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Zoo animals

Case Study: A Dexamethasone Suppression Test in Koalas (*Phascolarctos cinereus*)

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ABSTRACT

The hypothalamic–pituitary–adrenal axis responds to stress by releasing the adrenocorticotropic hormone which, in turn, stimulates the release of glucocorticoids (GCs). During acute stress events, the GCs' function is to maintain homeostasis. Short-term stress events trigger psychophysiological responses which are fundamental for survival in the natural world. Chronic stress occurs when the effect of stressors persists, and GCs secretion continues. The increase of GCs interacts with receptors in the brain triggering a negative feedback loop which inhibits the secretion of ACTH, consequently down-regulating GC production. Cortisol is the main GC in most mammals, including koalas. Cortisol is metabolised by the liver and bacterial enzymes in the intestine, and its metabolites are excreted via the faeces. In a previous study, where we used the tetrahydrocorticosterone) enzyme immunoassay (aka 50c EIA) for measuring faecal cortisol metabolites (FCMs) to assess stress in koalas, we did not detect a decrease in FCM values (negative feedback) after administration of prednisolone, an exogenous GC. Using the dexamethasone suppression test (DST), this study aimed at determining the ability of the feedback loop to decrease the concentration of plasma cortisol. This was achieved by measuring the values of plasma cortisol as well as FCMs using the 50c EIA in four koalas. No cortisol suppression was observed, rather an increase in plasma cortisol concentration in all koalas. This was also reflected in the increase of FCMs. An unresponsive feedback loop, and consequent prolonged high levels of plasma cortisol, is likely to increase koalas' susceptibility to diseases and it may impact their coping mechanism in nature.

1 | Introduction

The hypothalamic–pituitary–adrenal (HPA) axis responds to stress by releasing adrenocorticotropic hormone (ACTH) from the anterior pituitary gland which, in turn, stimulates the release of glucocorticoids (GCs), such as cortisol and corticosterone, produced by the adrenal cortex (Chu et al. 2024). During acute stress events, the GCs' function is to maintain homeostasis through a variety of metabolic processes, for example the metabolism of glucose, fat and proteins (Chourpiliadis and Aeddula 2023; Guilliams and Edwards 2010). Stress can either enhance or suppress the action of the immune system. Short-term stress events trigger psychophysiological responses which are fundamental for survival in the natural world (e.g., predator–prey interactions, dominance challenges and response to adverse weather events) (Dhabhar 2009; Romero 2004) and involve the redistribution of leukocytes to areas of the body where the immune response is needed for the organism to repair wounds or fight infections (Dhabhar 2009). Chronic stress, however, occurs when the effect of stressors persists, and the GC secretion continues. In human and animal

studies, the increased, constant output of GC, consequential to psychological, physiological, physical and/or environmental chronic stress, elicits a dysfunctional HPA response which can lead to a decline in overall health and exacerbate pre-existing conditions (Dhabhar 2014; Padgett and Glaser 2003). However, there are contradicting arguments on the effect of chronic stress on health in wildlife. As reported in a review by Boonstra (2013) one argument is that wildlife is not affected by chronic stress because if it were, it would increase the risk of diseases and be fatal for the individual and thereby impact species survival. The author concludes that chronic stress does not adversely affect wildlife health, as wildlife has evolved mechanisms to cope with chronic stress through adaptative response to their environment, which needs to be confirmed with well-designed field experiments (Boonstra 2013).

The increase of GCs interacts with receptors in the brain triggering a negative feedback loop which inhibits the secretion of ACTH, consequently down-regulating cortisol or corticosterone production. However, chronic stress increases the overall GCs response which can cause a deficit in negative feedback and a prolonged GC elevation (Romero 2004). For example, studies on chronically stressed subordinate baboons have shown that the effect of reduced negative feedback, and consequent elevated GC for long periods, was the cause of cardiovascular diseases (Sapolsky 1994 in Romero 2004). Assessing negative feedback using a synthetic GC is important to determine the physiological coping mechanism of animals in the natural environment as lack of suppression (return to baseline GC levels) may impact the response to acute stress (Romero 2004).

Cortisol is the main GC in most mammals, including koalas (Davies et al. 2013; Raff 2016; Santamaria, Barlow, et al. 2021). Cortisol is metabolised by the liver and bacterial enzymes in the intestine, and its metabolites are excreted via the faeces (Palme et al. 2005), hence, free cortisol is not found in faeces (Möstl et al. 2005).

Koalas are exposed to a broad range of stressors at both the individual and the population level (Department of Environment and Science 2023). The continuous decline in koala numbers is directly linked to anthropogenic actions related to loss or fragmentation of habitat and growing urbanisation (Gonzalez-Astudillo et al. 2017). In February 2022, koalas (*Phascolarctos cinereus*) were listed as *endangered* in Queensland, New South Wales (NSW) and the Australian Capital Territory under the Environment Protection and Biodiversity Conservation Act 1999 (EPBC Act) (Department of Climate Change, 2022).

Throughout our research, a tetrahydrocorticosterone (aka 50c) enzyme immunoassay (EIA) has been analytically, physiologically and biologically validated for measuring faecal cortisol metabolites (FCMs) and thereby stress in koalas (Santamaria, Barlow, et al. 2021; Santamaria, Palme, et al. 2021, Santamaria, Schlagloth, et al. 2021; Santamaria et al. 2023). Due to its cross-reactions, this EIA measures tetrahydrocortisol (THF), the main cortisol metabolite found in koala faeces (Santamaria, Barlow, et al. 2021). Previous studies (Davies et al. 2013; Narayan et al. 2013) have used an ACTH challenge to validate the use of FCMs to detect changes in plasma cortisol. However, it is also essential to use a dexamethasone suppression test (DST) to assess if the suppression of endogenous GC in plasma is reflected in the FCM values (Davies et al. 2013; Touma and Palme 2005).

Dexamethasone is a synthetic GC commonly used in the treatment of inflammation and diseases related to immune system dysfunction (Papich 2021). It is also used in evaluating the function of the feedback loop. Dexamethasone is used to assess the suppression of ACTH secretion by the HPA axis as it mimics the increase of endogenous GCs in the bloodstream. It is primarily used to diagnose hyperadrenocorticism, also known as Cushing's syndrome (Papich 2021) by identifying a failure by the dexamethasone to suppress ACTH production in affected individuals (Dogra and Vijayashankar 2024).

In our previous research on hospitalised wild diseased and /or injured koalas (Santamaria et al. 2023), the 50c EIA did not detect a decrease in FCM values (sign of negative feedback) after the therapeutic administration of prednisolone (an exogenous GC). We hypothesised that this could be a result of a lack of the negative feedback loop in this species, due to the low therapeutical dose (0.5 mg/kg) of administered prednisolone, or due to the effect of stress caused by pain and hospitalisation.

Here, we aimed to determine the ability of the feedback loop in inhibiting the release of ACTH by measuring the values of plasma cortisol in koalas using the DST. Cortisol metabolite values in the faeces were also measured using the 50c EIA for a period before and after DST.

2 | Methods

2.1 | Koalas

Between October and November 2023, a DST was carried out on four clinically healthy captive koalas (two females and two males) housed at Australia Zoo in Beerwah, Queensland, At the time of the study Marley (M) was 9 year old, Tala (F) 6 year old, Rove (M) 6 year old and Hazel (F) 3 year old. The koalas were taken from their enclosure at the zoo by their usual keeper and transported with a crate to the Australia Zoo Veterinary Department (located within the same facility) where the DST was performed. Zoo koalas are used to being health checked and are handled daily by their keepers.

2.2 | Dexamethasone Suppression Test

In most cases, DSTs, especially in domestic animals, but also in most wildlife species, are carried out without prior anaesthetic (Dickens et al. 2009; Eleftheriou et al. 2020). However, anaesthesia can be used to avoid stress during the procedure (Sapolsky and Altmann 1991).

All veterinary procedures were performed by the veterinarians at the Australia Zoo Veterinary Department.

General anaesthesia for up to 10 min was induced and maintained with isoflurane (1.5%) in oxygen (2 L/min) via facemask while an intravenous (IV) catheter was aseptically inserted into the cephalic vein and secured with adhesive strips (Leukoplast) and

TABLE 1 | Time of blood sampling (t0h), dexamethasone intravenous (IV) and weight of the four koalas.

Koala	Date	Time of blood collection at t0h	Time of dexamethasone IV administration	Koala weight (kg)
Hazel	30 October 2023	08:02 AM	08:05 AM	5.09
Marley	30 October 2023	08:16 AM	08:19 AM	8.47
Rove	2 November 2023	08:25 AM	08:27 AM	6.89
Tala	2 November 2023	08:46 AM	08:52 AM	4.96

the insertion site was covered with a non-adherent cohesive dressing (Vet Wrap). While koalas were anaesthetised, through the catheter, a blood sample (5 mL) was collected at t0h, followed by the IV administration of 0.1 mg/kg of dexamethasone (dexamethasone sodium phosphate 4.4 mg total dose—equivalent to dexamethasone phosphate 4 mg) for the DST (Papich 2021) (Table 1). Straight after the administration of dexamethasone, koalas were awakened by turning off the isoflurane flow and maintaining the flow of oxygen.

In domestic animals, where this procedure is routinely performed, the suppression test requires intravenously injecting dexamethasone and collecting blood at 0, 4 and 8 h post-injection (Papich 2021). However, due to the novelty of this study on koalas, the blood collection regime for the first male (Marley) and female (Hazel) koala was slightly modified to four blood samples (5 mL) taken at four timepoints (t0h, t3h, t6h, t8h). After the results of the first two koalas were received on the same day of the procedure, we noticed that there was an increase of plasma cortisol at t3h and no suppression at the time of the last collection (t8h). Hence, to ensure that we could detect any suppression in the subsequent female and male, we obtained an extra blood sample at t9.5 h. Thus, five blood samples were drawn from Tala and Rove.

During the day trial, while the IV catheter was in place and the blood samples were collected, the koalas were housed in separate enclosures at the Australia Zoo Veterinary Department to facilitate regular monitoring by veterinarians and nurses (Figure 1).

The IV catheter was removed after the last blood sample was collected, and haemostasis was achieved using a pressure bandage (gauze swabs and cohesive dressing) applied for 10–15 min. The catheterisation site was closely inspected after the dressing was removed to ensure complete haemostasis. This procedure was chosen (versus repeat conscious venipunctures for each blood sampling point) to minimise handling of the koalas (thereby reducing stress) and to minimise phlebitis and associated soft tissue swelling and inflammation caused by repeat venipuncture.

After each collection, blood samples were kept refrigerated at 4°C until collected and analysed for plasma cortisol concentrations using the Atellica IM Cortisol (Cor) assay by QML Vetnotics Pathology laboratory (Brisbane, Queensland). This assay is specifically designed for the detection of cortisol and used in DSTs, and cross-reactivity with dexamethasone is ≤ 1%.

FIGURE 1 | Marley in the Australia Zoo Veterinary Department enclosure after the dexamethasone injection (green Vet Wrap, securing IV catheter, can be seen on his left hind leg).

To establish the percentage change in cortisol concentration (suppression or otherwise) the following formula was used.

$$\%\text{change in [cortisol]} = \frac{\text{cortisol before dexamethasone} - \text{cortisol after dexamethasone}}{\text{cortisol before dexamethasone}} \times 100$$

2.3 | Faecal Sample Collection

After collection of the last blood sample, koalas were returned to their regular enclosures with no changes in daily husbandry and feeding routines and in interactions with their keepers.

The koalas were monitored by the researcher and zoo staff to ensure their wellbeing, and fresh faecal samples were collected by the researcher from every defecation event (between 08:00 AM and 4:00 PM) as soon as excreted, on the day before the injection of dexamethasone (baseline), on the same day of the dexamethasone injection and on the day after. A final fresh faecal sample was collected in the morning two days after the injection. No baseline faecal samples were collected for Tala as she replaced a female withdrawn from the trial due to possible early pregnancy from a recent mating event.

2.4 | FCM Extraction and Analyses

The extraction procedure and analysis are described in Santamaria, Palme, et al. (2021). In brief, 500 mg of wet faeces were placed into a 10 mL tube and 5 mL of 80% methanol were added. Samples were shaken for 30 min with an orbital rotator shaker, vortexed for 2 min with a hand vortex and centrifuged at 2500 × *g* for 15 min. Completely dried-down aliquots (0.25 mL) of the extracts in 1 mL Eppendorf tubes were shipped to the University of Veterinary Medicine (Austria), where dried sample extracts were resolubilised in 80% methanol and further diluted with assay buffer (1 + 9). Aliquots were analysed in duplicate with the 50c EIA (for details see Quillfeldt and Möstl 2003 and Santamaria, Barlow, et al. 2021).

3 | Results

3.1 | Dexamethasone Suppression Test

Initial concentrations of plasma cortisol (t0h) were close to 5 ng/mL for three out of four koalas except for Tala which was slightly higher (7 ng/mL). All koalas showed an increase in plasma cortisol concentration at t3h with large variation between koalas (Marley with 508%, Hazel with 208%, Rove with 193% and Tala 5% increase compared to t0h). None of the koalas showed any suppression after the dexamethasone injection (Figure 2). By the end of the trial, plasma cortisol concentrations for all koalas had decreased to baseline (Marley) or below (Hazel, Rove and Tala).

3.2 | Faecal Cortisol Metabolites

The plasma cortisol elevation at t3h was also reflected in the FCM values around 24 h after administration of dexamethasone. The greatest increase in FCM values was observed in Rove's faecal samples (142%) followed by Hazel's (125%), Marley's (49%) and the least increase in Tala's (25%), similar to her lower plasma cortisol increase. No suppression was shown in FCM values within the 24 h from dexamethasone administration (Figure 3).

4 | Discussion

Stress is associated with higher-than-normal plasma cortisol levels, which are also reflected in the faeces with an increased excretion of cortisol metabolites (Cope et al. 2022; Davies et al. 2013; Santamaria, Barlow, et al. 2021).

So far, our research has been mostly non-invasive, using the 50c EIA to detect variations in FCM values as an indicator of stress in diseased and injured wild koalas (Santamaria, Palme, et al. 2021; Santamaria et al. 2023). However, our previous findings in hospitalised wild koalas (Santamaria et al. 2023), where no decrease in metabolites was noticed after treatment with prednisolone, led to this investigation into exploring whether an artificial increase in GCs would trigger the suppression of adrenocortical activity (negative feedback loop). The immunosuppressive action of dexamethasone is six to seven times more potent than that of prednisolone (Papich 2021), which explains its use in assesssing the GC feedback loop 2021).

Due to the conservation status of koalas, any trials that may affect the individuals' wellbeing, whether in the wild or in captivity, need to be designed with the principles of the 3Rs (Replacement, Reduction and Refinement) in mind. As in previous studies (Davies et al. 2013; Narayan et al. 2013; Santamaria, Barlow, et al. 2021) here, we only used a small number of koalas and every precaution was taken to limit their stress. For this reason, the four koalas in this study were anaesthetised to reduce the stress caused by the handling events required for the trial. Isoflurane is a safe gaseous anaesthetic commonly used for a range of animal species (Preckel and Bolten 2005), including wildlife during field studies (Kocer and Powell 2009). It is fast-acting with a rapid anaesthetic recovery time (Flecknell 2023). However, its use to induce and/or maintain anaesthesia has been reported to rapidly elevate the concentration of endogenous GCs in rabbits and in humans undergoing gastric surgery, especially during maintenance of isoflurane anaesthesia for long procedures (González-Gil et al. 2006; Wu et al. 2019).

By performing a DST in koalas, we expected to observe a clear decrease in plasma cortisol as observed in healthy humans, dogs, wild subterranean rodents and male antechinus during the non-breeding season (McDonald et al. 1986; Naughton et al. 2014; Vera et al. 2019; Zeugswetter et al. 2021). In dogs, a decrease in plasma GCs occurs at around 4 h after the injection of dexamethasone and it is maintained at least for 8 h (Hoffrogge et al. 2020; Zeugswetter et al. 2021). Furthermore, a corresponding decrease in FCM values due to the suppressive action of dexamethasone has been observed in roe deer and other ruminant species (Dehnhard et al. 2001; Palme et al. 1999). However, to our surprise, rather than a decrease in plasma cortisol concentration (typical of a suppression test after an injection of dexamethasone in other species), we observed an increase in all four koalas. The magnitude of the increase in Tala was lower than the other three koalas, possibly indicating lower stress levels, but she also did not show suppression. The increase in plasma cortisol was possibly due to a direct effect of the anaesthetic agent, since isoflurane has been associated with increased endogenous GC plasma concentrations in other species. It could also have been caused by the stress of handling during anaesthetic induction, with previous studies showing a plasma cortisol increase in other species 3 min after a stressor (Romero et al. 2008). However, the captive koalas in this trial were accustomed to regular handling and so the effect of this may have been reduced compared with other captive and wild individuals. We could not determine

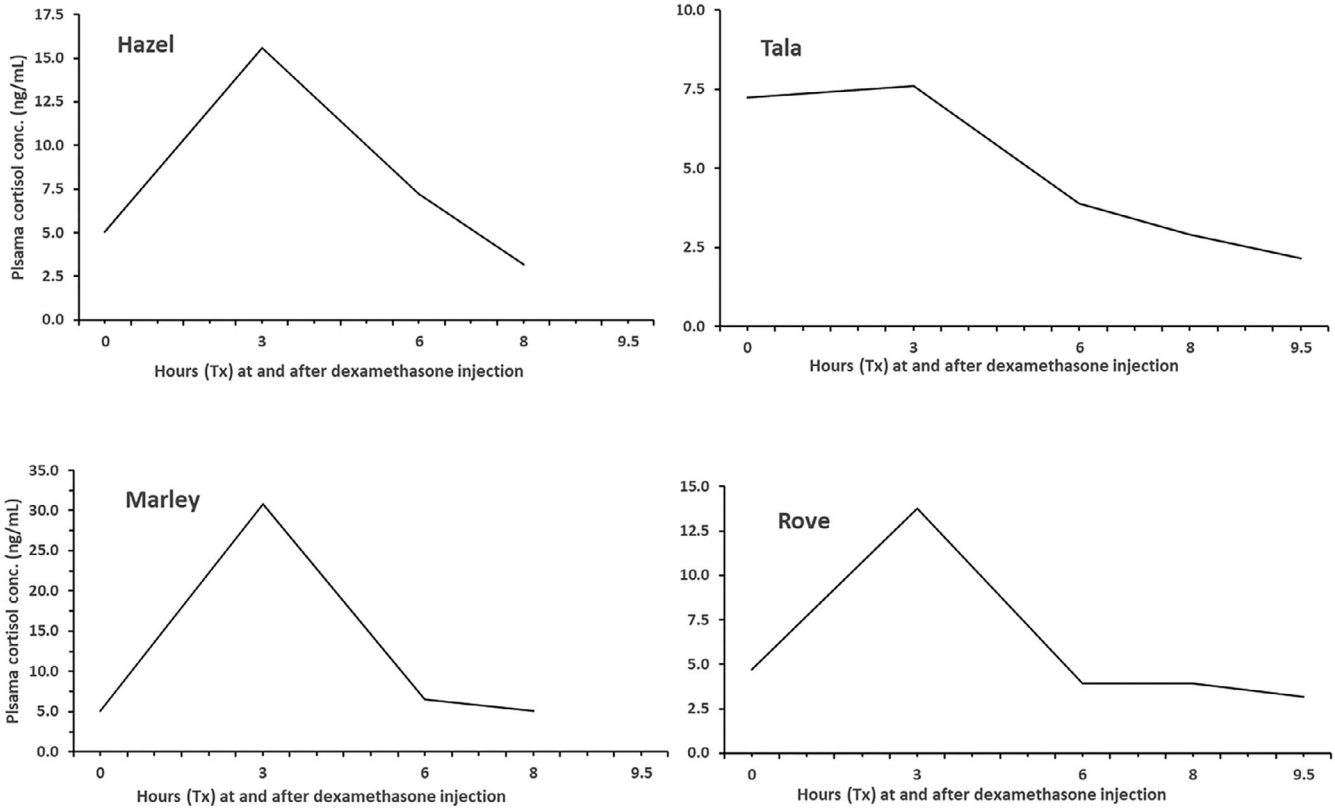

FIGURE 2 | Plasma cortisol concentrations of the four koalas obtained prior to and after dexamethasone suppression test (DST).

exactly when the increase in plasma cortisol started in the koalas in this study, as blood samples were collected at predetermined time points (Papich 2021). We also observed a return to baseline plasma cortisol values, or just below, in all koalas at the end of the trial.

The only baseline plasma cortisol value available before the DST was at t0h, when the first blood sample was collected and after the koalas had already been handled for transport to the hospital, hence, we cannot be sure whether we captured their true baseline cortisol values. It is reasonable to assume that, at the time of collection of the first blood sample, plasma cortisol concentrations may have already been slightly elevated. This also might account for the plasma cortisol values being slightly lower than t0h at the end of the trial for three of the four koalas.

The increase in plasma cortisol at t3h was also reflected in the increase of FCM values after around 21 h and a subsequent return to baseline values. Previous research shows that, accounting for the lag-time due to the transition in the intestinal tract, metabolites of cortisol are directly related to adrenocortical activity (Davies et al. 2013; Palme 2019; Palme and Mostl 1997; Santamaria, Barlow, et al. 2021).

Not all animal species respond to DST, and lack of suppression has been observed in highly stressed translocated chukar (Dickens et al. 2009), in chickens (Dehnhard et al. 2003), in chronically stressed subordinate baboons (Sapolsky 1993) and in breeding male antechinus. In the latter Australian native marsupial, this leads to the death of reproductively mature males after the mating season due to suppression of the immune system (McDonald et al. 1986). Also, seasonal variations in DST responses have been observed in some domestic horses and ponies (Bamford et al. 2020; Borer-Weir et al. 2013). Interestingly, a lack of suppression has also been observed in humans experiencing high levels of stress, while in most individuals suppression occurred straight after a 1 mg dose and continued for 24 h (Naughton et al. 2014).

This study was performed on a small number of animals to align with the ethical principles of the 3Rs. However, if future research confirms these findings regarding the function of the HPA axis in koalas, particularly their inability to suppress endogenous cortisol release during prolonged stress, its persistent elevation during chronic stress events, could significantly impair their immune system. (Padgett and Glaser 2003). It is unknown whether the arguments presented by Boonstra (2013) apply to koalas, but an unresponsive feedback loop with consequent prolonged high levels of plasma cortisol is likely to increase their susceptibility to diseases and it may impact their coping mechanism when facing acute and chronic stress in the wild (Romero 2004), thus hampering conservation efforts (Hofer and East 1998; McLaren et al. 2007). From a veterinary perspective, the results from this study may provide a rationale for wildlife veterinarians to reduce the use of the exogenous GC (prednisolone) for the treatment of inflammation in this species. In addition, given the findings of our previous study on the stress experienced by diseased and injured wild koalas (Santamaria et al. 2023), we recommend that exposure of koalas to stress be minimised wherever possible, and that monitoring of stress parameters be considered in the conservation, research and health management of this iconic

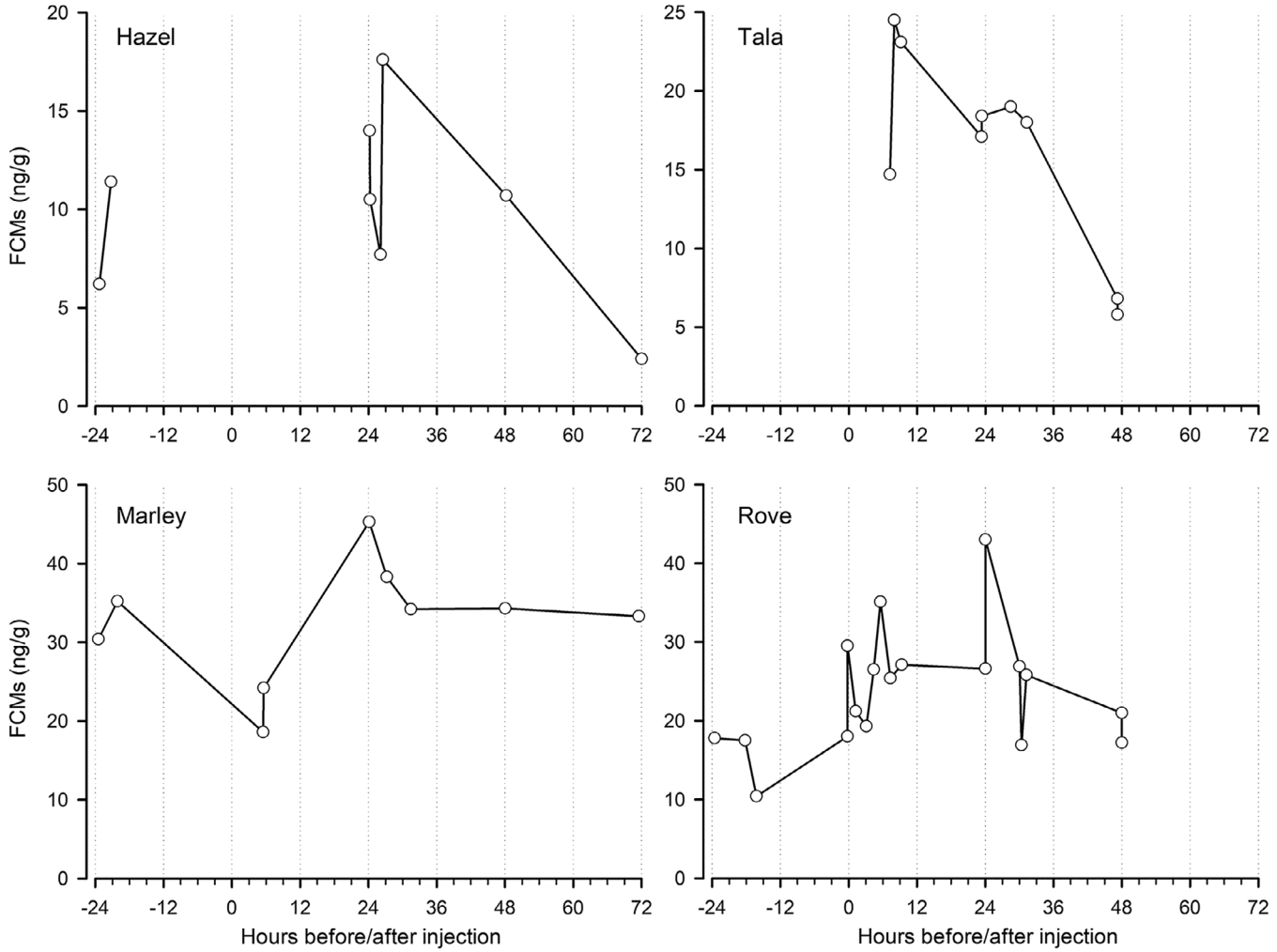

FIGURE 3 | Faecal cortisol metabolite (FCM) values of the four koalas before and after administration of dexamethasone. *Note:* Samples for Tala were not collected before the trial as she replaced another female withdrawn from the trial due to possible early pregnancy from a recent mating event *Note:* interrupted line for Hazel is due to a gap larger than 24 h between samples.

species (Schlagloth et al. 2018). Furthermore, we recommend that future research be focused on the effect of stress on the koala immune system.

Author Contributions

Flavia Santamaria: conceptualisation, methodology, formal analysis, investigation, resources, data curation, visualisation, supervision, project administration, writing – original draft, writing – review and editing. **Sam Young**: methodology, investigation, resources, project administration, writing – review and editing. **Ludovica Valenza**: methodology, investigation, resources, project administration, writing – review and editing. **Rolf Schlagloth**: investigation, resources, writing – review and editing. **Joerg Henning**: formal analysis, visualisation, writing – review and editing. **Rupert Palme**: conceptualisation, methodology, formal analysis, resources, visualisation, writing – review and editing.

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Ethics Statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. This project was approved by the CQUniversity Animal Ethics Committee, Approval No. 0000024229.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Peer Review

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