The Power of Koala Poo

The development and assessment of novel non-invasive technology for the assessment of Koala genetics, disease, and reproductive status

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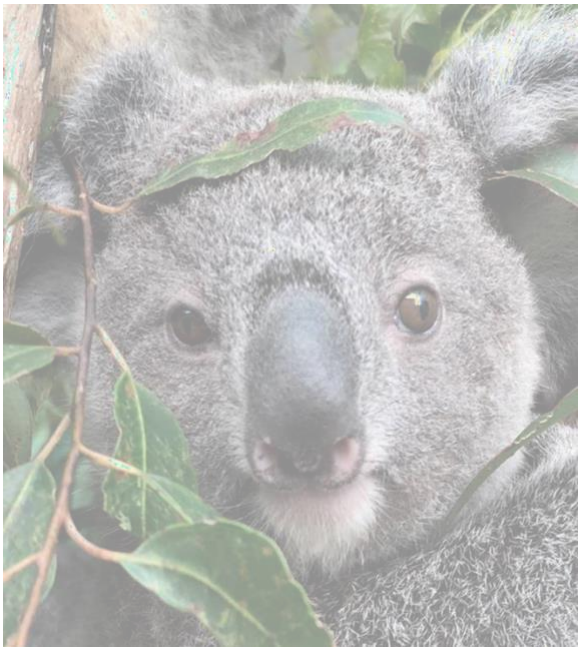

The University of Queensland

EXECUTIVE SUMMARY

The Power of Koala Poo research project was established as a research contract between the University of Queensland and Celestino Pty Ltd (as part of an offset obligation) to develop new generation technologies focused on the analysis of koala scat samples to improve our non-invasive assessment of koala genetics, disease and reproductive status, culminating with applications to the koala population residing in the surrounding area of the Celestino led Jimboomba Residential Development.

The first aim of the project was to develop a koala SNP (single nucleotide polymorphism) genetic marker array to be made available to all koala researchers and stakeholders via our project partner the Australian Genome Research Facility (AGRF), in order to facilitate provision of a standardized genetic marker panel for comparison of koala populations on the east coast of Australia. To this end, we have discovered and identified a final suite of 15,766 SNPs via mapping of genomes of koalas from south-east Queensland. Now that we have identified the SNPs, this information has been sent to Australian Genome Resource Facility (AGRF) who are in the process of manufacturing the array by mid-May 2024. The final step in the practical application of array will be its validation via the testing of koala DNA isolated from tissue samples of koalas from south-east QLD. Greater than 1000 koala tissue samples have been collected to date from koala admissions at Currumbin Wildlife Hospital and Australia Zoo Wildlife Hospital. While this project was initially based on DNA extracted from tissue samples, we have also identified SNPs that will be used to produce an array, specifically designed for poorer quality DNA obtained from faecal samples.

Secondly, this project aimed to validate and apply faecal hormone metabolite enzyme linked absorbent assays (EIA) for the koala, and included assays for progestogen, cortisol, corticosterone, oestrogens and testosterone. This suite of hormone assays was first applied to captive koalas (fresh and frozen samples) and wild koalas from the Gold Coast region for the purposes of validation and finally to the analysis of samples from the Jimboomba population. We report here the successful validation of a faecal testosterone metabolite assay for male koalas and a faecal progesterone metabolite assay for female koalas. Three different oestrogen metabolite EIAs were tested on female koala faecal extracts but none were suitable for oestrus detection (elevated levels of oestradiol). While further subsequent analysis of these samples from koalas of known reproductive status using liquid chromatography and mass spectrophotometry (LC/MS) and high pressure liquid chromatography/MS (HPLC/MS) was successful in isolating oestrogen metabolites, these values were not biologically relevant for characterizing oestrus.

In addition, we also applied our “in house” glucocorticoid (GC) EIAs to measure biologically relevant faecal concentrations of cortisol and corticosterone to captive and wild koalas. Results from the captive koalas showed adequate correlation of these GCs with perceived acute stressors. GC analysis of the koala faecal samples collected from the Jimboomba site in 2022 revealed cortisol and corticosterone metabolite levels that were generally very low; we attributed these results to poor sample quality associated with rain damage. Better quality samples collected in 2023, and which were cross-matched with genetic information, obtained from scat DNA, revealed a much more variable individual pattern of GC secretion. We conclude that while it is possible to measure GC in koala faeces, an interpretation of these values, as it relates to a dysfunctional stress response requires further validation. We have also commenced preliminary studies to explore other stress physiology biomarkers (e.g. Dehydroepiandrosterone [DHEA] and Thyroid hormone metabolites [T3 and T4]) that can be run in parallel with the analysis of faecal GCs in order to provide better context to the assessment of stress physiology.

The third aim of the research project was to conduct a field survey of the koala population in the Jimboomba Celestino site during its development to relate findings obtained from non-invasively collected faecal samples to conventional ecological surveys (transects and drone observations). The field survey of the Jimboomba site included 137 site visits by our field ecologist (Mr. Al Mucci) occurring between 03/01/22 to 27/07/23. Greater than 400km of line transects were walked within the bushland areas as part of this study. A total of over 200 plastic mats were laid under preferred koala food trees or trees associated with koala presence during the survey, with 74 scat collections. Drone surveys were also conducted across the study site on 14 occasions, with adverse weather activity playing a significant role in constraining drone flights during an extended wet period in 2022. Many of the koala scats collected in early part of the field survey were therefore rain damaged. Koala DNA (scat DNA with positive koala beta-actin PCR result) was detectable in 66% of faecal scats collected (49/74 scats) with none of the samples showing evidence of being infected with the pathogen *Chlamydia pecorum*. Genotype profiles based on 32 microsatellite genetic markers were generated from koala scat DNA; this genetic data revealed moderate genetic diversity of the Jimboomba population, with a moderate to high inbreeding value. Analysis of repeated genotypes within the dataset identified 7 individual koalas. Faecal DNA from the Jimboomba population will also be further analysed once the marker panel has been constructed by AGRF.

In addition to making our SNP markers publicly available to other research groups and government agencies via the Genomic data repository for the Threatened Species Initiative https://github.com/awgg-lab/australasiangenomes, we have also submitted for publication in the journal “Biology”, a review paper entitled “The utility of the koala scat”. We also presented the reproductive faecal hormone assay work to the Zoological and Aquarium Association Annual 2022 Conference. Further publications arising from our SNP validation studies will be ready for submission by late 2024.

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INTRODUCTION

The Power of Koala Poo research project was a collaboration between the University of Queensland and Celestino Pty Ltd to develop new generation non-invasive technologies based on the analysis of koala scats to improve our knowledge base and assessment in regard to koala genetics, disease and reproductive status, with the real-world application of these techniques to koalas residing in the surrounding area of the Celestino led Jimboomba Residential Development. The project encompassed 3 aims; (1) to establish a koala SNP array panel for genetic analysis, (2) to validate and apply faecal hormone assays to captive and wild koala populations and (3) to apply non-invasive technology to the local wild koala population at Jimboomba.

1.1. Development of a Koala SNP Array

Maintaining genetic diversity is a crucial component in conserving threatened or endangered species populations. For the iconic Australian koala, there is little genetic information on wild populations that is not either skewed by biased sampling methods (e.g., sampling effort skewed toward urban areas or animals entering veterinary hospitals) or which has been limited in usefulness due to the low numbers of genetic markers used. The ability to genotype DNA isolated from koala scats using next-generation sequencing technology will not only help to resolve location sample bias but also improve the accuracy, scope, and the utility of genetic analyses.

With an increase in the availability and affordability of next-generation sequencing technologies, genome-wide markers are becoming increasingly popular in evolutionary and ecological research. Such genotyping by sequencing methods allow for unprecedented ease of research into non-model organisms, such as the koala. Bi-allelic single nucleotide polymorphism (SNP) markers are less informative per marker than microsatellites but have the advantage of being highly abundant across the genome as well as being evenly spread across the genome.

Since the late 1990s, high-density DNA arrays have become a promising tool to assess genetic variability at a massive scale. After their first use in human genetics, they have been widely adopted for non-human research, including plant and animal breeding. Genome-wide SNP genotyping is now considered the preferred marker for population-based diversity studies in many species and serves as a versatile tool for providing insights into genetic structuring and micro-evolutionary processes in the koala.

These markers will be used to address critical questions for the species, including assessments of speciation, inter- and intraregional population diversity and relatedness, inbreeding, signatures of population reductions, estimates of effective population size, parentage, and evidence of adaptive variation. Aim 1 of this project was to develop a national koala specific array panel of genome-wide SNP markers that will serve as an important tool for the conservation of the species, and which will be used to provide insights into population structuring and variability across the species' range.

1.2. Assay of Koala Faecal Hormone Metabolites

The capacity to determine non-invasive physiological parameters of reproduction and stress physiology from scat samples represents a powerful tool for those making assessments of koala population health. For example, elevated progesterone levels tell us about koala pregnancy, elevated oestrogen levels inform us about female koala reproductive cyclicity and changes in testosterone levels are instructive with respect to male seasonality

and sexual maturity. While the relevance of elevate glucocorticoid metabolites secreted in the koala scat as a measure of stress physiology of the individual animal is still somewhat controversial, it may have application at the population level. Aim 2 of this project was to establish and validate faecal hormone metabolite assays for the koala including progestogens, cortisol, corticosterone, oestrogens, prostaglandin and testosterone metabolites. This suite of hormone assays was first applied to faecal samples previously collected from captive and wild koalas for the purposes of validation and to viable samples recovered from the Jimboomba koala population.

1.3. The Jimboomba Koala Population

Although our non-invasive technologies in this project were designed to have broad application to koala conservation throughout Australia, aim 3 of this project was directed at applying the results of aims 1 and 2 to the monitoring and assessment of the Jimboomba and surrounding koala populations. For this analysis, we combined conventional field ecology assessment (spotting, transects and using drones to identify koala location) with that of genetic, reproductive, stress physiology and disease (*Chlamydia pecorum*) data acquired from scat samples from the same habitat. Koala surveys were conducted over the development property and neighboring properties over a 1.5-year time period (2022-2023). Our analysis reports on the (i) the distribution and movement of individual koalas, (ii) an estimate of the number of koalas based on genetic analysis, (iii) genetic diversity of the population, (iv) the disease status (assessment of *Chlamydia pecorum* infection) and (v) the reproductive status of the Jimboomba koala population.

2. METHODS AND RESULTS

2.1 Development of a Koala SNP Array

Single nucleotide polymorphisms (SNPs) are single nucleotide base variations, caused by transitions (C/T or G/A) or transversions (C/G, C/A, or T/A, T/G), in the same position between individual genomic DNA sequences. SNPs are the predominant type of DNA polymorphism for genetic variation, which is ubiquitously located in genomes in the intergenic region (regions between genes), coding sequences of genes (exons), or non-coding regions of genes (introns). SNPs are extensively used as a molecular marker for analysing genotypes, with traditional SNP mining techniques being generally low in throughput and technically complicated. In this project, assembly and mapping of koala SNP markers has encompassed analysis of publicly available koala genomes, provided from the Koala Genome Consortium group (University of Sydney). (https://awgg-lab.github.io/australasiangenomes/species/Phascolarctos_cinereus.html).

2.1.1 Koala SNP discovery

The genome sequences of 63 koalas originating from south east Queensland were uploaded to the bioinformatics platform Galaxy Australia (https://usegalaxy.org.au/) and aligned to the koala reference genome (NCBI genbank reference genome GCA_002099425.1_phaCin_unsw_v4.1_genomic.fasta https://www.ncbi.nlm.nih.gov/assembly/GCA_002099425.1/) by the Burrows-Wheeler Aligner (BWA-MEM2 version 2.2.1). The tool Genomic File Manipulation was used to remove duplicate sequences and filter unmapped or unproperly mapped reads. DNA nucleotide variants (SNPs) were called for each of the 63 genomes using the tool FreeBayes, the Bayesian genetic variant detector enabling discovery and filtering of DNA nucleotide variants (SNPs) within the sample genome to produce a Variant Call Format (VCF) file for each sample. All 63 VCF files were merged to create one multi-sample file

containing total SNPs discovered specific for koalas in southeast Queensland.

A total of 79,642,434 SNPs were detected from DNA analysed in this project. VCFtools was employed to filter those SNPs with Minor allele frequency (MAF) lower than 0.05 and bedtools (version 2.30.0). SubtractBed was used to remove repeat regions of sequences and mitochondrial regions, leaving a final set of **15,766** filtered SNPs. These SNPs are currently being assembled for a custom designed SEQ specific koala SNP array panel to be implemented on the Allegro® Targeted Genotyping V2 (TECAN) platform. The custom SEQ koala SNP genome coordinates file required to initiate the array's custom design has been forwarded to our project partner Australian Genome Research Facility (AGRF) to initiate manufacture of the koala SNP array. Validation of the koala SNP array will include testing of koala DNA isolated from tissue samples collected from koalas located in south-east QLD. Greater than 1000 koala tissue samples have been collected from koala admissions at Currumbin Wildlife Hospital and Australia Zoo Wildlife Hospital.

2.1.2 Sharing of generated SNP Data: A national collaboration

The creation of a standardized suite of koala markers, available nationally to koala researchers and managers requires a collaborative contribution from koala genetic stakeholders to facilitate a direct comparative of the genetic health of regional koala populations. To this end, our project team have assembled and joined a national koala genetics working group consisting of researchers with a specific focus on koala genetics from University of Sydney (NSW), James Cook University (Townsville), University of Sunshine Coast (QLD) and Federation University (Victoria). Since the beginning of this project, the koala genetics working group have met three times to discuss the development of the koala SNP array, with design of the array to include specific SNP panels that will suit different purposes, e.g. region specific genotyping, immune genes, specific samples such as those associated with faecal DNA isolation.

With our UQ team participating as facilitators of this project, working group discussions have summarized the objectives of the project to include:

- Development of a koala SNP database (USyd: already in progress) to include
  - Mapping SNP loci
  - Keeping a record of the different sources the SNPs have originated from
  - Public accessibility to the web-based database

- Production of SNP panels
  - Collaborative agreement from the working group on SNP panels to be included
  - SNP groups for each panel can be different sizes and suit different purposes

- Establishment of a SNP genotype database (Uni of Sydney: already in progress)
  - Sharing/availability of genotypes
  - Central web-based koala genotype and metadata repository to be publicly accessible via the Genomic data repository for the Threatened Species Initiative https://github.com/awgg-lab/australasiangenomes

As a major component of the Power of Koala Poo project and as a contribution to the working group, the Australian Genome Research Facility (AGRF) have also joined the project as collaborators to physically manufacture the koala SNP array panel and to offer their genotyping of koalas as a service to all Australia researchers and conservation managers, enabling provision of a nationally standardized koala genetic marker panel. This was a primary motivation for the "Power of Poo" project.

2.1.3: A SNP array specifically for Faecal DNA

Furthermore, validation of a non-invasive sample koala SNP panel will include optimization of DNA isolated from koala faecal scats. While the initial aim of this project was to establish SNPs for DNA obtained from tissue, we have extended the research to incorporate faecal DNA, thereby increasing the "power of poo" analysis. DNA isolated from koala scats is inherently lower quality than the gold standard of DNA extracted from tissue. While DNA from faeces is easy to obtain non-invasively, it is nevertheless dominated by bacterial and other non-host DNA. The high proportion of non-host DNA drastically reduces the efficiency of high-throughput sequencing for host animal genomics. To address this issue, we have begun to investigate an inexpensive capture method for enriching host DNA from non-invasive faecal samples. This method utilizes natural differences in CpG-methylation density between vertebrate and bacterial genomes to preferentially bind and isolate host DNA from majority-bacterial samples. This method of DNA enrichment also isolates host DNA from faecal scats and essentially "cleans" the sample of microbial DNA that may inhibit downstream genetic analysis and increase the allele dropout rate.

2.2 Faecal Hormone Metabolites

2.2.1 Reproductive hormones

We have successfully validated in our laboratory a faecal testosterone metabolite assay for male koalas (see Figure 1) and a faecal progesterone metabolite assay for female koalas (see Figure 2). These assays have been used to confirm ovulation induction after natural mating as well as the monitoring the reproductive well-being and responses to changes in the management of captive and wild koalas housed in zoos. For wild koalas, these techniques have the potential to monitor the adaptation and acclimatization of koalas translocated to new habitat or returned to the wild after extended hospital treatment and rehabilitation, in addition to evaluating the health of wild populations under pressure (such as drought or bushfires).

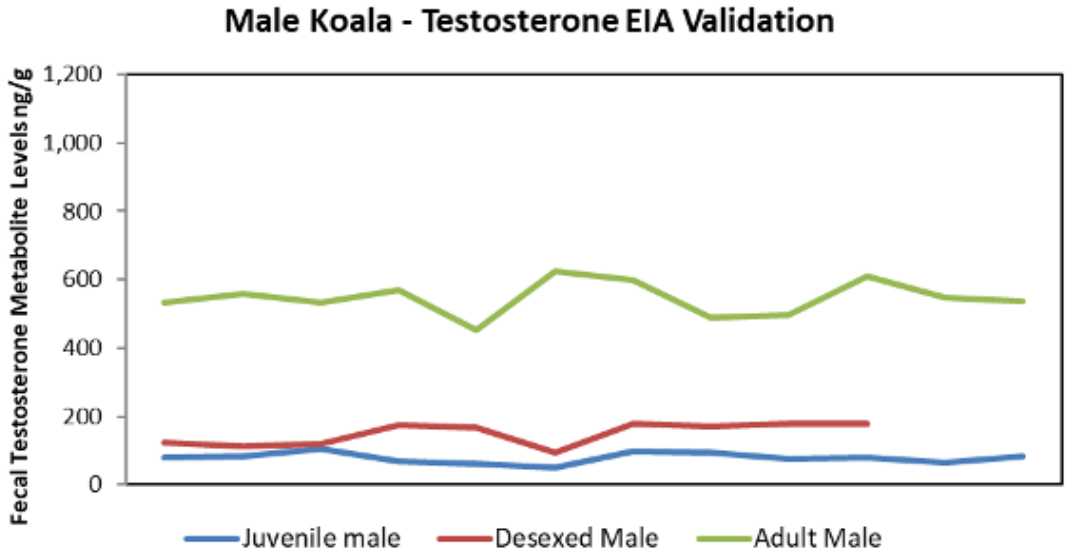

Figure 1: Comparison of the faecal testosterone metabolite levels of an adult male (green), juvenile male (blue) and desexed male (red) koala.

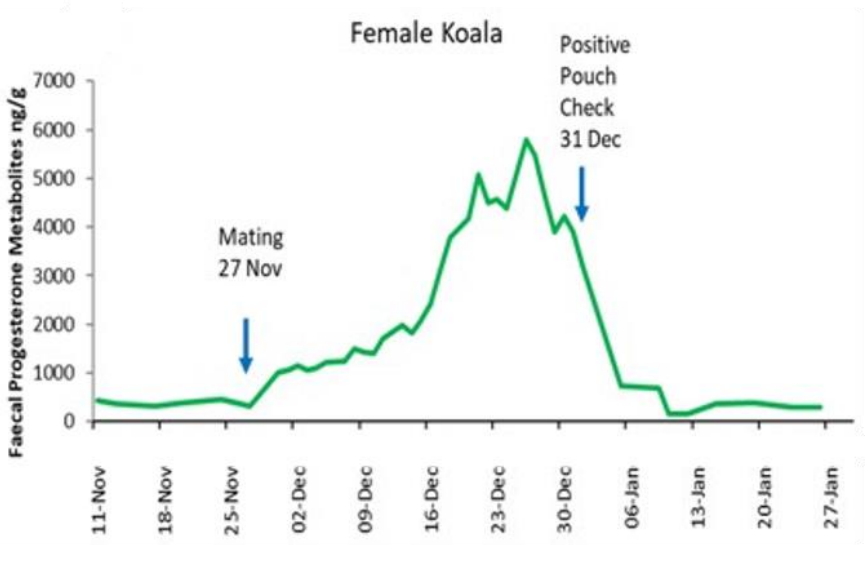

Figure 2: Changes in faecal progesterone metabolite concentrations after mating and through confirmed pregnancy in a female captive koala. Note elevated levels of faecal progesterone metabolite following ovulation and declining levels at birth.

Three different oestrogen metabolite assays were also tested for koala faeces but unfortunately none were shown to be suitable for detecting elevated concentrations of faecal oestrogens representative of female oestrus. To further investigate measurement of oestrogen metabolites, we arranged for the comparative analysis of plasma and faecal extracts by ultra-sensitive LC-MS/MS (liquid chromatography-mass spectrometry) by Dr. David Handelsman and Dr. Reena Desai (ANZAC Research Institute, University of Sydney) in order to quantify which reproductive steroid hormones might be circulating and excreted by the koala. We also sent similar faecal samples to the laboratory of Dr. Chris Barlow (Monash University) for analysis by high-performance liquid chromatography (HPLC), targeting oestradiol and oestrone and their metabolites. The ultra-sensitive LC-MS/MS picked up low but detectable 17α-oestradiol (3 of 6 samples) and 17β-oestradiol (2 of 6 samples) but not oestrone in the plasma samples of 6 koalas showing behavioural oestrus; with only one sample with detectable levels of both (Table 1). The ultra-sensitive LC-MS/MS picked up elevated oestrone levels in both faecal samples from koalas in oestrus with limited or no detectable oestrone in faecal samples post-ovulation, during pregnancy and post parturition (Table 2). Although detectable levels of 17α-oestradiol and 17β-oestradiol were found in most of the tested faecal extracts (9 of 10 and 8 of 10 samples, respectively), no clear pattern related to reproductive cycle of the koala was evident (Table 2). The HPLC analysis did not detect differences in oestrone between samples collected during oestrus,, and therefore, the results did not align to those of the ultra-sensitive LC-MS/MS.

We also attempted validation of an EIA for prostaglandin faecal metabolites (PGFM). While the initial analysis was encouraging in that a spike of PGFM in the faecal sample was noted at mating (most likely from semen) and parturition (typical for marsupials) we are currently not in a position to recommend it for use without further biological validation.

Table 1: Plasma ultra-sensitive LC-MS/MS analysis results for the detection of koala oestrogen faecal metabolites.

Koala Name	Reproductive Status	b-E2 (pg/ml)	a-E2 (pg/ml)	E1 (pg/ml)
Ruby	Oestrus	9.7	3.0	nd
Elsa	Oestrus	7.0	nd	nd
Margarita	Oestrus	nd	nd	nd
Nat	Oestrus	nd	14.7	nd
Buttercup	Oestrus	11.0	nd	nd
Etta	Oestrus	nd	nd	nd
Hazel	Unknown	6.0	nd	nd
Annabella	Low progesterone	nd	nd	nd
Annabella	Low progesterone	nd	nd	nd

Table 2: Faecal extract ultra-sensitive LC-MS/MS analysis results for the detection of koala oestrogen faecal metabolites.

Koala Name	Reproductive Status	b-E2 (pg/ml)	a-E2 (pg/ml)	E1 (pg/ml)
Poppy	Oestrus	40.6	48.8	180.4
Poppy	Post Ovulation	nd	161.1	nd
Poppy	Late Pregnancy	11.0	nd	nd
Poppy	Early Lactation	36.3	nd	nd
Maple	Oestrus	73.2	10.0	209.7
Maple	Post Ovulation	46.6	22.9	8.4
Maple	Late Pregnancy	28.5	66.2	nd
Maple	Late Pregnancy	6.4	22.9	nd
Hazel	Non-cycling	58.7	55.5	3.8
Hazel	Non-cycling	150.9	74.4	3.8

2.2.2 Glucocorticoids

Validation of faecal glucocorticoids (GC) in the koala has been conducted by previous studies using the administration of ACTH to induce a GC surge in the systemic circulation with subsequent secretion in the faeces (see Johnston et al. review in **Appendix 1**); however, the majority of these validations have not been correlated with biological validation. Using faecal samples from koalas entering Currumbin Wildlife Hospital we were able to biologically validate our “in house” cortisol and corticosterone faecal assays. We measured the cortisol and corticosterone secretion of a male koala that was suffered a trauma from a car strike, immediately after its presentation and initial veterinary assessment (including general anesthesia) to the hospital, and subsequently followed its rehabilitation; we also measured GC secretion following a further general anesthesia and electroejaculation procedure to collect semen from this animal. The results of the GC metabolite analysis are shown in Figure 3 and reveal clear acute elevations in both cortisol and corticosterone following the presumed “stress” of anesthesia and electroejaculation.

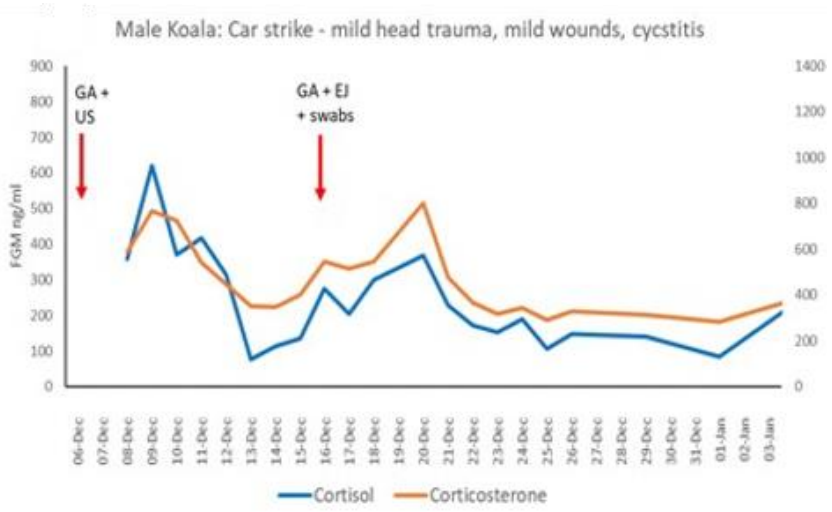

Figure 3: Changes in faecal glucocorticoid metabolite (FGM) levels after general anesthesia (GA) and electroejaculation for semen collection (EJ) in a male koala

2.2.3 Combined Faecal GC and Progesterone to Analysis Wild Koalas

Faecal GC (cortisol and corticosterone) and progesterone metabolite analysis techniques can be combined from the same faecal sample to provide physiological data to better understand and monitor the reintroduction of wild koalas back into natural habitat after extended periods of hospitalization or rehabilitation. To illustrate this application, we analysed the faecal samples from two wild female koalas in the Gold Coast region before and after their release after an extended period of human care (Figure 4A and B). Both females demonstrate normal, fluctuating patterns of faecal glucocorticoids metabolites (FGMs) confirming healthy adrenal responses to changes in their environment (Figure 4A and 4B). One of the females, “Fluffy” had elevated faecal progesterone metabolites (FPM) during the post-release monitoring period of similar amplitude and duration (estimated due to limited sampling during this period) to that of pregnant captive koalas, suggesting that this female was likely to be pregnant during this time (Figure 4B); this animal was subsequently confirmed to have a pouch young several months later, the size of which confirms that the elevated FPM detected was indeed her pregnancy/parturition. Both female koalas have settled in their respective wild habitats and appear to be thriving.

During a concurrent research study designed to quantify normal and abnormal GC hormone (cortisol, corticosterone) production in the koala (results not shown), it was found that some koalas in extreme poor health or that were unresponsive to hospital treatment, showed evidence of adrenal dysfunction, characterized by prolonged depressed GC excretion. As these abnormal, depressed levels of GC are similar to normal baseline GC excretion, we attempted further validation trials to find complementary faecal health biomarkers that might differentiate normal and abnormal GC production, and thereby attempt to establish from an endocrine standpoint, whether or not a koala is in a distressed health state. Initial validation trials have included faecal thyroid hormones (T3 and T4) and faecal dehydroepiandrosterone – sulphate (DHEA-S) (see below).

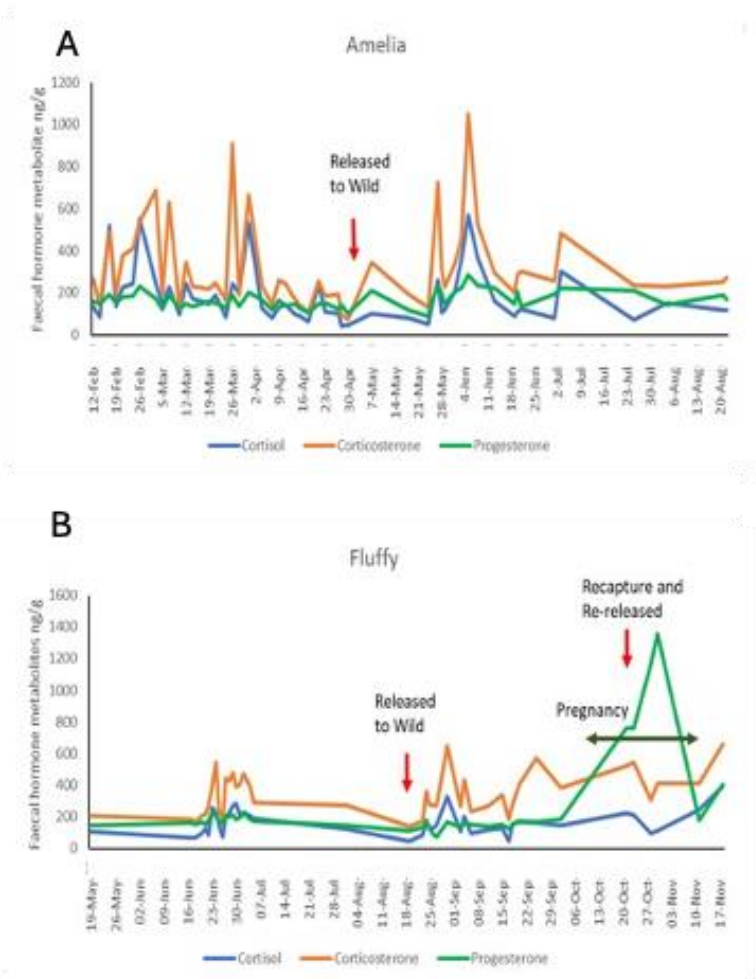

Figure 4: (A) Changes in faecal glucocorticoid and progesterone metabolite levels before and after release of a wild female koala back into natural habitat after an extended period under human care; (B) Changes in faecal glucocorticoid and progesterone metabolite levels before and after release of a wild female koala back into natural habitat after an extended period under human care. Note - Elevated faecal progesterone (green) metabolite levels detected in October were indicative of pregnancy - a joey was confirmed in the pouch several months later.

2.2.4 Additional Koala Health Biomarker - T3, T4

Thyroid hormones (thyroxine - T4 and tri-iodothyronine - T3) influence growth, maturation, metabolism and nutritional physiology. As thyroid hormones increase metabolism, a reduction in these hormones is associated with decreased metabolic rate, the conservation of energy, and a decrease in obligatory lipolytic activity (break down of fats) of energy stores and thereby an increased chance of survival when food availability is low. Consequently, an ability to non-invasively measure T3 and T4 in koala faeces and understanding the relationship of their production, could serve as useful biomarker to identify periods of nutritional deficiency due to depleted or poor food sources (e.g., bushfires or drought). Although not an initial aim of this project, we investigated the utility of T3 and T4 Arbor Assays® enzyme-immunoassay kits to detect and quantify secretion of T3 (triiodothyronine) and T4 (thyroxine). We investigated previously frozen-thawed faecal samples from koalas of varying clinical and body condition (body score 3-10) processed at the Currumbin Wildlife Hospital in an attempt to validate these EIAs. The initial analysis included 6 koalas (4 males, 2 females) of poor or good body condition to determine if biologically relevant levels and ratios of the two thyroid hormones could be detected (Figure 5). Our results revealed that the profiles of the faecal thyroid hormones for the initial koalas tested did not appear to demonstrate clear differences between

animals with poor or good body conditions when calculated to determine their respective overall levels of each hormone or a ratio of these hormones. The current dataset may be too small to make definitive conclusions, or it may be possible that one or both of the thyroid hormone EIAs are not suitable for the detection of biologically relevant changes.

2.2.5 Additional Koala Health Biomarker - DHEA

Exposure to repeated or chronic stress can cause dysregulation of the hypothalamic-pituitary-adrenal HPA axis resulting in pathophysiological effects such as a reduction in immune function and the inhibition of reproduction and growth. For an animal under chronic stress, elevated levels of GCs may not be effectively downregulated by the negative feedback loop, resulting in hypercortisolism (persistent elevated GC levels), which in turn causes damaging effects on cognition, well-being, immune function, growth and reproduction. While the HPA axis may initially be over-responsive, leading to hypercortisolism, hypercortisolism or “adrenal fatigue” may ultimately occur in some species under certain conditions. An exposure to chronic or repeated stressors, results in an adaptation within the HPA axis to protect the individual from chronically elevated GCs that threaten long-term survival; this then reduces GC signaling and alters the negative feedback loop, with reduced biosynthesis/depletion of ACTH resulting in reduced glucocorticoid production. Suppressed GC secretion is associated with numerous health issues including an increased risk of inflammatory diseases, a heightened susceptibility to certain pathogens (e.g., parasites, allergens, and toxins), impaired cognitive function, and behavioural issues. It can be difficult to differentiate between an adaptive stress response (normal fluctuations in GC production due to positive or transient events) and chronic stress or distress as both increases and decreases in baseline glucocorticoid levels can be associated with health issues and poor welfare. As such, a secondary measure of welfare or well-being should be used in conjunction with GC evaluation.

Dehydroepiandrosterone (DHEA) and its sulfate ester (DHEA-S) have been characterized as GC antagonists, immunostimulants, and neuroprotective hormones. DHEA and DHEA-S are the most abundant hormones produced by the adrenal glands in primates and helps produce estrogens in females and androgens in males by acting as a source of steroid hormone precursor but has also been shown to have a protective role by antagonizing the effects of cortisol. DHEA(S) may increase in response to acute stressors but dysregulation of the HPA axis due to repeated or chronic stressors which can lead to a reduction in DHEA(S), and it has been suggested that incorporating DHEA-S into the evaluation of GCs, specifically the ratio or interaction between DHEA-S:Cortisol may provide a better allostatic load index to identify physiological dysregulation.

The examination of the ratio of GCs to DHEA(S) in koalas may provide insight into how the HPA axis is functioning, and therefore, serve as an indicator of immune function. The goal of this preliminary validation test was to determine, for the first time in the koala, if DHEA-S:GC could provide a means of differentiating normal, low GC levels from hypercortisolism (adrenal fatigue/dysfunction).

The Arbor Assays® enzyme-immunoassay kit for DHEA-S was used to attempt to validate this EIA for the analysis of koala faecal DHEA-S levels. The initial analysis included 7 koalas (3 males, 4 females) of varying health conditions and responses to treatment to determine if biologically relevant levels and ratios of DHEA-S to GC could be detected. The results of this analysis are reported in Figure 6.

Figure 5: Results of T3 and T4 faecal assays from koala in poor (BS 3), improving (BS 7-8) and good (BS 9-10) body condition. No clear patterns or level differences between koalas with good versus poor body condition are evident.

Figure 6: Corticosterone, Cortisol and DHEA profiles of koalas in different states of health and treatment outcomes.

The profiles of the faecal DHEA-S hormones for the initial koalas tested demonstrated a difference in overall levels between males and females (with male values higher) which was not unexpected due to the role of DHEA-S as a precursor for testosterone and reported differences between sexes in other studies. It appears that in the male koalas, DHEA-S is higher and more variable in koalas with normal, fluctuating, and responsive GC levels (e.g., Felix – Figure 6A, Joseph – Figure 6B) but in Dale (Figure 6C) there appears to be a transition from fluctuating to nonfluctuating with a decrease in levels parallel to the decrease in GC through his treatment period. Dale did not respond well to treatment and was ultimately euthanised after which a number of chronic health issues were identified; consequently, his pattern and levels of GC:DHEA-S may be reflective of a transition from a functioning to non-functioning HPA axis.

Female Talle (Figure 6D) had higher GC levels and normal fluctuations in both GC and DHEA-S during her treatment period (and was ultimately released). The other females (Figures 6E-G) displayed lower GC levels and less variation in DHEA-S levels which may be reflective of HPA dysfunction. These early results suggest that it is likely that DHEA-S could be used to differentiate between normal, low GC levels and depressed GC levels due to HPA dysfunction, and therefore, may be a useful biomarker in the koala. Although not an initial aim of this project, these results suggest further validation of DHEA-S based on additional animals that do not respond to treatment compared to animals which improve during treatment and are released, would help to clarify the utility of DHEA-S as co-biomarker of stress physiology in the koala.

2.3 The Jimboomba Koala population

2.3.1 Study site

The study site was located on Teviot Road, Jimboomba approximately 40 km southwest of Brisbane City, and 17 km north of Beaudesert within the Logan City region. The site measured approximately 553 hectares (ha) in area (Celestino, 2023). Of the site, 415 hectares will be developed with 147 hectares of mixed open space and 70 hectares of vegetation retention and rehabilitation. Flora and fauna surveys undertaken by Saunders Havill Group (SHG) determined the site contains six Regional Ecosystems (REs) identified as Least Concern, Endangered or Of Concern (Celestino, 2023). The eastern boundary of the site is bordered by the Logan River. The site overall was fragmented from the surrounding vegetation due in part to neighboring rural residential properties to the southwest, and road infrastructure. The area running west to east through the middle of the site and north to south along Logan River had a high density of weed species through the understory. The canopy vegetation along these watercourses are dominated by *Eucalyptus tereticornis* (Forest Red Gum/Blue Gum) and dense understory of weeds such as *Lantana camara* (Lantana). The area of land to east and west of the unallocated state land contained forest red gums and *Corymbia citriodora* (spotted gum) with secondary koala food tree *E. siderophloia* (Ironbark) along with dense understory of *Acacia* species, *Alphitonia exelsa* (Red Ash), with evidence of cattle grazing and erosion. The area to the far west of the property bordering the council road reserve consisted mainly of regrowth vegetation and weeds. Dominant trees were *Lophostemon* and *Corymbia* species scattered with *E. tereticornis*. Cattle grazing, erosion and weeds were dominant in this area of the property.

2.3.2 Field Ecology Survey Methodology

The on-ground surveys to identify koalas and collect fresh scats prior to a clearing was the basis for the sampling survey analysis. The sampling technique used in this study to

assess the maximum amount on ground in the allocated time was determined by means of the Celestino clearing and retention plan (Figure 7). The sampling technique followed the clearing and retention plan, prior to clearing in each zone, general searches involved walked line transects in each of the identified zoned clearing areas, making use of the trails and wildlife tracks where they existed and avoiding thick areas infested with weeds.

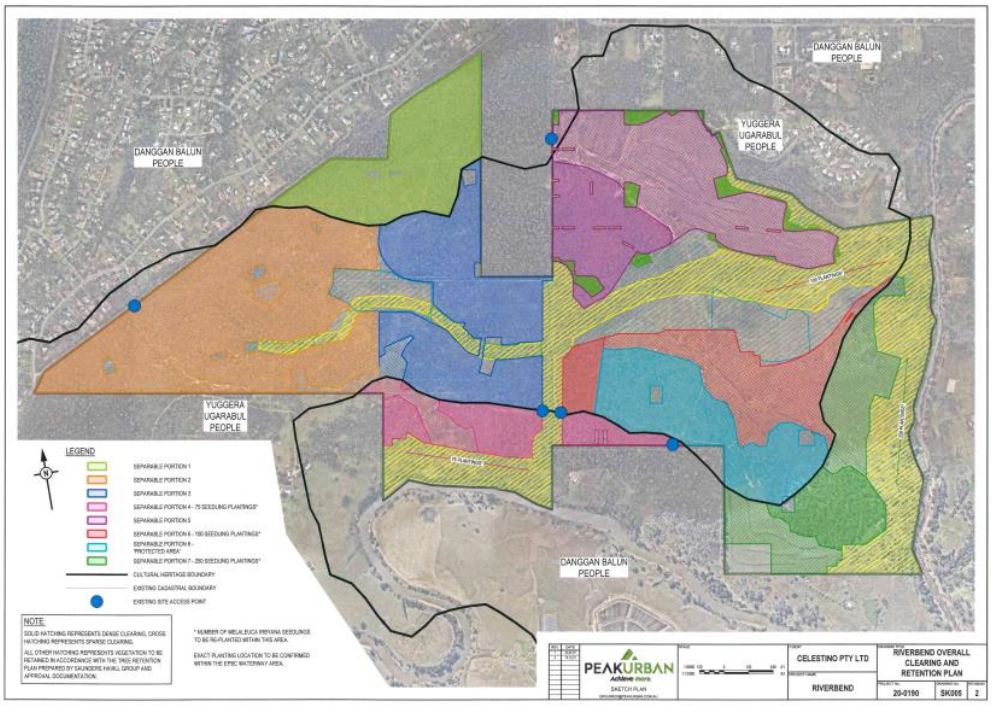

Figure 7: Original zone clearing schedule at Jimboomba development site.

Preferred koala food trees were targeted in each zone. Observation for secondary signs of koala usage were conducted throughout the site, including scat presence and scratch marks on the identified trees. This field survey was conducted under the University of Queensland Animal Ethics Permit 2022/AE000114 (ANFRA – Koala Power Poo v0.01). The survey team consisted of sub-contracted Field Ecologist, Mr. Al Mucci. Mr. Mucci's company subsequently sub-contracted Mr. Jamie Christian Holyoak from Ripper Corp (thermal koala detection specialist) to conduct multiple koala drone surveys.

Line transect surveys were conducted in dedicated zones of the development sites and surrounding habitat. Individual zones were surveyed based on the clearing schedule. Walked line transects approximately 20m apart were conducted in each zone during the early morning/twilight and koalas identified by means of handheld infrared device that detected body heat of the koalas. During each passage of each line transect an assessment comprised of (i) ground truthing the habitat areas of each zone prior to clearing, (ii) identification of preferred koala food trees, (iii) searching for scats around base (1m radius) and scratches on the trunk of each tree and (iv) searching the tree canopy using spotlight/bino/infrared handheld device for koalas. Survey site spatial data was recorded using preloaded site maps for the Bromelton region (9442-2) downloaded onto the Avenza Mapping Application available from Google Play (https://store.avenza.com/pages/app-features). Mapped transect data, including date and location of observed koala or scats, was overlaid onto Queensland Globe (https://qldglobe.information.qld.gov.au/) to provide an overview of the survey site.

Drone surveys were performed across each zone to identify koalas by means of thermal sensor spotting. CASA air law and RPA standard operating procedures applied to the

survey, which due to inclement weather sometimes constrained flight operations. Drone flights were scheduled to cover defined transect study zones on the site, staying ahead of the clearing plan. A generic survey grid was flown with specifications regarding flights. If a koala was observed in a tree, binoculars were then used in an attempt to ascertain: (1) gender, (2) external signs of chlamydial infection (conjunctivitis and/or “wet bottom” - a urinary tract infection causing cystitis), and (3) the presence of a joey.

The 2022-2023 field survey of the Jimboomba site included 137 site visits occurring between 03/01/22 – 29/07/24, and greater than 400km of line transects walked within the bushland areas. Over 200 plastic mats were laid under preferred koala food trees or trees associated with koala presence during the survey resulting in 74 scat samples (minimum of 10 scats per animal Table 3); many of these scats in 2022 were unfortunately rain damaged. Koala scat collection for 2022 at the Jimboomba site are reported in Table 3.

Table 3: Location estimated age of koala scats, ambient temperature at time of scat collection and koala sightings.

Date	Sample	Latitude	Longitude	Scat Age	T°C	Koala sightings
5/01/22	1	-27.826228	152.961397	4	28	1 koala (7-1-22) zone 1
17/01/22	2	-27.826247	152.961378	2	23	1 koala zone 2
1/03/22	3	-27.826295	152.96137	4	14	2 koalas (31-1-22) zone 2,3
1/05/22	4	-27.830147	152.965187	2	14	1 koala (13-4-22) zone 2
4/05/22	5	-27.8286172	152.9676898	2	14	2 koalas’ zone 3 and 5
7/05/22	6	-27.829481	152.967435	2	11	
9/05/22	7	-27.828212	152.967878	3	20	
7/06/22	8	-27.828328	152.968901	3	17	Male koala heard bellowing
7/06/22	9	-27.828152	152.969386	3	13	1 koala (1-6-22) zone 5
7/06/22	10	-27.828184	152.969281	2	14	
7/06/22	11	-27.829388	152.965883	3	11	
7/06/22	12	-27.829298	152.966277	3	11	
9/06/22	13	-27.8281477	152.9690829	3	14	
9/06/22	14	-27.828328	152.968905	2	12	
13/06/22	15	-27.82817704	152.9690829	3	14	
13/06/22	16	-27.82850784	152.9690038	3	14	
13/06/22	17	-27.8286172	152.9676898	3	17	
13/06/22	18	-27.82816976	152.9692968	2	18	
15/06/22	19	-27.828328	152.968901	2	10	
15/06/22	20	-27.828903	152.96953	3	11	
8/08/22	21	-27.82867	152.967563	3	6	
8/08/22	22	-27.829346	152.965827	2	8	
8/08/22	23	-27.828958	152.965242	2	8	
16/08/22	24	-27.825206	152.96431	3	19	Male koala heard bellowing
16/08/22	25	-27.822315	152.96624	3	16	
17/08/22	26	-27.82856	152.967723	4	15	1 koala corridor planting area 1 koala
4/01/23	27	-27.823200	152.965758	2	24	Lot 71
5/01/23	28	-27.823246	152.965775	1	22	
6/01/23	29	-27.829010	152.965278	3	22	
9/01/23	30	-27.825728	152.966006	3	24	Male koala heard bellowing Lot 71
10/01/23	31	-27.831472	152.966689	2	31	
10/01/23	32	-27.81974	152.967829	2	26	
10/01/23	33	-27.820348	152.966826	2	29	
10/01/23	34	-27.820749	152.967115	3	29	
11/01/23	35	-27.829459	152.966254	2	19	
11/01/23	36	-27.829549	152.96675	2	19	

Date	Sample	Latitude	Longitude	Scat Age	T°C	Koala sightings
11/01/23	37	-27.829066	152.966351	2	21	
17/01/23	38	-27.821545	152.960968	1	24	
17/01/23	39	-27.821254	152.961684	1	24	
18/01/23	40	-27.821698	152.960377	1	23	
18/01/23	41	-27.821097	152.968298	1	23	
18/01/23	42	-27.821394	152.968245	3	26	
23/01/23	43	-27.829642	152.967350	1	18	2 koalas Lot 71, corridor planting area
24/01/23	44	-27.829642	152.967350	1	23	
24/01/23	45	-27.831442	152.968825	1	24	
8/05/23	46	-27.822482	152.968510	2	8	1 koala Lot 71
8/05/23	47	-27.49593	152.58033	2	8	1 koala Lot 71
8/05/23	48	-27.822025	152.968103	2	9	1 koala Lot 71
8/05/23	49	-27.49228	152.58097	2	8	1 koala Lot 71
9/05/23	50	-27.820468	152.868266	2	3	
9/05/23	51	-27.820729	152.968351	2	3	1 koala Lot 71
9/05/23	52	-27.826550	152.967217	2	3	
22/05/23	53	-27.831742	152.980910	2	6	1 koala, corridor planting area
22/05/23	54	-27.820052	152.967204	2	13	1 koala Lot 71
22/05/23	55	-27.826942	152.965574	2	16	2 koalas (22-05-23) Lot 71
23/05/23	56	-27.820052	152.967204	2	5	
23/05/23	57	-27.824055	152.968012	2	5	
23/05/23	58	-27.826942	152.965574	2	8	
24/05/23	59	-27.831742	152.980910	2	6	
15/06/23	60	-27.831823	152.982077	1	17	1 koala, corridor planting area
16/06/23	61	-27.831817	152.982104	1	16	
16/06/23	62	-27.831528	152.982589	3	20	
19/06/23	63	-27.831700	152.982700	1	6	2 koalas, corridor planting area
20/06/23	64	-27.831700	152.982700	2	16	4 koalas, corridor planting area
27/07/23	65	-27.831970	152.968903	1	20	
27/07/23	66	-27.827281	152.979817	2	22	
27/07/23	67	-27.827212	152.979109	3	22	
27/07/23	68	-27.827173	152.980017	1	21	
27/07/23	69	-27.828798	152.980784	2	21	
28/07/23	70	-27.827173	152.980017	3	19	
28/07/23	71	-27.831700	152.982700	3	20	1 koala Corridor planting area
29/07/23	72	-27.831700	152.982700	2	22	
29/07/23	73	-27.831817	152.982104	3	22	
29/07/23	74	-27.831970	152.968903	3	24	

Site visits by the ecologist are listed in **Appendix 2** and include location, collection dates and age classification of koala scats collected in 2022. Koala scats were regularly collected beneath Blue Gum, Grey Box and Spotted Gum trees. Due to repeated inclement weather and flooding on the Riverbend region of the site, site clearing was paused between Feb-May and Sept-Oct 2022, with clearing restricted to elevated areas not subject to water inundation. Consequently, the schedule of site koala surveys of the designated zoned areas was modified to follow the adjusted site clearing plan. As of 2023, all zoned areas of the development site had been cleared, although koala surveys continued into surrounding neighboring properties, with the appropriate permissions.

Drone surveys were conducted across the study site on 14 occasions, again with adverse weather activity playing a significant adverse role in constraining drone flights during the first half of 2022. Table 4 reports the timing of the drone survey, weather conditions at time of flight, koalas sighted (Figure 8) and their location. The survey focal area was restricted to

vegetated areas of the study site and followed the zoned pre-clearing plan (Figure 7), which was subject to change because of inclement weather. The weather activity played a significant role in constraining drone flights during the wet period of 2022. Rain, low cloud, and fog restricted drone infra-red equipment and visually impairs the pilot, resulting in 5 flights being aborted. These site visits occurred in the early hours of mornings from 3am when koala presence can be best detected from an infrared handheld device and spotlighting.

Table 4: Drone Survey Activity during 2022

Date	Drones used	Weather	Koala sighted	Zone area surveyed
17/01/2022	1	Clear night	1	Zone 1, 2 and 3
24/01/2022	1	Partly cloudy		Zone 1, 2 and 3
31/01/2022	1	Partly cloudy	2	Zone 1, 2 and 3
Feb-April 2022	0	Poor weather - continued cloud coverage and heavy rainfall		N/A
4/05/2022	2	Partly cloudy	2	Zone 3, 5, 6, 7 and road areas
9/05/2022	2	Partly cloudy		Zone 5 and 6
1/06/2022	2	Clear night	1	Zone 2, 5, 6 and corridor areas
29/07/2022	2	Partially cloudy with some fog		Zone 2, 4 and corridor areas
8/08/2022	3	Slight fog in low lying areas		Zone 2, 7 and corridor areas
19/08/2022	3	Slight fog in low lying areas	1	Zone 3, 7 and corridor areas
Sept-Nov 2022	0	Poor weather conditions		N/A
23/01/23	2	Partly cloudy	2	Corridor planting area, Council Road Reserve and Lot 71 unallocated state land
8/05/23	2	Clear	4	Corridor planting area, Council Road Reserve and Lot 71 unallocated state land
22/05/23	2	Clear	5	Corridor planting area, Council Road Reserve and Lot 71 unallocated state land
5/06/23	0	Heavy fog	0	N/A
12/06/23	0	Heavy fog	0	N/A
19/06/23	2	Heavy fog	0	Attempted to fly, poor weather
26/07/23	1	Clear	4	Corridor planting area

Table 5: Tree species and height and zone location where koalas were observed.

Date	Koalas Observed	Tree species and height	Location (Peakurban)
7-01-22	1	*E. tereticornis - 30m*	Zone 1
17-01-22	1	*E. moluccana - 25m*	Zone 2
31-01-22	2	*E. tereticornis* -20m, *Corymbia citriodora* – 30m	Zone 1 and Zone 3
13-04-22	1	*E. tereticornis* – 30m	Zone 2
04-05-22	2	*E. tereticornis* - 25m, *E. moluccana* – 20m	Bushman Rd border of zone 3 & 5
01-06-22	1	*E. tereticornis* – 15m	Zone 5
19-08-22	1	*E. tereticornis* – 30m	Corridor area
04/01/23	1	*Alphatonia excelsa* – 10m	Lot 71 unallocated State land
23/01/23	2	*E. moluccana* – 15m, *E. moluccana* – 20m	Corridor planting area, Lot 71 unallocated state land
08/05/23	4	*E. moluccana 25m & 30m, Ironbark 20m & 35m*	Lot 71 unallocated state land
09/05/23	1	*Ironbark 20m*	Lot 71 unallocated State land
22/05/23	5	*Lophostemon suaveolens 15m, E. moluccana 25m & 30m*	Corridor planting area, Lot 71 unallocated State land
15/06/23	1	*E. moluccana 10m*	Corridor planting area
19/06/23	2	*E. moluccana 5m & 8m*	Corridor planting area
26/07/23	4	*E. tereticornis 10m & 20m, E. moluccana 25m*	Corridor planting area
28/07/23	1	*E. moluccana 20m*	Corridor planting area

Preferred koala food trees on the study site that were identified and included species such as *E. tereticornis* (Blue Gum), *E. moluccana* (Grey box), *E. siderophloia* (Northern Grey Ironbark), *C. citriodora* (Spotted Gum), while other species such *Corymbia intermedia* (Pink Bloodwood), *Lophostemon suaveolens* (Swamp Box) and *Alphitonia excelsa* (Red Ash) were found to be associated with koala presence. A total of 30 koalas were observed throughout the study site over the course of the study (Table 5). An additional 3 male koalas were heard bellowing but not directly observed.

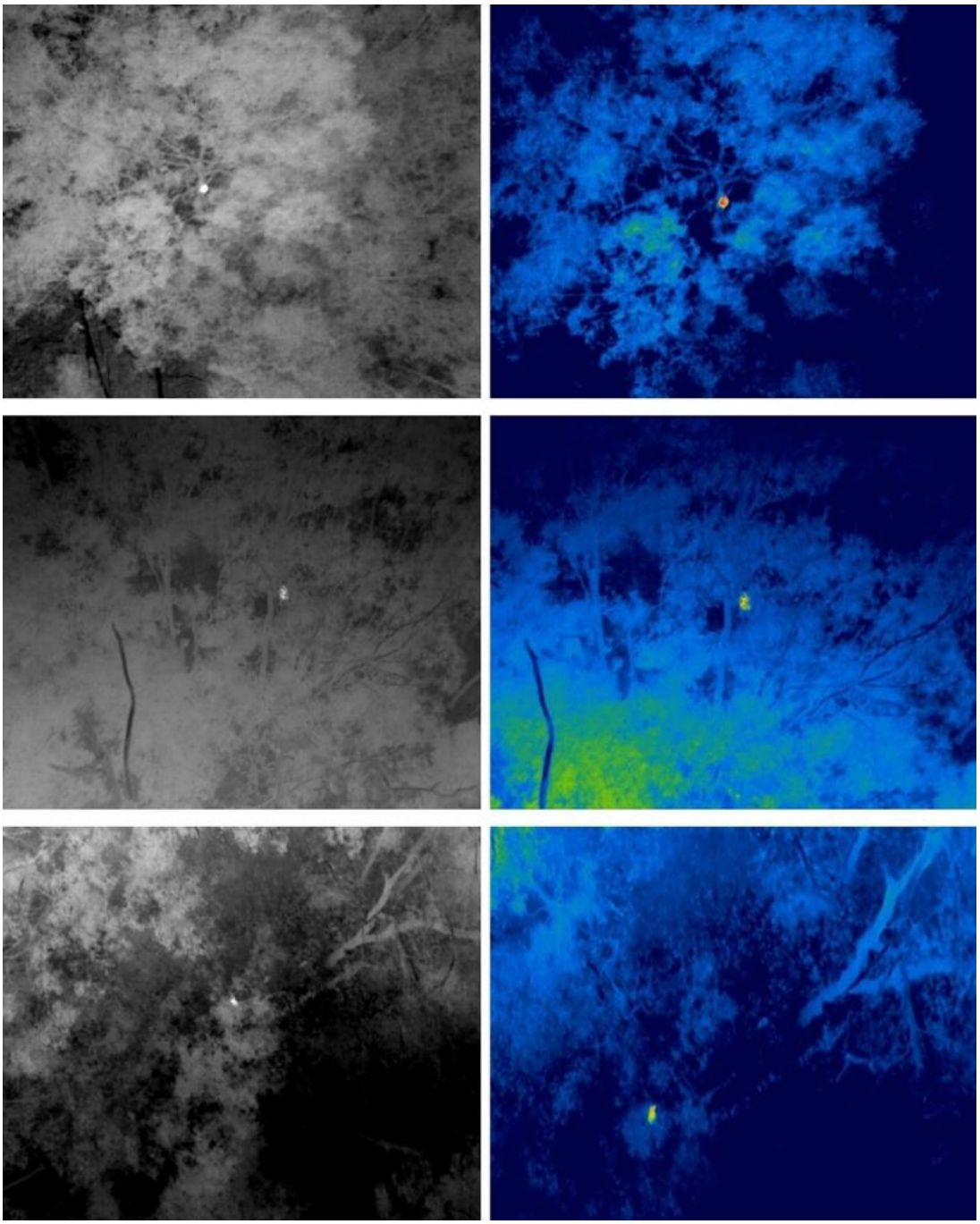

Figure 8: Thermal imagery detection of koalas during drone surveys.

2.3.3 Koala Scat Detection and DNA analysis

If koalas were identified by drone or following line transects, plastic mats were placed at the base of the tree as well as known surrounding food trees; these mats were checked 24h later or as quickly as practicable. When koala scats were found, their location was recorded with a hand-held GPS and the scat was classified by age (See Table 6). Fresh

scats were typically dark brown to black on the outside, had a shiny surface (mucus), bright green or yellow inside, and solid to the touch (or soft if extremely fresh, e.g. deposited within a few hours). Typically, an individual koala produces scats that are relatively consistent in both shape and size, although there may be significant variations in the shape and/or size of scats produced by different individuals. For example, they can be oblong or round in shape, and can range in size from approximately 10 mm to 40 mm. Koala scats (Figure 9) have the following characteristics: symmetrical and bullet-shaped (not jelly-bean shaped). They are generally about 1.5 cm in length and 0.5 cm in diameter (adult koala scat size), composed of compacted fine particles and lack insect.

Table 6: Koala faecal scat age categories (From Triggs, 1996)

Scat Age Category	Characteristics – approximate age
1	Extremely fresh (covered in mucus) – <24 hour
2	Fresh (shiny, smelly) – 24-48 hours
3	Medium fresh (shine, or smells when broken) – weeks old
4	Old (no shine, no smell) – months old
5	Very old and discoloured – many months to years old

Figure 9: Koala scats - Note freshest (Category 1) on right (Photo courtesy of R. Cristescu).

When viable koala scats were found, their physical condition was assessed in the field, and up to 10 scats were collected aseptically. The reason for collecting more than one scat per presumed individual at each location, was to provide several opportunities of isolating DNA of sufficient quality and quantity to enable downstream analysis of genetics, disease detection and endocrinological assessment. Individual scats were recovered from the substrate using forceps, to ensure no direct contact with human DNA, and not to disrupt or compromise genetic material located on the surface of the scat. Scats were placed into paper bags, stored in a small esky with freezer bricks (≈4 ^{o}C) in the field, until transport to a -20^{o}C freezer for longer term storage.

Fresh (<24 hr) koala faecal scats are preferable for successful isolation of DNA and downstream analysis such as molecular confirmation of host (koala) DNA, genetic profiling, pathogen (specifically *Chlamydia*) and for the detection of endocrine/metabolites. DNA degradation occurs over time and is expected to occur the longer biological samples are exposed to the environment (Matange et al., 2021). Faecal scats exposed to moisture and rain from inclement weather, heat and UV from sunlight have higher amplification failure, and genotyping failures compared to scats collected from weather-protected positions. The presence of volatile organic compounds and phenolics derived from the koala's diet of *Eucalyptus* leaves may also impede isolation and amplification of DNA.

Eucalypt molecules are excreted in koala faeces and are known to damage cell membranes, while phenolics can accelerate DNA degradation.

While the methodology for DNA isolation from koala scats has previously been developed (Schultz et al., 2018; Wedrowicz et al., 2013), consistency and the quality of DNA isolation from koala scats required further validation. This project explored reasons that may contribute to the unreliability of DNA isolation from koala faecal scats in order to make recommendations for a more reliable procedure. We investigated this hypothesis by assessing the quality (DNA purity) and quantity (concentration) of koala epithelial cell DNA surrounding koala scats collected after defecation (<24hr) by evaluating the variability within and between individual captive koalas.

A minimum of 5 koala faecal scats were collected from captive animals housed individually in 10 separate enclosures. Following afternoon cleaning of the enclosure, plastic mats were placed on the ground surrounding koala stands (perches) to catch scat samples overnight. Surface area (mm^2) of individual scats collected from each koala enclosure was measured and correlated with DNA concentration (ng/µL) as measured by spectrophotometry. Isolation of DNA from koala faecal scats collected was obtained by scraping the surface of faecal scats and using a column extraction method for purification of DNA. A combination of host (koala), microbial and other biological DNA is expected to be present in the DNA elute and was termed “total DNA”. DNA concentration (ng/µL) and purity (A260/280) was assessed using spectrophotometry. Samples were assessed for the presence of host DNA using the koala beta-actin gene. A Pearson correlation analysis was used to assess whether DNA yield was associated with the size of scat.

Comparison of faecal scats collected from individual koalas (intra-koala faecal scat variability) revealed no significant difference in scat size (mm^2) or isolated DNA concentration (ng/µL) of individual scats. A negative correlation between scat size (mm^2) and DNA concentration (ng/µL) was observed for all individual koalas (except Koala 2 and 10) suggesting the larger the size of the faecal scat, the lower the quantity of DNA isolated (Figure 10). This was an unexpected observation as one might consider that there would greater DNA quantity isolated from larger faecal scats. This phenomenon warrants further investigation.

Individual koala mean scat size and DNA concentration between koalas (inter-koala faecal scat variability) revealed a significant difference ($P = <0.0001$) in scat size excreted by Koala 2 compared with the koala cohort. There was a significant difference ($P = <0.0001$) in concentration of DNA isolated from faecal scats collected from Koalas 2 and 3 (Figure 11).

Figure 12 shows that the total DNA concentration isolated from Jimboomba koala faecal scats decreased with age of the scat. This supports the premise that DNA degradation occurs over time and is accelerated the longer biological samples are exposed to the external environment. Koala DNA (scat DNA with positive koala beta-actin PCR result) was detectable in only 66% of faecal scats (49/74 scats) (see Table 7). Many of the 2022 samples were also rain damaged and it clear from Table 7 that DNA quality improved in 2023 when the weather was not as inclement.

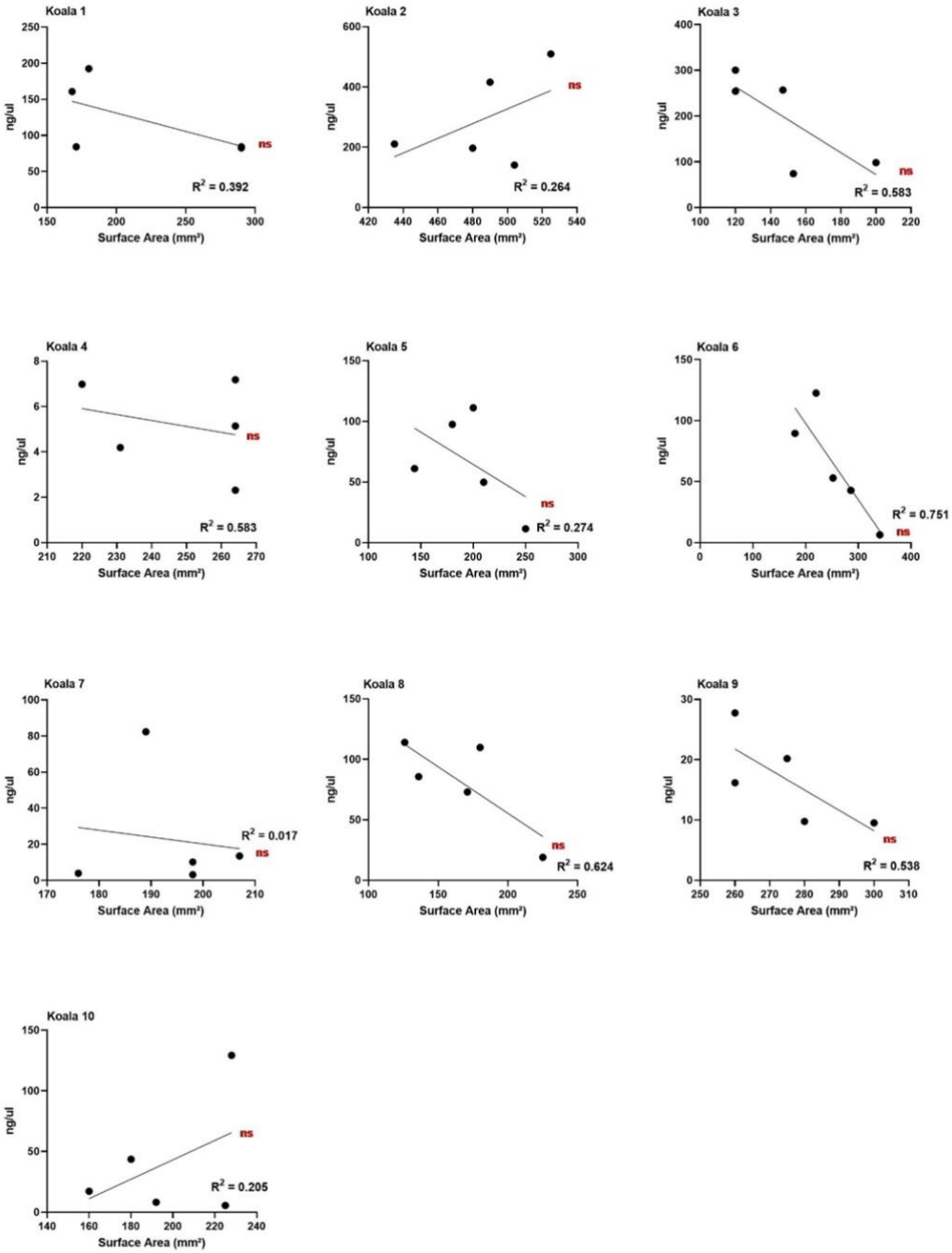

Figure 10: Intra-koala comparison of correlation between size of scat (mm2) and quantity of DNA isolated per scat (ng/µL).

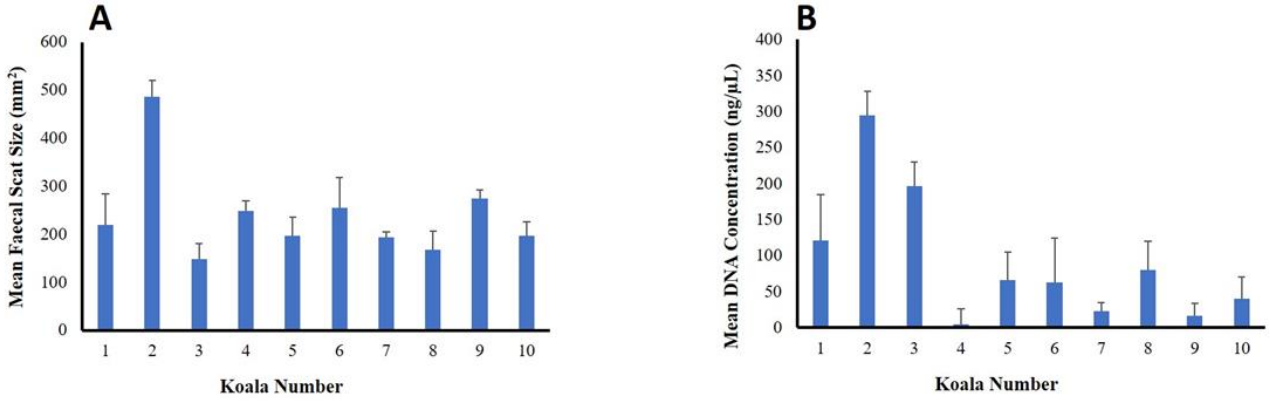

Figure 11: Inter-koala comparison of (A) mean (±SD) size of scat (mm^2) and (B) mean (±SD) DNA isolated per scat (ng/µL).

Table 7 reveals that a total of 74 scats of varying estimated age (Table 6) were found (latitude and longitude) in the study site and analysed for DNA quality in 2022/2023.

Table 7: Results of koala scat DNA analysis (DNA concentration and purity, detection of koala DNA and *Chlamydia* DNA).

Date	Sample	Latitude	Longitude	Scat Age Category	*DNA (ng/µL)	DNA Purity (A260/280)	Koala ß Actin	Presence *C. pecorum*
5/01/22	1	-27.826228	152.961397	4	2.06	1.55	-	NR
17/01/22	2	-27.826247	152.961378	2	5.30	2.1	-	NR
1/03/22	3	-27.826295	152.961370	4	5.40	1.83	-	NR
1/05/22	4	-27.830147	152.965187	2	79.25	1.88	+	-
4/05/22	5	-27.828617	152.967690	2	13.65	1.73	+	-
7/05/22	6	-27.829481	152.967435	2	32.71	1.92	+	-
9/05/22	7	-27.828212	152.967878	3	76.23	1.87	-	NR
7/06/22	8	-27.828328	152.968901	3	17.74	1.85	-	NR
7/06/22	9	-27.828152	152.969386	3	6.55	1.73	-	NR
7/06/22	10	-27.828184	152.969281	2	57.19	1.91	+	-
7/06/22	11	-27.829388	152.965883	3	12.85	1.49	-	NR
7/06/22	12	-27.829298	152.966277	3	27.78	1.76	+	NR
9/06/22	13	-27.828148	152.969083	3	17.46	2.09	+	NR
9/06/22	14	-27.828328	152.968905	2	18.21	2.03	+	-
13/06/22	15	-27.828177	152.969083	3	12.58	1.96	-	NR
13/06/22	16	-27.828508	152.969004	3	45.42	1.86	+	NR
13/06/22	17	-27.828617	152.967690	3	42.55	1.86	-	NR
13/06/22	18	-27.828170	152.969297	2	9.68	1.88	+	-
15/06/22	19	-27.828328	152.968901	2	12.62	1.8	+	-
15/06/22	20	-27.828903	152.969530	3	4.59	2.12	-	NR
8/08/22	21	-27.828670	152.967563	3	42.66	1.8	-	NR
8/08/22	22	-27.829346	152.965827	2	17.38	1.87	+	-
8/08/22	23	-27.828958	152.965242	2	9.87	1.73	+	-
16/08/22	24	-27.825206	152.964310	3	5.44	1.97	-	NR
16/08/22	25	-27.822315	152.966240	3	7.48	1.6	-	NR
17/08/22	26	-27.828560	152.967723	4	7.31	1.71	-	NR
4/01/23	27	-27.823200	152.965758	2	221.57	1.82	+	-
5/01/23	28	-27.823246	152.965775	1	158.66	1.82	+	-
6/01/23	29	-27.829010	152.965278	3	90.00	1.84	+	-
9/01/23	30	-27.825728	152.966006	3	58.49	1.82	+	-
10/01/23	31	-27.831472	152.966689	2	18.48	1.47	-	NR
10/01/23	32	-27.819740	152.967829	2	29.44	1.43	-	NR
10/01/23	33	-27.820348	152.966826	2	10.90	1.51	+	-
10/01/23	34	-27.820749	152.967115	3	83.06	1.79	-	NR
11/01/23	35	-27.829459	152.966254	2	46.89	1.76	+	-
11/01/23	36	-27.829549	152.966750	2	21.16	1.52	+	-
11/01/23	37	-27.829066	152.966351	2	66.87	1.59	-	NR
17/01/23	38	-27.821545	152.960968	1	18.59	1.41	-	NR
17/01/23	39	-27.821254	152.961684	1	29.23	1.71	-	NR
18/01/23	40	-27.821698	152.960377	1	45.20	1.70	-	NR
18/01/23	41	-27.821097	152.968298	1	144.54	1.70	+	-
18/01/23	42	-27.821394	152.968245	3	37.35	1.93	-	NR
23/01/23	43	-27.829642	152.967350	1	36.61	1.99	+	-

24/01/23	44	-27.829642	152.967350	1	114.47	1.41	+	-
24/01/23	45	-27.831442	152.968825	1	181.63	1.73	+	-
8/05/23	46	-27.822482	152.968510	2	34.33	2.03	-	NR
8/05/23	47	-27.495930	152.580330	2	97.41	1.92	+	-
8/05/23	48	-27.822025	152.968103	2	95.95	1.87	+	-
8/05/23	49	-27.492280	152.580970	2	34.63	2.09	+	-
9/05/23	50	-27.820468	152.868266	2	32.00	2.02	+	-
9/05/23	51	-27.820729	152.968351	2	32.00	1.92	+	-
9/05/23	52	-27.826550	152.967217	2	56.61	1.81	+	-
22/05/23	53	-27.831742	152.980910	2	307.16	1.58	+	-
22/05/23	54	-27.820052	152.967204	2	45.25	1.89	+	-
22/05/23	55	-27.826942	152.965574	2	87.04	1.86	+	-
23/05/23	56	-27.820052	152.967204	2	35.78	1.80	+	-
23/05/23	57	-27.824055	152.968012	2	51.84	1.70	+	-
23/05/23	58	-27.826942	152.965574	2	22.41	1.88	+	-
24/05/23	59	-27.831742	152.980910	2	70.24	1.62	+	-
15/06/23	60	-27.831823	152.982077	1	83.40	1.73	+	-
16/06/23	61	-27.831817	152.982104	1	107.08	1.67	+	-
16/06/23	62	-27.831528	152.982589	3	62.22	1.84	+	-
19/06/23	63	-27.883170	152.982700	1	131.52	1.93	+	-
20/06/23	64	-27.831700	152.982700	2	82.77	1.68	+	-
27/07/23	65	-27.831970	152.968903	1	121.08	1.86	+	-
27/07/23	66	-27.827281	152.979817	2	117.49	1.90	+	-
27/07/23	67	-27.827212	152.979109	3	57.40	1.86	+	-
27/07/23	68	-27.827173	152.980017	1	52.15	1.78	+	-
27/07/23	69	-27.828798	152.980784	2	33.36	1.90	-	NR
28/07/23	70	-27.827173	152.980017	3	33.48	1.65	-	NR
28/07/23	71	-27.831700	152.982700	3	36.02	1.90	+	-
29/07/23	72	-27.831700	152.982700	2	26.25	1.88	+	-
29/07/23	73	-27.831817	152.982104	3	135.42	1.85	+	-
29/07/23	74	-27.831970	152.968903	3	83.86	1.90	+	-

*DNA Purity – An A260/280 value of >1.8 or greater is indicative of acceptable quality; a value < 1.6 indicates contamination. Scat age categories are described in Table 6.

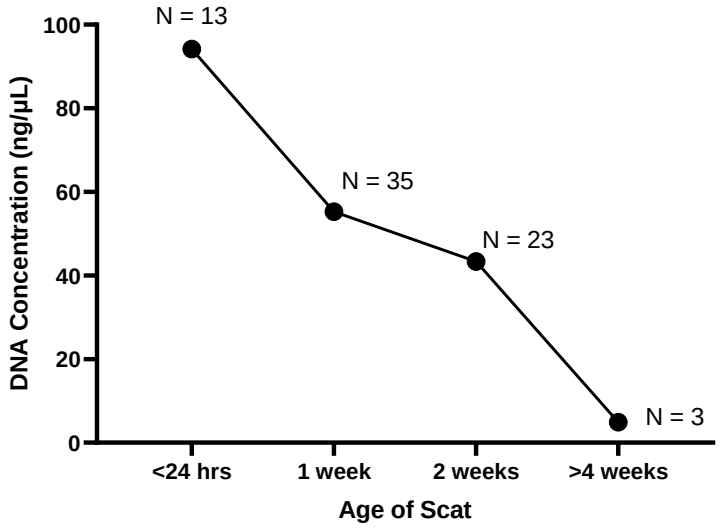

Figure 12: Jimboomba koala faecal scat mean DNA concentration over time.

2.3.4 Genetic analysis

Faecal scat DNA samples that confirmed positive for presence of koala DNA (PCR detection of koala beta-actin gene) were genotyped for 32 koala-specific microsatellite markers, providing a unique DNA profile per sample. This allowed for the identification of distinct individuals with a high degree of confidence (the probability that two individuals would share the same DNA profile by chance is less than 1 in 1,000,000,000). Analysis of repeated genotypes within the dataset to identify duplicate samples revealed samples with highly similar multilocus genotypes, suggesting these scats were from the same individual koala. Consequently, genetic analysis of faecal DNA recovered from the survey site was able to identify 7 individuals. These individuals were considered to be representative of the total population. Patterns of genetic diversity across the study site were then assessed using the following measures: (1) Expected heterozygosity (H_E); (2) Observed heterozygosity (H_O) and (3) Inbreeding coefficient (F_{IS}). Genetic diversity inferred from the limited sample set was estimated through heterozygosity value and revealed moderate genetic diversity (H_E = 74%; H_O = 63%). A moderate to high inbreeding value of local koalas was estimated (F_{INB} = 0.155). A positive inbreeding value indicates that individuals in a population are more related than you would expect under a model of random mating.

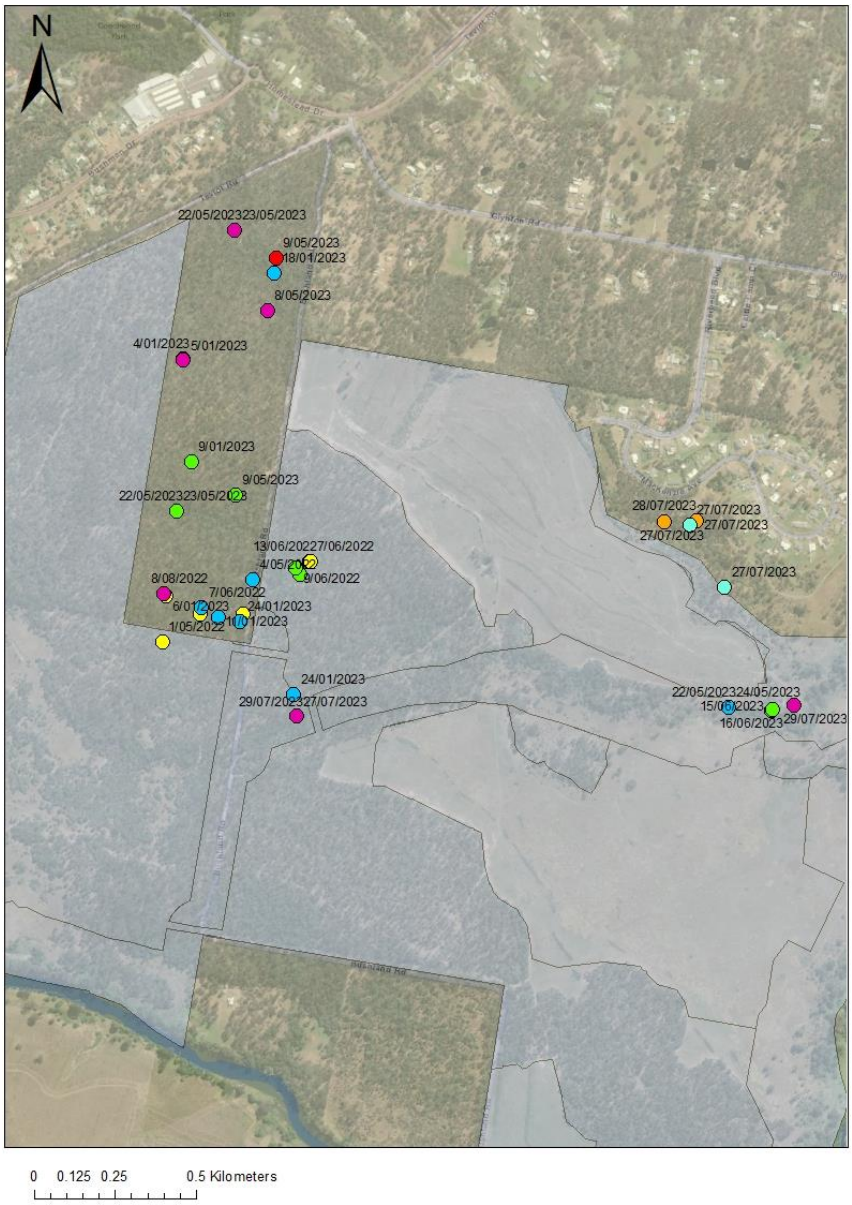

Figure 13: Distribution and movement of koalas over the survey period: 2022 - 2023. Each coloured circle denotes an individual koala.

2.3.5 Distribution and movement of koalas

The ability to distinguish individual koalas using genetic analysis allowed us to map the movement of individual koalas over time. Figure 13 presents the distribution and movement of 7 koalas over the survey period (2022 – 2023). It is clear from this analysis that the majority of the koalas were utilizing the surrounding bushland around the cleared site.

2.3.6 *Chlamydia pecorum* DNA detection in koala scats

Faecal samples were tested for *Chlamydia* infection, including speciation and quantitation of bacterial load using target-specific molecular markers as described in Hulse et al. (2018). No detection of infection with the pathogen *Chlamydia pecorum* was observed from faecal scats collected during the survey period (See Table 7). However, it should be noted that we have previously established (unpublished data) that detection of urogenital Chlamydial infection via koala faecal scats has a low sensitivity and specificity when compared to detection of the pathogen via koala urogenital swabs (sensitivity = 75%; specificity = 69%).

2.3.7 Faecal glucocorticoid metabolite analysis

The results from the glucocorticoid analysis of the first batch of samples collected from the Jimboomba site (n = 26 samples collected between January – August 2022) are shown in Figure 14. These results are reported without reference to individual animals.

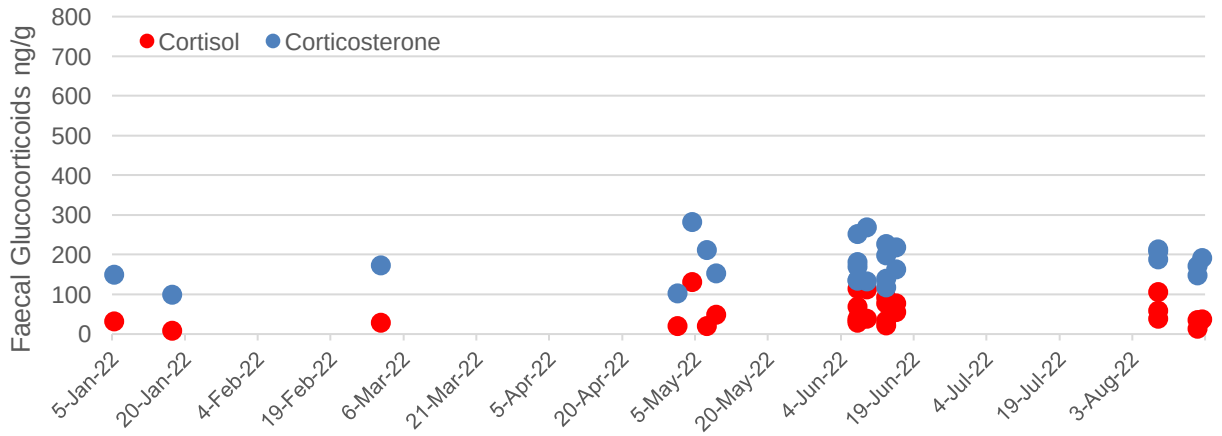

Figure 14: Cortisol and corticosterone concentration of all koala scat samples collected from Jimboomba study site in 2022.

The cortisol and corticosterone metabolite levels of the faecal samples were minimally variable and very low. Samples with values < 50 ng/g for cortisol and < 150 ng/g for corticosterone are lower than expected. Many samples also failed to have detectable koala beta actin, and therefore may have been subject to environmental degradation due to sun and/or rain exposure prior to collection. The remaining samples also had low values for both GC hormones with little variation making interpretation of the data difficult; some of these samples were affected by some level of environmental degradation. As such, it was not possible to provide an accurate evaluation of the faecal glucocorticoid metabolite levels for these animals.

Faecal samples collected between January and July 2023 (n = 48 samples) were also analysed for cortisol and corticosterone. These levels were within the expected range and show variability between samples and over time; with an overall an increase both faecal cortisol and corticosterone concentration over the sampling period (Figure 15).

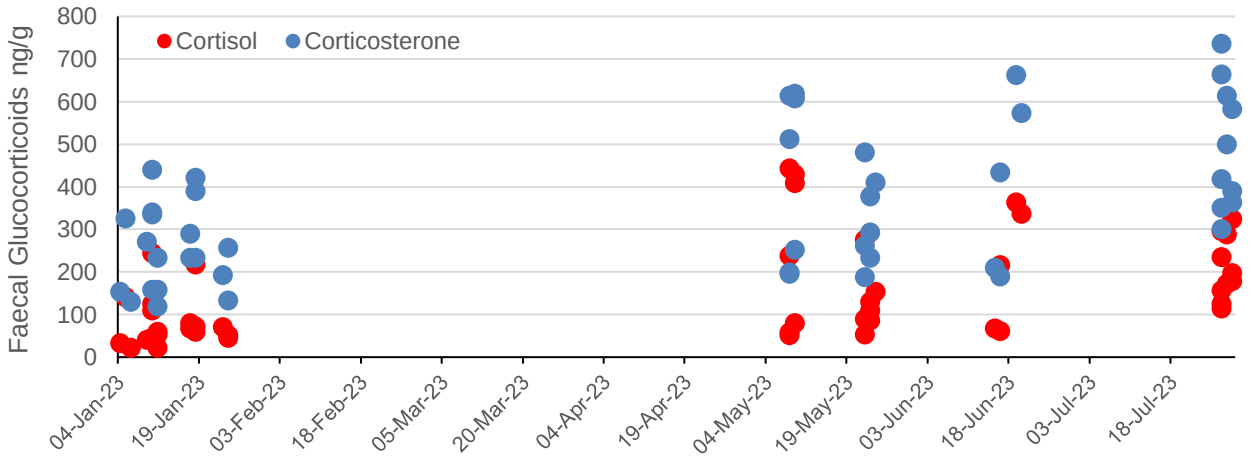

Figure 15: Cortisol and corticosterone concentration of all koala scat samples collected from Jimboomba study site in 2023.

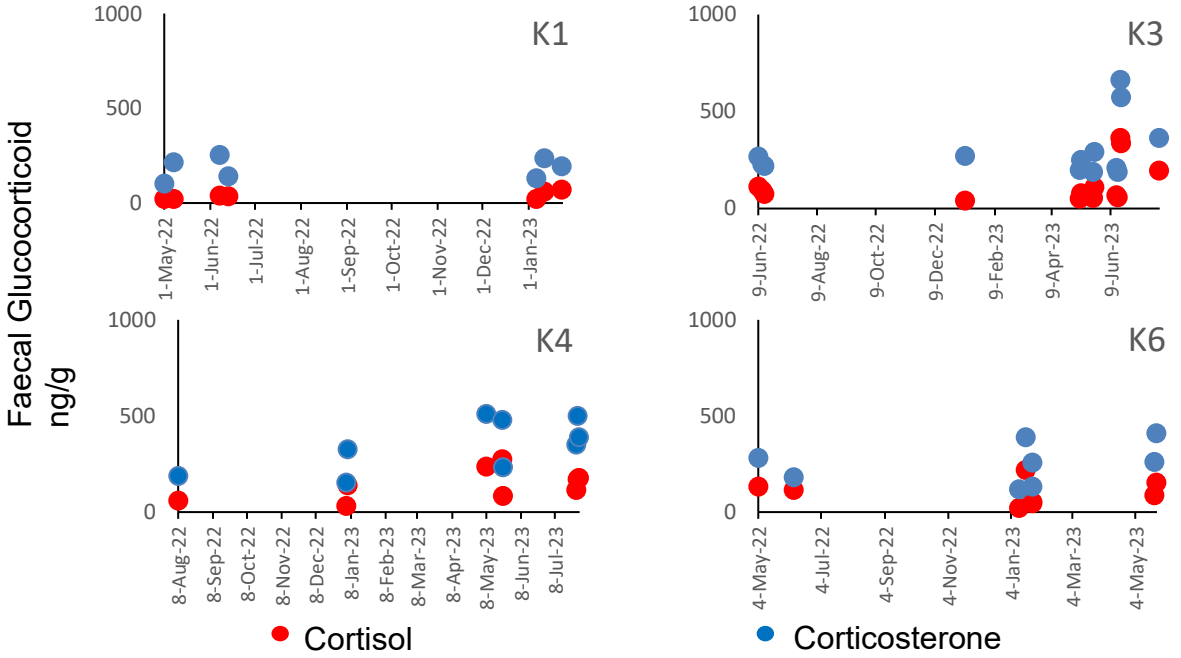

Figure 16: Cortisol and corticosterone concentration of scat samples from individual koalas identified by genetic analysis (K1, K3, K4 and K6) collected from 2022 – 2023.

The concentration of glucocorticoid recovered from samples collected in 2023 were reflective of a better quality faecal sample and indicative of a lower level of degradation and concord with a paralleled improvement in DNA quality from the same samples. When these results are considered as a population, without reference to individual animals, there appeared to be an increase in GC concentration over time. Based on positive faecal DNA identification of 4 koalas (K1, K3, K4 and K6) it was also possible observe individual animal changes in faecal GC concentrations (Figure 16).

2.3.8 Faecal progesterone metabolite analysis

All of the faecal samples collected over 2022-2023 were also analysed for faecal progesterone and testosterone metabolite concentration. Faecal progesterone and testosterone levels were within the expected range for koalas with a few samples demonstrating elevated levels of either progesterone or testosterone. Interestingly, three

faecal samples collected on 8 and 9 May 2023, confirmed by DNA to be from the same animal (K3), showed faecal progesterone levels of 594-682 ng/g, which are typical of levels found in the first week after ovulation and therefore suggestive of this female koala having been mated with in the last week of April or first week of May. There were also three other faecal samples (samples 31, 33 and 38 from the 10-17 January 2023) that had high faecal progesterone concentration of 988-1525 ng/g; there is a high likelihood that these samples were from the same female (not confirmed) and are representative of progesterone metabolite concentrations consistent with pregnancy. Unfortunately, there were a lack of samples collected during the peak of the koala breeding season (August – December 2022) so we were limited in providing any further reproductive data on this population.

3. CONCLUSIONS, OUTCOMES AND FUTURE STUDIES

3.1 A Koala SNP Array

The first aim of the project was to develop a koala SNP (single nucleotide polymorphism) genetic marker array to be made publicly accessible to all koala researchers and stakeholders via our project partner the Australian Genome Research Facility (AGRF). We have discovered and identified a final suite of 15,766 SNPs via mapping of genomes of koalas from south-east Queensland. Now that we have identified the SNPs, genome coordinates of each SNP have been sent to AGRF who are in the process of manufacturing the array, which will be ready for validation by mid-May 2024. We have also deposited our SNP suite into a public website for other koala managers and researchers to access in addition to including the data in a publication to be submitted to a peer-reviewed journal. Once the array is constructed by AGRF, the next step in the practical application of the technology will be its validation via the testing of koala DNA isolated from tissue samples collected from koalas located in south-east QLD region. Greater than 1000 koala tissue samples have already been collected from koala admissions at Currumbin Wildlife Hospital and Australia Zoo Wildlife Hospital and are waiting analysis. We are seeking to fund the production of two further koala array panels that will have significant benefit for koala conservation. The first of these will be a specific set of gene markers we have identified especially adapted for the poorer quality DNA obtained from faecal samples. The second additional array will be focused on immune genes and will allow us to explore the relationship between immune function and disease (Chlamydiosis and KoRV). Our research has paved the way for the production of a custom koala SNP array where submission of samples to a nationally commercial genotyping service provider, (AGRF) will now provide the opportunity to conduct a nationally coordinated approach to koala population genetic analysis, via a standardized suite of publicly accessible genetic markers to all koala researchers and managers; this will enable the ability to directly compare the genetic health of regional koala populations, as strongly recommended by the National Recovery Plan for the Koala (*Phascolarctos cinereus*) (DAWE, 2022) https://www.dcceew.gov.au/sites/default/files/documents/recovery-plan-koala-2022.pdf.

3.2 Faecal Hormone Metabolites

We report the successful validation of a faecal testosterone metabolite assay for male koalas and a faecal progesterone metabolite assay for female koalas; these assays are robust and reliable. The progesterone metabolite faecal assay will be useful in detecting ovulation and possible pregnancy in wild koalas, whereas the testosterone faecal metabolite assay may be used to help characterize reproductive seasonality, social hierarchy or used to assess sexual maturity in males. Three different oestrogen metabolite EIAs were tested on female koala faecal extracts but none were suitable for oestrus

detection (elevated levels of oestradiol). Further subsequent more detailed and sophisticated analysis based on LC/MS and HPLC/MS were able to detect oestrogen metabolites, but these could not be biologically validated to known reproductive status. Future studies should concentrate on establishing the primary excretory pathways of oestrogen metabolite in the koala.

We also applied our “in house” glucocorticoid (GC) EIAs to successfully measure biologically relevant faecal concentrations of cortisol and corticosterone to captive and wild koalas. Results from the captive koalas showed reasonable correlation of these GCs with induced or perceived acute stressors. GC analysis of the koala faecal samples collected from the Jimboomba site in 2022 revealed cortisol and corticosterone metabolite levels that were generally very low; we attributed these results to poor sample quality associated with rain damage. Better quality samples collected in 2023, and which were cross-matched with genetic information, obtained from scat DNA, revealed a much more variable individual pattern of GC secretion**.** We conclude that while it is possible to readily measure GC in koala faeces, an interpretation of these values, as it relates to a dysfunctional stress response still requires further validation. For example, a single elevated level of GC metabolite in a koala faecal sample is unlikely to differentiate a koala in the wild suffering from an acute or chronic stressor. Consequently, we also commenced preliminary studies to explore other stress physiology biomarkers (e.g. Dehydroepiandrosterone [DHEA] and Thyroid hormone metabolites [T3 and T4]) that might be run in parallel with the analysis of faecal GCs, in order to provide better context to the assessment of stress physiology; the analysis of these specific faecal biomarkers is a first for the koala, and our results would suggest that these metabolites are worthy of further investigation.

3.3 The Jimboomba Koala population

The third and final aim of the research project was to conduct a field survey of the koala population in the Jimboomba Celestino Development site, immediately before, during and after clearing and to compare these conventional ecological surveys to the data obtained from non-invasively collected faecal samples. Unfortunately, many of the koala scats collected in early part of the field survey in 2022 were severely rain damaged, such that it was not possible to recover quality DNA or data based on the analysis of faecal steroid metabolites. The adverse effect of rain damage represented the most significant limitation to the use of this non-invasive technology for studies of ecology and/or disease epidemiology. Essentially it restricts the application of scat based analysis to dry periods or to locations where substrates do not become water-logged after rain. We also showed how the quality of recovered DNA from the koala scat declined with time (environmental exposure), further highlighting the importance of obtaining fresh samples. We would recommend a combination of drone thermal signature technology to locate koalas in trees in the twilight hours (as used in this study) followed up by the use of detection dogs to home in on freshest scats.

Faecal samples collected from late 2022 and during 2023 were less affected by rain damage and provided valuable post clearance information on those koalas on or in the surrounds of the Jimboomba site; there was a marked increase in the number of scats from which koala DNA was successfully extracted based on positive koala beta-actin PCR results. None of the samples from which koala DNA was recovered showed evidence of being infected with the pathogen *Chlamydia pecorum*. Genotype profiles across 32 microsatellite genetic markers were also generated from faecal scats confirmed positive for presence koala beta actin; this genetic data revealed moderate genetic diversity of the population, with a moderate to high inbreeding value. Analysis of repeated genotypes within the dataset identified 7 individual koalas and was used to infer movement patterns

of individual koalas and habitat preference. Based on the production of a specific sNP array for koala faecal DNA, designed by our group, and produced by AGRF, we shall re-analyse the genetics of Jimboomba population and compare this with information to that which we obtained using microsatellites.

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Johnston SD, Hulse L, Keeley T, Mucci A, Seddon J, Maynard S (Submitted 28 Feb 2024) The utility of koala scat analysis. *Biology*

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Hulse L, Seddon J, Johnston SD (In draft 2024) Development of a high-density targeted SNP assay to promote standardized genotyping of the koala for comparative population genetic analysis in Australia. *Conservation Genetics Resources*

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biology

MDPI

Review

The utility of koala scat analysis

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Simple Summary: This review reports on the current and potential utility of the "koala scat" sample to provide a range of ecological and physiological assessments both at the population and individual animal level and do so in a non-invasive manner. DNA recovered from the scat sample has been shown to provide useful information on koala distribution, diet, genetics, disease, and physiology. While there are still limitations with respect to the decay of quality DNA (host, microbiome, and pathogen) over time related to climate (rain, humidity, temperature) and sample handling, some of these issues can largely overcome with timely sample collection (e.g. detection dogs). Other current limitations include an inability to detect and quantify particular hormone metabolites such estrogens and/or a correct biological interpretation of glucocorticoid metabolite secretion as measured in the fecal sample.

Abstract: The use of faecal samples or scats to provide important ecological, genetic, disease and physiology detail on free-range populations is gaining popularity as an alternative non-invasive methodology. Koala populations in SE Queensland and NSW have recently been listed as endangered and continue to face anthropomorphic and stochastic environmental impacts that could potentially lead to their extinction. This review examines the current and potential utility of the koala scat to contribute data relevant to the assessment of koala conservation status and decision making. Although we demonstrate that there is great potential for this methodology in providing details for both individual wild animal and population biology (distribution, abundance, sex ratio, immigration/emigration, genetic diversity, evolutionary significant unit, disease epidemiology, nutrition, reproductive status and stress physiology), the calibre of this information is likely to be a function of the quality the scat that is sampled.

Keywords: Koala; *Phascolarctos cinereus*; Faucal; Scat; Non-invasive; Methods; Ecology; Genetics; Hormone metabolites.

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1. Introduction

Koala populations are struggling to survive against the unyielding threats of habitat fragmentation, urban development, disease and stochastic catastrophic fires and drought associated with climate change. In 2022, the koala in Queensland, NSW and the ACT was officially listed as endangered under the *Environment Protection and Biodiversity Conservation Act 1999 (EPBC Act)* [1]. Despite conservation efforts over the last 20 years, koala numbers in SE Queensland and NSW continue to plummet, with extinction of further local populations, a real possibility. Compounding this bleak scenario, and particularly for the SE Queensland region, is the need to develop housing in order to accommodate an ever-increasing northward migrating human population. Queensland Government projections suggest a population density of 6 million people in SE Queensland by 2046 which represents an increase of nearly 160% on the current population numbers [2]; more people, means more housing supply, and this means more even pressure on threatened koala populations.

Clearly, regional planning will need to address, and if possible, attempt to mitigate this conflict. Koala management in South-east Queensland will not only require a thorough understanding of population abundance and distribution but a much more comprehensive understanding of the current and predicted threats and stresses to these populations. Consequently, both natural and anthropomorphic threats on koala populations such as those imposed by climate change (bushfire and heat waves), disease (Chlamydiosis and Koala Retrovirus) and habitat fragmentation (housing, infrastructure, vehicle strike trauma, and dog attack) will need to be carefully monitored and accounted for.

Methods for measuring koala population density and distribution have been evolving rapidly in the last decade such that there is now a wide range of protocols available for those conducting environmental assessment and impact; these include direct koala spotting, the detection of koala scats (often assisted by tracker dogs), the location of animals in the canopy facilitated by infrared cameras mounted on drones, or a combination of all these approaches. While all these methods have their pros and cons, costs, and benefits, one has particular utility, in that can potentially provide, and in a non-invasive manner, a plethora of additional information with respect to the individual animal and/or population as a whole.

An animal's fecal sample or "scat" provides valuable non-invasive information on a range of its physiological processes and health status (Figure 1). Traditionally, this has included an analysis of diet and an indication of parasite load or disease but with the further development of molecular and endocrine techniques, the scope of information can be dramatically expanded. When species defecate, they typically also shed epithelial cells from the lower intestinal tract that onto the surface of the scat as it is formed in the rectum. In herbivorous species that process their feces into individual pellets, it is common to observe a mucous layer over the surface of the scat that contains epithelial cells, some of which possess intact nuclei that contain genetic information. Once these nucleated epithelial cells have been washed or scraped from the surface of the scat and the isolated DNA confirms they have come from the actual host species, it is then possible to analyze the DNA for genetic information via genotyping using markers such as microsatellites and SNPs; this data can provide a range of detail including the sex and genetic identity of the individual animal and how this genetic information might relate to other individuals within the population or to other surrounding populations.

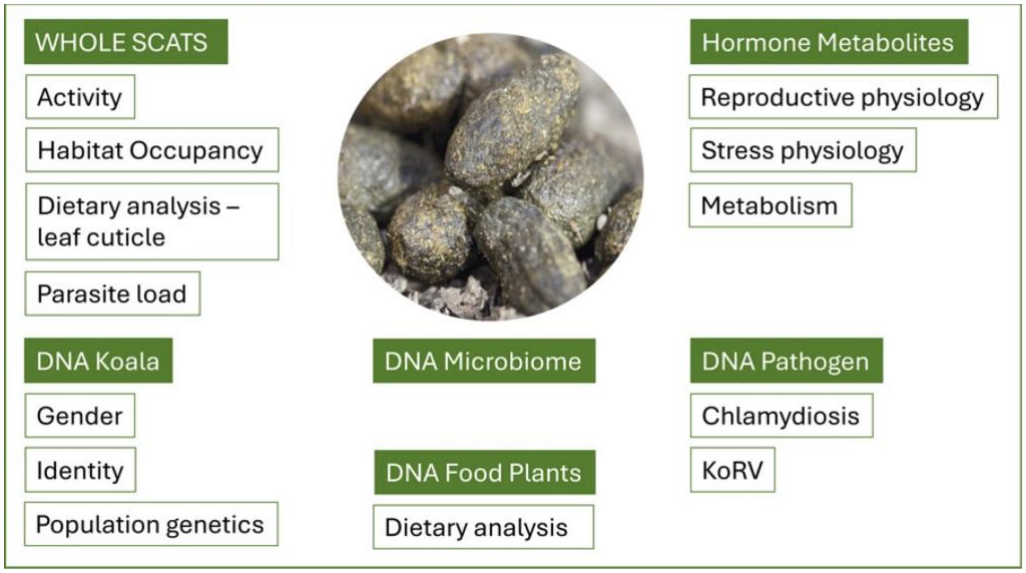

Figure 1. The "Power" of Koala Poo – The information that is available from a koala scat.

The same molecular technology can also be applied to the vegetal matter found in the pellet to further define what tree species the animal has been consuming and what gut microflora it might possess in order to metabolize the leaf; this microbiome may also change between populations, time of the year, and perhaps even between leaves on the same tree. Molecular technology can also determine whether the animal is shedding DNA from infectious pathogens (e.g. *Chlamydia* and Retrovirus infection) including determining pathogen load of these organisms. The use of fecal samples for determining endocrine detail has also become routine in the management and assessment of wildlife species health. The non-invasive nature of the sample collection and integrated secretion of the metabolites that are measured in the feces overcomes the confounding issue of the stress of taking a blood sample from a wild animal and the pulsatile secretion of hormones apparent in the systemic circulation [3]. Metabolites of hormones reflective of reproductive status, stress physiology and metabolism can all be measured in a fecal sample [4].

2. The Koala Scat

The koala scat has a characteristic morphology (Figure 1) and is therefore easily identified on the forest floor and persists in this form post-departure from the animal [5]. Koalas in captivity have been estimated to produce approximately 75-150 fecal pellets per day [6] and one may assume a similar level of productivity from wild animals. Locating koala feces in the wild has also become increasing more accurate and less labor intensive with the inclusion of detection dogs [7]. While the value of the fecal sample to koala monitoring has been postulated for decades, it has only been in the last 10 years that its utility for koala population assessment has become more fully realized [8]. The following review will examine the current use of fecal scat samples in koala conservation, highlighting the benefits and limitations of the information that can be obtained from its analysis. Unpublished data from our own ongoing studies will also be included for completeness.

3. The Non-Invasive Sample

The utility of sampling koala scats primarily stems from its collection as a non-invasively obtained biological. Particularly for the measurement of steroid hormones (e.g.

stress and reproduction) obtaining a blood sample in most wildlife species is most likely to require the animal being captured and anesthetized or restrained, the act of which may potentially confound the very physiological parameter being assessed. The most obvious example of this would be the measurement of glucocorticoid hormones (cortisol and corticosterone) in a blood sample wherein the act of actively capturing and restraining a wild koala would most likely stimulate an acute stress response, negating its value as a reliable indicator of stress physiology both for the individual and the population [9,10]. Additionally, the measurement of hormone metabolites in the feces typically represents an integrated sample of hormone secretion thereby also overcoming any inherent episodic secretion of the hormone in the systemic circulation (e.g. Testosterone) and negating the adverse effects on any altered hormone secretion associated with restraint or anesthesia.

4. Habitat Occupancy and Activity

Currently, the most common application of scats in koala conservation are their use as an indirect measure for monitoring koala habitat occupancy. As a survey technique the method is typically lower in cost to conduct and requires fewer resources than direct observation, and is therefore, popular for monitoring programs and environmental impact assessments [8]. Scat based koala surveys have been used in Queensland since 1996 [11]. Standardized scat surveys include various versions of the Spot Assessment Technique [12], the Koala Rapid Assessment Method [5,7] and Balanced Koala Scat Survey [13].

Each method has its own inherent limitations in terms of accuracy and effort especially when used to extrapolate estimates of abundance such that data collected from scat surveys should always be interpreted with caution [5]. False negative results can arise because of sampling technique but are also related to scat detectability, scat deposition and decay rates that in turn can all vary between sites [8,14]. Cristescu et al. ([7]2015) has demonstrated that false negatives and survey time can be greatly reduced when detection dogs are used to locate scats; detection dogs are 19 times more efficient and 153% more accurate than humans at locating scats. Nevertheless, scat surveys do have limitations; Ellis et al. [15] has indicated that scat searches are imprecise indicators of tree use. Scat surveys on their own also tell you nothing about the total number of animals, gender, health, diet, home range and movement patterns unless they can be supplemented with further laboratory analysis. Although scat surveys are likely to be suitable for determining koala activity levels or occupancy, alone they lack the robustness and accuracy as reliable estimates of density [8].

5. Dietary Analysis Using Cuticle Fragments

Koalas have also been shown to exhibit degrees of preference for certain food species [16]. Food quality and availability are fundamental to quality koala habitat [17] so it is critical that we understand what koalas eat and why. The stomatal complex and arrangement of subsidiary and guard cells on the cuticle of eucalyptus leaf fragments [18] that pass through into the koala scat have been used to determine dietary composition, and the technique found to be sufficient to separate out individual browse species [19,20]. The accuracy of this approach was examined by Ellis et al. [21] by feeding captive koalas with specified proportions of known browse species; this study revealed that the food species could be consistently detected in scat by microscopic analysis 34h after the browse was first presented and that they remained in the scat for up to 154h post-feeding. While the technique has subsequently been used by other researchers [22,23], the procedure is labor intensive, requires specific expertise and is unable to differentiate certain species of food trees [24].

6. Koala DNA Extraction from Faeces

The koala scat contains a range of host, dietary and pathogen DNA that can potentially be isolated via extraction and analyzed in the laboratory to obtain a range of ecologically important information, particularly useful in assessing environmental impact [8]. The scat contains only very small amounts of koala host DNA recovered from epithelial cells exfoliated onto the surface of the fecal pellet, whereas the bulk of the DNA in the scat is derived from the various eucalypt species it consumes, the gut microbiota and/or pathogens. While scats processed for DNA dietary analysis typically utilize the whole sample, isolation of host DNA is best achieved by carefully lavaging off the outer mucous layer containing the host's epithelial cells from the surface of the scat. The quantity and quality of the extracted DNA can then be assessed using spectrophotometry and confirmation of koala DNA achieved by reference to a house-keeping gene (e.g. Koala Beta actin).

Limitations with respect to this technique include that sufficient DNA is recovered for analysis [25] in addition to the presence of biological inhibitors, such as *Eucalyptus* tannins, that may interfere with downstream molecular analysis [26]. Wedrowicz et al. [25] has shown that the quality of the extracted DNA may also vary with commercial kits used for DNA extraction; consequently, it is recommended that pilot studies first be conducted to assess and optimize the quantity and quality of the extracted DNA in advance of any genetic analysis. Schultz et al. [27] has indicated, not unsurprisingly, that genetic sampling is best undertaken with fresh fecal pellets (less than 2 days old) as the quality of extracted DNA from the scat declines over time; hence, the use of scat detection dogs to ensure discovery of the freshest samples. Wedrowicz et al. [28] has also indicated that scats stored in paper bags or exposed to wet conditions (rain, condensation) may also result in lower DNA yields.

Downstream analysis of DNA isolated from fecal scats, such as PCR and next-generation sequencing can be challenging as the extracted DNA is often fragmented and low in quality. Fecal DNA extracts contains a preponderance of DNA from exogenous (non-host) sources such as gut microbes, digesta and environmental organisms. Gut bacteria pose a particular challenge as they account for the highest proportion of DNA in feces. To address this issue, a method to enrich host DNA from non-invasive fecal samples may be employed. One such method utilizes natural differences in CpG-methylation density between vertebrate and bacterial genomes to preferentially bind and isolate host DNA from majority-bacterial samples, technically separating host DNA from exogenous DNA. Studies have demonstrated that host DNA enrichment from fecal scats is a robust, efficient, and compatible with downstream molecular analysis [29].

7. Genetic Analysis

An understanding of population genetics is vital to understanding the long-term conservation management of any threatened wildlife species. At the population level, koala DNA isolated from scats has the potential to provide important detail such as sex ratio and effective population size, genetic diversity, and migration (gene flow); it can also be used to better define what constitutes a metapopulation or evolutionary significant unit. At the individual animal level, genetic information obtained from scat DNA may be used to explore behavioral ecology (e.g. paternity, mating strategy, animal-animal relationships). Having the ability to genetically identify an individual koala (genetic profile) also allows this information to be used to determine habitat occupancy and/or activity and when used for mark – recapture studies, a potential estimate of abundance.

Recently, Hogg et al. [30] have systematically reviewed population genetic papers examining koala populations in eastern Australia from 1996 to 2020, noting that the majority of early studies utilized between 6 – 17 microsatellite genetic markers for their analyses. While microsatellite markers have had their place in koala genetic analysis, they are not representative of genome wide diversity and are currently being progressively replaced by the analysis of single nucleotide polymorphisms (SNPs). When compared to the koala reference genome [31] the discovery of SNPs obtained from next generation sequencing can be used to determine genome wide diversity and/or explore the diversity in functional regions of the genome associated with different phenotypes.

As reviewed by Hogg et al. [30] there has been only a limited application of SNPs in studies of koala genetics to date [32-34]; while all these studies were conducted from DNA extracted from tissue, Schultz et al. [27] has also reported accurate SNP genotyping from koala DNA isolated from scats. The continuing decline in the cost of data acquisition of genomic studies will undoubtedly lead to the more widespread application of SNPs and/or whole animal genomic analysis, especially as the open database of the Koala Genome Survey continues to grow [30]. To date, 430 koala genomes have been released on Amazon Web Services Open Data program (https://awgg-lab.github.io/australasiangenomes/species/Phascolarctos_cinereus.html). If scat DNA is to make the best use of this database, then the quality of fecal DNA extraction procedures will need to keep up. In addition, if we are to better understand the genetic connectivity of koala populations then we need to implement a coordinated approach to population genetic analysis via the availability of a standardized suite of publicly accessible genetic markers to all koala researchers and managers; this will facilitate an ability to directly compare the genetic health of regional koala populations.

8. Dietary Analysis Based on DNA.

Molecular biology not only has an important role in koala genetics, but it can also be used to gain deeper insights into what koalas eat. Next generation sequencing of eucalyptus SNPs extracted from koala scat can be used as potential biomarkers of koala diet using a method known as DArTseq™. Schultz et al. [27] demonstrated the potential of SNPs to be used in this way. More recently Blyton et al. [24] have further refined this approach to focus on dietary species-specific SNPs using the plant DNA in the koala scat, resulting in a tool that allows a semi-quantitative analysis of what koalas eat. Blyton et al. [24] not only found general agreement with respect to what tree species koalas were already known to consume but also identified new species that could be contributing to their diet. This molecular approach to dietary analysis will facilitate comparison of koala food species and preference between locations, season and perhaps, even of browse types consumed within the same tree.

9. Microbiome

The microbiome is the collection of all microbes that naturally live on or in the body and represents the primary interface between the organism and its environment. In addition to what the koala eats, molecular biology conducted on DNA isolated from the koala scat is being used to identify the microbial diversity and abundance of the koala gut microbiome, how it differs in various geographical locations and season, and how it changes over life history. Dietary focused information based on microbiome analysis is likely to have an important role in helping to make population management decisions such as recommendations on koala translocation. In a similar manner, analysis of

changes in the microbiome is likely to have a therapeutic application following antibiotic treatment in order to prevent or manage dysbiosis.

Early studies used metagenomic analysis of 16S ribosomal RNA to profile the koala gut microbiome. Barker et al. [35] examined only 2 koalas at different sites along the digestive tract (caecum, colon, and scat) reporting a highly complex and diverse ecosystem with considerable intra-individual variation. Later Alfano et al. [36] also compared the microbiomes of 2 koalas from different regions of the body (eye, rectum, and scats) noting that the scat microbiome represented only a subset of that found in the rectum. Metagenomics was used by Shiffman et al. [37 to compare the respective microbiomes of the koala and wombat in order to contrast differences associated with dietary specialization; this analysis revealed that koala scat samples were dominant in the micro-organisms necessary for secondary metabolism, perhaps thereby indicating their role as an important player in the koala's ability to detoxify its diet.

The fecal microbiome was further characterized in wild koalas by Brice et al. [38]; targeting the bacterial 16S ribosomal RNA (rRNA) gene, these researchers found a strong association between the microbial community and host diet and concluded that even amongst individuals that a change in consumption of congeneric tree species to another, can significantly alter the gut microbiome. This finding was further supported by that of Blyton et al. [39] who showed that koalas feeding on different tree species (i.e. Messmate and Manna Gum) had different microbiomes based on 16S rRNA profiles, despite also showing some overlap. Blyton et al. [39] also demonstrated that the microbiome of the koala could be altered by means of fecal inoculation. Translational studies of the koala scat microbiome are likely to follow in the future, including the use of fecal inoculation capsules to overcome antibiotic induced gastrointestinal dysbiosis and their use in preparing koalas for translocation to assist individual koala gut microbiomes to adapt to shifts in diet [39]. Blyton et al. [40,41] have also examined changes in the scat microbiome to explore maturational changes between juvenile and adult assemblages of microorganisms associated with pap feeding in both captive and wild populations.

10. Chlamydiosis

Apart from the threat of natural disasters (drought, heatwaves, and bushfires) and anthropomorphic disturbance (habitat loss, road, and dog trauma), koalas are unfortunate enough to also be susceptible to debilitating diseases. *Chlamydia* spp. is an intracellular bacterium that causes significant inflammation of the conjunctiva and urogenital systems of both female and male koalas resulting in clinical blindness, cystitis, and infertility [42,43]. Although commonly associated with the urogenital tract and detection best sampled from swabs of this region, it is also possible to detect chlamydial DNA from fecal scat samples of infected individuals [44,45]. Using PCR, Wedrowicz et al. [44] reported a high concordance between *Chlamydia pecorum* in the DNA isolated from scats and urogenital swabs. Cristescu et al. [45] also compared the clinical efficiency (sensitivity and specificity) of a multiplex quantitative PCR (qPCR), next generation sequencing (DArTseq™) and a detection dog to correctly identify scats excreted by koalas that tested positive to *C. pecorum* from urogenital swabs and which showed observable clinical signs of the disease; all three methods showed 100% specificity but there was variable sensitivity (qPCR – 78%; DArTseq™ – 50%; Detection dog – 100%). The authors suggested that lower sensitivity of the qPCR and DArTseq™ compared to the detection was the fact that the molecular methods relied on the acquisition and extraction of quality DNA, whereas the dog is likely to be detecting volatile organic compounds released from the scat. Recent developments in the use of loop mediated isothermal amplification (LAMP) assays that can be readily conducted in the veterinary clinic and/or field [46,47] are providing a rapid diagnostic tool for detection of *Chlamydia* DNA from swabs but

have yet to be developed for scat samples. It also needs to be remembered that current molecular methods for both swabs and scats are only detecting chlamydial DNA and is unable to distinguish live or infectious elementary bodies.

11. Koala Retrovirus (KoRV)

Koala retrovirus is a gammaretrovirus that has been linked to koala neoplasia [48-50] with additional associations to immunosuppression [51-53]. To date, these links appear to be primarily correlative rathe r than causative [43]. Of all the KoRV subtypes, KoRV B is most commonly associated with lymphomas, leukaemias and neoplasia [53,54]. The ability to detect KoRV A subtype and determine copy number in the koala scat was first reported by Wedrowicz et al. [44] using real time PCR. Quigley et al. [43] has used DNA extracted from scats to conduct a validation between sample type (blood, swab, and scat) in preparation for a phylogenetic and geographical analysis of KoRV subtypes concluding general agreement between combined diversity profiles for all tissue types (blood – 100%, swab – 98%, scat – 90%) . More recently, Blyton et al. [55] successfully validated and applied PCR and deep sequencing to DNA extracted from the koala scat to characterize KoRV A and a range of other exogenous subtypes (B-M) in a geographical study; this work confirmed that subtype A appears to have endogenized in northern koalas and is progressively becoming endogenized into the southern koala genome. The other exogenous subtypes of KoRV (B-M) detected by Blyton et al. [55] only appear to be found in northern koalas and were geographically restricted, suggestive of sporadic evolution and local transmission.

12. Koala Reproductive Hormones

The measurement of hormone metabolites in fecal samples to assess reproductive status in wildlife has become increasing common practice in eutherian [56-58] and to a lesser extent marsupial species [59]. Hormone metabolites are typically extracted from faces using alcohol solvents and the reconstituted hormones measured following biological validation by enzyme immunoassays using antisera with appropriate cross-reactivity. Reproductive steroid hormones such as progesterone [60] and testosterone [61] and their metabolites have been successfully analyzed in koala feces with detection of the luteal phase and pregnancy in females and age and season changes in males. In fact, unpublished data from our group has shown how measurement of progesterone metabolites recovered from the koala scat can be used to precisely map the luteal phase or pregnancy with the same degree of resolution as that measured in plasma samples [62]. We also have unpublished data on androgen secretion in the male koala, which has direct application for the assessment of seasonality and sexual maturity.

However, despite numerous attempts using a range of different specific antibodies (e.g. estradiol, oestrone-3-glucuronide) to analyze fecal extracts of koalas of known reproductive status (e.g. estrus), and even multiple HPLC and mass-spectrophotometry analysis, all attempts so far to detect biologically relevant estrogen or estrogen metabolite levels in koala scats have failed thus far. Hence, the measurement of estrogen in feces remains a significant challenge in the koala. Schwarzenberger et al. [56] has indicated estrogen measurement in the feces of herbivores can be problematic not only because of the low levels in the plasma (picograms rather than nanograms) but the fact that the main route of excretion is often via urine. An inability therefore to measure estrogen readily in koala fecal samples means that we are currently limited to only identifying or monitoring animals that ovulate (luteal phase) or that are pregnant. Although reproductive steroids can be measured in feces it is not possible to measure protein-based gonadotrophins in the scat such as luteinizing hormone (LH) or follicle

stimulating hormone (FSH) as these molecules are excreted in the urine or denatured and degraded by digestive system by the time they are excreted in the feces [3].

13. Koala Glucocorticoid Hormones

Fanson et al. [63] have examined the utility of commonly used fecal hormone assays in marsupials and noted that the best performing assays varied with species, emphasizing the need to have thorough biological validation for each species and sample type. The measurement of glucocorticoid hormones (e.g. cortisol) from the koala scat has attracted significant attention in the literature (see Table 1 [63-78]). Numerous studies have attempted to validate the measurement of glucocorticoids (GC) using ACTH, exogenous cortisol or following a biological (physiological or behavioral) event in both plasma and feces and to apply the assessment of fecal GC levels as an index of stress. One study has incorporated liquid chromatography combined with mass spectrophotometry (LCMS) to attempt to specifically identify which glucocorticoid metabolites are present in the koala scat after administration of exogenous cortisol [72]. GC metabolites in koala scats have been used to try to assess stress in both captive and wild populations including the effects of habitat fragmentation, translocation, disease, trauma, hospitalization and the handling of captive koala during for photography (Table 1). Parker-Fisher and Romero [79] highlighted the importance of using multiple measures of stress physiology (such as behavior, immune function, health, weight changes), rather than restricting studies to single measure like GC, as this is likely to provide a better assessment of whether a species or individual is experiencing stress.

Table 1: A Summary of GC studies using koala scats

Metabolite	Type of Study	Assay	Reference
Cortisol	V - ACTH, Captive	EIA	[64]
Cortisol	A - Habitat (Aridity) (Semi-arid Zone), Wild	EIA	[65]
Cortisol	V - ACTH, A - Captive V Wild, Handling	EIA	[66]
Cortisol, CS	V – ACTH	EIA	[67]
Cortisol, CS, 72a, 37e	V – ACTH, Captive	EIA	[63]
Cortisol	A - Zoo visitor, Captive	EIA	[68]
Cortisol	A - Habitat clearing, Wild	EIA	[69]
Cortisol	A - Disease, Trauma, Hospital, Bushfire, Wild	EIA	[70]
Cortisol	A - Disease, Trauma, Hospital, Bushfire, Wild	EIA	[71]
Cortisol, 37e, 50c	V - Hydrocortisone, Captive	LCMS, EIA	[72]
Cortisol, 37e, 50c	V - Time decay, Water loss, Captive	EIA	[73]
Cortisol,37e, 50c	A - Seasonality, Captive	EIA	[74]
Cortisol	A - Hospital, Rehabilitation	EIA	[75]
Cortisol	A - Translocation, Wild	EIA	[76]

Key: A – Application, , CS – corticosterone, EIA – Enzyme Immunoassay, LCMS – liquid chromatography Mass Spectrophotometry, ACTH – Adrenocorticotropic hormone, V – validation, 37e - 5a-pregnane-3B,11B,21-triol-20-one, 50c - Tetrahydrocorticosterone, 72a – 11-Oxoaetiocholanolone-3-HS

The interpretation of fecal GC measurements is challenging and open to debate given the significant variation that may occur in GC hormone secretion between

individual animals , the role of GCs as mediators of the physiological stress response, as well as the maintenance of homeostasis and energy regulation among others [80,81]. Acute stress responses are normal and typically don't have a long term or significant impact on the individual as GC levels should quickly recover once they have served their role. Chronic stress responses may initially follow the same pattern as an acute response before GC dysregulation ensues (e.g. Loss of normal feedback regulation); hence ensuring sample collection frequency and extent is adequate to distinguish between the two is essential. Equally challenging is the fact that there is no "typical" chronic GC production pattern; in response to a chronic stressor, GC production can increase, decrease or be unresponsive [80]. This emphasizes the need to integrate other measures of stress to properly evaluate the koala's physiological state and response to the stressor of interest.

14. Koala Metabolic Hormones

Thyroid hormones are important for the regulation of metabolic activity in mammals and crosstalk with other hormone systems allows these hormones to coordinate metabolic changes and/or to modify growth and maintenance of an organism with respect to environmental conditions [82]. Thyroid hormones, therefore, have the potential to serve as biomarkers for ecological studies, energy allocation and growth and for the monitoring of physiological changes associated with food deprivation, food quality or reproduction. While no published studies currently exist for the measurement of nutritional fecal hormone metabolites in the koala, metabolites of thyroid (T3/T4) have been attempted in a range of eutherian wildlife species [82,83]. "Our group is currently attempting to test and validate fecal T3 and T4 analysis techniques for the koala and if successful, could provide valuable physiological data for in situ research.

15. Conclusions: The Power of Koala Poo

This review has revealed the current and potential utility of a "koala scat" sample to provide a range of ecological and physiological assessments both at the population and individual animal level. While there are still limitations with respect to the decay of DNA (host, microbiome, and pathogen) and hormone metabolites overtime related to climate (rain, humidity, temperature) and sample handling, these issues can be largely overcome with the use of detection dogs in order to find freshly deposited scats.

The ability to identify individual koalas from scat DNA has a range of applications for researchers, site managers and environmental assessment teams. It provides a powerful tool to obtain accurate estimates of population dynamics (size, sex ratio, birth, and death rate, immigration, and emigration rates), genetic diversity and habitat utilization and occupancy in a relatively short period of time. This data could be used to inform planning and environmental impact assessment processes providing a detailed and accurate picture of koala population dynamics and usage in areas identified for future housing or infrastructure development. With further improved methods of DNA isolation and application of next generation sequencing technology (SNPs and whole genome sequencing) it is not difficult to envision how scat DNA will also provide detailed information on behavioral ecology and disease susceptibility (e.g. MHC diversity). At the population level scat DNA could be used to define evolutionary significant units and as a tool for improve genetic management (e.g. translocation and genetic health). These techniques could be utilized in existing reserves and environmental offset sites to achieve improved outcomes for existing and new koala populations.

This review has also shown that disease (*Chlamydia* and KoRV subtype) in the population or individual koala can be assessed using scat DNA. The detection of *Chlamydia* DNA is already being utilized but is most likely to be that only associated with

urogenital infection (*C. pecorum*). While quantitative PCR has the capacity to quantify *Chlamydia* load in order to make comparisons between populations, it is currently not possible to differentiate infectious elementary bodies from that of degraded *Chlamydia* DNA, so it is not possible to identify those koalas with an active infection and those who are shedding chlamydial elementary bodies. Nevertheless, scat DNA may still be particularly useful for scanning populations prior to translocation or determining the efficacy of *Chlamydia* vaccination programs. Studies using scat DNA to assess the population for KoRV subtype has already provided valuable data on the epidemiology of the virus; an ability to identify KoRV in populations and individuals will also be important for disease management.

Studies of reproductive hormone metabolites from koala scats are highly instructive as markers for progesterone (ovulation and gestation) and testosterone secretion but further research is required to identify suitable analysis techniques that allow the successful monitoring of fecal estrogens and the koala estrous cycle. There has been a range of different studies validating suitable fecal glucocorticoid enzyme-immunoassays to attempt to evaluate the impact of stressors in captive and wild koala populations. However, the interpretation of these hormone profiles requires further research, both in terms of the normative and abnormal responses to acute and chronic stress, individual animal variation and a better understanding of koala adrenal physiology. It will also be important to not rely on single measures of stress physiology (e.g. GC secretion) but rather combine these with other assessments (e.g. behavior, health biomarkers). An ability to monitor koala metabolism through the measurement of fecal thyroid metabolites may allow managers to better assess the impact of reduced nutritional quality of eucalypt fodder associated with climate change (e.g. heat stress, increased sclerophylly and drought).

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APPENDIX 2

Date	Distance (km)	Koala Observed Y	Scats Collected Y	Drone Survey Y	Comments
3/01/22	5.2				weather hot and steamy
4/01/22	2.1				
5/01/22	3.71		Y		Older scats found around numerous E.tere trees - zone 1
6/01/22	3.2				odd scat found at base of E.tere(not enough for 1 sample)
7/01/22	5.58	Y (E.tere)			E. tere 30m. No koala image/laid mats around PKF trees - Zone 1
10/01/22	1.5				No scats on mats - unusual
11/01/22	2.8				
12/01/22	3.6				
13/01/22	4.2				
14/01/22	3.3				
17/01/22	2.7	Y (E.mol)	Y	Y	E.mol 25m Koala image,scats collected around base of tree, mats laid. Zone 2
18/01/22	4.3				nothing at base of tree, laid more mats around PKFT
19/01/22	4.1				nothing at base of laid mats around PKFT
20/01/22	2.9				
21/01/22	3.7				
24/01/22	4.4			Y	
25/01/22	2.2				
26/01/22	2.5				
27/01/22	3.5				Rain, slightly wet morning
28/01/22	4.2				
31/01/22	3.8	Y x 2 (E.tere, Spotted Gum)		Y	E.tere 20m, Spotted 30m, Koala images, scats around base of tree wet, mats laid. Zone 1 and 3
1/02/22	2.9				Wet morning , mats laid , collected mats from previous areas
2/02/22	2.1				Wet morning , pellets sitting in water on mats(not collected)
3/02/22	3.8				
4/02/22	2.9				
7/02/22	3.7				Mats laid have lots of water sitting on them
8/02/22	2.5				
9/02/22	3.6				
10/02/22	2.4				
11/02/22	2.8				
14/02/22	2.1				
15/02/22	3.5				
16/02/22	2.3				checking Mats laid are in areas 1 and 2
17/02/22	2.8				
18/02/22	3.6				
21/02/22	3.1				
22/02/22	1.1				Heavy rain, stay away from site due to inclement weather
1/03/22	1.6		Y		Boggy , scats collected from mat look old, very difficut weather and terrain
7/03/22	1.3				Boggy, difficult to traverse and heavy machinary on site clearing
8/03/22	1.8				Boggy
9/03/22	1.5				Boggy
10/03/22	1.9				Boggy
11/03/22	1.8				Boggy
14/03/22	1.5				Boggy, turned around due to diffculty in site access
15/03/22	2.1				Boggy
16/03/22	2.3				Boggy
17/03/22	1.9				Boggy
18/03/22	2.5				Boggy
21/03/22	2.1				Boggy
22/03/22	1.8				Boggy
23/03/22	2.3				Boggy
24/03/22	2.5				Boggy
25/03/22	2.1				Boggy
11/04/22	1.5				Wet
12/04/22	1.3				Wet
13/04/22	2.5	Y (E.tere)			Wet, E. tere 30m koala image , raining, scats sitting in water. Zone 2
15/04/22	1.7				Good Friday
19/04/22	2.6				
22/04/22	2.9				clearing plan is changing , will need to adapt
26/04/22	21.96				Driving around site where possible on trails and service road
29/04/22	3.2				
1/05/22	3.8		Y		collected scats on mat, wet weather persisting
2/05/22	4.1				laid more mats clearing plan changed
3/05/22	3.8				mats laid are generally have sitting water
4/05/22	10.81	Y x 2 (E.tere, E.mol)	Y	Y	E.tere 25m, E.mol 20m Driving around where possible, koala image, collected scats. Zone 3 and 5
7/05/22	4.2		Y		persistent wet weather impacting scats collection technique
9/05/22	3.51		Y	Y	persistent wet weather impacting scats collection technique
10/05/22	4.2				Couldn't get in to collect scats clearing plan has changed
13/05/22	3.8				
16/05/22	4.7				
17/05/22	3.9				
20/05/22	4.2				
23/05/22	4.5				
24/05/22	3.6				
27/05/22	4.9				
30/05/22	3.9				
31/05/22	4.8				
1/06/22	9.19	Y (E.tere)		Y	E.tere 15m, Driving around where possible, koala image. Zone 5
6/06/22	3.8				Wet weather and fog
7/06/22	1.48		Y		clear night and collected some dry scats from mats
9/06/22	7.34		Y		collected scats on mat, wet weather persisting
13/06/22	4.9		Y		collected scats on mat, wet weather persisting
15/06/22	4.5		Y		collected some scats oportunistically around PKFT
17/06/22	4.1				
24/06/22	3.9				clearing plan changing week to week. Adapting to the circumstances , some mats laid havnt been able to check as clearing has started in those zones.

Date	Distance (km)	Koala Observed Y	Scats Collected Y	Drone Survey Y	Comments
1/07/22	2.6				
5/07/22	3.8				
8/07/22	4.6				
11/07/22	2.1				
15/07/22	3.7				
21/07/22	4.2				
22/07/22	2.9				
26/07/22	2				
29/07/22	3.8			Y	Foggy
6/08/22	1.5				Boggy laid mats where I could multiple zones
7/08/22	2				Boggy, laid mats where I could multiple zones
8/08/22	4		Y	Y	Foggy, boggy and got bogged. Male koala heard calling
16/08/22	5.81		Y		collected scats on mat, wet weather persisting
17/08/22	9.8		Y		Driving around where possible
19/08/22	13.51	Y E.tere		Y	E.tere 30m, Driving around where possible, koala image-corridor zone
23/08/22	1				Laying mats in preserved areas
24/08/22	1				Laying mats in preserved areas
25/08/22	5.8				Laying mats in preserved areas
1/09/22	1.2				Cold and wet morning 8°C
2/09/22	1.1				Cold and wet morning 10°C
5/09/22	1				Cold and wet morning 7°C
9/09/22	1.1				Wet morning 12°C
12/09/22	1				Dry morning but too boggy 12°C
23/09/22	1.2				Wet morning 15°C
31/10/22	0				Onsite, unsafe due to clearing
14/11/22	0				Onsite, unsafe due to clearing and wet
3/01/23	7.68				Hot
4/01/23	5.52	Y (Alphatonia excelsa)	Y		A.excelsa 10m Hot, dry, 24°C, Lot 71, unallocated state land (koala image)
5/01/23	3.24		Y		Heavy rain
6/01/23	2.22		Y		Heavy rain
9/01/23	2.19		Y		Hot, humid E. mol, male calling
10/01/23	1.38		Y		Hot, partly cloudy, humid E.tere
11/01/23	1.01		Y		Partly cloudy, E.mol, E.tere
16/01/23	6.23				Dingoes observed
17/01/23	3.34		Y		Partly cloudy, rain last night, E.tere, council road reserve
18/01/23	3.03		Y		Partly cloudy, rained earlier, E.tere, council road reserve, cat skull
23/01/23	14.2	Y x 2 (E.mol)	Y	Y	Partly cloudy, E.mol 15m and 20m. Lot 71 USL, Corridor planting area
24/01/23	1.1		Y		Overcast, E.mol
31/03/23	0.1				Lots of works going on, difficult to get on site
8/05/23	6.8	Y x 4 (E.mol, Ironbark)	Y	Y	E.mol 25m and 30m, Ironbark 20m and 35m. Clear night, Lot 71 (koala images)
9/05/23	2.47	Y (Ironbark)	Y		Ironbark 20m, Clear cold morning, Lot 71 (koala image)
22/05/23	8.2	Y x 5 (Lophostemon suaveolens, E.mol)	Y	Y	L.suaveolens 15m, e.mol 25m & 30m. Clear cold morning, Corridor planting area (far eastern), Lot 71 (koala images)
23/05/23	3.51		Y		Clear cold morning. Lot 71, dingo adult and pup observed around E.mol
24/05/23	3.1		Y		Clear day. Corridor planting area
15/06/23	3.2	Y (E.mol)	Y		E.mol 10m. Clear day. Corridor planting area (koala image)
16/06/23	2.8		Y		E.mol, more scats under trunk than ever seen, Corridor planting area
19/06/23	4.4	Y x 2 (E.mol)	Y	Attempted	E.mol 5m & 8m. Mostly clear, heavy fog - couldn't drone. Corridor planting area. Koala image
20/06/23	1.16		Y		Partly cloudy. Corridor planting area
26/07/23	2.9	Y x 4 (E.tere, E.mol)		Y	E.tere 10m & 20m, E.mol 25m. Clear. Corridor planting area. Koala images
27/07/23	5.5		Y		Partly cloudy. Corridor planting area. E. tere, scats on mat, koala observed day before
28/07/23	2.13	Y (E.mol)	Y		E.mol 20m. Koala observed in same tree as per 19/06/23. Clear. Corridor planting area
29/07/23	7		Y		Scats collected from koala observed on 26/07/23