positive relationship between more koalas being admitted into clinical care over each year. Despite this, the proportion of koalas released decreased across all locations. During 1990, the Australian population was estimated at 17,041,431 people, and by 2018, was estimated at 24,772,247 people. The difference of 7,730,816 people over a 28-year period is dramatic especially as it is argued that the environment has reached the upper limits of human population growth (Smith, 1995). At the very least, the East-Coast of Australia, where koalas occupy their home ranges, are being encroached on as the number of humans increase. The presence of humans is closely tied to koala stress, and this is likely why our results indicated that more koalas were being admitted into clinical care over each year. These trends arise despite early detection as a result of growing awareness of koalas in Australia. Similarly, as facilities such as Port Stephens
Koalas, Port Macquarie Koala Hospital and Friends of the Koala receive slight increases in funding through donations and government grants, so too do their facilities and access to treatment improve.

**Conclusion**

The aim of this study was to perform a retrospective analysis of admission records for 12,543 wild, rescued koalas admitted into clinical care within New South Wales. Rescued, wild koalas were admitted to one of three locations, including Port Stephens Koalas in Port Stephens, Port Macquarie Koala Hospital in Port Macquarie, and Friends of the Koala in Lismore. Analysing admission records was performed with the aim of determining trends in clinical admissions and diagnosis over a period of 29 years. The first hypothesis, that there will be a difference between the prognosis and outcome of koalas based on year admitted into care (1989-2018), can be accepted. Until 2005, the most frequent occurring prognosis was disease, and the most frequent occurring outcome was released. From 2005 until 2012, the most frequent occurring prognosis fluctuated between disease and appearing healthy, and the most frequent occurring outcome was advice only. After 2012, the most frequent occurring prognosis continued to fluctuate between disease and appearing healthy, and the most frequent occurring outcome was record of sighting. The prognosis and outcome of koalas admitted into care continually changed between the years 1989 and 2018, and this clearly reflects societal awareness of koala presence as well as the increased prevalence of disease in koala populations over a 29-year period. The second hypothesis, that there will be a difference between the prognosis and outcome of koalas based on location admitted into care (Port Stephens, Port Macquarie & Lismore), can be accepted. The most frequent occurring prognosis for koalas admitted into care within Port Stephens and Port Macquarie was based on unsuitable habitat, whereas in Lismore disease was most common. The most frequent occurring outcome for koalas admitted into care within Port Stephens and Port Macquarie was released, whereas for koalas admitted into care within Lismore was advice only. The prognosis and outcome of koalas admitted into care continually changed between the three locations, and this clearly reflects how environment change affects koala populations differently based on individual locations over a 29-year period. The third hypothesis, that there will be a difference between the prognosis and outcome of koalas based on their gender (male & female), can be rejected. For both male and female koalas admitted into care, the most
frequent occurring prognosis was disease, while the most frequent occurring outcome was released. This clearly demonstrates that both male and female koalas are equally susceptible to disease and equally likely to be released after being admitted into clinical care. The fourth hypothesis, that there will be a difference between the prognosis and outcome of koalas based on their age (adult, joey, juvenile & mature), can be accepted. For adult koalas, the most frequent occurring prognosis was disease, while the most frequent occurring outcome was advice only. For joey koalas, the most frequent occurring prognosis was disease, while the most frequent occurring outcome was released. For juvenile koalas, the most frequent occurring prognosis was disease, while the most frequent occurring outcome was released. Finally, for mature koalas, the most frequent occurring prognosis and outcome were disease released respectively. The prognosis and outcome of koalas admitted into care remained the same for joey, juvenile and mature aged koalas, but differed for adult aged koalas, indicating that koala populations are affected differently based on their age bracket. In conclusion, this retrospective analysis effectively determined trends in clinical admissions and diagnosis for koalas admitted into care within New South Wales over a 29-year period. This enables any steps to mitigate the further decline of koala populations to be better informed, with the implementation of scientific based management strategies for effective koala conservation.
Chapter 3: Using blood as a method of measuring stress in rescued koalas

Abstract

The koala (*Phascolactos cinereus*) is currently listed by the IUCN as vulnerable to extinction with a decreasing population trend. This listing can be attributed to both the recent climate trends impacting ecosystems, and human induced environmental change from extensive land clearing and habitat fragmentation. These have both been proven to induce stress, which in turn influences the onset of disease. Stress induced disease can be detected using serum biomarkers in blood. This study analysed haematology in the koala - a traditional biomarker of immune function consisting of leukocyte counts, neutrophil to lymphocyte ratios, and urea levels. The outcome of this research delivers a better understanding between stress and disease detection in rescued wild koalas in order to meet their individual needs within a clinical setting, and therefore strengthening the outcomes of clinical intervention. Blood profiles were analysed for 30 koala patients which were admitted to the Adelaide Koala and Wildlife Hospital during January of 2017 through to November of 2017. Results indicated that the most common clinical disease diagnosed in patients on admission included renal failure, followed by calcium oxalate nephrosis and koala retrovirus. Furthermore, the results highlighted that leukocyte counts, neutrophil to lymphocyte ratios, and urea readings are all feasible measures of disease influenced by physiological stress. This is because the majority of koalas who were diagnosed with a disease had altered leukocyte counts, neutrophil to lymphocyte ratios, and urea readings. The use of haematological biomarkers is significant to the conservation of koalas as it helps to ensure population security by detecting and treating patients on an individual need basis, therefore strengthening the outcomes of clinical intervention.

Introduction

There are multiple sources of stress affecting koalas, predominately including anthropogenic driven environment change such as land clearing and habitat fragmentation (Narayan and Williams, 2016, McAlpine et al., 2017, Finn and Stephens, 2017). Anthropogenic driven environmental change is shown to be a contributing factor as to why koalas are listed as “vulnerable to extinction” with a decreasing population
trend (Woinarski and Burbidge, 2016). The definition of stress is highly contentious, however it can be broadly defined as a challenge to an animals regular capacity that activates the ‘fight or flight’ response, or a disturbance in an animals allostasis (discussed in chapter 1) (Hing et al., 2016). Responding to stress involves activating the HPA axis, which is a complex and essential negative-feedback system involving glucocorticoids among other neuro-endocrine mediators (Hing et al., 2016). The prolonged and excessive production of glucocorticoids in response to stress prevents the HPA axis reaching a recovery phase, and this results in the dysfunction of the negative feedback mechanism (Chrousos, 2009). When this occurs, subsequent health implications can develop such as diseases associated with growth, reproduction or immunity (discussed in chapter 1) (Chrousos, 2009).

Disease influenced by stress can be detected and indexed using conservation physiology tools such as measuring biomarkers of the immune system (Narayan and Williams, 2016, Rishniw et al., 2012). Leptospirosis is a bacterial disease that effects both domestic and wild animals, and humans (Sohail et al., 2017). One study took blood from horses, both infected and not infected with leptospirosis in order to further understand the biomarkers associated with this disease (Sohail et al., 2017). Results indicated that horses infected with leptospirosis showed a significant decrease in red blood cells, haemoglobin and platelets, while leukocytes, neutrophils, eosinophils, basophils and lymphocytes were significantly elevated (Sohail et al., 2017). Enteritis is a gastrointestinal disease encountered in all canine breeds (Bhat et al., 2013). One study took blood from dogs both infected and not infected with enteritis in order to further understand the biomarkers associated with this disease (Bhat et al., 2013). Results indicated that dogs infected with enteritis showed a significant decrease in blood plasma, and a significant elevation in urea (Bhat et al., 2013). Dysbiosis is an intestinal microbe imbalance encountered in feline breeds (Kathrani et al., 2017). One study took blood from cats both infected and not infected with dysbiosis in order to further understand the biomarkers associated with this disease (Kathrani et al., 2017). Results indicated that cats infected with dysbiosis showed a significant decrease in blood taurine (Kathrani et al., 2017). Biomarkers of the immune system (including red blood cells, haemoglobin, platelets, leukocytes, neutrophils, eosinophils, basophils and lymphocytes, blood plasma, urea, and
blood taurine) are very useful tools for diagnosing disease in animals admitted into veterinary clinics (Rishniw et al., 2012).

This study examined three biomarkers of the immune system that commonly effect immune function in koalas, including leukocyte counts, neutrophil to lymphocyte ratios, and urea readings. Leukocytes, also known as white blood cells, are the first component of the innate immune system which mediate a first-line defence against a microbial attack (Gordon-Smith, 2013). The primary role of leukocytes are to recognise, ingest foreign or degraded cells or proteins, kill pathogens, and to present specific pathogen antigens to assist in the immune response (Gordon-Smith, 2013). Additional to leukocytes, neutrophil to lymphocyte ratios are components of white blood cells that assist in the immune response through their susceptibility to stress (Gordon-Smith, 2013). Neutrophils divide and enter the blood stream to combat infection by eating foreign bacteria, whereas lymphocytes work to make two types of antibodies to fight foreign bacteria, T-type and B-type antibodies (Gordon-Smith, 2013). Increased levels of leukocytes, or dramatic neutrophil to lymphocyte ratios in blood are indicative of a disease that is acting to compromise immunity (Gordon-Smith, 2013). Chlamydia is a diseases associated with compromised immunity in koalas, and measuring levels of leukocytes and neutrophil to lymphocyte ratios in the blood of koalas admitted into veterinary clinics is useful for disease detection (Gordon-Smith, 2013). Urea is a nitrogen substance cleared from the body through the kidneys and flushed out of the body by urine (Zhao et al., 2018). The liver converts ammonia to a non-toxic compound, also known as urea, so it can be safely disposed of in the blood through the kidneys (Zhao et al., 2018). Increased levels of urea in blood is indicative of a disease that is acting to compromise kidney function (Speight et al., 2014). Oxalate nephrosis and renal failure are both diseases associated with kidney dysfunction in koalas, and measuring levels of urea in the blood of koalas admitted into veterinary clinics is useful for disease detection (Speight et al., 2014).

Using haematological biomarkers such as leukocyte counts, neutrophil to lymphocyte ratios and urea readings, this study aims to assess physiological stress in rescued koalas admitted into care at the Adelaide Koala and Wildlife Hospital throughout 2017. It is hypothesised that the three haematological biomarkers will be able to assist in
diagnosing disease influenced by stress, and therefore strengthen the outcomes of clinical intervention.

**Methods**

This project was performed under strict animal and human care guidelines. Animal ethics was granted by Western Sydney University (A12373). Admission forms and hospital records for 30 adult wild koalas (21 male, 9 female) admitted to the Adelaide Koala and Wildlife Hospital over an 11-month period (January 2017 to November 2017) were analysed. The information collected included each patient’s name, gender, age, location found, date of admission, reason for admission, demeanour, and diagnosis. Additionally, data was collected on blood samples that had been taken on the patient’s arrival to the hospital once they were placed under anaesthetic. All blood samples were analysed in a LaserCyte Haematology Analyser by nurses and veterinarians at the hospital. The blood biochemistry information included leukocyte counts, neutrophil to lymphocyte ratios, and urea readings in blood. Furthermore, blood profile reference ranges for healthy wild koalas were supplied by the Adelaide Koala and Wildlife Hospital.

Microsoft® Excel was used to statistically analyse the data. A between group analysis was conducted for the admission diagnosis of adult wild koalas (n=30) and this data was plotted in a pie graph. Leukocyte counts were plotted against the admission diagnosis and this was displayed as a column graph. Both high and low reference ranges were included as a line graph. Neutrophil to lymphocyte ratios were plotted against the admission diagnosis and this was displayed as a column graph. Normal or abnormal reference ranges were included through colour coordination. Urea readings were plotted against the admission diagnosis and this was displayed as a column graph. Both high and low reference ranges were included as a line graph.
Results

The most common diagnosis in adult wild koalas (n=30) admitted to the Adelaide Koala and Wildlife Hospital over an 11-month period (January 2017 to November 2017) was renal failure at 34% (figure 1). Following renal failure, the second most common diagnosis was retrovirus at 10%, and calcium oxalate nephrosis at 10% (figure 1).

**Figure 1:** The diagnosis that was given to each wild rescued adult koala patient (n=30) when they were admitted to the Adelaide Koala and Wildlife Hospital between January 2017 to November 2017.
The normal reference interval for a leukocyte reading in healthy koalas is anything between 2.8 and $11.2 \times 10^9$/L. Any reading less than $2.8 \times 10^9$/L is considered low, and any reading more than $11.2 \times 10^9$/L is considered high. The koala patients with a low leukocyte count included those diagnosed with retrovirus (figure 2). The koala patients with a high leukocyte count included those diagnosed with broken bones, head trauma and retrovirus (figure 2).

**Figure 2:** Leukocyte counts for each wild rescued adult koala patient ($n=30$) when admitted to the Adelaide Koala and Wildlife Hospital between January 2017 to November 2017.
The normal neutrophil to lymphocyte ratio in healthy koalas is 40% neutrophils to 55% lymphocytes (40:55). Any reading where the percentage of neutrophils is higher than 40, and the percentage of lymphocytes is lower than 55, is considered abnormal. The koala patients with an abnormal neutrophil to lymphocyte ratio included those diagnosed with bladder crystals, broken bones, calcium oxalate nephrosis, chlamydia, dental infection, head trauma, ligament cruciate, pyelonephritis, renal failure, retrovirus, and soft tissue damage (figure 3).

**Figure 3:** Neutrophil to lymphocyte ratios for each wild rescued adult koala patient (n=30) when they were admitted to the Adelaide Koala and Wildlife Hospital between January 2017 to November 2017.
The normal reference interval for urea in healthy koalas, is anything between 0.2 and 6.6 mmol/L. Any reading less than 0.2 mmol/L is considered low, and any reading more than 6.6 mmol/L is considered high. There were no koala patients with a low urea reading, however the koala patients with a high urea reading included those diagnosed with bladder crystals, calcium oxalate nephrosis, pulmonary edema, pyelonephritis, renal failure and retrovirus (figure 4).

Figure 4: Urea readings for each wild rescued adult koala patient (n=30) when they were admitted to the Adelaide Koala and Wildlife Hospital between January 2017 to November 2017.
Discussion

Evaluating biomarkers of the immune system in koalas such as leukocyte counts, neutrophil to lymphocyte ratios and urea readings is an effective method of assessing physiological stress (Davis et al., 2008).

Of the wild, adult koalas admitted to the Adelaide Koala and Wildlife Hospital between January 2017 and November 2017, 34% were diagnosed with renal failure (figure 1). Comparatively, another study has identified renal failure as the most common diagnosis in rescued wild koalas in South Australia (Narayan and Williams, 2016). Renal failure, also known as acute kidney injury, is characterised by the rapid loss of the kidneys excretory function (Bellomo et al., 2012). Typically, renal failure is diagnosed by the accumulation of nitrogenous end products such as urea in the body, or through decreased urine output (Bellomo et al., 2012). There has been minimal research conducted that can attribute an exact cause for the occurrence of renal failure in koalas; however, one study suspects that there is a link between the potentially nephrotoxic levels of aluminium ingested by South Australian koalas through their natural diet of eucalyptus leaves (Haynes et al., 2004). Electron dispersive x-ray analysis found aluminium present in both bone and kidney tissues in koalas that had died due to renal failure (Haynes et al., 2004). Aluminium toxicity is a major limiting factor in soils and plant production and aluminium absorption through eucalyptus leaves is increased due to the koalas herbivorous diet (Haynes et al., 2004, Silva et al., 2004). Aluminium accumulation is associated with impaired tubular function of the kidneys, thus increasing the likelihood of renal failure (Haynes et al., 2004).

Leukocytes are a white blood cell which functions to protect the body against infectious diseases and foreign invaders (Davis et al., 2004). It is common to use leukocyte counts to assess immune function, as the irregular production of white blood cells is indicative of host defence mechanisms (Davis et al., 2004). Of the koalas in this study, two out of three patients were diagnosed with retrovirus and had a low leukocyte count (figure 2). Comparatively, the single patient with broken bones had a high leukocyte count, the single patient diagnosed with head trauma had a high leukocyte count, and one out of three patients diagnosed with retrovirus had a high leukocyte count (figure 2). A study of the interaction between leukocyte readings and immune function in finches displays a
trend between irregularly high and low leukocyte counts and disease (Davis et al., 2004). In one study, finches were injected with a bacterial disease and were then found to have excessively high leukocyte counts compared to finches who had not been injected with the same bacterial disease (Davis et al., 2004). Furthermore, those finches who had been injected with the bacterial disease were susceptible to dramatic and irregular changes in their leukocyte readings once exposed to stress including capture and release (Davis et al., 2004). Those koalas (n=6) who exhibited irregularly high and low leukocyte counts presented with infectious diseases and foreign invaders, which was consistent with their diagnosis.

Neutrophils and lymphocytes are two components of white blood cells which when analysed as a ratio, are a clear representation of physiological stress (Narayan and Hero, 2011). A normal ratio in koalas is represented as a percentage of neutrophils to lymphocytes, and should be 40:55 (Dickens, 1975). Any reading of neutrophils which is higher than 40%, in addition to any reading of lymphocytes which is lower than 55%, can be classed as abnormal (Dickens, 1975). Neutrophil to lymphocyte ratios act as a physiological indicator of stress in its role through temporary redistribution of white blood cells to areas of the body where they are most needed during the stress response (Davis and Maerz, 2011). Examples of where white blood cells redistribute to include the epidermis to either fight an infection or close a wound (Davis and Maerz, 2011). The result of this redistribution includes the high production of glucocorticoids which increases the proportion of neutrophils and decreases the proportion of lymphocytes in circulation, making the neutrophil to lymphocyte ratio useful for assessing the degree of stress encountered by a koala (Davis and Maerz, 2011). Those koalas (n=19) who had an abnormal neutrophil to lymphocyte ratio included the one koala diagnosed with bladder crystals, one of two koalas diagnosed with broken bones, all koalas diagnosed with calcium oxalate nephrosis, the one koala diagnosed with chlamydia, the one koala diagnosed with dental infection, the one koala diagnosed with head trauma, the one koala diagnosed with ligament cruciate, one of two koalas diagnosed with nothing, the one koala diagnosed with pyelonephritis, five out of ten koalas diagnosed with renal failure, two out of three koalas diagnosed with retrovirus, and the one koala diagnosed with soft tissue damage (figure 3). A study of the interaction between neutrophil to lymphocyte ratios and immune function in frogs displays a trend between abnormal neutrophil to
lymphocyte ratios and disease (Gervasi et al., 2013). In one study, infectious diseases were studied by understanding the host-pathogen relationship using neutrophil to lymphocyte ratios in the blood of frogs with a bacterial disease (Gervasi et al., 2013). Those frogs who were infected displayed an abnormal neutrophil to lymphocyte ratio when blood samples were analysed, indicating an increase in circulating hormones such as glucocorticoids (Gervasi et al., 2013). Those koalas (n=19) with abnormal neutrophil to lymphocyte ratios all presented with physiological stress and disease as seen through their diagnosis.

Blood urea nitrogen is determined by the complex balance between urea production, urea metabolism, and urea excretion, and is moderated by a number of renal and non-renal dependent factors (Beier et al., 2011). Where blood urea nitrogen is not a direct factor in system dysfunction, it is associated with an increased severity of renal systematic illnesses (Beier et al., 2011). For example, elevated blood urea nitrogen is corelated with increased mortality in patients already suffering with heart related issues (Beier et al., 2011). Elevated levels of urea in blood profiles are an indicator of failing renal function (Lanyon et al., 2012). Of the koalas in this study, the only one diagnosed with bladder crystals, all three diagnosed with calcium oxalate nephrosis, the only one diagnosed with pulmonary edema, the only one diagnosed with pyelonephritis, five out of ten diagnosed with renal failure, and one of three diagnosed with retovirus had a high urea reading, and therefore presented with renal dysfunction (figure 4). A study of the interaction between urea readings and immune function in dogs displayed a trend between high urea readings and disease (Steinbach et al., 2010). In one study, the relationship between urea readings and disease was analysed through a number of healthy dogs and dogs who were diagnosed with spontaneous chronic kidney disease (Steinbach et al., 2010). The results proved a trend towards high urea readings in dogs with advanced chronic kidney disease compared to healthy dogs (Steinbach et al., 2010). Those koalas (n=12) with abnormal neutrophil to lymphocyte ratios all presented with renal systematic illnesses, as seen through their diagnosis.

**Conclusion**

Using haematological biomarkers such as leukocyte counts, neutrophil to lymphocyte ratios, and urea readings, this study aimed to assess the relationship between
physiological stress and disease in rescued, adult, wild koalas who had been admitted into care at the Adelaide Koala and Wildlife Hospital between January 2017 to November 2017. It was hypothesised that assessing three haematological biomarkers would assist in determining subsequent health implications such as those to the immune system. It can be accepted that blood biochemistry profiles are effective in the assessment of physiological stress and can be used in disease diagnosis on an individual/case needs basis. Leukocyte counts are effective in assessing immune function, neutrophil to lymphocyte ratios are a clear representation of physiological stress, and urea readings are associated with an increased severity of renal systematic illnesses. The current conservation status of koalas signifies there is much work to be done in the plight of their conservation. This study contributes to the detection and treatment of koala patients on an individual needs’ basis, and therefore has the ability to strengthen the outcomes of clinical intervention.
Chapter 4: Using fur as a method of measuring stress in rescued koalas

Abstract
The koala (*Phascolactos cinereus*) is currently listed by the IUCN as “vulnerable to extinction” with a decreasing population trend. This listing can be attributed to both the recent climate trends impacting ecosystems, and human induced environmental change from extensive land clearing and habitat fragmentation. These have both been proven to induce stress, which can have negative physiological effects including the suppression of growth, reproduction and function of the immune system. This study measured glucocorticoids in the koala – an effective biomarker of chronic stress consisting of hormones such as cortisol. Hair samples were analysed for 45 koala patients which were admitted to the Port Macquarie Koala Hospital during December of 2017 through to September of 2018. Measuring glucocorticoids has proven to be an effective method of stress assessment as seen in species including amphibians, marsupials and mammals. Despite this process already being successfully performed on a number of species, no such research has been performed to date where hormones such as cortisol are extracted from koala hair in order to assess chronic stress. The results of this study indicated that an enzyme immunoassay with an extraction solvent of 100% methanol can be used to successfully measure cortisol, an assessment of chronic stress in koalas. Furthermore, results indicated that although there was no significant difference in cortisol readings between each diagnosed prognosis, koalas suffering with a ‘disease’ produced the highest cortisol readings. The outcome of this research indicates that hair is a successful and non-invasive tool to measure chronic stress in koalas. Future studies will benefit as the methodologies used in this research can be adapted in healthy, non-stressed koalas to determine base-line measures of hair cortisol. This will contribute greatly to further studies in stress physiology and much needed conservation for the koala species.

Introduction
Australian ecosystems sustain human life through the provision of services including food and fibre among many other dynamic interactions (Sandhu et al., 2012). Ironically, it is human activity that operates as the dominant driver of ecosystem disruption through biodiversity change (Martinez-Ramos et al., 2016). Habitat fragmentation, known as the
process of dividing large continuous habitats into small patches of land, isolated from each other, is the major anthropogenic biodiversity change threatening Australian ecosystems (Martinez-Ramos et al., 2016). Habitat fragmentation modifies the structure and diversity of species composition in any given area, thus reducing the range of habitat necessary for these species to maintain viable populations (Martinez-Ramos et al., 2016). The result of this is increased competition between species for resources including food, fuel, fibre and water (Aukema et al., 2017). The destruction of Australian ecosystems through biodiversity change is having catastrophic consequences on many Australian native species, including koalas (Sandhu et al., 2012). These consequences include stress, which can have negative physiological effects including the suppression of growth, reproduction and function of the immune system (discussed in chapter 1).

Measuring an animal’s glucocorticoid production is a common indicator used to determine whether or not they are experiencing physiological stress (Narayan et al., 2013). The production of glucocorticoids is a physiological response to a stressor, and depending on the severity, glucocorticoids include either adrenaline or cortisol (Narayan and Parisella, 2017). The production of adrenaline is an indicator of acute stress, and is caused by a short-term negative situation where the individual is able to quickly and completely recover (Narayan and Parisella, 2017). The production of cortisol however, is an indicator of chronic stress, and is caused by a long-term stressor whereby the individual may never recover (Narayan and Parisella, 2017). Cortisol can be measured in blood plasma, blood serum, saliva, urine or faeces (Mastromonaco et al., 2014). However, evidence suggests that hair is a much more effective tool when measuring chronic stress, as opposed to the aforementioned (Mastromonaco et al., 2014). This is because hair is thought to incorporate blood-borne hormones during its growth phase, it is relatively stable, and any cortisol detected in hair reflects physiological stress experienced over the period of hair growth which can be weeks through to months (Mastromonaco et al., 2014). Acquiring samples to measure cortisol such as blood plasma, blood serum, saliva, urine, faeces and hair aren’t always a stress-free process due to the pressure associated with capture and handling of that animal (Mastromonaco et al., 2014). However, any stress experienced during capture and handling to acquire hair is not likely to impact glucocorticoid levels in hair samples so long as they are collected concurrently, thus
preventing further growth and integration (Mastromonaco et al., 2014, Cattet et al., 2014).

There have been many studies that explore the proficiency of using hair to measure long-term stress in a number of animals, and include studies on caribou, reindeer, grizzly bears, rhesus monkeys, muskoxen and brown bears (Ashley et al., 2011, Macbeth et al., 2010, Dettmer et al., 2012, Di Francesco et al., 2017, Cattet et al., 2014). For example, adrenaline was injected in Alaskan caribou and reindeer, and glucocorticoids were successfully measured through both their faecal and hair samples (Ashley et al., 2011). Free-ranging grizzly bears with high levels of cortisol due to being exposed to human altered landscapes had glucocorticoids successfully measured in their hair samples (Macbeth et al., 2010). Laboratory born rhesus monkeys exposed to a range of different rearing conditions and therefore having a range of altered cortisol levels successfully had glucocorticoids measured in their hair samples (Dettmer et al., 2012). Muskoxen exposed to unprecedented climate change and increased anthropogenic activities within their home range had cortisol measured successfully in samples of their hair (Di Francesco et al., 2017). And finally, brown bears with varying levels of cortisol due to body-condition related stressors had glucocorticoids successfully measured in samples of their hair in response to capture and handling (Cattet et al., 2014).

Consistent with the aforementioned studies, cortisol is extracted from hair by first washing the sample to ensure sweat and sebum-derived cortisol deposited on the surface of the hair is not co-extracted with glucocorticoids (Meyer et al., 2014). The sample is then pulverising in a bead-beater and dried to ensure there is a complete evaporation of solvents used in the wash (Meyer et al., 2014). The sample is then extracted and reconstituted to facilitate the assay of cortisol by using either ethanol, methanol or isopropanol of varying concentrations, depending on the hair in question (Meyer et al., 2014). The most common solvent used to extract cortisol from samples to measure stress is methanol (Carlitz et al., 2016, Cattet et al., 2014), however there are some studies that use ethanol and isopropanol in their cortisol extraction techniques. For example, a study that used ethanol was successful for extracting cortisol from human amniotic fluid (Aderjan et al., 1977). Additionally, a study that used isopropanol was successful for extracting cortisol from human hair and nails (Nejad and Ghaseminezhad, 2016). Our
preliminary studies found methanol produce the best results when used to extract cortisol in koala hair (Charalambous & Narayan, 2019). Furthermore, it is common to dilute solvents before use in sensitive immunoassays, as chemicals can interfere and cause results to become skewed (Palme et al., 2013). For this reason, the methanol used in this study was diluted by 10%.

To date, no such research has been performed where hormones such as cortisol are extracted from koala hair in order to assess chronic stress. This study aims to measure physiological stress in rescued koalas by using a pre-determined cortisol extraction technique in samples of hair from koalas admitted to care. This pre-determined extraction technique was published by me and Dr. Edward Narayan in JoVE in June 2019. It is hypothesised that there will be cortisol detected in all koala patients admitted into care, with no significant difference between each prognosis.

Methods

This project was performed under strict animal and human care guidelines. Animal ethics was granted by Western Sydney University (A12373). Additionally, a laboratory risk assessment and biosafety and radiation form were both submitted and accepted by Western Sydney University to safely undertake this research (B12366). Samples of koala hair used in this project were obtained from the Port Macquarie Koala Hospital, located on Lord Street, Port Macquarie New South Wales 2444. A sample of hair was shaved from 45 wild rescued koala patients who had been admitted to the hospital during December of 2017 through to September of 2018, and had all been either diagnosed with a disease (i.e. chlamydia, retrovirus etc), had been attacked by a dog, had been caught in a bushfire, had been hit by a car (i.e. when crossing a road), or had been in an unsuitable environment (i.e. being displaced by land clearing). On arrival to the hospital, each koala was placed into an enclosure so as not to increase their exposure to stress. Once settled in (several days depending on the koala), each koala was taken to the veterinary clinic to have a mandatory health screening. During this screening, approximately 1 gram of hair was shaved either from the nape of the neck or the left arm using standard animal clippers. The hair was shaved as close as possible to the skin, so as to ensure the skin was not cut. The hair was then placed in a pouch made of aluminium foil and stored below minus 20 degrees Celsius. While the hair was being shipped to the laboratory, it was
stored at an ambient temperature, until arrival to the laboratory where it was stored at minus 80 degrees Celsius.

Once removed from storage at minus 80 degrees Celsius, each hair sample was weighed on a laboratory analytical precision balance. Sixty milligrams from each hair sample was placed into a labelled 15 millilitre centrifuge tube. This process was repeated until a centrifuge tube was filled for each of the 45 samples. Five millilitres of 100% high-performance liquid chromatography HPLC grade isopropanol was added to each of the 45 centrifuge tubes using a pipette. Each of the centrifuge tubes were then place in an Eppendorf minni spin plus vortex mixer for 30 seconds. Once spun, the contents of each centrifuge tube was strained using a 0.5-millimetre micro precision sieve, so as to achieve complete separation between liquid and hair. The liquid was discarded into a waste container and the hair samples were placed into a small labelled plastic weighing boat. Each weighing boat was stored in a vacuum desiccator and the hair was left to dry for 3 days. Once dry, each hair sample was transferred from the weighing boat into a new, labelled 2ml centrifuge tube. Three 3.2-millimetre chrome steel beads were placed into each centrifuge tube, then propped into a Tissue Lyser bead mill and pulverised for 2 minutes at 30 shakes per second. Once each of the 45 hair samples were completely pulverised, they were placed into a new, labelled 1.5 millilitre microcentrifuge tube. A pipette was used to place 1.5 millilitres of 90% analytical grade methanol into each centrifuge tubes containing a hair sample. Each of the 45 centrifuge tubes were capped and incubated at room temperature with constant pulsating using a Bio Line orbital shaker for 3 hours. After 3 hours, the centrifuge tubes were removed, and the contents were strained using a 0.5mm micro precision sieve. The liquid from each hair sample was then transferred into a new, labelled 1.5 millilitre centrifuge tube with a pipette, and the hair was discarded.

An Arbor Assay DetectX Cortisol kit (product code K003-H1W, manufactured in Michigan USA) was used for processing and analysing the cortisol levels in each sample of hair. The cortisol standards were prepared by labelling 6 1.5 millilitre centrifuge tubes for the serial dilutions (labels include standard 1 through to 6). Using a micropipette, 450 microliters of assay buffer was placed into the centrifuge tube labelled standard 1, and 250 microliters of assay buffer into the centrifuge tubes labelled standard 2 through to 6
as per the kit’s instructions. Note, the serial dilution is performed to generate the standard curve. A pipette was used to place 50 microliters of samples and 50 microliters of standards into the well plate as per the kit’s instructions. A pipette was then used to place 75 microliters of assay buffer into the non-specific binding wells, and 50 microliters of assay buffer into the maximum binding zero wells as per the kit’s instructions. Note, all samples and standards were run in duplicate to allow for accuracy of results. A repeater pipette was then used to place 25 microliters of DetectX cortisol conjugate into every well on the well plate. A repeater pipette was again used to place 25 microliters of DetectX cortisol antibody into every well on the well plate except the non-specific binding cells. The well plate was next covered with a sealer and placed on an orbital shaker for 1 hour at room temperature. After an hour, the plate was taken off the orbital shaker and the sealer was removed. The well plate was then aspirated by washing each well with 300 microliters of wash buffer 4 times in a Bio Radi Mark microplate washer. The plate was then dried by tapping it on clean absorbent towels. A pipette was used to place 100 microliters of TMB substrate to each well in the well plate, then a new plate sealer was placed on the well plate so that it could be incubated at room temperature for a further 30 minutes. Finally, a pipette was used to place 50 microliters of stop solution to each well on the well plate. The well plate was placed in a Bio Redi Mark microplate reader at 450 nanometres to read the optical density of each well. The plate readers built-in 4PLC software capabilities were used to calculate the cortisol concentrate of each sample.

Microsoft® Excel was used to statistically analyse the data. A between group analysis was conducted for the admission prognosis of all 45 koalas admitted to the Port Macquarie Koala Hospital. The median cortisol reading was calculated for each prognosis (disease, dog, fire, HBC, unknown and unsuitable environment), and this was plotted in a column graph. The standard deviation for each prognosis category was calculated and then plotted in the same column graph to represent error bars. A single factor ANOVA was then run on the data to produce a P-value, which aims to determine if there is a significant difference between cortisol readings of each prognosis.
Results

Of all 45 wild rescued koala patients admitted to the Port Macquarie Koala Hospital, each were given a prognosis relating to why they were admitted into care. The varying prognoses for the koalas included suffering with a disease (i.e. chlamydia, retrovirus etc), being attacked by a dog, being caught in a bushfire, being hit by a car (i.e. when crossing a road) and being in an unsuitable environment (i.e. being displaced by land clearing). A hair sample taken from each koala patient was tested and produced a cortisol reading when analysed. The prognosis with the highest median cortisol reading was disease at 1370.99 ng/g (figure 1). Following disease, HBC had the next highest median cortisol reading at 1285.48 ng/g, with unknown prognosis following at 1258.9 ng/g (figure 1). A single factor ANOVA was run and produced a P-value of 0.97. This indicates that there was no significant difference between each prognosis and the associated median cortisol reading.

Figure 1: The median cortisol readings given to all 45 wild rescued koala patients based on their prognosis when admitted to the Port Macquarie Koala Hospital during December of 2017 through to September of 2018.
Discussion

There are multiple studies that use a range of techniques to detect cortisol in hair (Ashley et al., 2011, Macbeth et al., 2010, Dettmer et al., 2012, Di Francesco et al., 2017, Cattet et al., 2014). Despite the fact that this process has been successfully performed on a number of species already, no such research has been performed to date where hormones such as cortisol are extracted from koala hair in order to assess chronic stress. This groundbreaking study used hair from wild rescued koalas to measure physiological stress by using a pre-determined cortisol extraction technique in samples of hair from koalas admitted into care. Our preliminary studies found methanol to produce better results when used to extract cortisol in koala hair, compared with solvents such as ethanol and isopropanol. Ethanol, methanol and isopropanol are all primary alcohols that are bonded by hydrogen molecules and are commonly used as solvents in hormone extraction experiments (Kanse et al., 2014). Generally, polar substances dissolve best in other polar substances, whereas non-polar substances dissolve best in other non-polar substances. The alcohol group containing methanol are very polar solvents, whereas the alcohol group containing isopropanol are very non-polar solvents. Due to its molecular build, alcohol groups containing ethanol have the advantage of being both a polar and non-polar solvent. Steroid hormones such as cortisol are considered non-polar, meaning that cortisol should have a positive solvent relationship with both primary alcohol solutions ethanol and isopropanol. However, contrary to this, our preliminary studies proved that methanol was the most successful alcohol solvent for extracting the steroid hormone cortisol in koala hair. Furthermore, since it is common to dilute solvents before use in sensitive immunoassays, as chemicals can interfere and cause results to become skewed (Palme et al., 2013), we diluted the extraction solvent so that it was 90% methanol.

The production of excessive glucocorticoids such as cortisol can be indicative of some degree of chronic stress, as cortisol is produced when the body has reached over-exertion and the duration of a particular stressor extends beyond what an organism can handle (Beehner and Bergman, 2017). However, the body retains a baseline level of cortisol which must first be considered when determining if levels of cortisol are excessive in response to particular stressors (Narayan et al., 2013). Since there have been no previous studies that analyse cortisol in hair, there are no current baseline levels to compare the results of this research with. It is incorrect to compare baseline and elevated faecal
cortisol levels with baseline and elevated hair cortisol levels; however, trends can be analysed to allow for possible comparisons until proper baseline hair cortisol level are known. A previous study on captive koalas in Queensland, Australia was performed with the aim of determining the sub-lethal effects of environmental stressors on the physiology of koalas in captivity and in the wild (Narayan et al., 2013). Four koalas had faecal samples collected with the aim of determining baseline faecal cortisol levels additional to elevated faecal cortisol levels in response to glucocorticoid injections (Narayan et al., 2013). Before the koalas were injected with adrenocorticotropic hormone, their baseline faecal cortisol levels were as follows: Arthur (5.3 ± 0.31 ng/g), Irwin (7.1 ± 1.98 ng/g), Carrie (2.9 ± 0.45 ng/g) and Erna (3.3 ± 0.46 ng/g) (Narayan et al., 2013). After the koalas were injected with adrenocorticotropic hormone, their faecal cortisol levels were as follows: Arthur (8.2 ± 1.21 ng/g), Irwin (9.9 ± 1.24 ng/g), Carrie (3.6 ± 0.40 ng/g) and Erna (10.7 ± 2.15 ng/g) (Narayan et al., 2013). The percentage rise between baseline faecal cortisol levels and faecal cortisol levels after injections of adrenocorticotropic hormone were as follows: Arthur 54.7%, Irwin 39.43%, Carrie 24.13% and Erna 224% (Narayan et al., 2013). The elevated faecal cortisol levels in the Queensland, Australia koalas are significantly lower than the hair cortisol levels for the koalas in this study. This could be because the Port Macquarie, Australia koalas were significantly more stressed than the Queensland, Australia koalas, however as previously mentioned, it is unknown if faecal cortisol results and hair cortisol results are comparable.

Results indicate that each prognosis (disease, dog, fire, HBC, unknown and unsuitable environment) given to all 45 wild rescued koala patients admitted to the Port Macquarie Koala Hospital between December of 2017 through to September of 2018 produced a cortisol reading (Narayan and Parisella, 2017). Although these results cannot be compared to any baseline figures, the hair cortisol readings can still be analysed for their relationship with chronic stress. It is substantial that disease was the prognosis with the highest median cortisol reading as the presence of chronic stress does not allow the HPA axis to reach a recovery phase, resulting in dysfunction of the negative feedback mechanism and the onset of subsequent health impairments (Bonier et al., 2009). In terms of disease, the cost of the HPA axis not reaching a recovery phase involves increased susceptibility to disease, shedding of infectious agents and a shift in host-
parasite equilibrium (Martin, 2009). Furthermore, the prolonged exposure of glucocorticoids in the body can effect immunological processes via receptors on immune cells and changes in immune gene expression in target tissues (Martin, 2009). Therefore, it is not surprising that those wild rescued koala patients who were given the prognosis disease, had the highest median cortisol reading.

The wild rescued koalas who were given the prognosis of being hit by a car, being in an unsuitable environment, being attacked by a dog and being caught in a bushfire all returned cortisol readings, although they were not as elevated as disease. This indicates that these events and the consequences associated with them did not allow the HPA axis to reach a recovery phase, resulting in dysfunction of the negative feedback mechanism (Bonier et al., 2009). The rapid development, urbanisation and human population growth are dramatically altering the habitats in which animals dwell (Donnelly and Marzluff, 2006). The change in suitable habitat such as the decline of forest cover dramatically expands the likelihood of mortality and morbidity in koalas (Narayan and Williams, 2016). Koalas living in fragmented habitats are forced to increase their movement in search for food (Davies et al., 2013b, Davies et al., 2013a). This increases their vulnerability to predation, in particular to dogs, who view koalas as prey and therefore koalas are at a high risk at being attacked (Davies et al., 2013b, Davies et al., 2013a). Furthermore, as koalas are forced to move further for food, they increase the probability of falling victim to vehicle trauma as roads are being placed between interconnecting koala habitats (Davies et al., 2013b, Davies et al., 2013a). As koalas are increasingly needing to search for suitable food sources in locations further away, this species is finding itself in areas outside of their niche (Donnelly and Marzluff, 2006). Whether koalas are sustaining injuries from disease, being hit by cars, being attacked by dogs, or whether unsuitable habitat or bushfires are the source of morbidity or mortality, it is clear that koalas are exposed to an alarming number of stressors. Results clearly indicate that each wild rescued koala admitted to the Port Macquarie Koala Hospital encountered some degree of chronic stress.
**Conclusion**

The aim of this study was to measure glucocorticoids in koalas. Hair samples were analysed for 45 koala patients which were admitted to the Port Macquarie Koala Hospital during December of 2017 through to September of 2018. Despite the fact that this process has been successfully performed on a number of species already, no such research has been performed to date where hormones such as cortisol are extracted from koala hair in order to assess chronic stress. Results indicated that although there was no significant differences in cortisol readings between each diagnosed prognosis, koalas suffering from ‘disease’ produced the highest cortisol readings. Future studies will need to be adopted in healthy, non-stressed koalas to determine base-line measures of hair cortisol. However, this preliminary research will contribute greatly to studies in stress physiology and much needed conservation for the koala species.
Recommendations

There are a number of legislative policies aimed at assisting in koala conservation throughout Australia. Each state operates individually from each other by implementing their own management plans.

Within Queensland there is:

1) *The Planning Act 2016* which regulates new developments including areas of South East Queensland identified as having important koala habitat values.

2) *The Planning Regulation 2017* which prohibits the clearing of bushland habitat in these priority koala habitat areas, as well as areas outside the urban footprint to ensure koala habitat remains protected for the long-term.

3) *The Queensland Environmental Offset Policy* which is used to ensure environmental offsets for unavoidable impacts on high quality koala habitat, regulated by the Planning Act 2016, contributes to the rehabilitation, establishment and protection of koala habitat.

4) *Koala Conservation Policy* which outlines how state public sector entities will consider koala conservation outcomes in the planning and delivery of government supported infrastructure projects like roads, schools and hospitals.

5) *The Nature Conservation (Koala) Conservation Plan 2017* requires any clearing in certain areas to be undertaken sequentially, and in the presence of a suitably qualified koala spotter.

Within South Australia there is:

1) *The South Australian Koala Conservation and Management Strategy* which is committed to safeguarding the welfare of koalas by increasing the social, educational and economic benefits of having koalas in South Australia and reducing the negative impacts that over-abundant koala populations may have on broader ecological communities.

Within New South Wales there is:

1) *The NSW Koala Strategy* which aims to first stabilise and then increase koala numbers through four pillars, including habitat conservation, community action, safety and health of koala populations, and building knowledge and education.
2) *Saving our Species Iconic Koala Project* which aims to inform future koala conservation actions through a combination of threat mitigation, research, monitoring and community engagement.

3) *Rehabilitation of Protected Fauna Policy* which guides volunteer groups that rescue injured, sick or orphaned native animals, care for them and release them back into the wild.

It has been identified that the presence of disease is one of the primary reasons koalas are being admitted into clinical care throughout Australia, however there is only one policy among all three states in Australia which assists in guiding the volunteer groups responsible for the rescue, rehabilitation and release of koalas. Furthermore, the aforementioned Rehabilitation of Protected Fauna Policy was effected in 2001, and was last modified in 2010. Currently, the primary focus of this policy revolves around issuing and renewing licences to operate as a rehabilitation group, and ensuring that licence holders are not breaching procedures, guidelines and the code of practice. It is imperative that we continue to assist koalas who find themselves sick and injured, however there should be a heightened focus on preventing koalas from becoming sick and injured by protecting the habitat they depend on. Minimisation of chronic stress is the most effective means of safeguarding individual fitness, thus it is imperative that sources of stress are identified and mitigated.

In my educated opinion, the current legislative policies aimed at assisting in koala conservation is not enough. I would argue that a Koala Protection Act should be enacted to operate as a national legislation aimed at protecting koalas throughout Australia. This would ensure legislative action is consistent for all koalas in Australia, since they are all presented with the same stressors regardless of which Australian state they are in. Within this Koala Protection Act, there would be a renewed focus on the koala by focusing on protecting their habitat. In this act, if there is a development proposed within a predetermined geographic koala habitat range, applications would be denied unless it can be unequivocally proven that actions will be benign to the landscape. Good industry leaders should not be discouraged or threatened by sensible legislation. Additionally, the listing of koalas on the IUCN Red List of Threatened Species as “vulnerable to extinction”
is redundant. This study has explicitly shown that koalas are at risk of extinction, and thus their listing should be changed to “endangered”.

**Future Research**

In this Master of Research Thesis, a retrospective analysis was performed with the aim of identifying trends in clinical admissions and diagnosis of koalas. Thus far, the preliminary research to make chapter 4 possible was submitted and accepted to an academic journal as a methods paper (Title: Cortisol Measurement in Koala *Phascolarctos cinereus* Fur). The research performed in chapter 3 is currently under review for a book chapter, and the research performed in chapter 2 is being prepared to be submitted to another academic journal. Additionally, Dr Edward Narayan and I intend on furthering the research performed in this Master of Research Thesis by pursuing a PhD, whereby specific geographic koala ranges will be investigated, and a baseline hair cortisol range can be established to further investigate chronic stress in koalas.
Reference List


85


### Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>HPA</td>
<td>Hypothalamic-pituitary-adrenal axis</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotrophin-releasing hormone</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-Like Growth Factor-1</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle-stimulating hormone</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>EIA</td>
<td>Enzyme immunoassay</td>
</tr>
<tr>
<td>CSV</td>
<td>Comma-separated documents</td>
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<tr>
<td>Term</td>
<td>Description</td>
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<td>--------------------</td>
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</tr>
<tr>
<td>Disease</td>
<td>When a koala had a disordered or incorrectly functioning organ, part, structure, or system of the body resulting from the effect of genetic or developmental errors, infection, poisons, nutritional deficiencies or imbalance, toxicity or unfavourable environmental factors.</td>
</tr>
<tr>
<td>Unknown</td>
<td>When a koala had an unknown prognosis or when the prognosis was not recorded.</td>
</tr>
<tr>
<td>Appeared healthy</td>
<td>When the koala appeared to be in an ideal body condition with no visible injuries or diseases.</td>
</tr>
<tr>
<td>HBC</td>
<td>(HBC = hit by car) When a koala was physically impacted by a motor vehicle and sustained some level of injury.</td>
</tr>
<tr>
<td>Unsuitable environment</td>
<td>When a koala was found in an environment that was not suitable to live in. Some examples include being in a shop, restaurant, house, an area with cleared trees, road etc.</td>
</tr>
<tr>
<td>Dog</td>
<td>When a koala was either chased by or physically impacted by a dog.</td>
</tr>
<tr>
<td>Orphan</td>
<td>When a koala falls within the joey or juvenile age group and is not in the care of their mother.</td>
</tr>
<tr>
<td>Fire</td>
<td>When a koala was caught in a bushfire or escapes a bush fire. This often results in singed fur or other serious injuries.</td>
</tr>
<tr>
<td>Category</td>
<td>Description</td>
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<tr>
<td>------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Cattle</td>
<td>When a koala was either chased by or physically impacted by cattle.</td>
</tr>
<tr>
<td>Harassed by humans</td>
<td>When a koala was touched, handled, or picked up by a member of the public.</td>
</tr>
<tr>
<td>Collared for tracking</td>
<td>When a koala was caught, collared and then released back as part of a program to track koala movement.</td>
</tr>
<tr>
<td>Snake</td>
<td>When a koala was bitten or eaten by a snake.</td>
</tr>
<tr>
<td>DOA</td>
<td>(DOA = dead on arrival) When a koala was already deceased when a member of the public or rescue group attended a call out for a koala needing rescue.</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>-----------------------</td>
<td>---------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Signs of Chlamydia</td>
<td>When a koala was either diagnosed with the bacterial infection chlamydia, or they presented with signs of chlamydia including having a wet bum, or red swollen eyes.</td>
</tr>
<tr>
<td>Infection</td>
<td>When a koala was diagnosed with an infection, also known as an invasion of disease-causing agents. Infections included urinary tract infections, or infections from an open wound.</td>
</tr>
<tr>
<td>PBC</td>
<td>(PBC = poor body condition) When a koala was severely underweight, had multiple wounds on the body etc.</td>
</tr>
<tr>
<td>Organ damage</td>
<td>When one or many main organs are compromise or damaged in some way, including injury to the heart, brain, stomach, kidneys etc.</td>
</tr>
<tr>
<td>Eye injury</td>
<td>When one or both eyes are missing, or badly damaged to the point where vision is compromised.</td>
</tr>
<tr>
<td>Old age</td>
<td>When a koala is aged over 12 years old and is showing signs of old age including dirty teeth, worn down claws etc.</td>
</tr>
<tr>
<td>Head trauma</td>
<td>When a koala has any sort of injury to the brain, skull or scalp. These injuries can range from a mild bump or bruise to a traumatic brain injury.</td>
</tr>
<tr>
<td>Dehydrated</td>
<td>When a koala is seen drinking water directly on the ground or looks severely depressed.</td>
</tr>
<tr>
<td>Condition</td>
<td>Description</td>
</tr>
<tr>
<td>-------------------------</td>
<td>----------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Tick infestation</td>
<td>When a koala has more than a few ticks present on them. The koalas will usually look depressed as they will usually be suffering from severe anaemia.</td>
</tr>
<tr>
<td>KoRV</td>
<td>(KoRV = Koala Retrovirus) When a koala has an immune deficiency, much like AIDS, and is often seen through cancers.</td>
</tr>
<tr>
<td>Damaged claws</td>
<td>When a koala has one or many missing or broken nails on either of their four paws.</td>
</tr>
<tr>
<td>Leg injury</td>
<td>When a koala has any injury to one or more of their arm or leg limbs. These injuries can include damage to the muscles, ligaments or bones.</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>---------------------</td>
<td>--------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Released</td>
<td>When a koala was released back into the wild after being in care for a period of time.</td>
</tr>
<tr>
<td>Euthanised</td>
<td>When a koala was suffering from a disease or injury that compromised their welfare, so were injected with a substance that painlessly ended their life.</td>
</tr>
<tr>
<td>Advice only</td>
<td>When a member of the public made contact with a rescue group as they needed advice about a koala.</td>
</tr>
<tr>
<td>Record of sighting</td>
<td>When a member of the public made contact with a rescue group to report a sighting of a koala.</td>
</tr>
<tr>
<td>DOA</td>
<td>(DOA = dead on arrival) When a koala was already deceased when a member of the public or rescue group attended a call out for a koala needing rescue.</td>
</tr>
<tr>
<td>Died in care</td>
<td>When a koala died without assistance when in the care of a rescue group.</td>
</tr>
<tr>
<td>Relocated</td>
<td>When the koala was caught by a rescue group and moved to a habitat more suited to their survival.</td>
</tr>
<tr>
<td>Unknown</td>
<td>When a koala had an unknown outcome or when the outcome was not recorded.</td>
</tr>
<tr>
<td>Absent on arrival</td>
<td>When a member of the public reported a sighting of a koala but the koala was not present when the rescue group attended.</td>
</tr>
<tr>
<td>Transferred</td>
<td>When a koala was transferred from one rescue group to another.</td>
</tr>
<tr>
<td>In care</td>
<td>Refers to a koala still in the care of a rescue group at the time of data collection.</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>----------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Unable to capture</td>
<td>When a rescue group are not able to be catch a koala to admit into their care.</td>
</tr>
<tr>
<td>Self release</td>
<td>When a koala escapes from their enclosure when being cared for by a rescue group.</td>
</tr>
<tr>
<td>MOP would not hand over</td>
<td>When a rescue group attends a koala reporting but the member of public refuses to hand the koala over</td>
</tr>
</tbody>
</table>

**Age Groups**

<table>
<thead>
<tr>
<th>Joey</th>
<th>From birth to 6 months of age.</th>
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<tbody>
<tr>
<td>Juvenile</td>
<td>Between 6 months and 1 year of age.</td>
</tr>
<tr>
<td>Adult</td>
<td>From 1 year of age to 7 years of age.</td>
</tr>
<tr>
<td>Mature</td>
<td>Above 7 years of age.</td>
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<tr>
<td>Unknown</td>
<td>Unknown age or age was not recorded.</td>
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### Port Stephens

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### Port Macquarie Post

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### Lismore Postcodes

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$y = -0.6997x + 66.146$
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