Measuring the impact of the Woolgoolga to Ballina Upgrade on local koala populations: faecal cortisol metabolite concentrations before, during and after phased resource reduction and during clearing

Dr Carolyn Hogg, Ms Parice Brandies, Dr Belinda Wright & Dr Catherine Grueber

Australasian Wildlife Genomics Group

School of Life and Environmental Sciences

The University of Sydney

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Australasian Wildlife Genomics Group
Faculty of Science
School of Environmental and Life Sciences
Gunn Building (B19)
The University of Sydney NSW 2006 Australia

E soles.wildlife-genomics@sydney.edu.au
W sydney.edu.au/vetscience/research/wildlifegenomics/

ABN 15 211 513 464
CRICOS 00026A

Summary

The Woolgoolga to Ballina Pacific Highway Upgrade Koala Management Plan has recommended the use of phased resource reduction as a way of minimising stress-induced impacts on koala 'hotspots'. Here, we use faecal cortisol metabolite (FCM) concentrations in koala scat samples to measure these potential effects at two impact sites, Laws Pt and Wardell Rd near Wardell NSW. To account for other variables that may affect FCM concentrations in koala scat samples, samples were also collected from two control sites in the vicinity that had similar habitat and climate. Koala scat samples were collected at all phases: Phase 1, before resource reduction; Phase 2-4, during resource reduction and Phase 5, during clearing. As scat samples were for dual purpose, i.e. genotyping and cortisol analysis, they were air dried and then frozen at -20^{o}C prior to extraction. DNA yield from four different optimisation protocols gave poor results meaning genotyping could not be undertaken to identify individual scat samples. FCM concentrations were determined using a Demeditec Saliva Free Cortisol Kit. Mean FCM concentrations at Laws Pt across Phases 1-5 were 39.7 ± 38.8 ng/g (N = 38 samples; impact site) and 38.5 ± 29.1 ng/g (N = 44 samples; control site). At Wardell Rd, the mean FCM concentrations across Phases 1-5 was 56.5 ± 52.5 ng/g (N = 9 samples; impact site) and 42.3 ± 23.6 ng/g (N = 11 samples; control site). These concentrations did not differ between the impact and control sites and were no greater than those noted for captive koalas using the sample assay method. Due to small sample sizes, no statistical analyses were undertaken for Wardell Rd; nor comparison between the sexes, as a large portion of the samples did not have corresponding sex information. Health status of koalas producing the scats was documented in 96% of samples from Laws Pt and 90% of samples from Wardell Rd. There was no association between FCM concentrations and health status. Both the Laws Pt impact site and its corresponding control site had increases in FCM concentrations across time. The drivers of this increase could not be attributed to environmental factors (such as rainfall, minimum and maximum temperature or sun exposure) nor biological ones (such as health). Work is ongoing to develop a reliable and robust field method that will permit both genotyping and cortisol analysis of koala scat samples collected from the wild.

Introduction

Wildlife managers are frequently tasked with providing data on the health of wildlife populations, particularly in relation to determining cause and effect in species declines and assessing causal relationships between disturbance and persistence of populations (Baker et al. 2013). Glucocorticoids (GCs) are secreted by the adrenal glands and influence a range of physiological parameters, including energy regulation, homeostasis and stress (Sapolsky et al. 2000). Although GCs are commonly called "stress hormones", as they are involved in the flight or fight response (Sapolsky et al. 2000), they also have a role in regulating circadian rhythms (Sherman et al. 1985), reproductive function (Weingrill et al. 2004) and immune function (Buford and Willoughby 2008). Physiologists describe two types of stress: 1) acute stress, which is a result of a stimulus that activates the "flight or fight" response; usually defined as abrupt, short-lasting (seconds to hours timescale) and an isolated perturbation; and 2) chronic stress, which is a result of elevated or ongoing stimulus (persisting for several hours a day for weeks) and can have negative long-term health consequences for the individual (Dhabhar 2002). Even though they regulate a wide range of physiological functions, GCs (cortisol or its metabolite corticosterone) in non-invasively collected samples are used as a physiological measure of stress in many wildlife species (Romero and Wingfield 2015). Using cortisol as a definitive measure of stress can be problematic, because cortisol concentrations fluctuate in response to other inherent physiological processes, such as age and sex, as well as external influences such as time of day and season. The passage time of the gut can also influence faecal cortisol metabolite (FCM) concentrations among individuals and species. Therefore, species-specific assays need to be developed, and validated using an ACTH challenge, to determine the effectiveness of cortisol (or corticosterone) as a measure of the stress response.

Koalas are listed as a Vulnerable species under the NSW Biodiversity Conservation Act 2016. Because of this listing, a Koala Management Plan was developed for the proposed Woolgoolga to Ballina (W2B) Pacific Highway Upgrade. This Management Plan proposed a staged approach to clearing in two known koala hotspots situated at Laws Point and Wardell Road, NSW. This staged approach, referred to as 'phased resource reduction' (PRR), involved the gradual reduction of food resources by ring-barking and/or collaring trees to facilitate the voluntary movement of koalas into adjacent habitat. The NSW Roads and Maritime Services contracted Sandpiper Ecological to implement the PRR program. A component of this project also involved population surveys to monitor koala numbers throughout the PRR process.

Recently it has been shown that koala scat samples can be used not only to assess FCM concentrations in koalas in relation to particular events, e.g. handled versus non-handled koalas (Narayan et al. 2013), but that DNA can be collected from the scat samples to identify individuals (Wedrowicz et al. 2013). Different studies yielded different baseline FCM concentrations from koala scat samples, 29.47 ± 6.99 ng/g (Davies et al. 2013a), 72.8 ± 55.0 ng/g (Johnston et al. 2013) and 1.64 – 46.35 ng/g (Narayan et al. 2013). Koalas undergoing an ACTH challenge (induced stress response) had a FCM concentration of 261 ± 97.93 ng/g (Davies et al. 2013a), 127.0 ± 51.2 ng/g (Johnston et al. 2013) and 6.7 – 8.9 ng/g (Narayan et al. 2013). As part of the koala monitoring surveys during the PRR project, koala scat samples were collected from both impact sites at Laws Point and Wardell Road, as well as control sites located at Tucki Tucki and Hazlemount Lane (Sandpiper Ecological 2018b, a). Control sites were selected based on their similarity to the impact sites in regards to koala populations, suitable koala habitat and climate. As FCM concentrations are known to fluctuate due to both internal and external factors (Touma and Palme 2005), the impact populations were to be directly compared to control populations to determine whether any changes in FCM concentrations could be attributed to activities of the highway upgrade and not to other environmental factors.

The aim of this study was to determine FCM concentrations in koala samples across time at all four locations, two impact sites and two control sites. Genotyping of the scat samples was attempted, in order to identify the individuals that produced the scats and examine change in individual FCM concentrations across the phases.

Methods

Sandpiper Ecological undertook scat sample collection and monitored the koalas. Koala surveys were conducted during each phase of the PRR (see Sandpiper Ecological Reports for further details regarding the surveys; http://www.rms.nsw.gov.au/projects/northern-nsw/woolgoolga-to-ballina/koala.html).

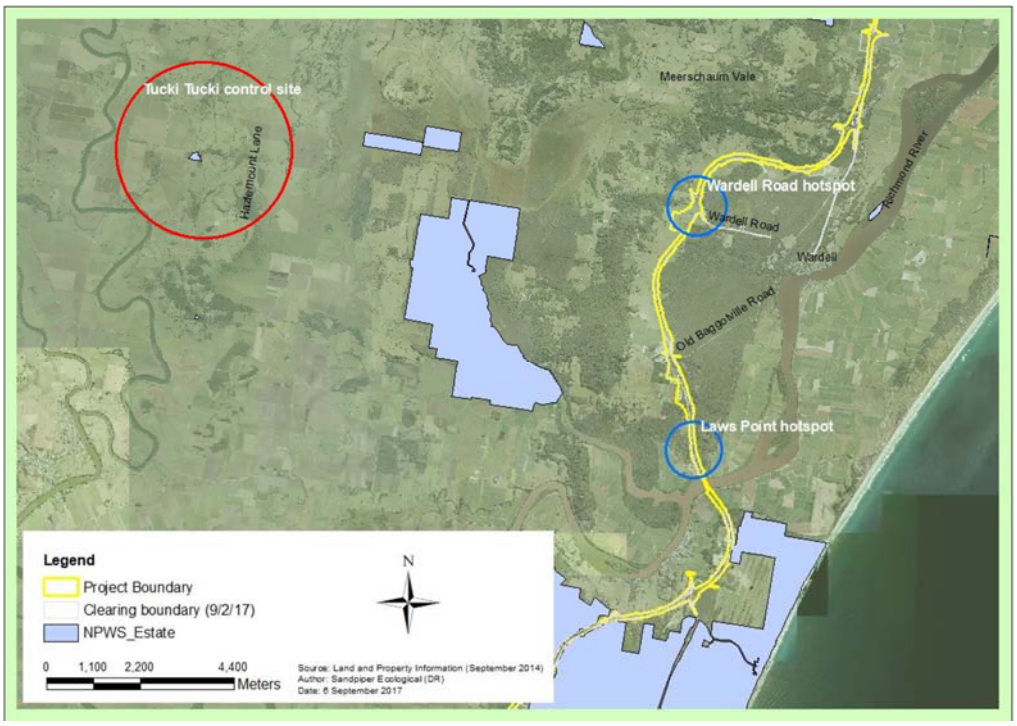
The five phases were:

- Phase 1 (Wks 1-3): Tag and map all trees to be collared/ring-barked and undertake six population surveys.
- Phase 2 (Wks 4-5): Collar 40%, ring-bark 20% of trees with continuous canopy to feed trees, ring-bark non-collared trees (DBH 100-300mm), and conduct two population surveys.
- Phase 3 (Wks 6-7): Collar a further 40% of trees, continue ring-barking non-collared trees (DBH 100-300mm), and conduct two population surveys.
- Phase 4 (Wks 8-10): Collar the remaining 20% of trees, finalise ring-barking, and conduct two population surveys.
- Phase 5 (Wks 11-17): Following clearing of the hotspot site undertake eight population surveys.

Study Sites and Sample Collection

There were two study areas as part of this project: Laws Point, which is located approximately five kilometres south of the town of Wardell NSW, and Wardell Rd, which is located east of Wardell (figure 1a). These sites were the impacted sites where phased resource reduction was to occur as part of the highway upgrade. In order to minimize the potential influence of other factors such as weather, samples were collected from the two impact sites at Laws Pt and Wardell Rd, and each of these sites had a control site located in similar habitat, with similar koala numbers and within the same climatic region as the impact sites. The two control sites were Tucki Tucki (Laws Point control) and Hazlemount Lane (Wardell Rd control) (figure 1a). Samples were collected across the five phases of PRR across May to November 2017 (figure 1b).

A



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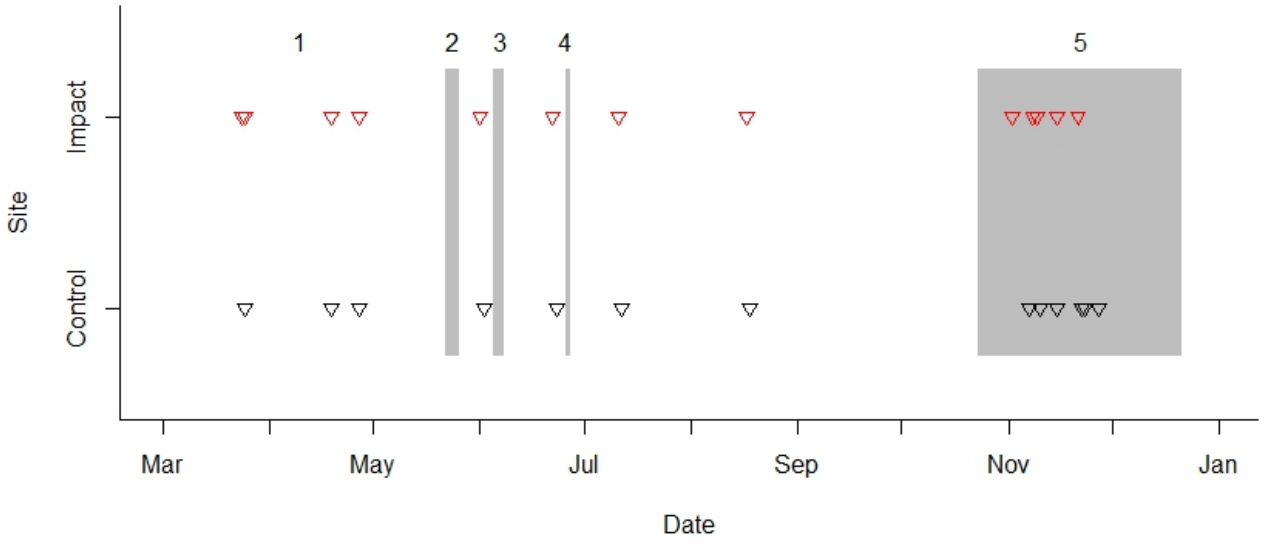


Figure 1: A) Locations of the impact and control sites for Laws Point and Wardell Road. A: Laws Point (impact), B: Tucki Tucki (control), C: Wardell Road (impact), D: Hazlemount Lane (control). Image provided by Sandpiper Ecological. **B)** Sample collection dates (open triangles) for all sites across all phases (multiple points may be overlaid for samples collected on the same day); grey bars indicate the timing of the tree collaring and/or ring-barking (Phase 2-4) and clearing (Phase 5).

As part of the koala monitoring, transect surveys were conducted where nocturnal surveys preceded diurnal surveys (Sandpiper Ecological 2018b, a). Scat samples were collected following each diurnal survey. Fresh scats were identified by their colour (paler green) and presence of moist coating. At the impact sites (Laws Pt or Wardell Rd), each tree containing a koala, or where a koala

was recorded the previous night, was revisited and a search conducted for fresh koala scats. The control sites (Tucki Tucki and Hazlemount Lane) were visited in the afternoon following the diurnal survey at the impact sites and trees containing koalas were marked. These trees were visited the following morning and scats collected. Scats taken from underneath a tree were assumed to have been produced by that koala, although koalas were not individually tagged for identification. The same number of scats from the same number of koalas, and possibly the same sex ratio, were collected between the impacted sites and the control sites. This led to the same sample sizes between impact and control sites for each phase, but sample sizes differed between phases. Scats were collected using a toothpick and placed in a Styrofoam block in a plastic container. Scats were stored in a cool dry location, open to the air, for a period of 2-3 weeks (Wedrowicz et al. 2013) before transport to the lab where they were stored at -20^{o}C before extraction and analysis. Data recorded at each scat collection site included location (easting & northing GDA 94), tree species, weather (temperature, cloud cover, rainfall), tree size, koala health and sex estimates, and observer (Sandpiper Ecological 2018b, a).

Faecal cortisol metabolite extraction and analysis

Koala FCM were extracted and analysed following the methods described in Davies et al. (2013a). Briefly, faecal pellet samples were oven dried and crushed through a fine sieve. For each sample, 200 mg of dry faecal powder was suspended in 2 ml of borate buffer (pH 6.5; 0.1 M) and incubated with ß-glucuronidase (50 µl containing 4000 units) for 4 hours at 37˚C to liberate free (unconjugated) cortisol (Möstl and Palme 2002). Following the addition of diethyl ether (3 ml), liquid nitrogen was used to freeze the lower aqueous phase and enable collection of the supernatant. The supernatant was evaporated to dryness and the extracted hormone was then re-dissolved in 200 µl of diluted (1:10) zero cortisol calibration solution and incubated for 1 hour at 37˚C. FCM concentrations of each sample were measured using the Demeditec Saliva Free Cortisol Kit (Demeditec DES6611, Demeditec Diagnostics GmbH, Kiel, Germany), following the manufacturer's instructions. Samples were run in duplicate. The specificity of the assay was as follows: 100% cortisol, 5.2% corticosterone; 0.2% cortisone; 10.4% 11-deoxycortisol; 0.4% 11-deoxycorticosterone and 63.4% prednisolone; with all other steroids of a similar structure having a specificity of <0.10%. Intra- and inter-assay variation was determined from internal controls included in the assay. Intra-assay variation was 7.1% coefficient of variation (CV), while inter-assay variation was 12.1% CV. The analytical sensitivity of the assay was 0.024 ng/ml. Serial dilutions of faecal extracts run against the assay kit calibrator standards demonstrated good parallelism of the assay. A four-parameter logistic fit was employed to analyse assay data using MyAssays Analysis Software Solutions (www.myassays.com) and quantify cortisol concentrations in ng/g.

DNA extraction and analysis

We trialled four methods of DNA extraction from koala scats to determine which methodology would enable sufficient concentration and quality of DNA from scats collected in the field, as most of the optimized protocols in the literature are based on fresh scats from captive animals (Wedrowicz et al. 2013). The proposed DNA extraction protocol (1) Qiagen QIAamp DNA stool mini kit following the optimisations described in Wedrowicz et al. [2013]) did not produce viable results for genotyping. We therefore trialled koala DNA extraction from scat using three further methods: 2) Bioline isolate II Genomic DNA kit following the standard protocol; 3) Qiagen QIAamp DNA stool mini kit following the "Isolation of DNA from stool for human DNA analysis" protocol; and 4) Qiagen QIAamp DNA stool mini kit following optimisations described in Schultz et al. (2018). Following extraction, microsatellite analyses were performed to genotype koalas and determine whether the same koalas were sampled over consecutive phases. We targeted thirteen microsatellites using primers from a previous study (Dennison et al. 2017), split into 3 multiplexes using a fluorescently labelled (6-FAM) CAG-tag (Schable et al. 2002) following Brandies et al. (2017). This genotyping assay has been performed using DNA extracted from koala tissue samples with high quality results (Brandies et al. 2017).

PCRs were carried out using Qiagen Type-it Microsatellite PCR Kit with a modified total reaction size of 10 µl and the following modified primer concentrations: 0.06 µM tagged primer, 0.6 µM untagged primer and 0.6 µM 6-FAM labelled CAG tag. Thermocycling conditions followed a protocol of 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 60°C for 90 s and 72°C for 30 s with a final extension of 60°C for 30 min. Capillary electrophoresis was undertaken at the Australian Genome Research Facility (AGRF) using MCLAB DSMO-100 Orange Size Standard. Alleles were manually called using GeneMarker v2.7 (Hulce et al. 2011). Controls included a negative control whereby water instead of DNA was added to the PCR reaction, and a positive control using DNA from a koala that was successfully genotyped in a previous study (Brandies et al. 2017).

Despite trialling four different methods of DNA extraction, genotyping success rates were extremely low: 46.7% for the Bioline kit and 7.7% (overall) for the Qiagen kit (individual genotyping success rates were 1.4% for method 1, 16.7% for method 3 and 11.1% for method 4). DNA analysis of the scat samples used in this study was therefore unable to be performed with sufficient precision to identify individuals.

Dataset overview

A total of 83 samples were collected from Laws Pt with equal numbers at control and impact samples for each phase, but varying numbers of samples among phases (figure 2a). A total of 20 samples were collected from Wardell Rd, with equal numbers of samples from control and impact sites (figure 2b). Sample sizes at Wardell Rd were much smaller, precluding detailed statistical analysis; we therefore focussed our statistical analysis on the samples from Laws Pt and its control site at Tucki Tucki. Because identification of koalas using DNA from scats was not achievable, all samples were analysed together to represent the koala populations. We note that some replicate samples from individuals may therefore be included in the analysis.

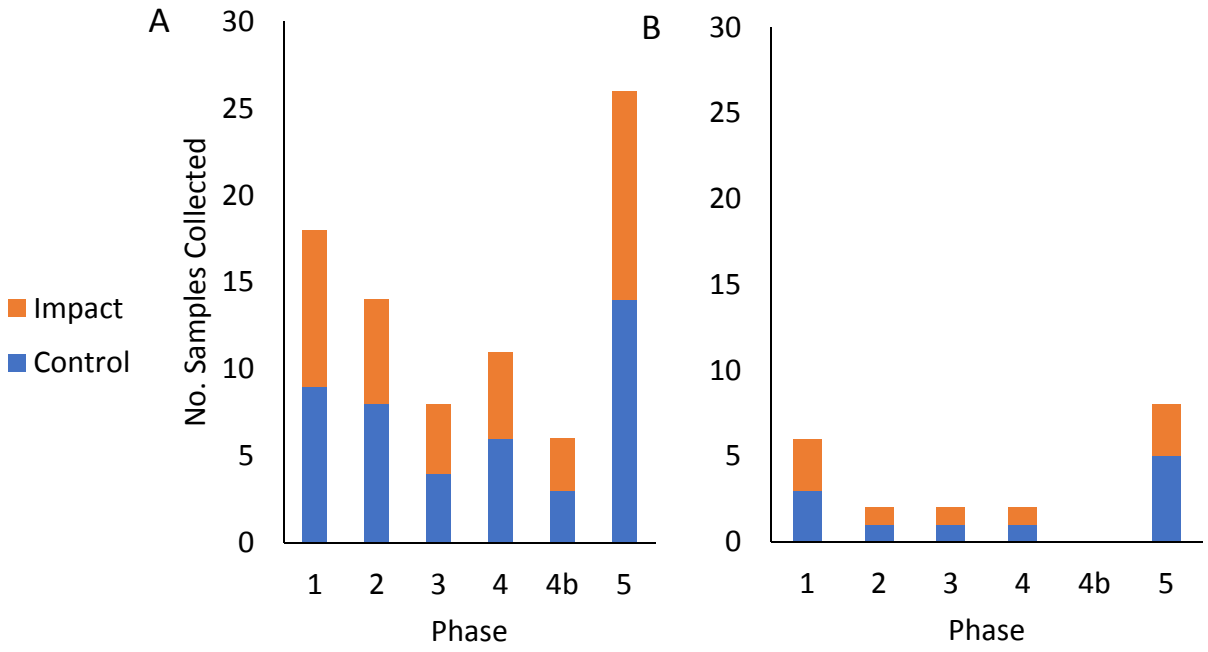
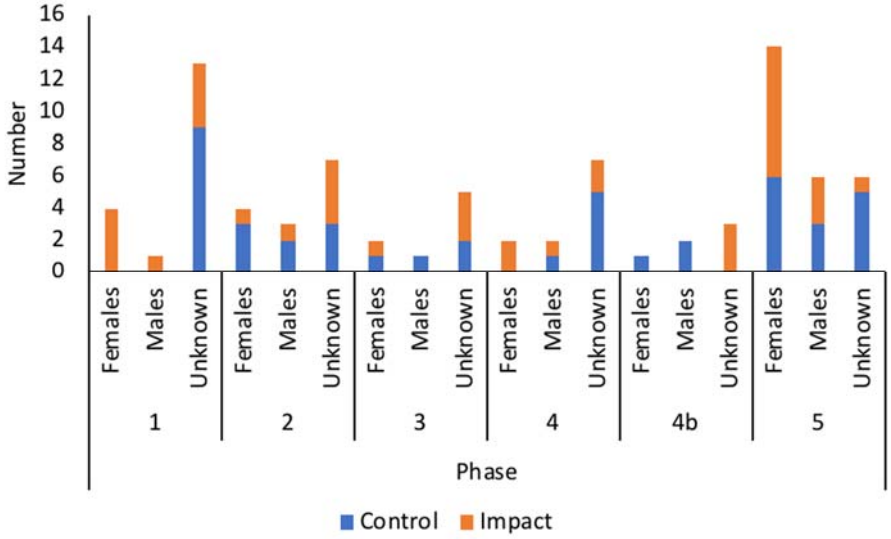


Figure 2. Number of faecal samples collected from the control and impact sites at Laws Pt (A) and Wardell Rd (B) across each phase.

Field teams provided details about the individual koalas from which scats were collected, including estimates of sex and health status. Due to difficulties in sexing koalas at all locations, a comparison of FCM concentrations between the sexes was not undertaken. Sex was allocated for only 52% of scat samples collected at Laws Pt (impact & control sites; figure 3a) and 20% of samples from Wardell Rd (impact & control sites). The health status of animals was determined by cleanliness of the rump and clarity of the eyes. Health status was assigned for 96% of samples at Laws Pt (impact & control sites; figure 3b). At Wardell Rd, 90% of samples had a health status assigned with 50% of these (9/18) listed as healthy versus unhealthy across the entire study. Because of this, health status was included as a predictor in our model.

A)



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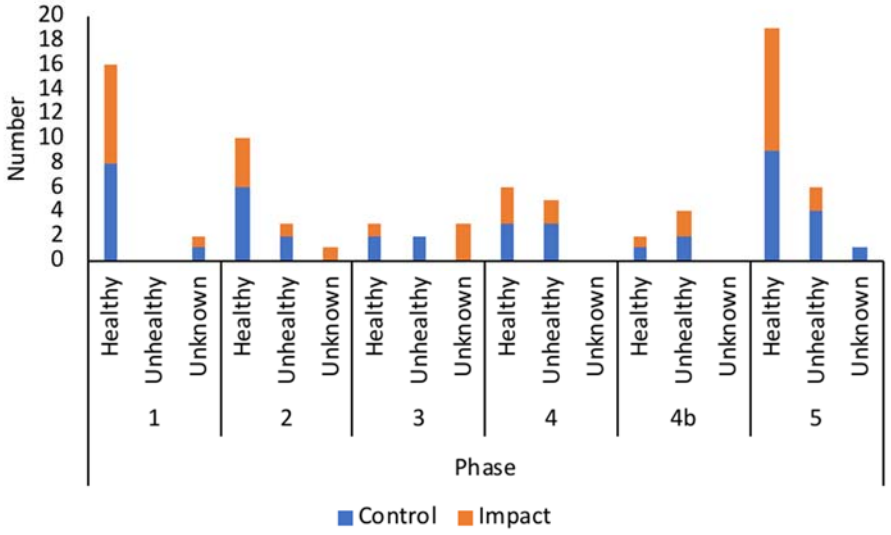

Figure 3. Comparison of individual variables, sex (A) and health status (B), across Laws Pt impact site (orange) and Tucki Tucki control site (blue) each phase of the program.

Weather data were incorporated into the model, as these may affect FCM concentrations (Davies et al. 2013b). Weather data were obtained from the Australian Bureau of Meteorology website (www.bom.gov.au). The closest weather station to all impact and control sites was Ballina Airport (located 15-20 km from all impact and control sites), which recorded daily rainfall (mm), minimum and maximum temperatures (^{o}C) and solar exposure (kWh m^{-2}). It is estimated that the delay time of FCM appearance in koala faeces is 36 hours (Davies et al. 2013a). Daily weather observations were averaged across the three days prior to each scat sample collection to account for weather patterns that may have influenced FCM concentrations. Weather variables are typically highly correlated with one another, so we used principal components analysis (PCA) to reduce the number

of weather variables included in the model. We used the first two principal components (PC), which together explain 79.3% of the variation in the weather data.

Statistical analysis

It is possible that cortisol levels may change across phases at both control and impact sites (for example, in response to an unmeasured environmental factor). To determine whether the PRR impacted koala faecal cortisol concentrations, we therefore tested whether the effect of phase differed between impact (Laws Pt) and control (Tucki Tucki) sites by fitting an interaction in a linear regression. The main effects in the model were site type (a binary predictor, 0/1 for control/impact) and phase (continuous predictor with each phase [1, 2, 3, 4, 4b, 5] coded as values 1 – 6). In this analysis, we would interpret a statistically significant (at $\alpha = 0.05$) interaction between site type and phase (labelled as Type×Phase in the model output) as evidence that the effect of PRR on faecal cortisol differs between the impact and control sites. If the interaction is not significant, then we cannot reject the null hypothesis, and would infer that there is no evidence of a difference in the effect of phase timing on FCM across the impact and control sites. We also fitted health status (healthy, Y = 1, N = 0), because healthy animals may have different FCM concentrations from unhealthy animals (McKenzie and Deane 2005), and the first two weather PCs (labelled as w.PC1 and w.PC2). Our response variable, FCM measurement, was log transformed for analysis (Davies et al. 2013a). Models were fitted using R (R Core Team 2017).

It is also plausible that the sex of individual koalas may impact FCM concentrations, as previous studies have found sex related differences in koala FCM concentrations (Narayan et al. 2013). However, because the sex data was not definitive for our sample set, we did not fit this variable in our model.

Results

The mean FCM concentration recorded at Laws Pt across Phases 1-5 was 39.7 ± 38.8 ng/g (N = 38 samples) for the impact site and 38.5 ± 29.1 ng/g (N = 44 samples) for the control site (Table 1). At Wardell Rd, the mean FCM measurement across Phases 1-5 was 56.5 ± 52.5 ng/g (N = 9 samples) for the impact site and 42.3 ± 23.6 ng/g (N = 11 samples) for the control site.

Table 1. Summary of Laws Pt FCM data including the sample size (N), range, average and standard deviation of FCM concentrations (ng/g) for each phase.

	Impact (Laws Pt)			**Control (Tucki Tucki)**		
Phase	**Average (ng/g)**	**Range (ng/g)**	**N**	**Average (ng/g)**	**Range (ng/g)**	**N**
1 (Mar-Apr)	35.8 ± 18.5	18.3 – 77.0	9	24.1 ± 22.8	7.4 - 82.7	9
2 (Early June)	12.6 ± 4.0	6.9 - 18.7	6	28.2 ± 19.5	9.9 – 74.0	8
3 (Late June)	10.9 ± 3.15	7.9 – 14.9	4	29.2 ± 7.3	19.3 – 36.0	4
4 (July)	45.8 ± 42.4	18.9 – 119.8	5	33.8 ± 25.4	6.5 – 73.7	6
4b (Aug)	63.0 ± 85.2	12.7 – 161.4	3	46.1 ± 34.4	26.2 – 85.9	3
5 (Nov)	59.9 ± 41.2	3.7 – 131.2	11	56.6 ± 34.9	14.7 – 135.0	14
Overall	39.7 ± 38.8	3.7 – 161.4	38	38.5 ± 29.1	6.5 – 135.0	44

There was an upward trend in FCM concentrations at both Laws Pt (impact) and Tucki Tucki (control) across the phases (figure 4). The driver of this change over time could not be determined. After controlling for weather and health status, our data did not detect any statistically significant difference in this trend between the impact and control sites (the interaction term Type×Phase is not statistically significant; i.e. there is no evidence that there is a difference in the trend, Table 2). However, as Phase is statistically significant (Table 2) there is a gradual increase in FCM concentrations across the Phases, but this occurs across both the impact and control sites (figure 4).

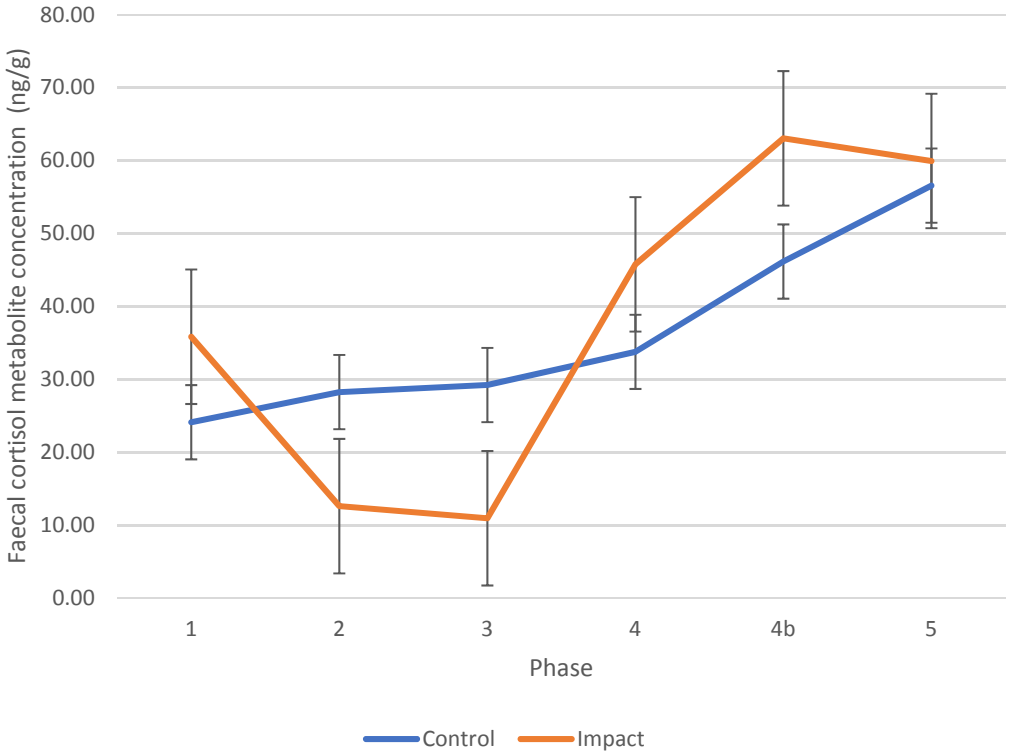

Figure 4. Mean FCM concentrations (ng/g ± SE) of koalas from the impact (orange, N = 38 samples) and control (blue, N = 44 samples) sites at Laws Pt across each of the 6 phases. Phase 1: Pre-phase reduction, phase 2: 40% of trees collared, phase 3: 80% of trees collared, phase 4: 100% of trees collared, phase 4b: post-phase reduction, phase 5: clearing.

Table 2. Linear modelling of changes in koala FCM concentrations (log-transformed); N = 78 (samples from 4 individuals excluded due to missing health data).

Predictor	Estimate	Std error	t statistic	p value
(Intercept)	2.799	0.330	8.491	0.000
Type[1]	0.029	0.360	0.082	0.935
Phase[2]	0.174	0.067	2.597	0.011
w.PC1[3]	-0.078	0.068	-1.153	0.253
w.PC2[3]	0.103	0.080	1.294	0.200
Healthy[4]	0.036	0.201	0.177	0.860
Type×Phase[5]	-0.047	0.087	-0.542	0.589

[1] The control site was the reference category

[2] Fitted as 1:6 for the phases as indicated on x-axis of figure 4.

[3] Weather principal components w.PC1 and w.PC2 together explain 79.3% of the variation in 3-day average rainfall, maximum temperature, minimum temperature and sun exposure.

[4] Healthy shows the effect of healthy animals relative to unhealthy animals

[5] The interaction is the parameter of interest, as this variable shows whether the trend in FCM over the phases differs between the two site types (i.e. differs between the impact and control sites)

All sites sampled at Laws Pt, Tucki Tucki, Wardell Rd and Hazlemount Lane had some koalas that exhibited signs of ill health. There was no trend in the FCM concentrations between healthy and unhealthy koalas (figure 5; Table 2). That is, some individuals that had obvious signs of ill health, i.e. cloudy eyes and a brown wet rump, did not have higher FCM concentrations on average than those individuals recorded as healthy. It should be noted however, that some of the highest FCM concentrations observed in this study were found in koalas that were observed to be unhealthy (figure 5).

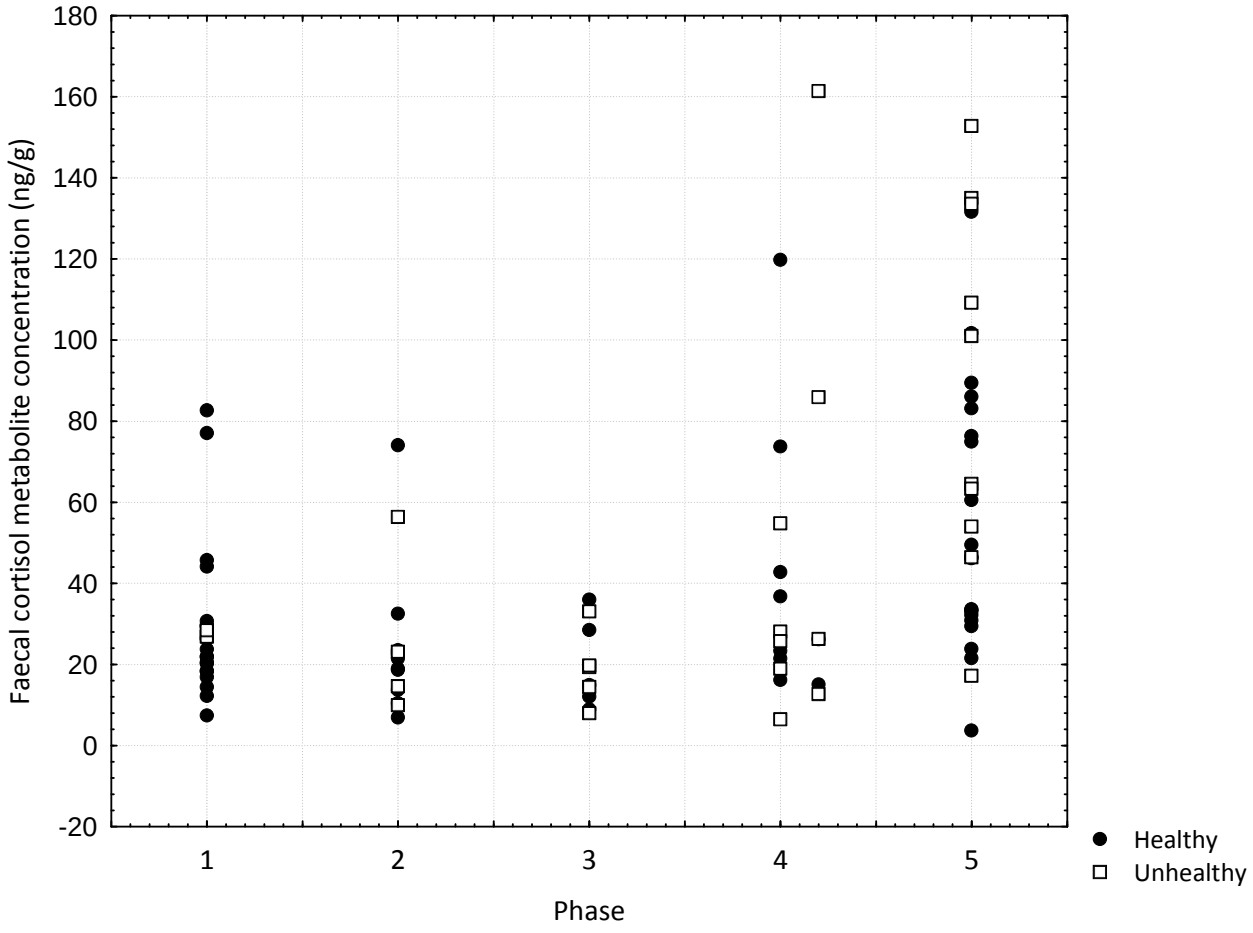

Figure 5: Scatterplot of FCM concentrations across all sites (two impact and two controls) for koalas recorded as healthy and unhealthy across all Phases.

Discussion

The Koala Management Plan for the Woolgoolga to Ballina (W2B) Pacific Highway Upgrade recommended phased resource reduction at the koala 'hotspots' as a "way of reducing any stress-induced impacts on koalas associated with clearing activities" (NSW-RMS 2016). A number of different studies have shown an association between FCM concentrations and an increase in ACTH in koalas (Davies et al. 2013a, Narayan et al. 2013). In this study, FCM concentrations in koala scat did not show a significant difference between the impact and control sites across the different phases of the project. There was however, an upward trend in FCM concentrations at both the impact and control sites across time (figure 4). We were unable to identify the drivers of this upward trend but they could be environmental factors (Davies et al. 2013b) or biological ones, such as breeding season (Millspaugh and Washburn 2004), sex of individuals (Narayan et al. 2013), or a combination of these.

Unfortunately, the published DNA extraction methods used in this study (Wedrowicz et al. 2013, Schultz et al. 2018) did not yield suitable DNA from the koala scats for genotyping in triplicate. Due to the low DNA yield for these samples, our genotyping assay had unacceptable levels of error, so we were unable to identify individuals as part of this study. The koala monitoring surveys undertaken across all phases report approximately 8 individuals in the Laws Pt area (Sandpiper Ecological 2018b). It should be noted that a proportion of scars from Laws Point were from the east site of the study area, well away from the PRR. Three individuals were removed from the primary impact study site between phases 4 and 5 for health reasons (Sandpiper Ecological 2018b). Two of these individuals relied on tress inside the PRR site. Work is ongoing to determine the stability of DNA from koala scat samples that are collected for dual purpose for non-invasive analyses such as genotyping and hormone analysis. The results of the DNA stability trials will be provided to NSW RMS to permit a more robust sampling design into the future if monitoring samples are to be used for both genotyping and cortisol analysis.

Other studies have shown there to be a difference in FCM concentrations between male and female koalas (Narayan et al. 2013). The range of FCM concentrations in this study (Laws Pt, 38.5 ± 29.1 ng/g, N = 44 samples; Tucki Tucki, 39.7 ± 38.8 ng/g, N = 38 samples) were slightly higher than those documented by Davies et al. (2013a); 29.74 ± 6.99 ng/g, N = 5 koalas (4 males; 1 female; all captive) using the same cortisol assay. The concentrations reported here, and in Davies et al. (2013a), differ from other reported concentrations using different assay methods. Other studies have reported FCM concentrations of 72.8 ± 55.0 ng/g, N = 5 koalas (1 male; 4 females; all captive) (Johnston et al. 2013) and 1.64 – 46.35 ng/g, N = 39 koalas (19 male [10 captive; 9 wild]; 20 female [13 captive; 7

wild]) (Narayan et al. 2013). However, FCM concentrations in this study are not outside the range observed for captive koalas. Our results are difficult to interpret relative to other wild samples (Narayan et al. 2013), because we used a method which employed enzymatic cleavage prior to solvent partitioning, and as a result measured concentrations of extracted cortisol rather than metabolites (Davies et al. 2013a). We chose this method, which uses a commercial cortisol assay kit (Davies et al. 2013a), to ensure our results would be comparable with future studies.

Johnston et al. (2013) have raised concerns that koala scat samples do not reflect the acute stress response in koalas, although faecal cortisol has been used to ascertain chronic stress responses in other species in the wild, such as European starlings (*Sturnus vulgaris*) (Cyr and Romero 2007) and cheetah (*Acinonyx jubatus*) (Terio et al. 2004). There is no one-size-fits-all approach to non-invasive monitoring (Fanson et al. 2017), but as two other studies have shown a FCM response to an ACTH challenge in koala scat samples (Davies et al. 2013a, Narayan et al. 2013), we feel that the method employed here was suitable for monitoring a long-term, chronic change as a result of phased resource reduction (if any). It should be noted that the koala monitoring surveys were undertaken a week, or more, after the activity (collaring and ring-barking; figure 1b). As the time lag for cortisol, and its metabolites, in koala scat has been shown to be up to 36 hours (Davies et al. 2013a), the koala scat samples collected as part of this study are not suitable for assessing an acute stress response, as acute stress responses may have subsided by the time samples were collected.

As the individual identity of each koala producing the scat samples could not be determined, all samples were grouped together for analysis. Although individual identity was not determined at either the impact or control sites, sample sizes for all sites (Laws Pt, Tuck Tucki, Wardell Rd and Hazlemount Lane) were still low throughout this study (N = 6 to 16 individuals at each site, figure 2). Across all koala monitoring surveys it is estimated that 16 individuals were residing within the Laws Pt study area and four individuals at the Wardell Rd study area (SandpiperEcological 2018b, a). Thus, it is likely that some of the scat samples included in our analysis are from replicate individuals, although we would need genetic data to confirm whether this impacts our statistical analysis. For example, we expect individual sex to impact FCM concentrations (Millspaugh and Washburn 2004), but because we could not individualise the scat samples, nor confirm sex from field observations, we are unable to determine whether there are sex ratio differences between study populations, nor how sex ratio differences might impact our interpretation of the effect of the PRR or differences between the impact/control sites. Nevertheless, from the field reports from Sandpiper Ecological we expect that the sex ratio of impact and control sites to be similar.

Conclusions & Recommendations

In conclusion, the results of this analysis show there to be no difference in FCM concentrations in koala scat samples collected during phased resource reduction at Laws Pt, relative to the control site. Due to small sample sizes at Wardell Rd, we were unable to undertake any statistical analyses for that population.

1. Due to low DNA yield from koala scat samples collected in the field, individual identification of koalas was not possible and so all findings are at the population level. Investigation into developing a robust field DNA protocol is ongoing at this time.
2. There was no statistically significant difference in the change in FCM concentrations between koalas at Laws Pt and Tucki Tucki.
3. An upward trend in FCM concentrations was noted across time at both the impact and control sites (figure 4). Drivers of this trend could not be determined.
4. Ongoing monitoring of the koala populations at both the impact and control sites will ascertain if any change occurs as a result of the construction phase.
5. Future sample collection should be undertaken using established collection protocols used in this study and the literature. The protocol used here should be modified so scat samples are collected on toothpicks in a container during collection and then immediately placed in a -20^{o}C freezer once returning from the field day. Samples should be shipped frozen to the lab. Stability experiments are required to ascertain if long-term -20^{o}C storage is problematic for both cortisol and DNA extracts from koala scat samples. In the absence of the stability experiments, cortisol and DNA from scats should be extracted as soon as possible and extracts stored at -20^{o}C before analysis.

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