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Non-invasive evaluation of physiological stress hormone responses in a captive population of the greater bilby *Macrotis lagotis*

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ABSTRACT: Captive breeding programs are increasingly being used as a management option for threatened mammals. The greater bilby *Macrotis lagotis*, for example, is a vulnerable species which is maintained in captivity at several facilities in Australia. Non-invasive evaluation of stress hormones (cortisol in mammals) via excretory metabolites can be used to monitor physiological stress responses of captive individuals. In this study, we validated an enzyme-immunoassay (EIA) to measure cortisol metabolites in fresh faecal samples of adult male and female bilbies (n = 7) held in captivity at the Dreamworld Theme Park, Queensland, Australia. The faecal cortisol EIA was validated via parallelism and the recovery of exogenous cortisol added to pooled faecal extracts (>99% recovery). Female bilbies had higher average faecal cortisol metabolite concentrations and higher day-to-day variation than male bilbies; however, there was no relationship with bilby age. Cortisol metabolites for most individuals varied widely through time, with numerous peaks and troughs in response to long-term stressors (illnesses, injury and reproductive issues) and short-term stressors, such as use in shows at Dreamworld or public displays in local schools, manual restraint and short-term veterinary procedures (e.g. general anaesthesia). Overall, the higher mean cortisol metabolite concentrations of individuals suffering long-term stress was related to a greater response to short-term stressors. This suggests an interaction between responses to short-term and long-term stressors which is perhaps due to habituation and/or facilitation of long-term stressors. Non-invasive faecal monitoring of stress hormones could provide further information on the implications of captive breeding programs and the release of animals reared in captivity.

KEY WORDS: *Macrotis lagotis* · Captive breeding · Capture · Reintroduction · Stressor · Health

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INTRODUCTION

Translocation programs and captive breeding programs are key to species recovery (Fischer & Lindenmayer 2000, Narayan et al. 2009). Minimising the impact of multiple stressors on animals should be a major consideration when translocating animals from the wild into captivity and when maintaining animals in captivity (Narayan & Hero 2011). Thus, it is important to understand the relationship between short-term and long-term stressors (environmental stimuli that affect homeostasis) and animal health, because recurring stressors can culminate in decreased disease resistance (immunosuppression) and may reduce the animal's capacity to cope with novel stressors (Von Holst 1998, Romero 2004). During a physiological stress response, activation of the stress axis, or more specifically the hypothalamo-pituitary adrenal axis (HPA), stimulates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland, which then stimulates the release of stress hormones (cortisol in mammals) from the adrenal

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cortex (Young et al. 2004, Bayazit 2009). The metabolites of cortisol can be found mainly in urine and/or faeces (Bayazit 2009). Stress hormone release is considered a hallmark of the vertebrate stress response, which protects animals from short-term stressors (e.g. sight of a predator) through several actions such as changes in metabolism, cardiovascular tone and behaviour (Sapolsky et al. 2000). However, long-term activation of the stress hormone response to chronic stressors (e.g. illnesses or injury) could be detrimental to the animal. Chronic or prolonged cortisol concentrations have been associated with a number of consequences including hyperglycemia, neuronal cell death and suppression of the immune and reproductive behaviour in mammals (Sapolsky 1992, Wingfield & Romero 2001). Thus, it is important to develop stress hormone monitoring tools for threatened animals because conservation biologists and managers could then use stress hormones as physiological indices to describe the health of animals, as well as for improving captive husbandry techniques for enhancing captive management and breeding.

The greater bilby *Macrotis lagotis* (Thylacomyidae: Marsupialia) is one of Australia's most vulnerable mammals, with an estimated 600 bilbies remaining in the wild (Dunwoody et al. 2009). It is presently listed as 'Vulnerable' (C1 Ver. 3.1) on the International Union for Conservation of Nature (IUCN) Red List (Friend et al. 2008). This burrowing marsupial was once widespread across 70% of mainland Australia, but has disappeared from 90% of its historical range. Presently, it exists only in fragmented populations in arid and the least fertile parts of its former range, including small isolated areas in the deserts of Western Australia, the Northern Territory and patches in far Western Queensland (Smith et al. 2009). Queensland's greater bilby population is the most threatened and genetically distinct population in Australia (Southgate et al. 2000). This major contraction in range is primarily attributed to predation pressure by introduced mammals such as red foxes *Vulpes vulpes* and cats *Felis catus*, habitat destruction through agriculture and heavy grazing, and competition for habitat space with herbivores such as the European rabbit *Oryctolagus cuniculus* (Dunwoody et al. 2009, Smith et al. 2009). There is a long history of captive management programmes for the greater bilby, and, while information is widely available regarding the greater bilby's reproductive biology, anatomy and behaviour (Southgate et al. 2000, Gibson 2001), the impact of captive management on the health/well-being of this species has not been investigated. In particular, there has been no evaluation of their physiological stress responses to short-term and long-term stressors. This leaves a major gap in our knowledge of the effects that husbandry practices and management initiatives might have on the greater bilby.

Recent studies have utilised faecal monitoring of stress hormone metabolites (such as cortisol metabolites) in large mammalian species (Goymann et al. 1999, Metrione & Harder 2011). This non-invasive sampling technique reduces undue stress as no capture or handling is required to obtain faecal samples. Most importantly, faecal cortisol metabolites represent pooled fractions of plasma cortisol, providing an integrated measure of the physiological stress response (Goymann et al. 1999). The use of faecal material is preferred, particularly when dealing with endangered and managed populations (Wielebnowski et al. 2002) and also when samples can be matched to individuals housed collectively in captivity using non-toxic faecal markers (Hogan et al. 2011b). There are only a handful of published studies on non-invasive faecal monitoring of cortisol metabolites in marsupials; these include a population of the Gilbert's potoroo *Potorous gilbertii* at a captive breeding facility in Western Australia (Stead-Richardson et al. 2010), the tammar wallaby (McKenzie & Deane 2005) and the honey possum (Oates et al. 2007). Most recently, faecal cortisol metabolite enzyme-immunoassay (EIA) was validated and used to assess the physiological stress responses of captive wombats *Lasiorhinus latifrons* against a handling stressor (Hogan et al. 2011a). The latter study reported that faecal cortisol metabolite concentrations consistently increased in reaction to a handling procedure involving forced human contact (indicating a lack of habituation).

In our study, we investigated whether faecal cortisol metabolites are a useful indicator of physiological changes (i.e. stress hormones) to short-term and long-term stressors in a captive population of the greater bilby at the Dreamworld Theme Park, Queensland, Australia. We validated faecal cortisol EIA for evaluating cortisol metabolite concentrations in adult male and female bilbies. We compared the daily cortisol metabolite concentrations with bilby sex and with known short-term and long-term stressors in captivity. The bilbies are exposed regularly to short-term stressors such as manual restraint, veterinary checks and use in educational programs such as public shows on site and at local schools, which are part of Dreamworld's conservation goal. We hypothesise that these short-term stressors are likely to cause a physiological change in plasma cortisol concentrations, hence resulting in elevated faecal corti-

sol metabolite concentrations (lasting several days). Long-term or prolonged stressors include breeding events, as well as ongoing health problems such as illness or injury. We hypothesise that these long-term stressors are likely to result in elevated faecal cortisol metabolite concentrations (lasting weeks or months).

MATERIALS AND METHODS

Captive population

A total of 7 adult bilbies *Macrotis lagotis* were studied. The animals were housed individually in the bilby breeding chamber (separate from other captive animals and away from human visitors) at Dreamworld, Queensland. The bilbies were fed daily with whole grains and carnivore dental chews supplemented with whole insects. Each bilby enclosure was similar in size, and 2 hide boxes and a deep sand substrate were provided in each enclosure. The Dreamworld bilby husbandry database was used to obtain information about sex, age and long-term stressors (illness, injury, or reproductive issues) associated with each bilby (details in Table 1). The bilby database also provided information regarding the short-term stressors, including school visits, public displays or shows, manual restraint and veterinary checks. Data on baseline or unmanipulated faecal cortisol metabolite concentrations from healthy individuals (that were not affected by any short-term or long-term stressors prior to the study period) were unavailable due to some logistical constraints (time limitation and small sample sizes). Hence, for this study, we only report on individual and mean faecal cortisol metabolite concentrations in male and female bilbies during short-term and long-term stress situations over the study period.

Faecal collection method

In order to evaluate daily changes in faecal cortisol metabolite concentrations of captive bilbies and to ascertain whether faecal cortisol EIA measures are useful for assessing the welfare of bilbies in captivity, fresh faeces were collected routinely from all available individuals (n = 3 adult males and n = 4 adult females) daily over a period of 21 d in August 2011. One faecal sample (comprising on average 5 fecal pellets) per day per animal was collected during routine cleaning of the enclosure (08:00 to 13:00 h), with minimal disturbance to the animal. Faecal samples were immediately stored in resealable plastic bags in a −20°C freezer prior to processing. All faeces were removed from each enclosure each day so that we could ensure that fresh faeces were collected during the study.

Faecal hormone extractions

Cortisol metabolites in bilby faeces were extracted in ethanol (90% vol/vol) using a method previously described for other mammals (Millspaugh et al. 2001, Wielebnowski et al. 2002). The mean (±SEM) dry weight of faecal samples collected daily over the study period from each sex was as follows: males (23.2 ± 3.22 g) and females (15.3 ± 2.34 g).

Faecal cortisol enzyme-immunoassay

All consumables for the cortisol EIA were obtained from Sigma-Aldrich, and the cortisol antibody and reagents were obtained from the Clinical Endocrinology Laboratory, School of Veterinary Medicine, Uni-

Table 1. *Macrotis lagotis*. Sex, age and potential long-term stressors of the greater bilbies evaluated during the study

Name	Code	Age (yr)	Long-term stressors
Allira	Female 1	2	At the time of the study this animal had arthritis, first noted in March 2011. Following the study, a dental check on 14 September 2011 revealed a dental infection that was possibly active and affecting health at the time of the study
Carina	Female 2	4	This animal has had recurrent upper respiratory tract infections since 2010
Josie	Female 3	4	This animal has had life-long skin issues. Two biopsies were taken under general anaesthesia, one from the upper lip, another from the skin of the tail
Molly	Female 4	5	This female had a joey die in her pouch on 25 July 2011. Mammary gland was checked with a dental check involving a general anaesthetic on Day 1
Beelar	Male 1	2	At the examination on Day 5 (12 August 2011) arthritis of unknown duration was noted. This would probably have been present for at least a number of weeks
Billy	Male 2	2	No medical issues reported throughout the sampling period
Kalana	Male 3	2	This animal was anaesthetised for a dental check on Day 2. No other medical issues reported

versity of California, Davis, USA. The faecal cortisol metabolite EIA was validated for the greater bilby by demonstrating (1) parallelism between dilutions of pooled faecal extracts and the cortisol standard curve and (2) extraction efficiency/significant recovery of exogenous cortisol added to faecal extracts. The validity of the faecal cortisol metabolite EIA was confirmed by parallel displacement of serially diluted pooled bilby faecal extract to those of cortisol standards (Fig. 1). This parallelism curve was used to determine the dilution factor at which to run the samples for the entire assay, which was 1:3 for bilby faecal extracts based on the 50% binding point on the parallelism curve (Fig. 1). Extraction efficiency was calculated as the amount of hormone observed relative to the amount expected, and was expressed as a percentage (mean ± standard error of the mean [SEM]). Extraction efficiency was presented as a linear regression equation: $y = mx + b$, where y is the concentration of the hormone observed, x is the concentration of the hormone expected, b is the y intercept, and m is the slope of the line that was expressed as a percentage representing extraction efficiency (Narayan et al. 2010). The resulting equation was $y = 0.98x + 1.26$, $r^2 = 0.997$; n = 7. Thus, the extraction efficiency was >99%. The sensitivity of the faecal cortisol metabolite EIA was 1.2 ± 0.2 pg well^{-1} (n = 10). The intra- and inter-assay coefficients of variation were 2.3 and 6.4% for the high-binding internal control, and 1.9 and 14.3% for the low-binding internal control, respectively (n = 15).

Faecal cortisol metabolite concentrations were determined using a polyclonal anticortisol antiserum

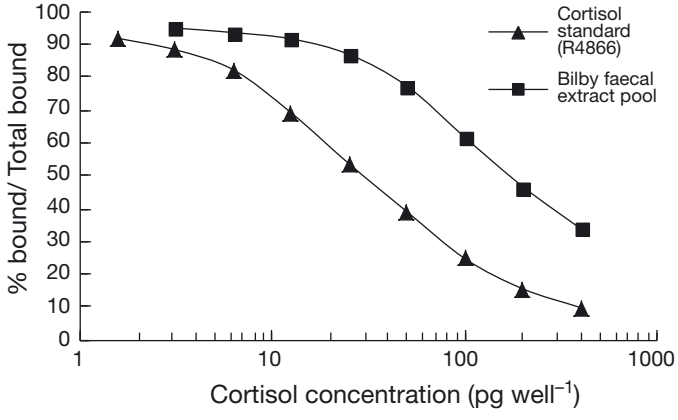

Fig. 1. *Macrotis lagotis*. Binding displacement curves of serially diluted faecal sample pools from male greater bilby (parallelism curve for female greater bilby was the same) against standards used in the corticosterone enzyme-immunoassay. The *y*-axis shows the percent of labeled hormone bound/ total bound measured at 450 nm (reference 630 nm)

(R4866) diluted 1:15 000, horseradish peroxidase conjugated cortisol label diluted 1:80 000 and cortisol standards (1.56 to 400 pg well^{-1}). Cross reactivity of the R4866 anticortisol antiserum was reported to be 100% with cortisol and <10% with the other steroids tested (Narayan et al. 2010). Samples were assayed in duplicate on Nunc MaxiSorp plates (96 wells). For each assay, the Nunc MaxiSorp plates were coated with 50 µl of antibody diluted to the appropriate concentration in a coating buffer (50 mmol l^{-1} bicarbonate buffer, pH 9.6) and incubated for at least 12 h at 4°C. Plates were washed using an automated plate washer supplied with phosphate-buffered saline containing 0.5 ml l^{-1} Tween 20 to rinse away any unbound antibody. Stocks of standards, high- and low-binding internal controls, faecal extracts (diluted 4 times in assay buffer based on 50% binding on the parallelism curve), and horseradish peroxidase labels were diluted to the appropriate concentration in assay buffer. For each EIA, 50 µl of standard, internal control and diluted faecal extract was added to each well. For all assays, 50 µl of the corresponding horseradish peroxidase label was then added to each well, and the plates were incubated at room temperature for 2 h. Plates were washed, and 50 µl of a substrate buffer (0.01% tetramethylbenzidine and 0.004% H_2O_2 in 0.1 M acetate citrate acid buffer, pH 6.0) was added to each well. Stopping solution (50 µl of 0.5 mol l^{-1} H_2SO_4) was added based on the visual inspection of plates so that the optical density of the zero wells would read between 0.7 and 1, usually after 7 to 20 min incubation at room temperature. Plates were read at 450 nm (reference 630 nm) on a microplate reader. All hormone data were expressed as faecal cortisol metabolite concentration (pg g^{-1}) on a dry weight basis.

Statistical analysis

Data are presented as individual or mean (±SEM) values. Statistical analyses were conducted using Prism Graphpad Software Inc. (GraphPad Prism Version 5.02 for Windows). All data were tested for normality using Bartlett's test, and data were log transformed to meet the assumptions of equal variances. Two-way repeated-measures ANOVA was used to compare mean faecal cortisol metabolites between individuals with sex and time (days into study) as the sources of variation. A post hoc comparison between mean cortisol metabolite concentrations of male and female bilbies was done using the Mann-Whitney *U*-test. Spearman

rank correlation was used for comparing the mean faecal cortisol metabolite concentrations and age of each bilby. Variation in faecal cortisol metabolite concentrations for each bilby over the study period was quantified by calculating coefficients of variation (CV). The deviation or level of change (pg g^{-1}) in cortisol metabolites was calculated for each known short-term stressor using the formula: (cortisol level on 1 d after known stressor−cortisol level on the day of stress event). The current study was limited by multiple short-term stress events taking place within an average of 2 d after a short-term stressor for these bilbies; thus, a comparison between pre- and post-single stressors could only be done for 1 d before and 1 d after stressor occurrence (this assisted in ensuring a level of independence of sampling given the supposed 24 h time lag of glucocorticoid excretion in faeces). For all analyses, significance was assessed at the 0.05 level.

RESULTS

Faecal cortisol metabolite profiles of male bilbies

The daily faecal cortisol metabolite concentrations of the 3 adult male bilbies *Macrotis lagotis* (Fig. 2) ranged from 30.7 to 666.0 pg per g of dry faecal matter for Male 1 (Beelar), 23.08 to 127.9 pg g^{-1} for Male 2 (Billy) and 24.1 to 139.2 pg g^{-1} for Male 3 (Kalana). There was a significant difference in mean faecal cortisol metabolite concentrations between the 3 male bilbies over the 21 d of sampling ($F_{2,53}$ = 6.384, p = 0.002). The mean faecal cortisol metabolite concentration of Male 1 (202.3 ± 48.26 pg g^{-1}) was significantly higher than the mean faecal cortisol concentrations of Male 2 (60.20 ± 7.14 pg g^{-1}) and Male 3 (65.34 ± 9.35 pg g^{-1}) (p < 0.05 for both comparisons). However, mean faecal cortisol metabolite concentrations of Males 2 and 3 were not significantly different from each other (p > 0.05).

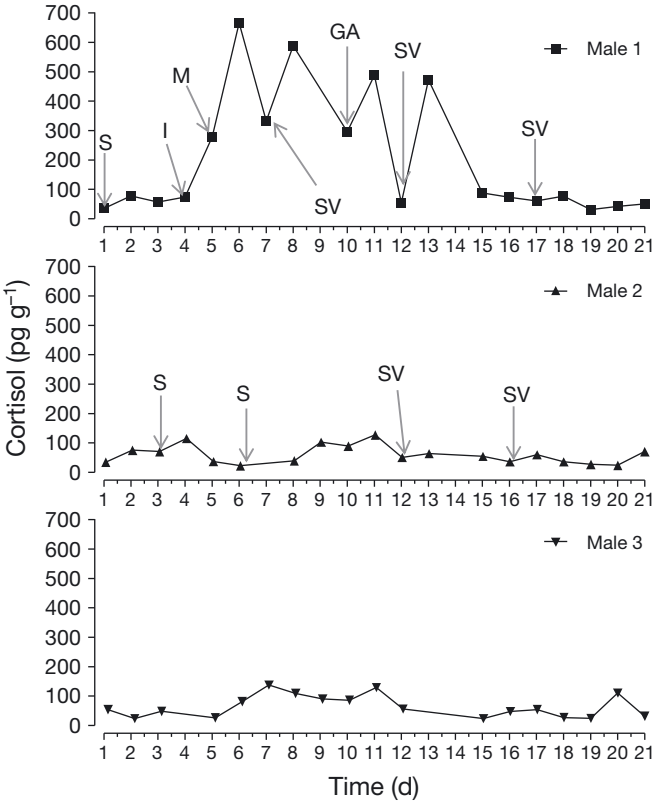

Fig. 2. *Macrotis lagotis*. Individual faecal cortisol metabolite profiles of 3 male greater bilbies illustrating inter-individual variation in cortisol metabolite concentrations (in pg per g of dry faecal matter). Arrows indicate the day of known short-term stress event (levels of deviation in cortisol metabolites are provided in Table 2). SV: school visit; S: shows; M: manual restraint; I: injury; GA: general anaesthesia

Faecal cortisol metabolite profiles of female bilbies

The daily faecal cortisol metabolite concentrations of the 4 female bilbies (Fig. 3) ranged from 80.5 to 1272.0 pg g^{-1} for Female 1 (Allira), 193.6 to 1192.0 pg g^{-1} for Female 2 (Carina), 100.1 to 693.6 pg g^{-1} for Female 3 (Josie) and 70.2 to 384.2 pg g^{-1} for Female 4 (Molly). There was a significant difference in mean faecal cortisol metabolite concentrations between the 4 female bilbies over the 21 d of sampling ($F_{3,71}$ = 11.34, p < 0.0001). Mean cortisol metabolite concentration of Female 2 (577.1 ± 76.5 pg g^{-1}) was significantly higher than mean faecal cortisol metabolite concentration of Female 1 (267.3 ± 65.5 pg g^{-1}) and Female 4 (191.9 ± 19.5 pg g^{-1}) (p < 0.05 for both comparisons). However, mean faecal cortisol metabolite concentrations of Females 2 and 3 were not significantly different from each other (577.1 ± 76.5 cf. 340.9 ± 44.9, p > 0.05).

Variation in faecal cortisol metabolites of bilbies

There was a significant effect of sex ($F_{1,17}$ = 11.32, p = 0.02) and time (sampling days) ($F_{1,17}$ = 14.58, p < 0.0001) on the faecal cortisol metabolite concentrations of bilbies in captivity. The mean faecal cortisol metabolite concentrations of female bilbies were significantly higher than those of male

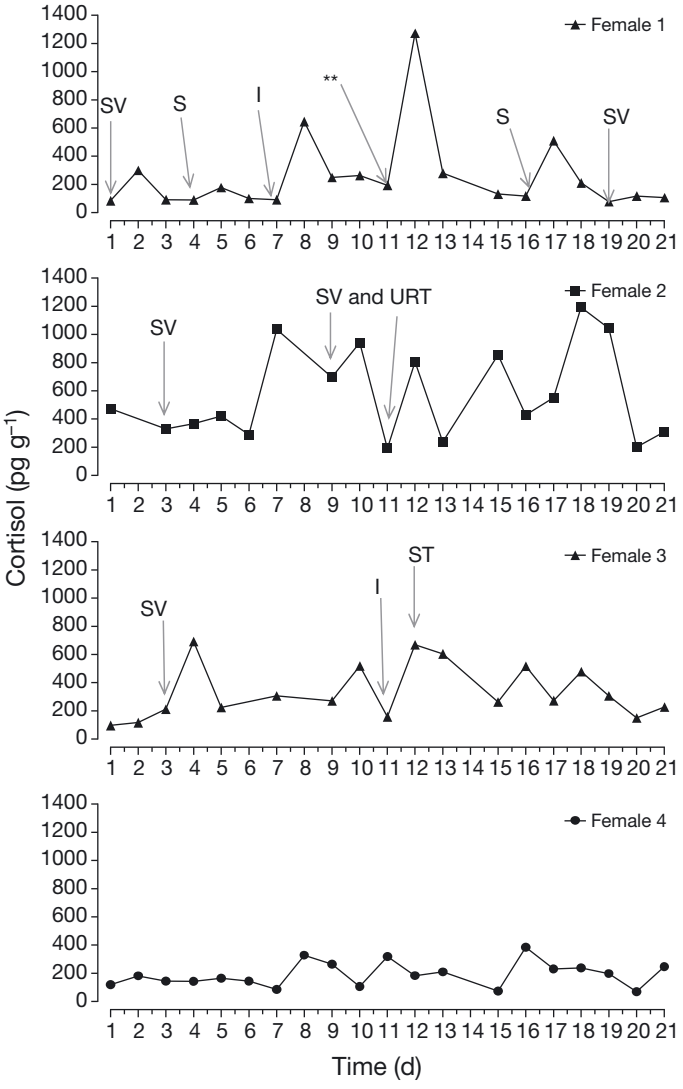

Fig. 3. *Macrotis lagotis*. Individual faecal cortisol metabolite profiles of 4 female greater bilbies illustrating inter-individual variation in cortisol metabolite concentrations (levels of deviation in cortisol metabolites are provided in Table 3). **: unknown stressor; URT: upper respiratory tract infection; ST: suture; other conventions and abbreviations as in Fig. 2

bilbies (Fig. 4; 419.1 ± 67.8 pg g^{-1} cf. 87.4 ± 11.8 pg g^{-1}; U = 372, $p < 0.0001$). There was no significant correlation between mean faecal cortisol metabolite concentrations and age (years) of bilbies ($p > 0.05$). Variation in the daily faecal cortisol metabolites over the study period was the highest for Male 1 (103.99%) followed by Male 3 (58.78%) and Male 2 (51.70%). For the female bilbies, day-to-day variation in the faecal cortisol metabolites over the study period was the highest for Female 1 (106.79%) followed by Female 2 (56.27%), Female 3 (55.95%) and Female 4 (45.43%).

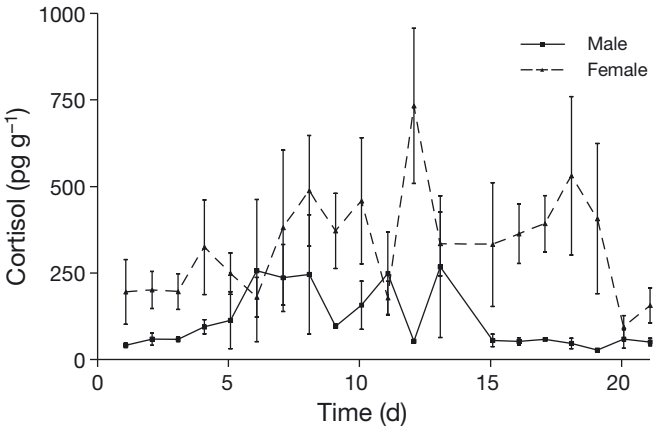

Fig. 4. *Macrotis lagotis*. Mean (±SEM) faecal cortisol metabolite profiles of male (n = 3) and female (n = 4) greater bilbies over the 21 d sampling period

Physiological stress responses of bilbies to short-term and long-term stressors

We examined the known history of each individual and their faecal cortisol metabolite concentrations. The individuals with elevated hormone concentrations (3 of the females and 1 of the males) had some illness or injury during the study period (see Tables 2 & 3). Male 1 had chronic arthritis (Table 1); however, Males 2 and 3 showed no signs of illness or injury (Table 2). These broad patterns follow the higher faecal cortisol metabolite concentrations observed in Females 1, 2 and 3, and also in Male 1. The amount of change (deviation) in faecal cortisol metabolite concentrations to known short-term stressors is shown in Tables 2 & 3. These data are variable because most animals took part in more than one activity, on several days, which would mask any subtle stressors, since responses can last over several days after a stress event (see Figs. 2 & 3 for individual responses to known short-term stressors). It is clear from Figs. 2 & 3 that some individuals (e.g. Male 1 and Females 1 and 2) showed a greater response (higher amplitude in faecal cortisol metabolite concentration) to the known short-term stressors. Figs. 2 & 3 show that the individuals with long-term health issues have higher pre-stressor faecal cortisol metabolite concentrations and substantially higher magnitudes of short-term stress responses in comparison to individuals with few long-term health problems (such as Male 3 and Female 4). The overall higher mean cortisol metabolite concentration of long-term stressed individuals appears to be, at least partially, due to their greater response to short-term stressors.

Table 2. *Macrotis lagotis*. Short-term stressors recorded during the study period for male greater bilbies. Deviation in faecal cortisol metabolites calculated based on the level of change in concentration (pg g^{-1}) of cortisol (1 d after stress event — day of stress event). –: no sample

	Known short-term stressors (multiple stress events)	Deviation (pg g^{-1}) in cortisol metabolites
Male 1		
Day 1	Show	42.48
Day 4	Right leg injury	203.62
Day 5	Manual restraint, non-steroidal anti-inflammatory	369.65
Day 7	School visit	294.5
Day 10	General anaesthesia, right leg injury healed	–
Day 12	School visit	160.73
Day 17	Show	12.20
Male 2		
Day 3	Show	24.69
Day 6	Show	–
Day 12	School visit	13.2
Day 16	School visit	12.2
Male 3		
Day 2	General anaesthesia	24.69

Table 3. *Macrotis lagotis*. Short-term stressors recorded during the study period for female greater bilbies. Deviation in faecal cortisol metabolites calculated based on the level of change in concentration (pg g^{-1}) of cortisol (1 d after stress event – day of stress event). Day 1 is 8 August 2011. –: no sample

	Known short-term stressors (multiple daily stress events)	Deviation (pg g^{-1}) in cortisol metabolites
Female 1		
Day 1	School visit	214.60
Day 4	Show	86.64
Day 7	Vet check: right hind leg, nail avulsed	552.74
Day 12	Vet check: right hind leg, nail settled	–
Day 16	Show	390.91
Day 19	School visit	40.88
Female 2		
Day 3	School visit	35.91
Day 9	Congested sinus, abnormal pouch discharge, sneezed blood, swollen eyes, severe upper respiratory tract infection including tear duct, eye and nose; school visit	244.33
Day 12	Sneezed blood, congestion as normal for her, low-grade nasal cavity infection	–
Day 15	Sneezed blood in morning	–
Female 3		
Day 1	Follow up from previous 2 wk. Hyperkeratosis and associated infection on tail	–
Day 3	School visit	479.24
Day 11	Lesions on snout bled heavily; show	509.40
Day 12	Snout lesions sutured and gingivitis found; biopsy on tail showed dermal infection (general anaesthesia)	–
Day 22	Sutures removed	–
Female 4		
	No stressors reported	–

DISCUSSION

We successfully established faecal monitoring of the physiological stress response in greater bilbies as a means of assessing their well-being in captivity. We also evaluated the effects of various types of stress on captive individuals. The results of our study demonstrate that faecal monitoring of cortisol metabolites can provide a useful tool for evaluating stress in greater bilbies, and can thus provide a powerful tool for monitoring the physiological stress responses of bilbies when used in combination with analyses of health, behaviour and other relevant captive husbandry information. Individuals that were facing long-term or ongoing health issues generally had higher mean faecal cortisol metabolite concentrations and larger variation in faecal cortisol metabolite concentrations throughout the study period. This is an interesting feature, suggesting an interaction between the various physiological stress responses to short- and long-term stressors.

We have shown that increased stress hormone concentrations reflect a physiological stress response, which could be part of a chronic stress response in an unhealthy animal. However, stress hormone concentrations may also be elevated in otherwise healthy animals coping with recovery. Thus, it is essential to accurately interpret the stress hormone profiles of individual animals. In an earlier review, Romero (2004) provided detailed theoretical explanations on why stress hormone concentrations differ between and among individuals. A total of 4 key explanations were provided that are also highly relevant to the present study: (1) habituation to a long-term stressor; (2) facilitation of a novel short-term stressor by an ongoing, long-term stressor; (3) individuals that are chronically stressed and have not become habituated to a long-term stressor; and (4) individuals have just

experienced a short-term stressor (e.g. manual restraint or shows), which causes their stress hormone concentrations to rise. The above 4 key theories were demonstrated in the current study. For some individuals, we could not find any significant impact of short-term stressors, such as shows or school visits on changes in stress hormones. This could be explained by Theory 1, in which a chronic stressor masks the impact of a short-term stressor through habituation (Rich & Romero 2005). A closer inspection of individual faecal cortisol metabolite profiles shows that some individuals had higher mean cortisol concentrations and the amplitude of their stress hormone response to short-term stressor(s) was much higher in comparison to that of other individuals. This is a good example of a long-term stressor underlying or facilitating a short-term stress response (Theory 2). Those individuals that were undergoing long-term stressors associated with illnesses showed higher variation in their faecal cortisol levels; these were most probably linked to the lack of habituation to a long-term stressor (Theory 3). Theory 4 was clearly exemplified through the short-term stress responses of bilbies to physical stressors such as general anaesthesia and suture. Thus, the identification of chronically ill or stressed animals in a captive breeding colony is vital for understanding the complexities of physiological stress responses; it is also important for evaluating the adaptation of animals to captivity. Such interpretations of our data are limited by low sample sizes and individual bilbies undergoing multiple short-term stressors (e.g. show and vet check performed on the same day) during the study period. Thus, future studies within captive populations should explore the impact of each stressor individually and over time so that individuals serve as their own controls. For example, the effect of general anaesthesia or shows should be examined in a controlled experiment, whereby the faecal cortisol of bilbies is first tested daily under baseline conditions (such as off roster and in the absence of any threatening stressors for 3 wk) and then daily over 3 wk after the show or educational event. This approach would also reduce the possible biases associated with the inter-individual differences observed in this study and allow researchers to clearly interpret the changes in stress hormones in relation to long-term and short-term stressors and individual well-being. Furthermore, intrinsic within-day variation of cortisol concentrations should be considered; this would also provide important information regarding the best sampling times if huge diel variations in faecal cortisol metabolite concentrations exist for bilbies (Bosson et al. 2009).

Another important consideration is that the amount and quality of human interaction and events that have occurred during the animal's life prior to the study could also influence the variation in cortisol concentrations (Pedersen 1994, Hemsworth et al. 2000). Such factors may profoundly affect animal well-being in captivity (Hemsworth et al. 2000). Human interventions in captivity, such as short-term capture and procedures like general anaesthesia, are known to influence stress hormones (Ram et al. 2005, Narayan et al. 2011). Previously, it has been shown that faecal cortisol metabolite concentrations in the endangered Indian blackbuck *Antelope cervicapra* were higher during increased zoo visitation, which suggested that zoo visitor density affected the behaviour and cortisol secretion in Indian blackbucks (Rajagopal et al. 2011). Similarly, zoo visitor numbers significantly affected urinary cortisol metabolite concentrations in spider monkeys *Ateles geoffroyii rufiventris* (Davis et al. 2005). Age can also affect physiological stress responses in vertebrates (Blas et al. 2006, Wilcoxen et al. 2011). Granted we had a small sample size (n = 7 animals), but we found no correlation between bilby age and faecal cortisol metabolite concentrations. However, we did find a significant difference between sexes in faecal cortisol metabolite concentrations, with females producing, on average, higher faecal cortisol metabolite concentrations than males. Gender differences in faecal cortisol excretion are not uncommon and have been found in other mammals such as Stellar sea lions *Eumetopias jubatus* (Mashburn & Atkinson 2007) and clouded leopards *Neofelis nebulosa* (Wielebnowski et al. 2002). Differences between sexes could be an evolutionary adaptation of females to increase watchfulness (i.e. an increased 'fight-or-flight' response), with the purpose of protecting and rearing young (Gray 1987) and avoiding aggression from dominant males (Vandenheede & Bouissou 1993). Thus, monitoring of behaviour, together with stress physiology and health, should be an integral part of the bilby captive breeding program.

Faecal cortisol metabolite EIAs allow for non-invasive assessment of stress hormone concentrations in captive animals (Bayazit 2009). Repeated sampling of free-living individuals may be difficult under field conditions; hence, faecal stress hormone monitoring can only provide population-specific rather than individual estimates for wild populations. An important technical consideration is that there is a time lag of glucocorticoid excretion in faeces following exposure to a stressor, which is approximately 5 h in small mammals (Bosson et al. 2009) and up to 50 h in larger

mammals (Goymann et al. 1999, Young et al. 2004). It is important to know this time lag, because it allows the researcher to pinpoint the biological response to a stressor and it also provides the researcher with a valuable time window for faecal sample collection if disturbance cannot be avoided (Bosson et al. 2009). In the future, objective information on the transit times for bilbies could be measured, as done by Hogan et al. (2011b) with wombats, by adding indigestible markers to the food. To our knowledge, there have been at least 2 similar studies on the bandicoots *Perameles nasuta* (Moyle et al. 1995) and *Isoodon macrourus* (Waring et al. 1966), but no study of food transit times for bilbies. The study by Moyle et al. (1995) showed a strong effect of diet on transit time (8 h on insect diet vs. 21 h on a tuber/root diet). This provides an 'approximate figure' for bilby food transit time (<24 h) that should be confirmed by a future study with indigestible markers. However, we acknowledge that the type of digestive system, the degree of dependence on microbial fermentation and the diet would also affect the specific transit time. Furthermore, the amount of time between secretion of cortisol and its subsequent transfer through the bloodstream, target organs and to the digestive system for excretion would influence the excretion time lag. Thus, in addition to measuring food transit times, the only other way to validate the excretory lag time of cortisol metabolites in faeces is to perform a biological challenge using ACTH, with both blood plasma and faecal cortisol metabolite responses measured in the hours (or days for faecal measures) following a particular challenge. Thus, it is crucial to biologically validate the immunoassay to show that the hormonal measures accurately reflect the physiological events of interest. Currently, we are working on a method to improve bilby faecal glucocorticoid monitoring by using an ACTH challenge.

Non-invasive faecal stress hormone monitoring is an important tool for assessing the physiological status of greater bilbies, and it should be incorporated with behavioural observations and other measures of animal health and physiology to more accurately determine what constitutes well-being for this threatened marsupial species maintained in captivity. Ultimately, this combination of physiological and behavioural measurements may help to manipulate and improve captive breeding programmes that would eventually lead to improved reproductive success and a higher chance of establishing self-sustaining captive populations. Captive breeding is now a common strategic practice that supports species conservation programmes involving reintroductions. Successful reintroductions and translocations using captive-bred individuals are rare (Seddon 1999, Moro 2003) and, with few exceptions, improvements over time are not apparent (Fischer & Lindenmayer 2000). Translocated animals may be particularly vulnerable because of a lack of acquired immunity from previous exposure to infections, and stress-induced immunosuppression. Baseline data on stress hormones are lacking for most species of conservation concern. Detailed monitoring of physiological stress responses in captive animals is very important because these responses can be used to understand the impacts of interventions such as capture, restraint and transportation on the animals. Such interventions could lead to adverse effects such as capture myopathy, which is a syndrome that occurs in wild (free-ranging and captive) mammals and birds, and is associated with the stress of capture, restraint and transportation (Montané et al. 2007). This is particularly important because some animals raised in captivity may become accustomed to handling over time, hence potentially reducing their physiological capacity to respond to a natural stressor (such as being chased by a predator). In conclusion, non-invasive faecal monitoring of stress hormones in the greater bilby offers promise for improved monitoring of captive populations and wildlife interventions that will increase the success of captive breeding and reintroduction programmes.

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