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Influence of environmental conditions and facility on faecal glucocorticoid concentrations in captive pygmy rabbits (Brachylagus idahoensis)

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Abstract

*The objective of this study was to determine if housing conditions, specifically pen size and soil enrichment, had an effect on faecal glucocorticoid concentrations in the endangered pygmy rabbit (*Brachylagus idahoensis*). The success of the captive breeding programme has been limited, so one hypothesis is that chronic stress due to sub-optimal housing conditions may be responsible for poor fecundity. Faecal glucocorticoid concentrations were assessed in 50 females housed among several pen types at two breeding facilities. The highest glucocorticoid concentrations were found in females housed in 0.37 m^2 crates as compared to enclosures ranging from 0.96 to 75 m^2 in size. Results also indicated that enrichment of enclosures with soil had a significant influence on adrenal activity, based on a reduction in glucocorticoid excretion for females moved from non-soil pens to those with soil. Last, a significant facility effect on glucocorticoid concentrations was observed, suggesting that factors other than housing influenced adrenal activity in these rabbits. In conclusion, based on measurements of faecal glucocorticoids, pygmy rabbits are best managed in enclosures that contain soil for digging burrows. Pen size had little effect on stress hormones, except for crates where limited space and/or absence of soil was associated with higher glucocorticoid concentrations. These results underline the importance of monitoring glucocorticoid concentrations in captive breeding programmes to identify optimal husbandry and management practices.*

Keywords: *animal welfare, captive breeding, environmental enrichment, faecal glucocorticoids, husbandry, pygmy rabbit*

Introduction

For zoo animals, the number of potential stressors in captive environments can be numerous, and the effects often are species-specific. Identifying what captive conditions are associated with high levels of stress is critically important for effective population management and ultimate success of captive breeding programmes. The biological stress response is defined as a physiological reaction to an animal's perception of threat or uncertainty in its environment (Seyle 1976; Sapolsky 2002). One of the main components of the stress response is activation of the hypothalamic-pituitary-adrenal axis, which results in the release of glucocorticoids (ie stress hormones). These steroids cause the mobilisation of energy and the temporary suppression of non-essential functions, such as the reproductive and immune systems, so that an animal can respond adaptively to a threat. Long-term exposure to a stressor, however, can turn temporary suppression into chronic inhibition, which has negative consequences for reproduction and health (Sapolsky *et al* 2000; Sapolsky 2002; Young *et al* 2004; Boonstra 2005). In many species, temporary increases in glucocorticoids can be used to identify acute stressors, whereas long-term elevations of glucocorticoids are more likely to indicate the existence of a chronic stressor (Young *et al* 2004; Reeder & Kramer 2005). For endangered species management, an optimal way to assess adrenal activity is to quantify the amount of glucocorticoids excreted in faeces under various conditions. The non-invasive nature of this approach allows evaluation of adrenal activity without handling the animals, which can compromise the accurate assessment of stress (Millspaugh & Washburn 2004). In addition, faecal hormone data provide a pooled estimate of hormone production from the previous 12–24 h, thus averaging across acute fluctuations in secretion (Millspaugh & Washburn 2004).

Between 2002 and 2012, a captive breeding programme for the endangered Columbia Basin pygmy rabbit (*Brachylagus idahoensis*) was established to produce animals for restoration to native habitats from which they had been extirpated (Hays 2001, 2003; United States Fish and Wildlife Service [USFWS] 2007). Since little was known about the biology of pygmy rabbits when the programme began, the three captive breeding facilities (Washington State University, Oregon Zoo and Northwest

Trek Zoological Park) used adaptive management over the course of a decade to continually improve survival and reproduction. A research programme also was established to characterise the reproductive and stress physiology of pygmy rabbits to aid this conservation effort (USFWS 2007). One finding was that female pygmy rabbits that did not conceive excreted significantly higher concentrations of faecal glucocorticoids compared to those that produced surviving offspring (Scarlata *et al* 2011), which suggested that stressful conditions in captivity may be decreasing reproduction and, potentially, welfare.

In the wild, pygmy rabbits spend the majority of their time within approximately 30 m of their burrows (Wilde 1978; Gahr 1993; Janson 2002). Although some females travel up to 300 m away from their burrow, most use approximately 3,000 m^2 as their core home range during the breeding season (Gahr 1993). Captive enclosures for pygmy rabbits at the breeding facilities ranged from 0.37 to 75 m^2 in size, limiting the range to only a fraction of that in the wild. Advocates of animal welfare commonly cite pen size as a potential captive stressor due to its limiting effect on natural behaviours, such as exploring, foraging, hiding and mating (Clubb & Mason 2007; Morgan & Tromborg 2007). Thus, one goal of this study was to determine the effect of pen size on stress hormone levels in pygmy rabbits at two of the breeding facilities, Washington State University and Oregon Zoo, USA. Another factor that can affect stress and reproduction in captivity is enrichment, and several studies have shown that low reproductive success is linked to stress ensuing from a lack of appropriate environment enrichment (Mellen 1991; Carlstead & Shepherdson 1994; Wielebnowski *et al* 2002). Pygmy rabbits dig their own burrows, including underground natal burrows, so deep, loose soil is a critical feature of their natural habitat (Green & Flinders 1980; Rachlow *et al* 2005; Elias *et al* 2006). At the breeding facilities, most captive enclosures for pygmy rabbits were filled with 0.5 to 1.0 m of soil each year. However, because soil can harbour infectious agents and is difficult to remove and replace, concrete pens without soil were built at one facility to stop the spread of coccidiosis and mycobacteriosis (USFWS 2007). During the breeding season, these concrete pens were half-filled with soil to allow females to dig natal burrows, but there was not enough soil to create deep, natural tunnel systems. In addition, when larger pens were not available, some pygmy rabbits were held in small crates without soil. Therefore, we also explored the relationship between rabbit stress levels and soil enrichment, a housing element that can provide additional space for movement, opportunities for burrowing, as well as a favorable microenvironment (Morgan & Tromborg 2007).

The overall objectives of this study were to use non-invasive hormone monitoring to explore relationships between faecal glucocorticoid concentrations and two potential sources of captivity stress in the pygmy rabbit, pen size and soil availability. We hypothesised that female pygmy rabbits would have lower concentrations of faecal glucocorticoids when housed in larger, soil-filled pens than in smaller pens with no soil.

Materials and methods

Study animals and facilities

Animals in this study (n = 50) were all captive-born, adult females (aged 1–3 years) housed at one of two facilities: Washington State University, Pullman, WA (WSU, Facility 1) or Oregon Zoo, Portland, OR (OZ, Facility 2). Pygmy rabbits were provided water, balanced grain-forage rabbit pellets and a variety of fresh greens (big sagebrush [*Artemisia tridentata tridentate*], lettuce [*Lactuca sativa*], dandelion [*Taraxacum officinale*], Italian parsley [*Petroselinum crispum var neapolitanum*] and clover [*Trifolium repens*]) daily. The pre-breeding season was defined as January–February, the breeding season as March–June, and post-breeding season as October–December. For this study, the non-breeding season included samples only collected between October and February (Scarlata *et al* 2011).

Together, the two facilities used seven pen types: 1) crates; 2) oval pens; 3) circular pens; 4) rectangular pens; 5) non-soil pens; 6) half-soil pens; and 7) carport pens (Table 1, Figure 1). Crates consisted of 60 × 60 × 45 cm (length × width × height) stainless steel cages surrounded by hardware cloth. Oval and circular pens were constructed from galvanised steel water tanks filled with 0.5–1.0 m of compacted soil. Rectangular pens were also filled with 0.5–1.0 m of soil, but were surrounded by wire-mesh siding and a plastic barrier to keep soil in. Non-soil and half-soil pens were rectangular enclosures with a concrete floor covered in wood-shavings. During the breeding season, the non-soil pens were half-filled with less than 0.5 m of soil that sloped down to the centre of the pen; these are referred to as half-soil pens during this period. The carport pens were constructed from a carport surrounded by wire-mesh and filled with several mounds of soil (~1 m in height). All pens were exposed to natural fluctuations in temperature and photoperiod. In addition, all pens were covered by a corrugated greenhouse roof or carport roof and so they were partially shielded from changes in precipitation. For this study, oval pens and crates were used only during the non-breeding season, whereas carport pens were used only during the breeding season.

With the exception of crates and carport pens, all enclosures were enriched with one plastic nest-box, one or two artificial burrows (7.6-cm diameter plastic drainage tubes) and sagebrush branches, so the number of potential hiding places was relatively consistent among these pens. Carport pens each had two nestboxes and at least eight artificial burrows, whereas each crate contained an unsealed plastic nest-box and Dri-Dek mats (Kendall Products, Naples, FL, USA) that allowed faeces and urine to fall into the tray below. Carport pens housed one female and one male throughout the breeding season. For the other enclosures, females were housed singly to avoid aggressive interactions, except during 1–6 day pairings when a male was introduced into the female's pen. During the breeding season, keepers maintained detailed records of pairings, births and emergence of

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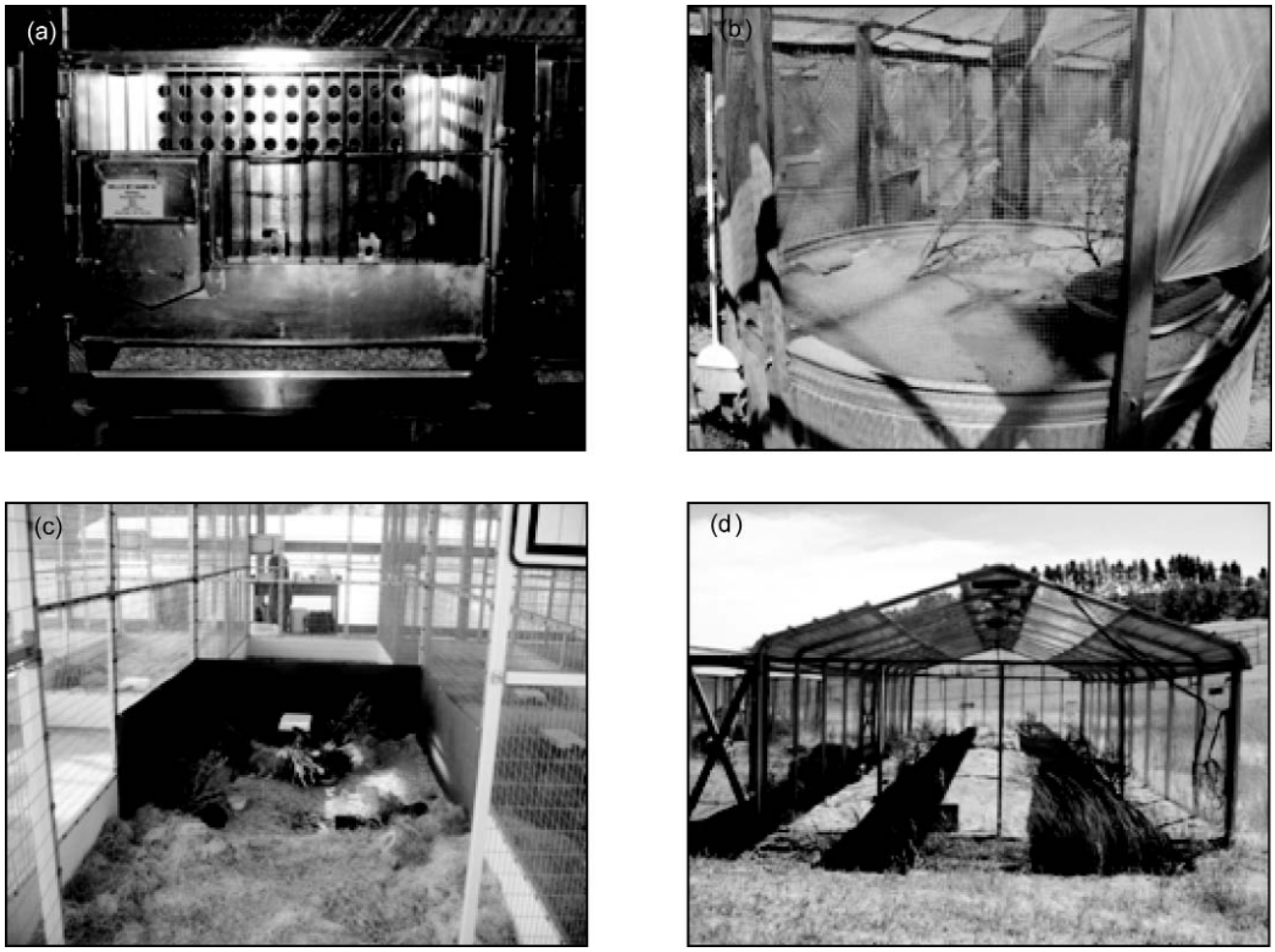
Table 1 Characteristics of pens used to house captive pygmy rabbits (*Brachylagus idahoensis*) at the two facilities.

Pen type	Size (m^2)	Soil	Facility	Non-breeding	Breeding
Crate	0.37	None	1	n = 7	
Oval	0.96	0.5–1.0 m	1	n = 7	
Rectangular	4.0	0.5–1.0 m	1	n = 6	n = 6
Circular	4.7	0.5–1.0 m	1	n = 17	n = 20
Circular	4.7	0.5–1.0 m	2	n = 7	n = 15
Non-soil	6.7	None	2	n = 12	
Half-soil	6.7	0.5 m in half of pen	2		n = 11
Carport	75	1.0 m mounds	1		n = 3

Facility 1: Washington State University, Pullman, WA, USA; Facility 2: Oregon Zoo, Portland, OR, USA and sample sizes (n) collected during each season.

Figure 1



Pen types (a) crate, (b) circular soil, (c) non-soil and (d) carport used to house pygmy rabbits (*Brachylagus idahoensis*).

young from the natal burrows, which occurred approximately 14 days after birth (Elias *et al* 2006). This project and all husbandry and animal care techniques were approved by Institutional Animal Care and Use Committees at Washington State University and the Smithsonian Conservation Biology Institute, and followed the animal care guidelines of the American Society of Mammalogists.

Study design

Faecal collections took place between January 2006 and December 2008. During the non-breeding season, fresh faecal samples were collected 2–4 times a week for 2–3 months from four housing groups at Facility 1 and two housing groups at Facility 2. During the breeding season, samples were collected 4–7 times a week for 3–4 months from three housing groups at Facility 1 and two housing groups at Facility 2. Pen types and sample sizes for each season are summarised in Table 1.

Fresh faecal samples (< 24 h old) were collected around the same time every day and keepers avoided the collection of those contaminated with urine. Sample age was determined by observation, colour and/or condition. Samples older than 24 h are lighter in colour due to drying and were usually covered in dirt. Most samples were collected within 12 h of defaecation because pen cleaning removed all day-old samples. When males and females shared pens, female faecal samples were verified by observation of defaecation and/or comparison of progesterone and testosterone levels. If hormone analyses could not verify the sex, the samples were removed from all analyses. Samples during pregnancies were excluded.

Pen size comparison

During January and February, several females at Facility 1 were housed in crates (0.37 m^2; n = 6) or oval pens (0.96 m^2; n = 7) for several months to allow workers to empty and refill future breeding pens with soil. Then, in late February and March, these females were moved to larger breeding pens (≥ 4 m^2) at Facility 1. To compare changes in glucocorticoid concentrations within females moved between different pen sizes, samples were collected for 1–2 months from females housed in the smaller pens (crates and oval pens) and for 3–4 months after being moved to a larger pen (≥ 4 m^2). In addition, a control group of females (n = 9) that were moved in late February from circular pens to identically sized circular pens were monitored before and after the move. Post-movement faecal collections were longer to account for acclimation to new pen environments during the first few weeks; samples during pregnancies were excluded.

Soil enrichment comparison

At Facility 2, ten females were housed in non-soil pens during January and February. Then, during late February or early March, females were moved into identically sized pens that were half-filled with soil for the breeding season. Faecal samples were collected for 1–2 months in the non-soil pens and for 3–4 months in the half-soil pens. As a control, seven females housed in circular soil pens at Facility 2 were moved to identical circular soil pens and faecal samples were collected before and after being moved as described above. Post-movement faecal collections were longer to account for acclimation to new pen environments during the first few weeks; samples during pregnancies were excluded.

Faecal sample processing

Each faecal sample consisted of ~20–50 faecal pellets that were placed in a re-sealable plastic bag and immediately frozen at –20°C until processed. Samples were lyophilised (Labconco Lyophilizer, Kansas City, MO, USA) and crushed into a fine powder. For steroid extraction, 0.1 g of dried faecal material was added to 5 ml of 90% ethanol using a method similar to that described by Brown *et al* (1994b) except that vortexing for 40 min was used instead of boiling. Samples were centrifuged for 20 min at 1,300 g and the supernatant extract was poured into a second set of tubes. The remaining faecal pellet was re-suspended in 5 ml of 90% ethanol, re-vortexed for 1 min and re-centrifuged at 1,300 g. Extracts were combined, evaporated to dryness and re-suspended in 1 ml phosphate buffer (0.2 M NaH_2PO_4, 0.2 M Na_2HPO_4, 0.15 M NaCl; pH 7.0). Steroid extraction efficiency averaged 91% (range 82–99%) as determined by recovery of tritiated cortisol added to faeces before extraction. Samples were diluted 1:3 in buffer and glucocorticoid metabolites were quantified using a glucocorticoid enzymeimmunoassay (EIA) validated for pygmy rabbits (Scarlata *et al* 2011).

The glucocorticoid EIA used a polyclonal cortisol antibody generated against cortisol-3-CMO (R4866, 1:20,000 dilution; C Munro, University of California, Davis, USA), a horseradish-peroxidase conjugated cortisol label and cortisol standards (Young *et al* 2004). The sensitivity of the assay was 3.90 pg per well and intra- and inter-assay coefficients of variation were less than 10%. The glucocorticoid EIA was validated by demonstrating: 1) parallelism between binding inhibition curves of dilutions of faecal extract and the cortisol standard curve; and 2) significant recovery of exogenous steroid added (> 90%) to faecal extracts before analysis. Physiological validation of the glucocorticoid assay was demonstrated by showing a significant increase ($P < 0.05$) in glucocorticoid concentrations above baseline within 48 h after transfer of an animal to a new facility (n = 8 rabbits), a presumed stressful event (Scarlata 2010).

Statistical analysis

Faecal glucocorticoid concentrations are reported as the overall and baseline mean (± SEM). Overall means were calculated for each female and included all data points analysed for an individual within the specified time-period. Individual baseline means were calculated for each female using an iterative process where all peak values two standard deviations above the mean were excluded and means were recalculated until extreme values were excluded (Brown *et al* 1994a). Baseline means provide an estimate of basal hormone secretion that excludes temporary increases in hormone secretion due to reproductive or stressful events. Assumptions of normality were checked by examining normal probability plots and calculating a Shapiro-Wilks statistic. For all analyses, signifi-

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cance was assessed at the 0.05 level. All statistical analyses were conducted with the aid of Microsoft Excel 2003 (Seattle, WA, USA) and SPSS Version 15.0 for Windows (SPSS Inc, Chicago, IL, USA).

For the initial 'housing factors' analysis, a general linear model was used to investigate the effects of pen size, soil enrichment, facility, age and animal on baseline glucocorticoid concentrations among females housed in different pen types during the non-breeding season. Data from the non-breeding season were used because they included the most variety of pens in terms of soil enrichment and pen size. Because this test was significant, a *post hoc* LSD multiple comparison analysis was conducted to determine what housing groups differed significantly and in what direction. For this analysis, both full factorial and additive models were explored, but none of the models had significant interaction terms. In subsequent tests, each significant factor was analysed using both an 'among-females' and 'within-females' comparative framework that minimised the effects of the other factors. For example, pen size and soil enrichment comparisons were conducted using only females housed at one of the facilities, whereas facility comparisons used only females housed in identical pens.

For the 'among-females' analysis of pen size, glucocorticoid overall and baseline means were compared among females housed in different sized pens at Facility 1 during the pre-breeding season and breeding season using a general linear model. For the 'within-females' analysis of pen size, glucocorticoid overall and baseline means were calculated for each female (n = 20) before and after being moved to a new pen and comparisons between the two pen types were conducted using a paired one-tailed *t*-test.

For the 'among-females' analysis of soil enrichment, glucocorticoid means were compared between females housed in either circular pens or half-soil pens at Facility 2 using a two-tailed Student's *t*-test. Since all pen types during the breeding season contained soil, this analysis explored whether differences in the quantity of soil provided had an effect on adrenal activity during the breeding season. For the 'within females' analysis of soil enrichment, glucocorticoid means were calculated for each female (n = 17) before and after being moved from a non-soil to a half-soil pen. Comparisons between the two pen types were conducted using a paired one-tailed *t*-test.

For the analysis of facility differences, a student's *t*-test was used to compare faecal glucocorticoid overall and baseline mean concentrations among females housed in circular pens at the two facilities. To account for possible seasonal effects, glucocorticoid data were compared during three time-periods: 1) pre-breeding season; 2) breeding season; and 3) post-breeding season. In addition, one female was transferred from one facility to another and monitored during consecutive breeding seasons, so a two-tailed *t*-test was used to compare daily glucocorticoid concentrations between the two facilities.

Table 2 General linear model results predicting the effect of facility, pen size, soil enrichment, age, and animal on faecal glucocorticoid concentrations in pygmy rabbits (*Brachylagus idahoensis*) at two facilities from 2006–2008.

Variable	β	SE (β)	*t*	*P*-value
Intercept	33.16	16.39	2.02	0.048
Soil (no = 0, yes = 1)	–26.09	8.82	–2.96	0.005
Pen size	–5.59	2.36	–2.37	0.022
Facility	30.53	11.44	2.67	0.010
Age	–0.87	6.75	–0.13	0.898
Animal	–0.12	0.27	–0.44	0.660

Results

Effect of housing factors

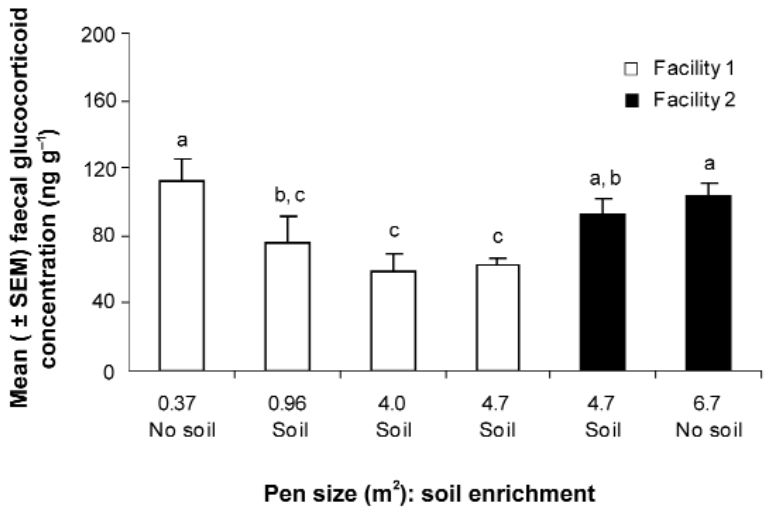
An initial exploration of factors that could affect adrenal activity found that pen size, soil enrichment and facility, but not age or animal, had a significant effect on faecal glucocorticoid baseline concentrations (n = 55, $F = 6.553$, $P < 0.001$, $R^2 = 0.401$; Table 2). Glucocorticoid concentrations were lower in soil-enriched pens ($P = 0.005$), larger pens ($P = 0.022$), and Facility 1 ($P = 0.010$). A *post hoc* analysis determined that females housed in oval, circular and rectangular pens had significantly lower glucocorticoid baselines than females housed in crates and non-soil pens (Table 2, Figure 2). Females housed in crates showed the highest glucocorticoid baselines, and because crates were the smallest pens available and lacked soil enrichment, both of these factors were explored in more depth using a comparative framework.

Effect of pen size

Comparisons among the four pen sizes used during the non-breeding season at Facility 1 showed that there was a relationship between pen size, and overall ($F = 6.08$, $P = 0.002$, n = 35) and baseline ($F = 6.67$, $P = 0.001$, n = 35; Figure 2) concentrations of glucocorticoids. However, when crates were removed from the analysis to eliminate the confounding effect of soil enrichment, the pen size effect disappeared for overall ($F = 0.11$, $P = 0.90$, n = 28) and baseline ($F = 0.82$, $P = 0.45$, n = 28) means. Comparisons among the three pen sizes used during the breeding season at Facility 1 also revealed no pen size effects on glucocorticoid overall ($F = 1.66$, $P = 0.21$, n = 33) or baseline ($F = 1.38$, $P = 0.27$, n = 33; Figure 3) means.

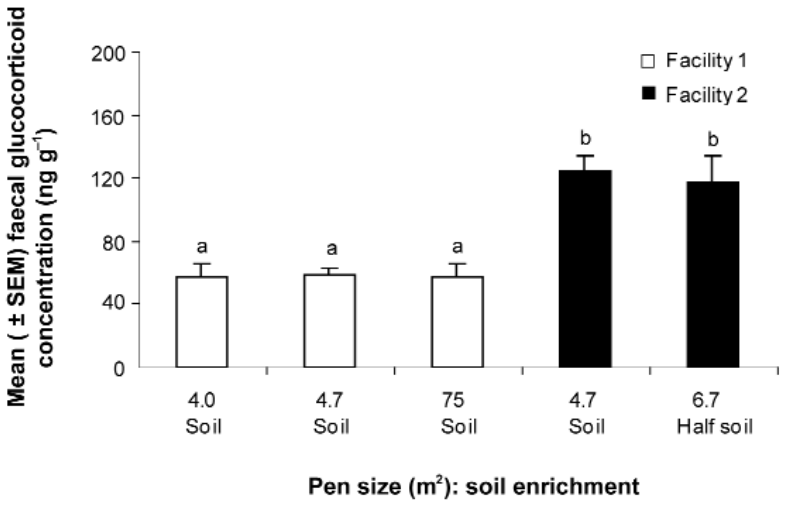
Glucocorticoid excretion was also evaluated in several females that were moved between pens. For the control group (animals moved between identical circular pens), no changes in glucocorticoid overall or baseline means were observed after transfer (overall mean: $t = 1.31$, $P = 0.11$, n = 9; baseline mean: $t = 1.58$, $P = 0.08$, n = 9; Figure 4). For the crate group (animals moved from crates to larger pens), higher glucocorticoid overall mean and baseline concentrations were observed in females while housed in

Figure 2



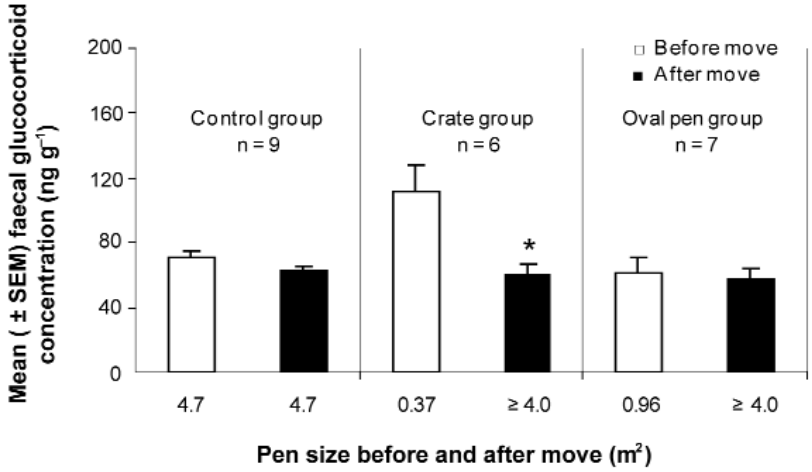
Mean (± SEM) faecal glucocorticoid baseline concentration of female pygmy rabbits (*Brachylagus idahoensis*) housed in different pen types during the non-breeding season, ordered by increasing pen size (m^2) and separated by facility (Facility 1: Washington State University, Pullman, WA, USA; Facility 2: Oregon Zoo, Portland, OR, USA).
Superscripts based on *post hoc* comparisons among pen types, means with the same letter do not differ significantly ($P > 0.05$).

Figure 3



Mean (± SEM) faecal glucocorticoid baseline concentration of female pygmy rabbits (*Brachylagus idahoensis*) housed in different pen types during the breeding season, ordered by increasing pen size (m^2) and separated by facility (Facility 1: Washington State University, Pullman, WA, USA; Facility 2: Oregon Zoo, Portland, OR, USA). Superscripts based on *post hoc* comparisons among pen types, means with the same letter do not differ significantly ($P > 0.05$).

Figure 4



Pen size comparison of mean (± SEM) faecal glucocorticoid baseline concentrations of female pygmy rabbits (*Brachylagus idahoensis*) before and after being moved from one pen size to another at Facility 1: Washington State University, Pullman, WA, USA. For the control group, 16 females at Facility 1 were moved from circular pens to identical circular pens.
* Indicates significant differences within females between baselines for 1–2 months before and 3–4 months after the move ($P < 0.05$).

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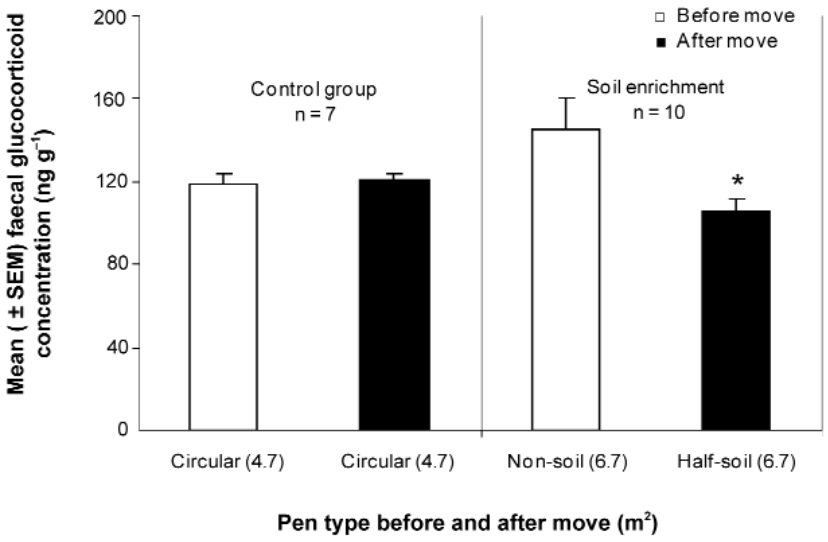
Figure 5



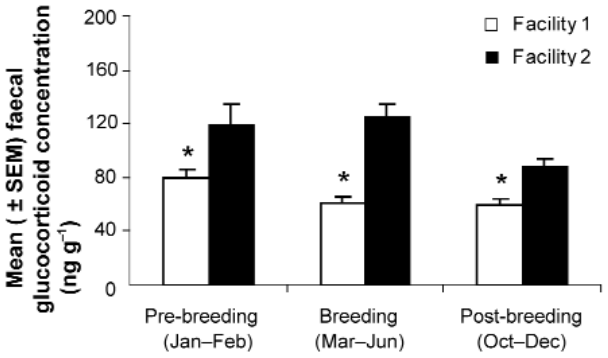
Soil enrichment comparison of mean (± SEM) faecal glucocorticoid baseline concentrations of female pygmy rabbits (*Brachylagus idahoensis*) before and after being moved from one pen to another at Facility 2: Oregon Zoo, Portland, OR, USA. For the control group, seven females at Facility 2 were moved from circular pens to identical circular pens.
* Indicates significant differences within females between baselines before and after the move ($P < 0.05$).

crates than after they were moved to the larger pen types (overall mean: $t = 4.94$, $P = 0.002$, n = 6; baseline mean: $t = 4.405$, $P = 0.003$, n = 6; Figure 4). However, because crates did not contain soil, this factor may have confounded the results for the crate analysis, and thus differences may not be indicative of a direct pen size effect. For the oval pen group (animals moved from oval pens to larger breeding pens), no differences were found in glucocorticoid overall or baseline means (overall mean: $t = 0.298$, $P = 0.388$, n = 5; baseline mean: $t = 0.266$, $P = 0.399$, n = 5; Figure 4). In addition, three females moved from pens ≤ 4.0 m^2 to large carport pens were monitored. Although the sample size was too small for a powerful statistical test, glucocorticoid concentrations were elevated in the large carport pens (overall mean: 98.92 [± 11.66]; baseline mean: 57.74 [± 8.68] ng g^{-1}, n = 3) as compared to the smaller pens (overall mean: 55.70 [± 11.36]; baseline mean: 40.67 [± 4.67] ng g^{-1}, n = 3) (overall mean: $t = -2.29$, $P = 0.07$, n = 3; baseline mean: $t = -2.01$, $P = 0.09$, n = 3).

Effect of soil enrichment

A comparison between females housed in half-soil and circular pens at Facility 2 revealed that the amount of soil enrichment did not influence glucocorticoid overall mean ($t = 1.75$, $P = 0.094$, n = 24) or baseline ($t = 0.568$, $P = 0.576$, n = 24; Figure 3) concentrations. However, comparisons within females at Facility 2 showed that higher glucocorticoid concentrations were observed in females while they were housed in non-soil pens as compared to half-soil pens (overall mean: $t = 1.98$, $P = 0.040$, n = 10; baseline mean: $t = 2.25$, $P = 0.026$, n = 10; Figure 5). By contrast, there were no changes in glucocorticoid means within females in the control group (overall mean: $t = 0.379$, $P = 0.359$, n = 7; baseline mean: $t = 0.118$, $P = 0.45$, n = 7; Figure 5).

Figure 6



Facility differences in mean (± SEM) faecal glucocorticoid baseline concentrations by season for pygmy rabbits (*Brachylagus idahoensis*) housed at Facility 1: Washington State University, Pullman, WA, USA and Facility 2: Oregon Zoo, Portland, OR, USA between 2006 and 2008. * Indicates significant differences between facilities ($P < 0.05$).

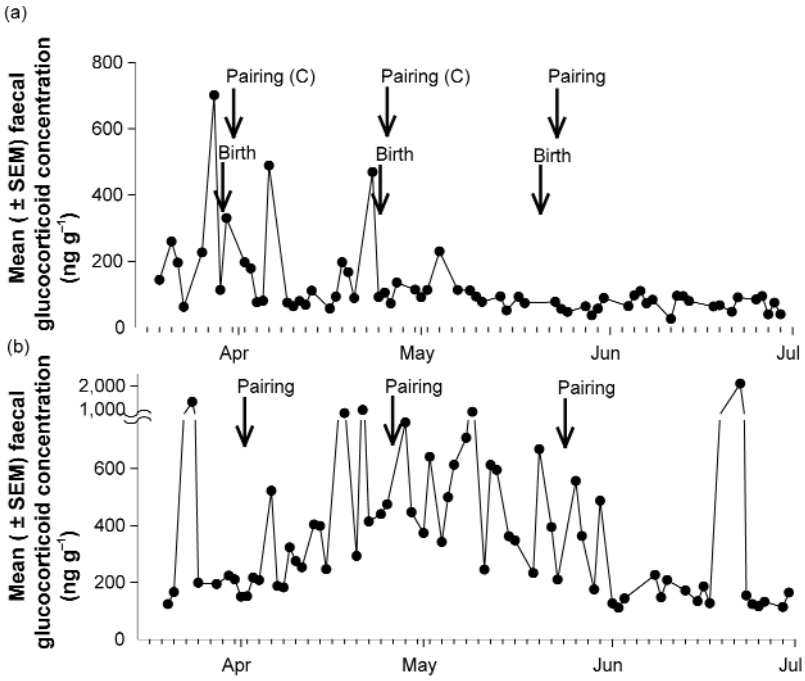
Facility differences

An analysis of differences between females housed at the two facilities in identical circular pens found that glucocorticoid overall and baseline means were higher at Facility 2 than Facility 1 during all three time-periods (Figure 6, Table 3). In fact, during the breeding season, glucocorticoid baselines at Facility 2 (124.89 [± 9.42] ng g^{-1}, n = 15) were twice as high as those at Facility 1 (61.28 [± 3.57] ng g^{-1}, n = 20). Figure 7 illustrates this difference through a representative glucocorticoid profile of a pygmy rabbit that was transferred from

Table 3 Mean (± SEM) differences in faecal glucocorticoid concentrations (ng g^{-1}) between female pygmy rabbits (*Brachylagus idahoensis*) housed in circular pens (4.7 m^2) at two facilities (Facility 1: Washington State University, Pullman, WA, USA; Facility 2: Oregon Zoo, Portland, OR, USA).

Season	N	Overall mean (± SEM)				Baseline mean	
		Facility 1	Facility 2	*t*	*P*-value	*t*	*P*-value
Pre-breeding	16	96.23 (± 10.50)	160.23 (± 21.49)	2.87	$P = 0.01$	3.12	$P = 0.01$
Breeding	28	56.22 (± 3.55)	185.52 (± 19.81)	6.88	$P < 0.001$	5.97	$P < 0.001$
Post-breeding	16	83.18 (± 8.63)	211.27 (± 56.96)	4.29	$P < 0.001$	3.95	$P = 0.001$

Figure 7



Faecal glucocorticoid profiles of a female pygmy rabbit (*Brachylagus idahoensis*) while housed at (a) Facility 1: Washington State University, Pullman, WA, USA and (b) Facility 2: Oregon Zoo, Portland, OR, USA during subsequent breeding seasons (2007–2008). Dates of pairings and conceptions (C) are indicated by arrows and pairings that resulted in a pregnancy are indicated as 'Pairing (C)'. (Note: Y-axes are different).

Facility 1 to Facility 2, and monitored during two consecutive breeding seasons. For this female, glucocorticoid concentrations at Facility 1 (overall mean: 118.85 [± 14.09] ng g^{-1}; baseline mean: 78.65 [± 3.06] ng g^{-1}) were consistently lower than when she was housed at Facility 2 (overall mean: 367.64 [± 32.13] ng g^{-1}; baseline mean: 178.58 [± 7.39] ng g^{-1}; $t = -14.14$, $P < 0.001$, df = 84). This female successfully produced three litters at Facility 1, but failed to produce any at Facility 2, despite numerous pairings.

Discussion

This was the first study to examine the effect of captive environmental factors on adrenal stress status in an endangered rabbit species. Overall, this study suggests that housing factors such as soil enrichment and facility-specific conditions may affect welfare in pygmy rabbits, as evidenced by changes in faecal glucocorticoid concentrations. Animal welfare refers to how well an individual copes with its environment, both mentally and physically; failure to cope can lead to detrimental physiological and behavioural changes, including compromised health and reproduction (Broom 1991; Hewson 2003). Although our study was not designed to show a direct cause and effect relationship between stress hormone levels and animal welfare, previous studies suggest that elevated glucocorticoid concentrations can be a physiological indicator of stress, with long-term elevations indicating an inability to cope with environmental stressors (Barnett & Hemsworth 1990). In addition, previous studies in female pygmy rabbits have shown that elevated glucocorticoids were associated with decreased reproductive success, which is an indicator of reduced welfare (Scarlata *et al* 2012). However, the possibility remains that changes in glucocorticoids reflect physiological responses to the environment that do not involve altered mental states.

Across two breeding facilities, six pen sizes ranging from 0.37 to 75 m^2, and three soil treatments (non-soil, half-soil, and soil), we found that rabbits exhibited higher faecal glucocorticoid concentrations when kept in crates (0.37 m^2) with no soil, compared to the other pen types. Since pen size was confounded with soil treatment for this type of pen, we cannot determine with certainty if it was the small pen size,

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lack of soil enrichment or a combination of the two that was responsible for this effect. In other species, confinement in smaller pens is associated with increases in glucocorticoid excretion and behavioural indicators of stress, such as increased stereotypies, reduced activity levels, retarded growth, decreased incidence of mating behaviour, or increases in aggression (Line *et al* 1987; Carlstead *et al* 1993; Cassinello & Peters 2000; Shepherdson *et al* 2004; Morgan & Tromborg 2007; Moriera *et al* 2007). We suspect that for pygmy rabbits there may be a point where limited space severely compromises the ability to perform natural behaviours and cope with stressors, but further increases in enclosure size do not cause a detectable decrease in glucocorticoid levels. For example, the transfer of pygmy rabbits between small oval pens and larger pens failed to show a noticeable change in glucocorticoid concentrations. Consistent with these findings, Crockett and co-workers (1993, 2000) found that moderate changes in cage size for female longtailed (*Macaca fascicularis*) and pigtailed macaques (*M. nemestrina*) were not significantly related to glucocorticoid excretion. Although our initial 'housing factors' analysis found that pen size was a significant factor in predicting glucocorticoid concentrations, our 'pen size' analyses did not find a such an effect at the individual level. Thus, our data suggest that beyond small crates, an increase in pen size did not have a substantial effect on adrenal activity in pygmy rabbits.

On the other hand, our analyses found that enrichment of a female's enclosure with soil did have a significant effect on adrenal activity. Females moved from non-soil enclosures to soil enclosures exhibited a decrease in faecal glucocorticoid concentrations. The quantity of soil, however, was less influential; there was no difference in glucocorticoid excretion observed in rabbits housed in half-soil vs full-soil pens. These data suggest that the mere presence of soil during the breeding and non-breeding seasons can potentially lead to reduced glucocorticoid excretion. Many studies have explored the effects of environmental enrichment on animal welfare, such as the addition of hiding structures, novel objects, nesting materials or new foraging opportunities (Carlstead & Shepherdson 1994), but few have looked specifically at soil as a possible source of enrichment (Swaisgood & Shepherdson 2005). Given the fossorial nature of pygmy rabbits, soil is an important component of their environment as it provides opportunities for natural behaviours such as digging, exploring and hiding. Soil also plays a vital role in pygmy rabbit reproduction because females give birth in underground natal burrows (Rachlow *et al* 2005; Elias *et al* 2006). In addition, the presence of soil in captive environments may reduce slipping and provide a more comfortable substrate than plastic mats or concrete. Several studies have shown that animals prefer flooring that provides softer or more solid footing (Morgan & Tromborg 2007). For example, golden hamsters (*Mesocricetus auratus*) and rats (*Rattus norvegicus*) showed a behavioural preference towards cages with solid floors and bedding over stainless steel cages with wire-mesh flooring (Arnold & Estep 1994; Manser *et al* 1995), while pigs (*Sus scrofa domesticus*) showed preference towards soil-like substances such as peat, compost or sawdust, as compared to wood bark, straw and concrete (Beattie *et al* 1998). An added benefit to soil enrichment is the positive effect it could have on reintroduction efforts, by facilitating the development of skills needed to survive after release into the wild. For example, for black-footed ferrets (*Mustela nigripes*), another fossorial mammal, animals reared in large, semi-natural pens where they could dig their own burrows had higher survival after reintroduction than ferrets reared in indoor cages with artificial burrows (Vargas & Anderson 1999).

Beyond pen size and soil enrichment, our study suggests that other aspects of housing and husbandry may influence adrenal hormones in captive pygmy rabbits. For example, moving females from small pens to much larger carport pens was associated with an increase in glucocorticoid concentration means, although animal numbers for this experiment were small (n = 3). The carport pens were the largest pens available and represented the closest approximation to the natural home range of pygmy rabbits. Clubb and Mason (2007) reported that a species' natural home range is a good predictor of how an animal responds behaviourally to different enclosure sizes and thus we predicted that glucocorticoid concentrations would be the lowest in the largest pens. Their analysis, however, was conducted only on carnivore species. One theory is that because the pygmy rabbit is a prey species, its response to the larger pens may have been associated more with limited hiding opportunities than the actual size of the enclosure. Large carport pens in our experiment had only two nest-boxes, one for the female and one for the male, so the number of hiding opportunities did not increase with size. Several studies have found that the addition of hiding spaces to enclosures can result in decreased glucocorticoid secretion as well as reduced expression of stereotypic behaviours (Carlstead *et al* 1993; Shepherdson *et al* 2004). Thus, for this as in other species, it may not be the quantity, but rather the quality of space that is most important, including the availability of species-appropriate hiding and burrowing opportunities (for a review, see Morgan & Tromborg 2007). Another contributing factor may have been that the large carport pens housed a male and female together at all times, so social interactions could have influenced glucocorticoid production. Pygmy rabbits in the wild have limited social interactions except during the breeding season, and were often aggressive to pen-mates in the captive breeding facility (Adams *et al* 2001; Elias *et al* 2006). In other species, social variables such as aggression, dominance rank, proximity to predators or conspecifics and abnormal social groups have been shown to alter glucocorticoid excretion (Creel *et al* 1996; Creel 2005; Morgan & Tromborg 2007). Thus, future studies should be conducted to determine the optimal social environment for pygmy rabbits.

Table 4 Characteristics of husbandry practices at two captive breeding facilities for pygmy rabbits (*Brachylagus idahoensis*) (Facility 1: Washington State University, Pullman, WA, USA; Facility 2: Oregon Zoo, Portland, OR, USA).

Facility 1	Facility 2
Weekly nest-box cleaning	Daily nest-box cleaning
Pens swept of faeces 2–5 times a week	Pens swept of faeces 5–7 times a week
Keepers lean into cage to clean	Keepers stand inside cage to clean
Proximity to cages: 1–2 h per day	Proximity to cages: 3–4 h per day
Keepers near pens for short time-periods	Keepers work near pens for several hours
Trapping frequency: < once per month	Trapping frequency: 1–3 times per month
Animals trapped in nest-box or live-trap	Animals trapped by hand or in nest-box
Seasonal enrichment of pens with sagebrush bushes; barren in the winter	Cages enriched with pots of fresh sagebrush clippings year-round

Of particular interest was the difference observed in faecal glucocorticoid concentrations between the two captive breeding facilities. Although they differed somewhat in the availability of different pen types, a comparison among females housed in identical pens at both facilities found that rabbits at Facility 2 excreted significantly higher glucocorticoid concentrations, both at the population level and in individual females that were transferred between facilities. Differences in glucocorticoid concentrations may be related to differences in husbandry or other aspects of the captive environment (Table 4). In general, Facility 2 had a more intensive cleaning schedule, which may have reduced mortality and illness from disease, but increased stress in these animals. Routine cage cleaning removes odours associated with marking a territory or signalling reproductive status. Cleaning can also introduce novel stimuli, such as new nest-boxes or nesting materials that may be perceived as stressors (Morgan & Tromborg 2007). Among group-housed male mice (*Mus musculus*), the removal of scent marks increased aggression, which was subsequently reduced when some of an animal's scent-marked nesting materials were transferred to the newly cleaned cages (van Loo *et al* 2000). Additionally, husbandry routines such as cage cleaning or feeding can result in a forced proximity between animals and their keepers, something that may be particularly stressful for a prey species (Morgan & Tromborg 2007). In other species, increased exposure to the public, numbers of visitors or keepers, and/or time spent in proximity to humans has been shown to increase physiological and behavioural indicators of stress (Wielebnowski *et al* 2002; Carlstead & Brown 2005). For example, Wielebnowski *et al* (2002) found that faecal glucocorticoid concentrations in clouded leopards (*Neofelis nebulosa*) were positively associated with the number of keepers per facility. Future studies should look at how pygmy rabbits are affected by changes in husbandry routines or interactions with keepers.

Animal welfare implications and conclusion

One of the challenges faced by captive breeding programmes is determining what housing conditions will maximise animal reproduction and well-being. The design of captive enclosures must take into account species-specific physiological and behavioural needs and balance these with costs, space availability and ease of care. For example, although larger enclosure sizes may be more likely to promote natural behaviours, they may be more difficult to clean or observe animal health. We found that with pygmy rabbits, the highest glucocorticoid concentrations were associated with females housed in crates, where both small pen size and absence of soil may have influenced the expression of natural behaviours and contributed to elevated concentrations of glucocorticoids. It was also clear that the presence of at least some soil was beneficial to lowering stress levels. Therefore, we offer several management recommendations for housing pygmy rabbits in captivity. First, keepers should make every attempt to provide soil to rabbits, even during the non-breeding season. Second, there was no stress-linked advantage to housing rabbits in large carport enclosures, so space allocated for rabbit housing could be focused on providing rabbits between 1 and 7 m^2 of space. Large carport areas could be divided into several medium-sized pens, thus avoiding the need to use crates during the non-breeding season. Last, future studies should explore the effect of different husbandry routines and social factors on the stress levels of captive pygmy rabbits so that mitigating strategies can be developed to promote animal welfare.

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