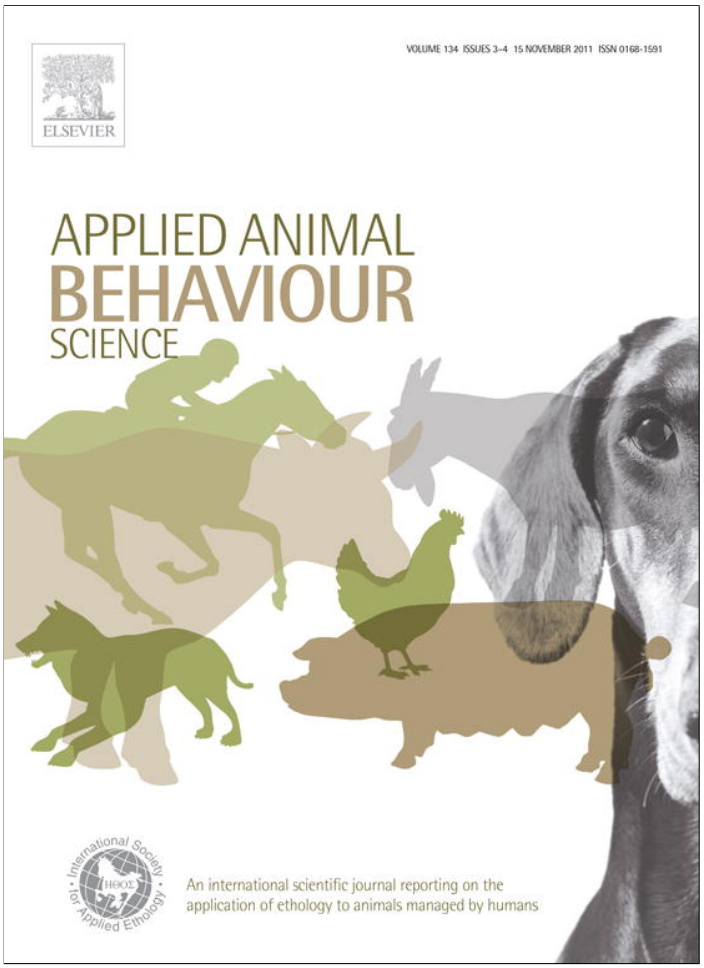

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Behavioural and physiological responses of captive wombats (*Lasiorhinus latifrons*) to regular handling by humans

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ABSTRACT

The response of animals to handling by humans has been extensively evaluated in domesticated livestock, but rarely examined in wildlife species. Twelve captive wombats (*Lasiorhinus latifrons*) were subjected to two treatments in a replicated design: (1) daily handling, involving 15 min of tactile contact 5 d/wk for 12 wk and (2) no-handling, involving no contact apart from that received during routine husbandry. The effect of handling was assessed via overt responses to human approach and touch, a stressor, and a novel stimulus. Daily handling reduced the wombat's flight distance in response to human approach; more in the first handling replicate (-0.16 ± 0.02 m/wk) than in the second (-0.06 ± 0.02 m/wk). A behavioural reactivity score also declined faster in the first than second handling replicate. Synthetic ACTH was used to validate the measurement of faecal cortisol metabolites in *L. latifrons* by EIA. Faecal cortisol metabolite secretion consistently increased in reaction to a handling procedure involving forced human contact (indicating a lack of habituation) but the magnitude of this response was not reduced by regular handling. Regular handling therefore changed the human–wombat relationship by lowering reactivity to and avoidance of the human handler, but did reduce the stress response, suggesting that the wombats entered into a state of learned helplessness.

1. Introduction

In nature, wild animals rarely come into contact with humans and generally show marked avoidance of people. In captivity, however, contact with humans is often frequent and unavoidable. Unlike domesticated species, wild animals have not undergone selective breeding for milder temperaments and ease of handling (Maciejowski and Zieba, 1982). Hence, the constant presence of humans is a difficult adjustment for wild animals in captivity, and a high or persistent fear of people can be major source of psychological stress, which can transpose into behavioural problems that make handling difficult (Shepherdson et al., 2004). Positive (or gentle) handling has been successfully used to reduce fear towards humans through desensitisation (Jones, 1994); a process in which fear is diminished by pairing positive rewards with actions or objects that normally induce fear (Rushen et al., 1999). Positive handling has been shown to (1) diminish fearfulness and avoidance of people, (2) facilitate easier capture

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and reduce risk of injury during transport and (3) increase reproductive performance, resistance to pathogens, feed conversion efficiency, growth rates and interest in novel stimuli (Carlstead et al., 1993; Barnett et al., 1994; Lensink et al., 2000a). To date, most handling studies have been conducted on domesticated species, with few studies investigating the effects of handling on wild animals. Mellen (1991) and Carlstead et al. (1993) both found a positive correlation between the amount of positive keeper interaction and reproduction in wild felids, whilst Pedersen (1994) discovered that the reproductive performance of fox vixens is greatly reduced by aversive handling. Additionally, Waitt et al. (2002) found that more positive interactions from caretakers resulted in reduced stress-related responses during routine husbandry in macaques, and Baker (2004) showed that increased interactions with humans reduced intra- and inter-specific aggression in chimpanzees.

Emotional reactivity is often used to investigate responses to handling and includes concomitant behavioural changes and physiological manifestations that occur as a result of fear and/or anxiety (Fureix et al., 2009). Behaviours such as avoidance (i.e. flight) and aggression (i.e. fight) serve to protect animals from aversive stimuli, and the intensity of the behavioural response is considered to reflect the degree of aversiveness of the triggering situation (Veissier and Boissy, 2007). Two physiological responses commonly associated with aversive stimuli are elevated glucocorticoid (GC) secretion and heart rate (Carlstead, 1996). GC and heart rate responses of animals to human contact have been successfully used in conjunction with behavioural data to assess the level of fear of human beings in a variety of species (Hargreaves and Hutson, 1990a; Carlstead et al., 1993; Barnett et al., 1994; Pedersen, 1994; Waiblinger et al., 2004). Conventionally, fear assessment from GC concentrations has relied on blood sample collection. In wildlife, regular blood sampling is difficult and introduces variables (such as stress caused by capture and handling) that may alter the results. Hence, faecal sampling has become an increasingly popular substitute for the analysis of GCs. The primary advantages of faecal sample collection are that there is no need to handle the animals and faecal GC metabolites represent pooled fractions of plasma GCs, providing an integrated measure of adrenal activity (Goymann et al., 1999). To date, measurement of faecal GCs in marsupials has only been validated in three species, the tammar wallaby (McKenzie and Deane, 2005), honey possum (Oates et al., 2007) and Gilbert's potoroo (Stead-Richardson et al., 2010).

In this study, a colony of wild-caught southern hairy-nosed wombats (*Lasiorhinus latifrons*) at Rockhampton Zoo was utilised to examine whether regular handling could modify the emotional reactivity, measured by behavioural and physiological responses, of a captive marsupial to humans. This species was chosen because little is known about the behavioural biology of wombats and previous research has indicated that stress caused by regular blood sampling can impair their reproductive function (Druery, 2004). The hypothesis tested was that regular handling would reduce (a) the wombats' behavioural reactivity to and avoidance of humans and (b) cortisol responses following forced human contact.

2. Methods

Animal ethics approval for this study was granted by The University of Queensland's animal ethics committee (SAS/609/04/UQ/EPA).

2.1. Animals and study area

The history and captive husbandry of the wombats used in this study has been previously described in detail (Hogan et al., 2010). Briefly, 12 (4♂, 8♀) wild-caught adult *L. latifrons*, housed at Rockhampton Zoo in groups of three (1♂:2♀) were remotely monitored from March 2006 to February 2007. Each group had access to an indoor and outdoor enclosed area. The indoor areas (i.e. artificial burrow system) were temperature-controlled, between 18 and 25 °C and contained two separate sleeping dens with a series of interconnecting tunnels. The outdoor areas, of dimensions 163–250 m^2, were exposed to local weather conditions and were provided with grass, trees, rocks, logs and digging chambers.

2.2. Experimental design

The wombat groups ($n = 4$) were randomly allocated to one of two treatments: handling (treatment; T) and no-handling (baseline; B). The T animals received interaction with the human handler for 15 min/d (a five-part handling procedure) for five consecutive days a week for 12 weeks, whilst the B animals received no such interaction. The experiment had a switch-back design with each animal receiving two replicates of each treatment, either TBTB or BTBT. In the first (February–April 2006) and third (September–December 2006) test periods, groups 2 and 3 received T, whilst groups 1 and 4 received B; the reverse occurred during the second (May–August 2006) and fourth (January–March 2007) test periods.

All of the animals were unavoidably exposed to human contact during routine health checks. Rockhampton Zoo's standard health check regime comprised one brief (~2 min) physical examination per day (08:00 h) and one weighing (~5 min) per fortnight (11:00 h). During the daily examination, the wombats were confined (using a sliding door) to a den (or section of tunnelling) and a zookeeper would physically inspect them for signs of illness and/or injury. During weighing, the wombats were individually manoeuvred from their dens, through a series of tunnels, into a blind tunnel containing a large animal digital platform scale. The wombat was coaxed onto the scale, its weight recorded and it was then manoeuvred back to its original den. The wombats were moved using a series of sliding doors and gentle physical contact, i.e. patting or gentle pushing with open hands. The entire weighing procedure took about 1-h and involved zookeepers and volunteers.

2.2.1. The handling procedure

Five days a week, six wombats were manoeuvred (17:00 h) from their dens and confined to the outdoor areas.

Each was, in turn, exposed to three handling trials, approximately 5 min in duration, at 30 min intervals. The handling trials encompassed five separate human-to-animal interactions, which were derived from a temperament test used by the Royal Society for the Prevention of Cruelty to Animals (RSPCA, 2004; Poulsen et al., 2010). For interactions 1–4, each wombat was approached head on, until the handler was within touching distance. Interaction 1 (Stroking), the wombat was stroked along its back (neck to rump). Interaction 2 (Scratching), the wombat was scratched around the base of the ears and/or on the rump. Interaction 3 (Massaging), a patch of skin on the neck, flank and pouch of the wombat was 'massaged' (i.e. gently squeezed and lifted). For these interactions, the animal was stroked, scratched or massaged for a maximum of 30 s unless it walked away, ran off, or responded aggressively to the handler, at which point the interaction ceased. Interaction 4 (Noise), the handler jingled a set of keys for 5 s next to one of the wombat's ears. Interaction 5 (Following), the handler stood 0.5 m behind the wombat and followed the animal around the outdoor area for 60 s. The wombats were verbally communicated with during the handling trials, in a positive manner, except during interaction 4. In order to standardise the procedure, handling followed a fixed order, i.e. wombats were allocated a number (1–6) and were exposed to trials in the same order.

2.2.2. Overt response test

For every handling trial the wombat's behavioural response was rated from 0 to 5 (RSPCA, 2004; Poulsen et al., 2010): 0 = no response to stimulus, no movement away from handler; 1 = flicking of ears and/or head turning in response to stimulus, no movement away from handler; 2 = tensing of neck and shoulder muscles in response to stimulus, steps one or two paces away from handler; 3 = flinching in response to stimulus, walks quickly away from handler; 4 = jumps in response to stimulus, runs away from handler; 5 = aggressive in response to stimulus, lunging at and/or biting of handler. A rating (0–5) was assigned to each wombat prior to, during and after interactions 1 and 2, and during interactions 3, 4 and 5. Ratings attributed to each interaction are hereafter termed 'interaction scores'. A total score, encompassing the nine ratings was calculated for each wombat at the end of every trial. If a wombat refused to be touched and repeatedly walked or ran away, continual approach attempts were made until the handler was able to touch the animal with her hand; only then was a rating assigned to the interaction.

2.2.3. Human approach test

A human approach test, based on the method used by Uetake et al. (2002), estimated the aversiveness of the human handler. Once a week, on a non-handling day, all 12 wombats were manoeuvred (17:00 h) from their dens and confined to the outdoor areas. Each was, in turn, approached from the front at approximately 0.5 m/s (1 step/s). As soon as the wombat began to move away, the handler stopped. The positions of the handler and the wombat were then marked with metal spikes, and the distance between the spikes was taken as the flight distance (FD). The test was done three times, at 5-min intervals, and the mean FD used for individual weekly scores.

2.2.4. Stressor test

Routine weighing was utilised to measure the wombat's physiological response to forced human contact. Each wombat was weighed 24 times, at fortnightly intervals, and two faecal samples were collected from all animals every fortnight: one on the day of weighing (D_0) and one three days later (D_3). Freshly excreted, marker-labelled (Hogan et al., 2011b) faecal pellets were collected from the ground and stored at −20 °C until extracted. Cortisol was extracted from faecal samples using established methods for steroid hormones (Graham et al., 1993). Briefly, 0.5 g of thawed wet faeces was mixed with 5 mL of 80% methanol, vortexed and placed overnight on a rotating shaker. The following morning, samples were centrifuged for 15 min at 2500 rpm and the supernatant decanted; 500 μL of the supernatant was then evaporated to dryness and reconstituted using 500 μL of phosphate buffer (pH 7.0). All extracted samples were stored at −20 °C until analysis.

Cortisol concentrations were analysed in duplicate (50 μL/well) using enzyme-immunoassay (EIA) procedures previously reported for adrenocortical steroids (Young et al., 2004). The assay employed a cortisol horseradish-peroxidase (HRP) ligand (diluted 1:20,000) and antiserum (No. R4866; University of California, Davis, CA) (diluted 1:9000). Assay detection limits for plasma and faecal extracts were 0.016 and 0.019 ng/well, respectively. Serial dilutions of plasma and faecal extract pools yielded displacement curves parallel to those of the cortisol standard. Plasma and faecal extract inter- and intra-assay coefficients were 3.8% ($n = 20$) and 2.9% ($n = 4$), and 4.9% ($n = 212$) and 4.7% ($n = 47$), respectively.

2.2.5. Novel stimulus test

At the end of each month the aversiveness of an unfamiliar stimulus was measured using a procedure similar to Lensink et al. (2000b), on a day without handling or other testing. All 12 wombats were manoeuvred (17:00 h) from their dens and confined to the outdoor areas. Each was approached, in turn, until the handler was approximately 2 m away and then a spring-loaded umbrella, held at arm's length, was pointed towards the wombat's muzzle and opened. A rating (hereafter termed 'reaction score') was given for the distance the wombat moved in response to the opening of the umbrella, from its position immediately prior to the umbrella opening to where it eventually stopped: 0 = no movement; 1 = moved ≤ 100 cm away; 2 = moved 101–200 cm away; 3 = moved 201–300 cm away; 4 = moved 301–400 cm away; 5 = moved ≥ 401 cm away. To prevent habituation an umbrella of different size and colour was used each month.

Overt response, human approach and novel stimulus testing always commenced at 17:00 h because this was when the captive wombats began to emerge from their dens (Hogan et al., 2011a). To minimize any effects of novelty, the same handler conducted all overt response, human approach and novel stimulus testing and wore similar clothing over the entire testing period.

Table 1
Behaviours recorded for 12 captive *L. latifrons*.

Major behaviour	Sub-behaviour	Description of behaviour
Courtship and mating		Appetitive sexual behaviour and coitus between male and female
Digging		Removal of soil from (a) the digging chamber or (b) around enclosure furnishings, e.g. logs
Exploring		Investigation or examination of inedible objects
Feeding		Consumption of food items
Foraging		Searching and locating of food items
Grooming		Scratching of body part with hind limbs or rubbing against an object
	Body rubbing	A body part is pressed up again an object and rubbed back/forth, up/down or sideward
	Scratching	Back and forth motion of foot claws across a section of body in standing or sitting position
Lying resting		Lying awake (ears up, eyes open) in sternal recumbency, whilst doing nothing else
Sitting-at-rest		Sitting on hind quarters and front paws, whilst doing nothing else
Sleeping		Lying with neck recumbent on the ground, ears relaxed, eyes closed
Stereotypic behaviour		Unvarying, repetitive behavioural patterns with no apparent goal or function
	Gate pawing	Repeated fore-limb pawing/scratching at a closed gate/door
	Pacing	Repetitive walking in a straight line or figure 8 pattern or around the enclosure parameter (repeated > 20 times in a 10 min period)
	Wall climbing	Repeated attempts to climb up a wall in one of the sleeping dens; animal falls backward after each attempt
Sun-basking		Lying awake in direct sunlight in lateral recumbency, whilst doing nothing else
Minor behaviour		
Approach		Movement towards another wombat
Bite		A light nip of another wombat, usually along the flank or on the rump
Defaecation		Deposition of faecal pellets on the ground, dropped from the cloaca
Drinking		Consumption of water
Huddling		Occurs during sleep; two or more wombats will curl their bodies around each other
Retreat		Movement away from another wombat

2.3. ACTH challenge

Four wombats (2♂, 2♀) were randomly selected for an ACTH challenge in September 2005. They were individually restricted to a den in order to hand inject 10 mg/kg of Zoletil 100 (Virbac Laboratories, VIC, Australia) IM using a 21 ga needle. Following recumbency, the animals were transported to Rockhampton Zoo's veterinary surgery and maintained by mask on 2–5% gaseous isoflurane at a flow rate of 0.3 L/min oxygen. Approximately 2 mL of blood was collected from the cephalic vein using a 25 ga wing infusion set (Baxter Healthcare; Provet, Australia) with a 3 mL syringe and dispensed into 2 × 1.3 mL lithium-heparin tubes (Sarstedt AG & Co.; Provet, Australia). Heparinised samples were refrigerated at 4 °C until the end of sampling period when they were then centrifuged at 3000 rpm for 10 min. The plasma supernatant was removed, transferred to centrifuge tubes and stored at −20 °C until analysis. Blood samples were taken at −15, 0, 15, 30, 45, 60, 75, 90, 105 and 120 min in relation to the injection of ACTH (Synacthen, 250 μg IM) (Novartis Australia Pty Ltd.; Australia). After the procedure, each wombat was placed back into a den and monitored by video surveillance until they had fully recovered. Faecal samples were collected daily from each wombat for three days prior to and for seven days following ACTH administration.

2.4. Data collection: behaviour

A digital video surveillance system was used to monitor wombat behaviour. The system's component parts, installation and modes of operation have been previously described in detail (Hogan et al., 2009). In brief, 4 × 4 channel digital video recording cards were installed onto a desktop computer equipped with surveillance software (Skyview Model: PCSSUPER; OzSpy, Australia). This functioned as a central recording station to receive input from 16 cameras; eight colour-bullet cameras with inbuilt infrared (840 nM) LED's (Sony Model: N11368; OzSpy, Australia), one positioned within each sleeping den and eight day/night cameras (Sony Model: B480-312-TA; OzSpy, Australia) accompanied by separate custom-built infrared (926 nM) lights, positioned at opposite corners of each outdoor area.

An ethogram was constructed prior to formal behavioural analysis (Table 1). Each behaviour was defined as 'major' ($n = 11$) if it had a mean bout length ≥ 5 min or 'minor' ($n = 6$) if it had a mean bout length < 5 min (Table 1). For recording purposes, 'grooming' was divided into two sub-behaviours (body rubbing and scratching), whilst 'stereotypic behaviour' was divided into three (gate pawing, pacing and wall-climbing) (Table 1). Each wombat was observed for 12 × 24 h during each treatment period, totalling 1152 h of observations for each individual. Animal identification was aided by light reflective, patterned collars (Hogan et al., 2009). Behaviours were recorded using both continuous and 1-0 sampling methods, at 5 min intervals ($n = 288$). Major behaviours were measured in reciprocal units of time (i.e. whether it was the most predominate behaviour expressed within each interval), whilst minor behaviours were measured as frequencies (i.e. whether it occurred during each interval).

2.5. Data analysis

Residual data sets were tested for normal distribution and when the original scale violated the homoscedasticity

assumption a logarithmic transformation was used to achieve a normal distribution. Regression analysis was used to fit an exponential decay model ($S_t = S_0 10^{-RT}$; where s = score, R = slope and T = time in days) to the mean weekly interaction (1–5) and total scores, for each animal period combination; with half-lives being estimated from these results. For interactions 1–4, the models were statistically significant ($P < 0.01$) and a good fit, with adjusted r^2 between 0.58 and 0.99. Goodness of fit was weaker for interaction 5 (Following), with 7/24 individual models leading to non-significant regression ($P > 0.05$), and adjusted r^2 ranging from 0.00 to 0.63. The remaining 17 models had adjusted r^2 values from 0.32 to 0.91. Due to this weakness, only the estimated half-life of interaction 5 is reported, not its rate of decay.

A linear regression of $\log_{10}$ score was used to measure the difference in the mean rate of change of interactions 1–5 and total scores between the two handling trials (H1, H2). In addition, a linear regression of change in $\log_{10}$ faecal cortisol (from D_0 to D_3) was used to measure effects of treatment (T vs. B). Only the change in faecal cortisol (from D_0 to D_3) had to be transformed for analysis, pre- and post concentrations of faecal cortisol and all other data sets were found to be normally distributed. The calculation of statistical tests was carried out using the programs Minitab (Version 15, 2007) and SAS (SAS®/STAT, Version 8.2, 2001). Significance levels for all tests were set at $P \leq 0.05$ and means are given with standard errors (SE) unless otherwise noted.

2.5.1. Handling procedure

For the purposes of analysis, behavioural and physiological responses were subdivided into three categories: first handling trial (H1), second handling trial (H2) and no-handling (B). The combined responses of the handled animals ($n = 6$) in (a) periods 1 (Grps 2 and 3) and 2 (Grps 1 and 4) formed the category of H1 and (b) periods 3 (Grps 2 and 3) and 4 (Grps 1 and 4) formed the category of H2. Responses from animals receiving no-handling ($n = 6$) in all periods (1–4) were combined to form the category of B.

2.5.2. Overt response test

For interactions 1 and 2 the three ratings were combined into a mean composite score, as they were highly related ($r \geq 0.91$, $P < 0.01$, $n = 285$). Individual mean daily interaction (1–5) and total scores were calculated by taking the mean value of the three handing trials performed each day. Overall mean daily interaction (1–5) and total scores were calculated from the mean daily scores from all handled animals. An exponential rate of decay model ($S_t = S_0 10^{-RT}$) was used to calculate the overall mean rate of decay and half-lives of each interaction (1–5) and total score during H1 and H2. A mixed model ANOVA with REML estimation was used to test for differences between the two handling trials in total score and half-life. The same model was used for interaction scores, except that interaction score components were nested within each individual handling period combination. Between group and between subject effects were random, whilst sex, handling trial and sex * handling trial effects were fixed.

2.5.3. Human approach test

A mixed-model ANOVA with REML estimation was used to analyse the rate of change in FD, with mean weekly reductions per treatment (H1, H2 and B) being calculated on a collective rather than individual level. Between group and between subject effects were random, whilst sex, treatment and period effects were fixed.

2.5.4. Stressor test

Changes in plasma and faecal cortisol concentrations after ACTH injection and changes in faecal cortisol concentrations after stressor test exposure were analysed using a Student's paired t-test. Repeated measurements of changes in $\log_{10}$ faecal cortisol concentration were analysed using a mixed-model ANOVA with REML estimation. Between group and between subject effects were random, whilst sex, treatment, period and date effects were fixed. The relationship between pre- and post-stressor test cortisol secretion was tested using a Pearson's correlation.

2.5.5. Novel stimulus test

A mixed-model ANOVA with REML estimation was used to analyse the change in reaction score, with mean estimates being calculated on a collective rather than individual level. Between subject and group effects were random, whilst sex, period and handling trial effects were fixed.

2.5.6. Behavioural data

Major behaviour intervals were combined to determine the total time (in 5 min units), whilst minor behaviour frequencies were combined to determine the rate of occurrence over each 24 h day. Mean T and B treatment daily totals were calculated from an integration of data from six animals, from all sampling days ($n = 24$) of that treatment. Repeated measurements of behaviour were analysed using a mixed-model ANOVA with REML estimation. Between subject, within subject and group effects were random, whilst sex, period and treatment (T vs. B) effects were fixed.

3. Results

No significant sex, group or period effects were detected, so only treatment effects are presented.

3.1. Overt response test

There was a significant difference among interactions (1–5) in initial mean score in both H1 ($F_{4,10} = 27.08$, $P < 0.01$) and H2 ($F_{4,10} = 20.97$, $P < 0.01$). Interaction 3 (Massaging) had the highest mean initial score in both handling trials, whilst interaction 5 (Following) had the lowest (Table 2). Mean initial interaction (1–5) scores ($F_{1,10} \geq 36.72$, $P < 0.01$) and total score ($F_{1,10} = 75.19$, $P < 0.01$) varied significantly between the handling trials, with H2 values being less than those recorded for H1 (Table 2). Mean interaction (1–5) scores and total score significantly declined over H1 ($F_{1,10} \geq 15.07$; $P < 0.01$) and H2 ($F_{1,10} \geq 14.80$; $P < 0.01$). There was a significant difference between interactions (1–5) in half-life in both H1 ($F_{4,10} = 18.60$, $P < 0.01$) and H2 ($F_{4,10} = 26.61$, $P < 0.01$). Interaction 5 (Following) had

Table 2
Initial score, half-life (i.e. time taken to half score) and rate of decay of each human–animal interaction (1–5) scores and total score, for 12 *L. latifrons*, during two series of handling treatments (H1, H2).

Score	Initial H1	Initial H2	Half-life[a] H1	Half-life H2	Rate of decay[b] H1	Rate of decay H2
Interaction 1: Stroking	3.18 ± 0.12b	2.44 ± 0.11a	50.79 ± 3.45c	68.21 ± 2.79c	−0.00641 ± 0.00044a	−0.00453 ± 0.00045a
Interaction 2: Scratching	3.11 ± 0.13b	2.39 ± 0.10a	57.98 ± 4.11b	82.00 ± 3.58b	−0.00551 ± 0.00034c	−0.00377 ± 0.00035b
Interaction 3: Massaging	3.35 ± 0.11a	2.57 ± 0.11a	54.77 ± 6.51bc	86.61 ± 5.73b	−0.00560 ± 0.00028c	−0.00381 ± 0.00025b
Interaction 4: Noise	2.91 ± 0.13c	2.38 ± 0.09a	49.93 ± 2.97c	68.60 ± 2.69c	−0.00616 ± 0.00026b	−0.00450 ± 0.00024a
Interaction 5: Following	2.76 ± 0.11c	2.05 ± 0.10b	95.04 ± 7.67a	186.70 ± 7.66a		
Total	27.72 ± 10.06	21.51 ± 8.89	56.89 ± 3.17	78.56 ± 3.03	−0.00540 ± 0.00023	−0.00394 ± 0.00022
SED	0.148	0.169	6.395	8.624	0.0010	0.0004
P-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Different letters within the same column indicate significant differences between mean interaction scores.
[a] Time taken to half score (in days) using an exponential rate of decay model.
[b] Slope of exponential rate of decay model $S_t = S_0 10^{-RT}$; where s = score, R = slope and T = day; H1 = first handling trial; H2 = second handling trial.

the longest half-life in both handling trials, whilst interaction 4 (Noise) had the lowest in H1 and interaction 1 (Stroking) the lowest in H2 (Table 2). H2 interaction (1–5) scores ($F_{1,10} \geq 14.80$, $P < 0.01$) and total score ($F_{1,10} = 31.73$, $P < 0.01$) half-lives were significantly longer than those of H1. Individual and group changes in mean daily total score during each experimental period (1–4) are shown in Fig. 1. There was a significant difference between interactions

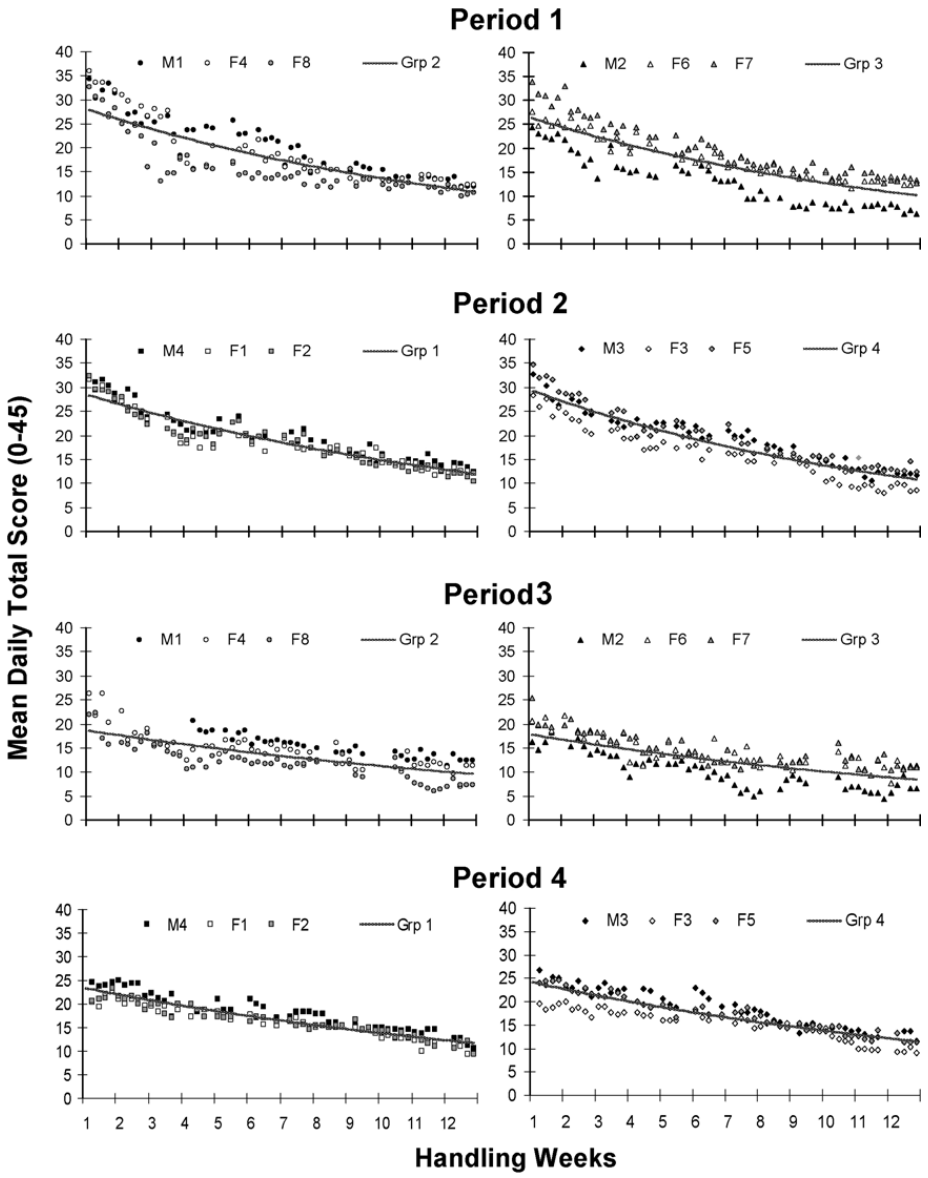

Fig. 1. Individual and group changes in mean daily total score for the wombats ($n = 6$) receiving the daily handling treatment in periods 1–4.

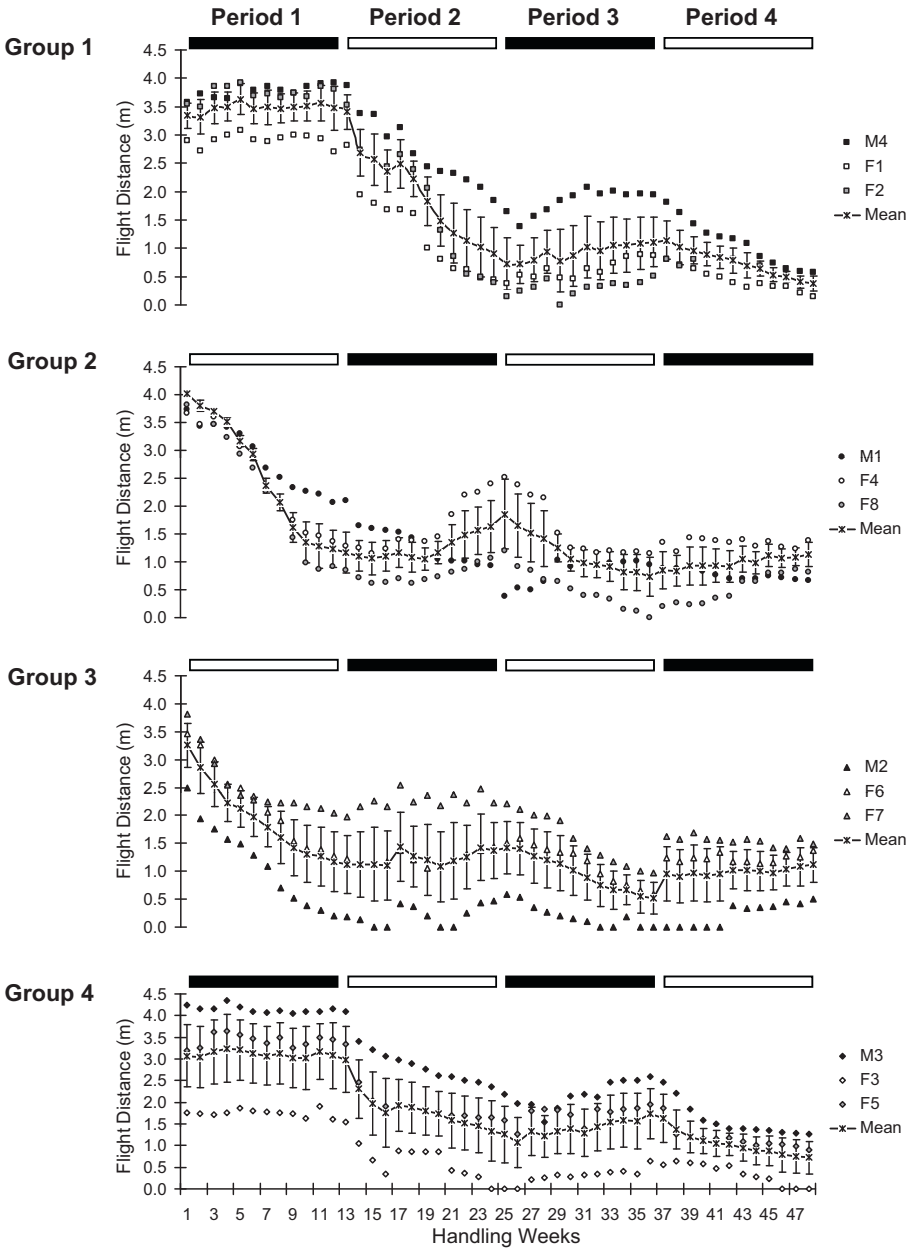

Fig. 2. Individual and group (mean ± SE) changes in mean weekly flight distance for the wombats in groups 1–4, in four separate handling periods. Key: (■) non-handling and (□) daily handling.

(1–4) in rate of decay in both H1 ($F_{3,10} = 25.01$, $P < 0.01$) and H2 ($F_{3,10} = 17.55$, $P < 0.01$). Rates of decay were faster for interactions 1 (Stroking) and 4 (Noise) than for interactions 2 (Scratching) and 3 (Massaging) (Table 2). Rates of decay for interaction (1–4) scores ($F_{1,10} = 15.07$, $P < 0.01$) and total score ($F_{1,10} = 27.30$, $P < 0.01$) during H2 were smaller than those recorded for H1.

3.2. Human approach test

Individual as well as group changes in FD during the four handling periods are shown in Fig. 2. There was a significant ($F_{2,31} = 111.61$, $P < 0.01$) change in FD over the handling trials, with the mean reduction per week being -0.16 ± 0.02 m for H1 and -0.06 ± 0.02 m for H2. The mean weekly rate of change in FD in H1 was greater than in H2 ($F_{1,31} = 32.02$, $P < 0.01$), with no change in FD occurring during B ($F_{1,31} = 0.90$, $P = 0.37$). Overall total change in mean FD over the handling trials was significant ($F_{1,31} \geq 12.38$; $P < 0.01$), decreasing from 3.31 to 1.27 m in H1 and from 1.64 to 0.67 m in H2.

3.3. Stressor test

3.3.1. ACTH challenge and cortisol response

Compared to pre-injection values (T_0, 0.40 ± 0.71 ng/mL; D_0, 6.82 ± 3.16 ng/g), administration of 250 μg Synacthen resulted in a significant increase in the mean (±SE) (a) plasma cortisol concentrations T_{30}, T_{45}, T_{75}, T_{90}, T_{105} and T_{120} after injection ($t \geq 4.07$, $P \leq 0.05$, $n = 4$), with maximal secretion (5.94 ± 2.34 ng/mL) occurring at T_{45} (Fig. 3A) and (b) faecal cortisol metabolite concentra-

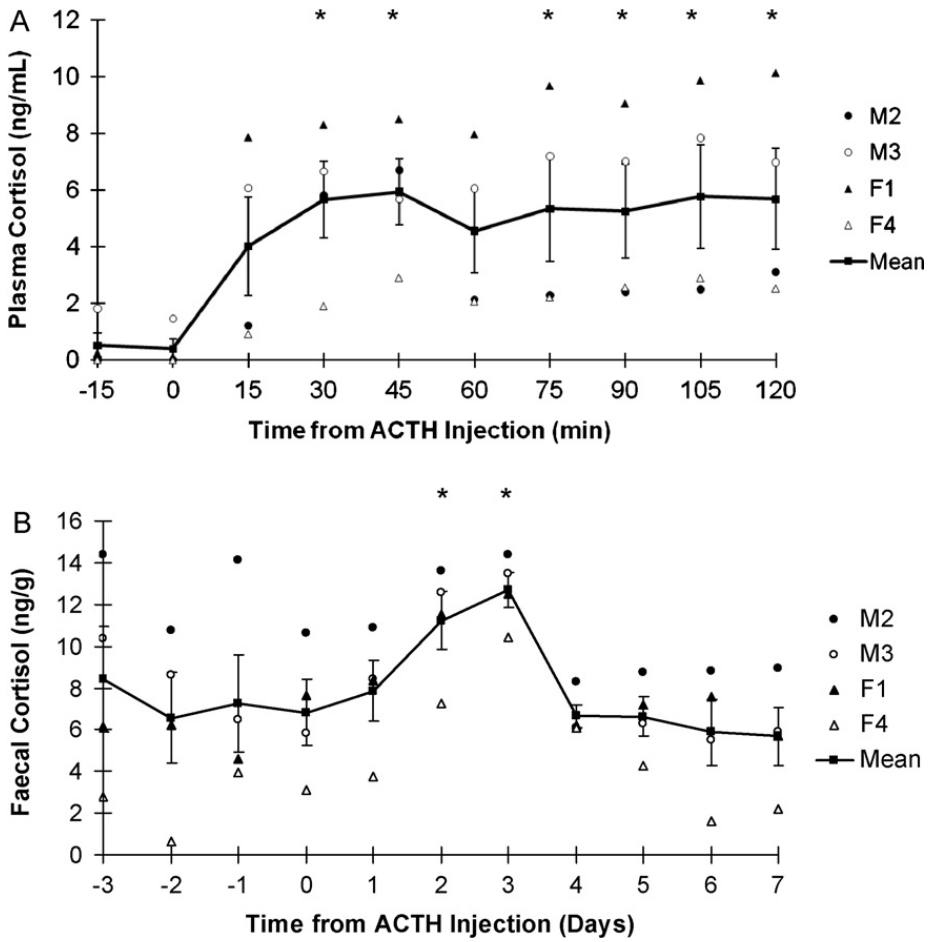

Fig. 3. Mean ± SE and individual *L. latifrons* (A) plasma cortisol concentration following administration of 250 μg of synthetic ACTH IM; (B) faecal cortisol metabolite concentrations following administration of 250 μg synthetic ACTH. *Indicates significant rises in plasma and faecal cortisol levels.

tions 2–3 days (D_2–D_3) after injection ($t \geq 5.14$, $P \leq 0.05$, $n = 4$), with peak secretion (12.71 ± 1.69 ng/g) occurring at D_3 (Fig. 3B). By D_4 faecal cortisol concentrations returned to pre-injection (D_0) levels. The mean (±SE) change in plasma and faecal cortisol concentration (Δ[C]; where $\Delta C = [C]C_X - [C]C_0$) when calculated over all significant time periods after ACTH injection was +5.26 ± 0.09 ng/mL and +5.15 ± 0.74 ng/g, respectively.

The comparison of mean initial (D_0) and post (D_3) faecal cortisol concentrations over 24 stressor tests is shown in Fig. 4. Mean post-stressor levels (13.53 ± 1.32 ng/g) of cortisol were significantly ($t = 4.90$, $P < 0.01$, $n = 24$) greater than mean pre-stressor levels (10.84 ± 0.95 ng/g) and were strongly correlated with initial values ($r = 0.85$, $P < 0.01$, $n = 24$). There was no treatment (H1, H2 and B) difference in the change in faecal cortisol between D_0 and D_3 ($F_{2,249} = 0.17$, $P = 0.84$).

3.4. Novel stimulus test

The collective monthly changes in reaction score over the four handling periods for all wombats ($n = 12$) are shown in Fig. 5. There was a significant ($F_{3,8} = 65.92$, $P < 0.01$) change in reaction score over the experimental period, with mean score decreasing by 3.56 (from 4.12 to 0.56). Mean reductions were −2.39 ± 0.39 for period 1, −1.75 ± 0.08 for period 2, −1.21 ± 0.06 for period 3 and −0.85 ± 0.12 for period 4. There was no difference in reaction score change between H1, H2 and B ($F_{1,8} = 2.23$, $P = 0.17$).

3.5. Behavioural response to handling

In response to handling, mean time spent exploring ($F_{1,30} = 6.62$, $P = 0.02$) and stereotyping ($F_{1,30} = 8.18$,

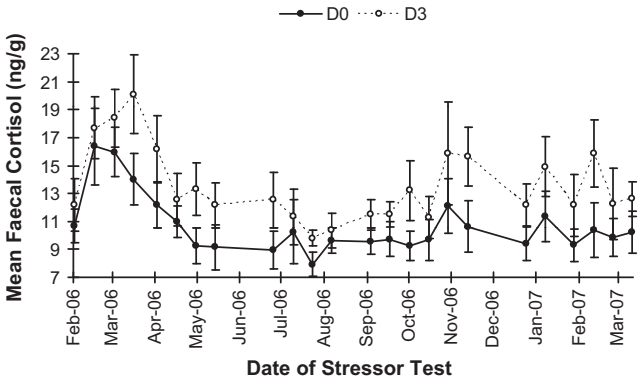

Fig. 4. Initial (D_0) (–●–) and post-stressor (D_3) (–○–) changes in *L. latifrons* ($n = 12$) mean faecal cortisol metabolite concentration over 24 stressor tests.

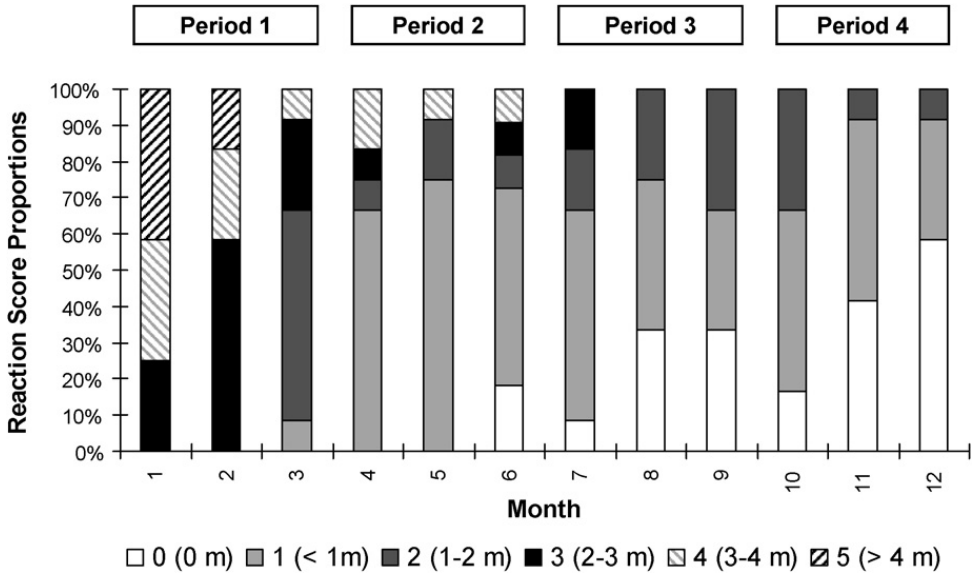

Fig. 5. Collective changes in the reaction scores of 12 *L. latifrons* in response to monthly novel stimulus tests.

$P < 0.01$) increased by 11.1% and 32.5%, respectively, over the 24 h period. The increase in stereotyping was of gate pawing ($P < 0.01$), rather than pacing ($P = 0.49$) or wall-climbing ($P = 0.70$). There was no significant handling effect ($F_{1,30} \leq 1.90$, $P \geq 0.18$) on the other nine major behaviours (Table 1). Mean daily occurrence of defecation ($F_{1,38} = 5.91$, $P = 0.02$) and retreat ($F_{1,38} = 105.99$, $P < 0.01$) also increased in response to handling, by 44.4% and 200.0%, respectively, over the 24 h period. Drinking also tended to increase in response to handling (15.4%; $F_{1,38} = 2.83$, $P = 0.10$), but there were no other handling effects on the occurrence of other three minor behaviours (Table 1; $F_{1,38} \leq 1.09$, $P \geq 0.30$).

4. Discussion

The results of this study indicate that regular handling reduced the wombats' avoidance of and reactivity towards the human handler, but had no effect on their responses to routine husbandry or novel stimulation. As in farm animals (Hargreaves and Hutson, 1990a; Hemsworth and Barnett, 2000; Petherick et al., 2009) daily handling resulted in a reduction in flight distance in response to human approach, which is the first demonstration of such handling effects on a wild-caught, captive marsupial. Daily handling also reduced the behavioural reactivity of the wombats to close human proximity and physical contact. This is consistent with earlier studies, where regular positive handling was successfully used to reduce the defensive, escape and/or fear responses of sheep (Hargreaves and Hutson, 1990b,c), pigs (Gonyou et al., 1986), chickens (Jones, 1994), rabbits (Podberscek et al., 1991), cattle (Lensink et al., 2000a,b) and primates (Clay et al., 2009), to their human handlers.

The decrease in avoidance and reactivity may have arisen from habituation to the handler's presence. Habituation is the process whereby an animal becomes non-reactive to a certain stimulus as a result of its repeated occurrence (Hemsworth and Barnett, 1986). It has been successfully applied before to reduce the emotional reactivity of a number of species (Barnett et al., 1994; Breuer et al., 2003; Clay et al., 2009; Lensink et al., 2000b). Over time, the wombats may have learnt to associate the handler with non-harmful interactions, becoming accustomed to approach/physical contact through repeated exposure and thus showing diminished reactivity to later encounters with the handler.

However, it is more likely that the reduced responsiveness of the wombats was due to the development of 'learned helplessness', a phenomenon where a lack of response-reinforcement during handling resulted in non-responsiveness. Although learned helplessness was originally associated with inescapable electric shocks (Seligman and Beagley, 1975), this mechanism could also be relevant to inescapable contact. In this study, the wombats were submitted to forced contact (i.e. confined to their outside pens and repeatedly approached until touch was achieved) and there was no reinforcer for flight or defensive behaviour. Hence, they may have learned that such behaviour had no effect on their situation and therefore, developed non-responsiveness. Jezierski et al. (1999), Simpson (2002) and Lansade et al. (2004) have all advocated the learned helplessness mechanism in foals exposed to forced human contact, and our behavioural results support the learned helplessness theory. Normally, habituation to the handler results in a decrease in behaviours linked to stress, such as vocalisations, defecation, self directed (e.g. grooming) and abnormal (e.g. stereotypic) behaviour (Fureix et al., 2009). However, in the present study, there was an increase in stress-related behaviours (e.g. defecation and stereotypies) in response to handling, indicating that the wombats did not habituate to the human handler. Furthermore, the handling treatment did not lead to a reduction in cortisol response to the stressor.

The differing rates of decay for the five interaction scores also support the theory of learned helplessness. Interactions 1–3 (Stroking, Scratching and Massaging) had the highest mean initial scores and the faster mean rates of decay. Initially, these three interactions would have probably induced the greatest reactivity, as these types of

physical contact were new to the wombats. The wombats also had no choice but to submit to these three interactions and the faster decays in score may have been due to the wombat learning they had no control, i.e. they gave up trying to avoid these interactions the fastest. In contrast, interaction 5 (Following) was the one interaction in which the wombats had some control, as they could choose to move away from the handler, and the handler never got closer than 0.5 m. This interaction had the lowest mean initial score and the longest half-life. The low initial score was probably due to the wombats being accustomed to being followed (from daily incursions of keepers into their enclosures for health checks) and the slow rate of decay may have been the result of the wombats exerting some control (i.e. choosing to repeatedly move away) during the interaction.

It is acknowledged that our behavioural results must be interpreted cautiously because of the small sample size and lack of corresponding physiological data. Whilst our samples sizes were small compared to farm animal experiments, we did investigate handling effects on the world's largest *in situ* wombat population. Also, the collection of samples for adrenocortical steroids during the handling procedure would not have been possible without highly invasive procedures (e.g. blood sampling), as defecation during handling was not reliable. It is also acknowledged that the reduction in responsiveness may have been amplified due to the fact that the wombats were exposed (1) as groups, rather than individually to the handling procedure and (2) to multiple tests, each involving some form of human contact. The wombats, may have (through social learning) responded more quickly to the handling procedure, due to their exposure in groups (Range et al., 2009). However, previous research has shown that social animals display increases in behavioural arousal/stress in response to isolation from conspecifics (Boissy and Le Neindre, 1990; Jones and Harvey, 1987). Due to the gregarious nature of *L. latifrons*, separation for testing would have caused isolation-induced distress, and thus it was concluded that testing as a group, rather than individually, would cause less behavioural interference. It is possible that the four tests used in this study had some residual effects on each other, as all were repeated and involved some form of human contact. We attempted to reduce these residual effects by temporal separation (i.e. each test was performed on a day with no other testing) and only the overt response test involved a large amount of human–animal interaction (300 min/month; whereas the other tests involved < 15 min/month).

This study was the first to validate the measurement of faecal cortisol metabolites in *L. latifrons*. Synacthen (250 μg) resulted in an almost immediate (T_{15}) rise in plasma cortisol secretion (peak at T_{45}) and a corresponding rise in faecal cortisol metabolites three days later. Collectively these results confirm the biological validity of the cortisol EIA used in this study. For all wombats, cortisol concentrations were higher following weighing, a result similar to other studies involving weighing of animals (Broom and Johnson, 1993; Simpson, 2002). These findings indicate that the wombats experienced some form of stress (psychological) and/or arousal in association with this procedure. Without corresponding behavioural data (e.g. time to complete weighing, how much coaxing necessary) it is difficult to know how the wombats perceived the weighing procedure. It is possible that they found certain aspects of the procedure to be aversive and stressful, which would account for the elevation in cortisol. Alternatively, it is also possible that the cortisol increases were a response to elevated heart rate as a result of general arousal (either positive or negative) and/or physical activity (DEFRA, 2005). Given that this is the first report of cortisol levels in *L. latifrons*, we cannot be certain whether our values are indeed high for this species and therefore, indicative of a significant stress event.

There was no reduction in the size of the cortisol spike following weighing, despite 24 repetitions of this procedure. Some farm animals have been shown to habituate to certain management procedures, showing declines in GC secretion with repeated exposure (Andrade et al., 2001; Hargreaves and Hutson, 1990a). However, other studies have found that GC concentrations do not decline, despite regular handling, if the procedure involves forced movement and restraint (Lensink et al., 2000a; Pedersen, 1994). It is possible that the wombats did not show the same decline in GC secretion, as seen in some farm animals, because wild animals do not habituate as readily to humans as domestic species (Maciejowski and Zieba, 1982). Alternatively, regular handling may not have been an effective method for lowering GC responses to forced human contact. For instance, the decline in wombat behavioural reactivity was probably specific to the handler only and not a generalized response to all human beings. Thus forced contact with unfamiliar humans (e.g. volunteers) during weighing could have been fear-inducing (Waiblinger et al., 2004). Alternatively, other factors such as arousal and activity may have been responsible for the cortisol elevation. It is reasonable to assume that the wombats would have been used to the weighing procedure as they underwent weighing every two weeks during the experimental period and had been routinely weighed by zoo staff for several years prior to the experiment commencing; thus the reason behind the constant elevation in cortisol following weighing is unclear. What can be concluded with some certainty is that the cortisol response was probably not due to novelty. There was a gradual reduction in the degree of reaction to the novel stimulus test over the experimental period, with repeated exposure reducing the novelty value of the umbrella and the arousing properties of the test.

5. Conclusions

This study has demonstrated that regular handling can lower wombats' reactivity to, and avoidance of, a human handler. Despite evidence that these reductions may be due to learned helplessness, the observed behavioural responses to handling suggest that 'gentling programs' have the potential to improve the captive management of wombats (and perhaps other marsupials) by making the animals easier to catch and handle. This study has also reported the successful validation and application of a method for monitoring faecal cortisol metabolites in *L. latifrons* via EIA. Our results however, did not support

the hypothesis that regular handling would lower GC responses following forced human contact; a constant elevation in cortisol was observed in all animals following weighing. Since this is the first report of cortisol levels in *L. latifrons* and no corresponding behaviour data accompanied the weighing procedure the reason for this consistent elevation remains obscure. The most probable explanation is that it was due to stress associated with the procedure, rather than novelty, as the wombats habituated quickly to a novel stimulus test used in this study.

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