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Research

Quantification of faecal glucocorticoid metabolites as a measure of stress in the rock hyrax *Procavia capensis* living in an urban green space

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Despite the abundance of rock hyrax *Procavia capensis* within South Africa's urban areas, there is not much information available about the effect of anthropogenic activities on rock hyrax wellbeing. To determine the potential impact of anthropogenic disturbance on adrenocortical activity, we conducted an ACTH challenge to identify a suitable enzyme-immunoassay (EIA) for measuring faecal glucocorticoid metabolite (fGCM) concentrations in the rock hyrax. This study identified an 11β-hydroxyaetiocholanolone EIA as the most suitable assay in this regard. The fGCM levels measured, indicate the physiological stress response in different rock hyrax populations, living in an area with varying degrees of anthropogenic activity (low, medium, high) within the National Botanical Garden of Pretoria, South Africa. The species' habituation to human numbers (weekly mean number of people) was examined by determining individual flight initiation distance (FID). Seasonally, there were overall higher fGCM concentrations in late spring compared to winter. The fGCM concentrations, although not significantly different but possibly biologically relevant, in the section with the lowest anthropogenic disturbance were ~10% higher compared to those in the section with medium disturbance, and ~20% higher compared to those in the section with the highest disturbance. Animal FID did not differ significantly between seasons but they did differ significantly between sections, and decreased in accordance with fGCM concentrations. The non-invasive approach established in this study provides a foundation for assessing rock hyrax wellbeing, and can help better understand how anthropogenic presence is perceived as a stressor in this species.

Keywords: camera traps, faecal glucocorticoid metabolites, flight initiation distance, rock hyrax, urban wildlife

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Introduction

As urbanisation continues to transform pristine habitats into grey and green spaces, several wildlife populations have become urban adapters in order to survive (McKinney 2006). An abundance of food and shelter, as well as a decrease in predators, entails larger population sizes and an increase in negative human–wildlife interactions (Fernández-Juricic and Tellería 2000, Coleman and Barclay 2011, Hubert et al. 2011, Duduś et al. 2014). As a result, certain wildlife species are labelled as pests (Wiid and Butler 2015), but several studies also define wildlife survival and habituation to the urban landscape as positive (Coleman and Barclay 2011, Hubert et al. 2011, Duduś et al. 2014). Unregulated human activities can lead to several deleterious effects on urban populations, including a change in habitat use, animal behaviour, metabolic equilibrium/homeostasis and individual physiology, while increasing human–animal conflict and biodiversity loss (Müllner et al. 2004, McKinney 2006, Rogala et al. 2011, Steven and Castley 2013, Larson et al. 2016).

The rock hyrax (*Procavia capensis*) is an African urban exploiter inhabiting urban areas of South Africa, where shelter and food are abundant and where natural predators have been eradicated (Wiid and Butler 2015). In this type of environment, it is often considered a pest species for gardens and cultivated land, requiring authorities to use control measures, like electric fences or removal of colonies (Kershenbaum et al. 2011, Wiid and Butler 2015, Visser and Wimberger 2016). Although this is an abundant species in metropolitan areas in South Africa (Wiid and Butler 2015), there is not much information about how anthropogenic factors influence its physiology and behaviour (Gill et al. 1996, Koren et al. 2002). Different techniques, including camera trapping, 'flight initiation distance' (FID) (Ydenberg and Dill 1986) and endocrine measurements, can be useful to assess changes in animal behaviour and physiology in response to human activities (Gill et al. 1996, Sheriff et al. 2011, Buxton et al. 2018). Monitoring the distribution and magnitude of human presence in urban green spaces is an important factor to quantify the different impact activities might have in a defined space (Miller et al. 2017). The use of camera traps to assess visitor numbers and animal behaviour is an efficient technique that reduces observer bias and provides continuous data without disturbing the animals or visitors (Buxton et al. 2018). In many instances, human presence is perceived as a predator stimulus, possibly leading to behavioural changes, which can be quantified to assess the impact of humans (Gill et al. 1996). While performing routine activities in the presence of people may indicate habituation, avoidance or flight behaviour is generally seen as a reaction to threats (Lima and Bednekoff 1999). The FID depends on a) the closeness of the subject to a threat (Blumstein 2003), b) the nature and direction of the threat, the prey's physical fitness (Stankowich and Blumstein 2005) and c) the distance from shelters (Engelhardt and Weladji 2011). Repeated exposure to a low-risk stimulus usually reduces an animal's behavioural response (Shulgina 2005, Rankin et al. 2009), a process frequently observed in species living in and around human settlements (Samia et al. 2015, Mbise et al. 2020). Wildlife usually react to disturbance by altering flee episodes and vigilance, as well as feeding time and habitat selection, subsequently reflected in energy loss (Fernández-Juricic and Tellería 2000, Larson et al. 2016). Measuring FID is also functional for comparisons between 'disturbed' and 'undisturbed' (or 'less disturbed') populations belonging to the same species, whose differences should be ascribed to variation in habituation levels (Blumstein and Runyan 2004). The FID measurements are often used by wildlife and conservation managers to set intermediary zones to avoid or minimize human impact on animals (Rodgers and Smith 1997, Fernández-Juricic et al. 2005).

Stress can be defined as a state that develops in response to any external or internal stimulus that is, or is perceived as, a threat (Selye 1936, Touma et al. 2003). To restore the stress-induced disruption of homeostasis, an individual relies on behavioural responses and the neuroendocrine and immune systems (Moberg 2000). An initial step in the physiological stress response is the activation of the hypothalamic–pituitary–adrenal (HPA) axis, resulting in the production and secretion of glucocorticoids (GCs) into the bloodstream (Möstl and Palme 2002, Sheriff et al. 2011). Short-term activation of the HPA axis can be beneficial in nature, leading to the return in homeostasis by mobilizing energy, stimulating the immune system and participating in behavioural and physiological modifications activated to restore the body's equilibrium (Touma and Palme 2005, Dhabhar 2009). Conversely, the long-term activation of the physiological stress response can lead to several deleterious effects such as compromising fitness and reproduction rates, behaviour and cognition, suppressing immune function and degrading body tissues (Möstl and Palme 2002, Ganswindt et al. 2010, Sheriff et al. 2011). After being released into the bloodstream and unfolding its effect, GCs are metabolised and excreted via urine or faeces (Möstl and Palme 2002, Hodges et al. 2010). Due to the importance of GCs in the physiological stress response and the numerous deleterious effects of homeostatic overload, GCs can cause in an individual; researchers often monitor GCs and their metabolites as a robust marker of stress experienced by an individual or population (Sheriff et al. 2011). Quantifying GCs or their metabolites can be realized invasively or non-invasively (Ganswindt et al. 2010). Invasive and semi-invasive methods, usually involving blood or saliva collection, have mostly been used in a captive setting where animals can be frequently and safely accessed (Sheriff et al. 2011, Ganswindt et al. 2012). For free-roaming animals, non-invasive methods involving collecting alternative matrices such as faeces or urine, and more recently, feathers or hair, have become popular (Ganswindt et al. 2003, Sheriff et al. 2011, Palme et al. 2013). However, GC metabolism is species- and sex-specific (Touma et al. 2003, Palme et al. 2005), and the composition of GC metabolites in faeces can change at different rates over time post-defecation depending on environmental conditions (Palme et al. 2005). Therefore, if when the freshness of faecal samples collected can not be guaranteed by the direct observation of the deposition act, it is fundamental

to define the stability of fGCM concentration post defecation to interpret respective hormone values reliably (Touma and Palme 2005). Thus, it is fundamental to carefully validate assays for non-invasive hormone monitoring, ensuring its applicability for the species-specific hormone matrix of interest and as a reliable quantification of respective glucocorticoid metabolites (Ganswindt et al. 2012). So far, however, endocrine studies involving rock hyraxes have only been conducted using hair as a hormone matrix, comparing androgen and GC levels in relation to social rank (Koren et al. 2002, 2008, 2019). Therefore, we aimed to examine the suitability of five enzyme immunoassays (EIAs) for monitoring adrenocortical function in rock hyraxes, thereby investigating the relationship between anthropogenic disturbance and faecal glucocorticoid metabolite (fGCM) concentrations in urban rock hyraxes. More specifically, the study aimed to a) determine stress-related physiological responses in captive rock hyraxes by performing an adrenocorticotropic hormone stimulation test (ACTH challenge test), b) examine the rate at which fGCM concentrations change over time post-defecation and c) examine the relationship between the quantified seasonal variation of fGCM concentrations and different levels of anthropogenic disturbance in rock hyraxes, living in an urban green space.

Material and methods

ACTH challenge test

Study sites and animals

For this study, we conducted an ACTH challenge test at the South African National Biodiversity Institute's (SANBI) Mokopane Biodiversity Conservation Centre (24°10′17.77″S, 29°01′02.44″E) in October 2019, utilizing one male (2 years, 3.4 kg) and two females (F1: 9 years, 3.6 kg; F2: 8 years, 3.7 kg) rock hyraxes. Prior to the research, the three rock hyraxes formed a social group within a single enclosure. To acclimatise the study animals to individual housing, individuals were separated into adjacent enclosures three weeks before the anticipated challenge test, allowing for visual, olfactory and auditory contact. Each enclosure (3.50 × 1.70 m) contained vegetation and enrichment (climbing platforms/rocks/shelter), ensuring the necessary enrichment and resource availability for each animal. Experienced animal keepers provided daily fresh food and water and clean enclosures. The conservation staff conducted regular checks to ensure that no potential impacting behaviours (e.g. pacing, excessive vocalisation) occur.

ACTH challenge test

Following the acclimatisation to single housing, we conducted an ACTH challenge. To determine baseline fGCM concentrations in the study animals, the enclosures were checked for fresh faecal samples every 3 h between 06:00 and 24:00 h for 3 days and a half (3–5 fresh samples were collected per individual each day), while for 1 day and a half the collections were more frequent (every 2 h, between 06:00 and 24:00 h). A sample was considered fresh when the consistency was soft, the colour was a shiny dark brown, the sample was still warm and the smell was persistent. On day five, the animals were trapped and placed into an isoflurane gas-induction chamber (Longley 2008) for anaesthesia (ISO 0.5%, 0.1–0.2 ml l^{-1}) for 10 min. Once sedated, a veterinarian weighed each animal and injected intramuscularly 0.1 IU kg^{-1} of Synacthen. To confirm effective ACTH administration, we collected four blood samples (0.25–0.4 ml) into plain serum tubes, with a 21-gauge needle, from the brachiocephalic vein from each individual; one immediately before and three at 15, 30, 45 min post-injection. The veterinarian and assisting staff constantly monitored all animals' condition until full recovery at the end of the experiment. Following the ACTH injection, we checked the cages hourly for 46 h post injection and all recognised fresh faecal material was collected. All blood (n = 15) and faecal (n = 52) samples were collected using clean, sterile latex gloves, stored in single labelled tubes, and immediately frozen at –20°C, until transferred for analysis to the Endocrine Research Laboratory (ERL) at the University of Pretoria. Following the conclusion of the study, the animals were re-introduced to their original enclosures and monitored for 10 days by experienced caretakers to ensure there was no harmful conflict between individuals.

Stability of fGCM concentration post-defecation

To determine alteration in fGCM concentrations post-defecation, in order to get an indication of suitable collection intervals in the field without compromising adrenocortical activity analysis reliability, fresh faeces were collected on one day from a communal latrine at the SANBI Mokopane Biodiversity Conservation Centre. Faeces were collected using sterile gloves, homogenized into one single sample, subsequently divided into 21 sub-samples, and stored outside (20–27°C, 40–50% humidity, no precipitation) in clearly labelled open plastic containers. Due to this form of sample collection, it was not possible to determine the sex of the sampled animals or individual samples. Following the methodology explained by Webber et al. (2018), sub-sample triplicates were frozen at –20°C at 0, 1, 2, 4, 8, 16 and 24 h. All frozen samples were sent to the ERL for enzyme immunoassay analysis.

SANBI pretoria national botanical garden

Study site and animals

The second phase of the study focused on free-roaming colonies at the SANBI Pretoria National Botanical Garden (PBG), South Africa (28°16′19.8″E, 25°44′18.2″S). The PBG is an urban and circumscribed area surrounded by busy roads and neighbourhood fences that generally prevent hyrax migration, thus producing a partially closed system. It offers rocky shelters and varied vegetation to feed on, with natural predators being absent. An unknown number of colonies of rock hyrax live at the PBG (Willis 2015). In the PBG, different activities such as trail running events, guided walks,

educational tours, birdwatching, garden parties, art exhibitions, music concerts and markets frequently take place (Willis 2015).

As visitor numbers were not evenly distributed, due to various activities offered in distinct areas of the park, we divided PBG into three different sections (1–3) based on human activity present (low, medium, high). We could identify one rock hyrax colony in each section, utilizing multiple dens and at least three active latrines (to the best of our effort, no additional colonies with active latrines could be identified in any of the three sections during the study period of 60 days). The colony in section 1 (highest human density) was found about 360 m away from the colony in section 2 (medium human density, located on a 35 m high quartzite outcrop). The third colony in section 3 was located approximately 900 m away from the one in section 1, and 1360 m away from the colony in section 2. The size of the colonies or size variation among each section could not be determined at any time. Animal movements across colonies were never observed. Individual samples could not be collected, and there are no marking programs or animal capture carried out at the PBG, allowing for any identification. Thus, representative fresh samples of each population within the study area were collected from communal latrines only.

Camera trapping

To evaluate the assumption of different levels of human numbers in different sections of the PBG, we used digital motion activated cameras (Cuddeback digital C.5.1- 1347 – Wisconsin, USA), with a trigger time of 0.25 s, and a detection zone of 20 m, to quantify human activity in the respective areas. Cameras were set up following Miller and colleagues (2017). Pictures were taken from 06:00 to 18:00 h over a total of 50 days, split into two sampling periods (Period 1: 35 days in winter, July–August, and Period 2: 25 days in late spring, October–November 2019). One camera trap was placed at knee height, in each of the three study sections, around the den site, close to a human pathway, to estimate the number of pedestrians walking and/or stationing in the proximity of the dens. The camera traps were set up at a height and angle that did not allow individual identification of the humans walking by.

Flight initiation distance (FID)

Following Chapman and colleagues (2012), during the same study period of camera traping and faecal sampling (35 days in winter, July–August, and 25 days in late spring, October–November 2019), we also took FID measurements from animals living in each section, using a laser range finder (Nikon Forestry Pro laser range finder, accuracy: +18.2 mm, distance: 10–500 m). A total of 132 individual total measurements for colony one, 123 for colony two and 115 for colony three were collected, and daily median were calculated (n = 15) for each colony during both study periods. The researcher randomly selected individuals from the three study colonies and walked at a constant speed of about 1.2 m s^{-1}, stopping immediately when the animal moved away. A starting distance from the animal (maximum 25 m, minimum 4 m), physically orientated towards the source of disturbance, thus aware of the researcher's presence, was ensured before approach. Measurements took place throughout the day, and the order of section was shuffled. The maximum starting distance from an animal was at 25 m, and the minimum starting distance was at 4 m from the study object. As starting distance for measurements tends to vary when an encounter occurs in a natural scenario, and encounters are not predictable, the researcher tried to keep an equal average starting distance for each colony during FID measurements. The researcher also collected measurements opportunistically by visually chose between different animals on a daily basis, and randomly select one to measure. The range finder was pointed on the ground, right in front of the hyrax, measuring the FID when the focal animal moved away from the observer. FID measurements were taken between 06:00 and 18:00 h. The same researcher took all the measurements to avoid inter-observer bias. The observed had to estimate FID when the distance was less than 10 m, as the range finder could not assess shorter distances.

Faeces collection at the PBG

We collected only fresh faeces from the middens of the three study colonies. All samples were collected over 35 days in winter (July–August 2019) and over 25 days in late spring (October–November 2019), from 07:00 to 08:00 h. We avoided collecting portions contaminated by external elements (e.g. soil, leaves). We used sterile gloves, and stored the faeces in labelled sterile plastic containers. Each sample was stored at −20°C within 20 min of collection and frozen until further processing at the ERL. A total of 90 faecal samples were collected during the study.

Blood sample processing and analysis

The ERL analysed 15 serum samples using a Cortisol EIA, originally described by Palme and Mostl (1997) and following procedures previously described (Ganswindt et al. 2012). Palme and Mostl (1997) provide detailed assay characteristics, including antibody cross-reactivities. The sensitivity of the assay was 25 pg ml^{-1}. The inter-assay coefficient of variance (CV), determined by repeated measurements of high- and low-value quality controls, was 3.80% and 15.15% and the intra-assay CV was 5.84% and 7.45%.

Faecal steroid extraction and analysis

The lab extracted and analysed a total of 163 faecal samples (ACTH challenge test: n = 52; degradation study: n = 21 sub-samples; from animals at the PBG: n = 90). The samples from the ACTH challenge test included all collected material from 26 h prior injection until 46 h after ACTH administration. Additional collected material prior or post to that time interval from all animals was not analysed (82 samples). Frozen faeces were lyophilized, and the resulting dry faeces pulverized and sifted with a wire filter to remove any

undigested material (Ganswindt et al. 2002). Steroid extraction was performed by dissolving 0.050–0.055 g of faecal powder in 3.0 ml of 80% ethanol, with each sample being subsequently vortexed for 15 min and then centrifuged for 10 min at 1500 g (Ganswindt et al. 2002). Lab technicians decanted supernatants into 1.5 ml safe-lock microcentrifuge tubes, labelled and frozen at −20°C until further analysis. To determine the suitability of the EIAs for fGCM quantification, a subset of the ACTH challenge faecal extracts (n = 13 per animal), were measured for immunoreactive fGCM concentrations using five different enzyme-immunoassays (EIA): 1) a 11-Oxoaetiocholanolone I EIA (detecting 11,17 dioxoandrostanes) (lab code 72a), 2) a 11-Oxoaetiocholanolone II EIA (detecting fGCMs with a 5β-3α-ol-11-one structure) (lab code 72T), 3) a 5α-pregnane-3β,11β,21-triol-20-one EIA (detecting fGCMs with a 5α-3β,11β-diol structure) (lab code 37e), 4) a Cortisol EIA (lab code CSL) and 5) a 11β-hydroxyaetiocholanolone EIA (detecting fGCMs with a 5β,3α,11β-diol structure) (lab code 69a). Detailed assay characteristics, including cross-reactivities, can be found in Palme and Mostl (1997) for the 11-oxoetiocholanolone I and cortisol EIAs, Möstl et al. (2002) for the 11-oxoetiocholanolone II EIA, Touma et al. (2003) for the 5α-pregnane-3β,11β,21-triol-20-one EIA, and Frigerio et al. (2004) for the 11β-hydroxyaetiocholanolone EIA. The sensitivity of the EIAs used were 1.2 ng g^{-1} dry weight (DW) (11-oxoaetiocholanolone I, 11-oxoaetiocholanolone II and cortisol), 2.4 ng g^{-1} DW (11β-hydroxyaetiocholanolone) and 4.8 ng g^{-1} DW (5α-pregnane-3β,11β,21-triol-20-one). Intra-assay coefficients of variation (CV) of high and low-concentration and controls were 5.31% and 6.78% (11-oxoaetiocholanolone I), 4.37% and 5.67% (11-oxoaetiocholanolone II), 5.84% and 7.45% (cortisol), 6.57% and 7.28% (11β-hydroxyaetiocholanolone) and 4.93% and 7.32% (5α-pregnane-3β,11β,21-triol-20-one). Inter-assay CVs of high and low-concentration and controls were 13.26% and 17.15% (11-oxoaetiocholanolone I), 11.17% and 14.78% (11-oxoaetiocholanolone II), 12.35% and 13.95% (cortisol), 14.04% and 14.69% (11β-hydroxyaetioc holanolone), 10.12% and 15.25% (5α-pregnane-3β,11β,21-triol-20-one). The individuals showed a clear increase > 100% above baseline in fGCM concentrations within comparable times post injection (15–22 h) when analyses were conducted with the 11β-hydroxyaetiocholanolone EIA. Although the Cortisol assay discriminated best between baseline and peak values for two of the tested individuals (male M1 and female F2), the 11B-hydroxy-assay also performed well in both the females (F1 and F2) of our study cases (Table 1).

Based on these initial results, we used the two most suitable assays (the cortisol and 11β-hydroxyaetiocholanolone EIA) for analysing the steroid extracts from the fGCM stability post-defecation test. Serial dilutions of faecal extracts gave displacement curves that were parallel to the respective standard curves (relative variation (%) of the slopes of respective trend lines < 5%) for both assays. Subsequently, faecal samples collected at the PBG were analysed by using only

Table 1. Baseline and peak fGCM concentrations post-ACTH administration for one male and two female rock hyraxes. We used five EIAs to analyse samples, and determined percent increases for peak values post-injection. The numbers in bold indicate relevant results (>100).

	Enzyme immunoassay											
Animal ID (and no. of samples collected and analysed pre and post ACTH)	11-Oxoaetiocholanolone I			11-Oxoaetiocholanolone II			5α-Pregnane-3β, 11β,21-triol-20-one			Cortisol			11β-Hydroxyaetiocholanolone		
	Baseline fGCM conc. (ng g^{-1} DW)	Peak conc. (ng g^{-1} DW)	Increase (%)	Baseline fGCM conc. (ng g^{-1} DW)	Peak conc. (ng g^{-1} DW)	Increase (%)	Baseline fGCM conc. (ng g^{-1} DW)	Peak conc. (ng g^{-1} DW)	Increase (%)	Baseline fGCM conc. (ng g^{-1} DW)	Peak conc. (ng g^{-1} DW)	Increase (%)	Baseline fGCM conc. (ng g^{-1} DW)	Peak conc. (ng g^{-1} DW)	Increase (%)
M1 (pre = 10, post = 14)	82.18	182.85	**122**	124.27	145.71	17	355.94	485.76	36	40.03	128.89	**222**	98.56	172.84	75
F1 (pre = 27, post = 23)	43.29	72.28	67	206.91	308.75	49	379.71	527.04	39	42.75	46.77	9	285.05	584.53	**105**
F2 (pre = 17, post = 13)	62.1	95.98	55	146.69	231.91	58	413.24	512.06	24	42.89	184.72	**331**	147.26	393.5	**167**

the 11β-hydroxyaetiocholanolone EIA. All hormone analyses were conducted at the ERL.

Statistical analysis

We used descriptive statistics to analyse the pattern in serum GC and fGCM concentrations following ACTH administration and to describe alterations in fGCM concentrations post-defecation. Changes in fGCM concentrations post-defecation, determined with the cortisol and 11β-hydroxyaetiocholanolone EIA, were further analysed using one-way analysis of variance (ANOVA), followed by Holm–Sidak's t-test as post hoc. Individual serum GC concentrations, collected at the point of ACTH injection (time 0 h), and individual median fGCM concentrations determined from samples collected up to 26 h pre-ACTH administration, were set as baseline (100%). Subsequently, we calculated relative changes (%) in serum GC concentrations for individual samples collected at 15-, 30- and 45-min post-injection. Differences in peak fGCM concentration 15–22 h post-ACTH administration were identified by comparing individual peak values with individual median pre-injection (baseline) values and normalised those by expressing these differences in percent. Peak values with an increase of > 100% above baseline were deemed considerable (Young et al. 2017). Variations in human numbers and FID for each study period were determined by using a generalised linear model (GLM), using the weekly mean number of people and, calculating daily median FID for each colony, as a response variable and section and season as predictor variables. An interaction between season and section was included to the model. The GLM analyses were followed by a Tukey's post hoc analysis, conducted at 95% family wise confidence levels. A GLM was also used to calculate whether the covariates section (1, 2, 3) and season (late spring and winter) significantly influenced variations in fGCM concentrations. Subsequently, the use of a Tukey's post hoc test, conducted at 95% family wise confidence levels, followed by Bonferroni adjustment, helped to determine whether significant differences in seasonal fGCM concentrations for each section occurred. Data are presented as mean ± standard deviation (SD), and significance was taken at 0.05. Data were statistically analysed using R Studio software (<www.r-project.org>).

Results

ACTH challenge test

We could obtain sufficient serum samples 15-, 30- and 45-min post ACTH administration from all the individuals. Each of the animals showed a substantial increase in serum glucocorticoid (GC) concentrations 15 min post Synacthen administration, with only animal M1 showing a continuous rise in serum GC levels until 30 min post-injection. Individual baseline concentrations varied over 3-fold (range: 19.91–66.08 ng ml^{-1}). The three animals showed an increase post injection between 79% and 85%.

An increase in fGCM concentrations (> 100%) between 15 h and 22 h post-injection was detected; with at least one of the five different EIAs evaluated (Table 1). For animal M1, a peak in fGCM concentration post-injection was detected with the 11-oxoaetiocholanolone I and cortisol EIA at 18 h (122% increase) and at 24 h (222% increase) post-injection, respectively. Further, the 11β-hydroxyaetiocholanolone EIA revealed an increase of 75% at 16.5 h post ACTH administration. For animal F1, a 105% increase in fGCM concentrations was detected 20 h post-injection, using the 11β-hydroxyaetiocholanolone EIA. For animal F2, the 11β-hydroxyaetiocholanolone EIA revealed a peak in fGCM concentrations 19 h post-injection (167% increase), while the cortisol EIA detected a 331% increase in fGCM concentrations 23 h post injection. Two of the three individuals showed a clear concentration increase (> 100%) within comparable times post injection (15–20 h) when conducting analysis with the 11β-hydroxyaetiocholanolone EIA. We used these specific peaks to express the maximum increase in faecal hormone metabolite concentrations post ACTH-administration.

Stability of fGCM concentrations post-defecation

The cortisol and the 11β-hydroxyaetiocholanolone EIA demonstrated an overall comparable trend of fGCM concentrations for the first 8 h post-defecation (Fig. 1). From 8 h post-defecation onwards, fGCM concentrations determined with the cortisol EIA increased significantly and continuously ($F_{6,14} = 20.149$, $n = 21$, $p < 0.01$; 16 h: $t_{20} = 3.137$, $n = 21$, $p < 0.05$; 24 h: $t_{20} = 8.436$, $n = 21$, $p < 0.01$) with a final increase of over 80% after 24 h. Conversely, fGCM concentrations detected with the 11β-hydroxyaetiocholanolone EIA, decreased significantly and continuously from 8 h post-defecation ($F_{6,14} = 15.771$, $n = 21$, $p < 0.01$; 16 h: $t_{20} = 3.328$, $n = 21$, $p < 0.05$; 24 h: $t_{20} = 5.492$, $n = 21$, $p < 0.01$) with a final decrease up to 42% below $t = 0$ levels after 24 h.

Human numbers

Section of the garden had a significant influence on human numbers, while season and the interaction of season and section did not (Table 2 and Supporting information). Human numbers were highest in section 1 (298 ± 138; mean ± SD), intermediate in section 2 (110 ± 40) and lowest in section 3 (10 ± 6). Section 1 showed the highest variability during late spring (weekly mean = 463.25 range: 40–870), and section 2 showed higher variability in winter (weekly mean = 186 range: 42–498) compared to late spring (weekly mean= 83.5 range: 63–103).

Flight initiation distance (FID)

A total of 15 measurements for each colony for each study period was calculated, with 132 individual total measurements

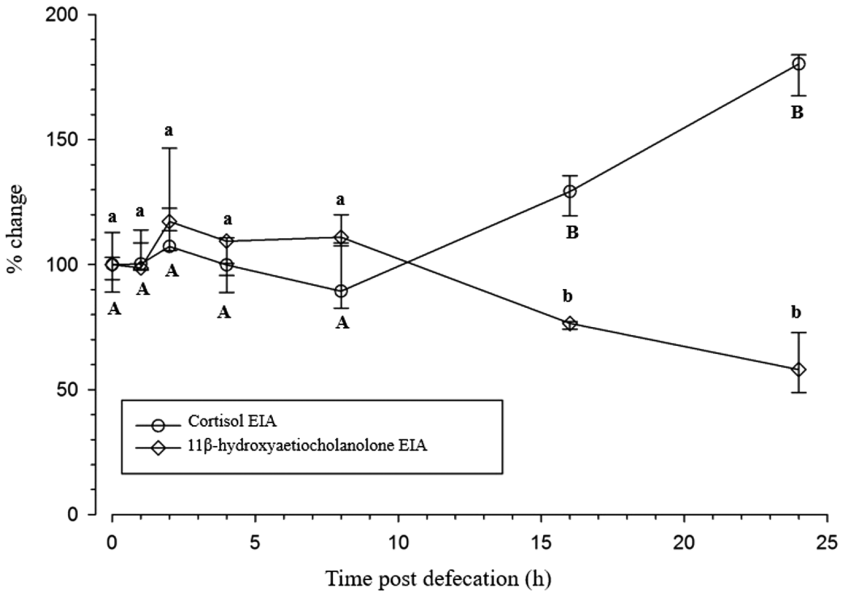

Figure 1. Change in fGCM concentrations of rock hyrax (median ± SD in %) post-defecation (0–24 h) determined using a cortisol and 11β-hydroxyaetiocholanolone EIA. Superscripts (capital: cortisol EIA; lower case: 11β-hydroxyaetiocholanolone EIA) indicate significant differences in % change of GCM concentrations against respective t = 0 values.

for colony one, 123 for colony two and 115 for colony three. Section had a significant influence on FID, while season did not. The interaction term of season and section demonstrated a significant influence (Table 2). We registered the greatest FID value in animals within section 3 (22.70 ± 2.91 m), followed by animals in section 2 (15.80 ± 2.16 m) and section 1 (8.58 ± 2.11 m), respectively. FID did not differ between seasons in the same section ($p \geq 0.05$). Overall, section 3 showed the highest variability in FID in late spring compared to winter, while FID variability remained constant for animals in sections 1 and 2 during both study periods (Fig. 2 and Supporting information).

Comparison of fGCM concentrations between season and sections

Season had a significant influence on fGCM concentrations while section and the interaction term of season and section did not (Table 2). Overall, mean fGCM concentrations of animals in section 3 (426.20 ng g^{-1} DW ± 215.20 ng g^{-1} DW) were ~10% higher compared to respective fGCM concentrations of animals in section 2 (381.56 ng g^{-1} DW ± 226.27 ng g^{-1} DW) and ~20% higher compared to respective hormone metabolite concentrations of animals in section 1 (340.43 ng g^{-1} DW ± 163.93 ng g^{-1} DW) (Fig. 3 and Supporting information).

Discussion

This is the first study to establish an EIA for measuring fGCM concentrations in the rock hyrax. After performing an ACTH challenge test, we successfully validated the 11β-hydroxyaetiocholanolone EIA. Subsequently, we used it to determine the stability of fGCM concentration post-defecation, as well as fGCM patterns in rock hyraxes occupying a green urban landscape.

Out of the five EIAs tested, the 11β-hydroxyaetiocholanolone EIA assay allowed a reliable quantification of fGCMs in rock hyrax faeces up to 8 h post-defecation. Following ACTH administration, serum glucocorticoid levels increased between 79% and 85% within 15–30 min in all three study animals. This is in line with previous findings for African buffalo (*Syncerus caffer*, Ganswindt et al. 2012), samango monkeys (*Cercopithecus albogularis erythrarchus*, Scheun et al. 2020), bat-eared foxes (*Otocyon megalotis*, Le Roux et al. 2016) and cattle (Veissier and Le Neindre 1988) that observed elevated serum glucocorticoid levels 10–60 min post injection, confirming that the adrenal cortex was sufficiently stimulated. Baseline serum glucocorticoid levels varied by over 300% between study animals, which could result from individual differences in response to the stimulus (Koolhaas et al. 2007, 2010) or the fact that some individuals were already affected prior to

Table 2. Results (χ^2, p values and degrees of freedom) of season, section and the interaction term between season and section within human numbers, FID and fGCM concentrations data collected at the PBG, during the study period.

Study aims at the PBG	Season (χ^2, p value and df)	Section (χ^2, p value and df)	Interaction season and section (χ^2, p value and df)
Human numbers	$\chi^2 = 0.032$, $p \geq 0.05$, df = 1	$\chi^2 = 169.218$, $p \leq 0.001$, df = 2	$\chi^2 = 1.602$, $p \geq 0.05$, df = 2
FID	$\chi^2 = 0.01$, $p \geq 0.05$, df = 1	$\chi^2 = 466.83$, $p \leq 0.001$, df = 2	$\chi^2 = 630$, $p \leq 0.001$, df = 2
fGCM concentrations	$\chi^2 = 18.7585$, $p \leq 0.001$, df = 1	$\chi^2 = 2.8019$, $p \geq 0.05$, df = 2	$\chi^2 = 3.1120$, $p \geq 0.05$, df = 2

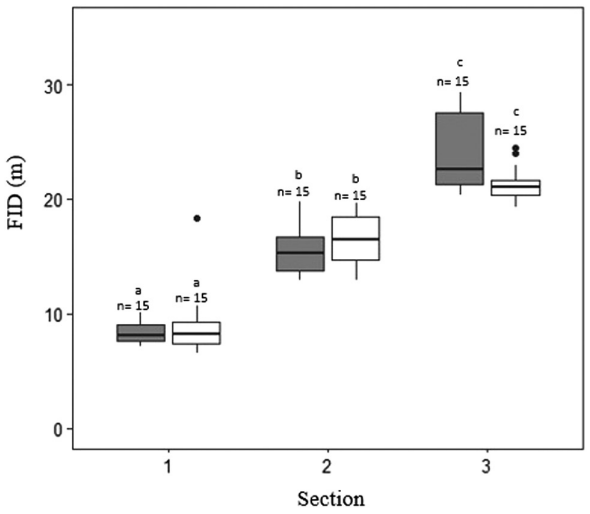

Figure 2. Boxplot representing FID of rock hyrax in the three study sections during the two study periods of late spring (grey) and winter (white) 2019. Boxes indicate median, 25 and 75 percentiles, and whiskers indicate 10/90 percentiles, and dots represents outliers. Different superscripts indicate statistically significant differences between groups (Supporting information). The n numbers indicate total faecal samples collected per season.

injection. Blood samples should be collected within 3 min of the stress event in order to obtain baseline serum cortisol concentrations (Romero and Reed 2005). Thus, prolonged handling procedures involving the male M1; may have influenced its rise in serum glucocorticoid levels. Studies on African elephants *Loxodonta africana* (Stead et al. 2000) and ponies

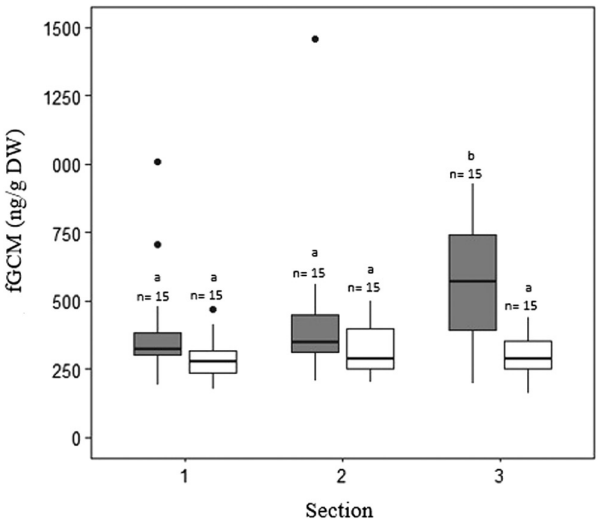

Figure 3. Boxplot representing fGCM metabolite concentrations (ng g^{-1} DW) of rock hyrax in the three study sections during the two study periods of late spring (grey) and winter (white) 2019. Boxes indicate median, 25 and 75 percentiles, and whiskers indicate 10/90 percentiles and dots represents outliers. Different superscripts indicate statistically significant differences between groups (Supporting information). The n numbers indicate total faecal samples collected per season.

(*Equus ferus caballus*, Möstl et al. 1999) also found variation in baseline serum glucocorticoid levels between 400–700% and 209–514%, respectively. Other studies (Ludwig et al. 2013, Sieber-Ruckstuhl et al. 2015) measured individual differences in serum glucocorticoid concentrations in peak values which could be caused by individual differences in adrenocortical response to the injection. Compared to our three study females, the male showed a delayed response with peak serum glucocorticoid concentrations at 30 min post-injection. It was also possible to recognize individual time-related variation in peak responses in a study on domestic cats *Felis catus* attributed to the individual variance of perceived handling procedures (Sparkes et al. 1990).

The analysis of related faecal material collected during the ACTH challenge experiment underlined individual differences in the activation of the HPA axis. After examining five enzyme-immunoassays, our study detected a considerable response in fGCM output post ACTH-administration for the male when using the 11-oxoaetiocholanolone I and cortisol EIA, and to some extent when using the 11β-hydroxyaetiocholanolone EIA. In contrast, the cortisol EIA only provided suitable results for one of the females, while the 11β-hydroxyaetiocholanolone EIA performed adequately in both females (we could not include the third female in the analysis due to inadequate availability of sample material). These findings indicate possible sex-related differences in glucocorticoid metabolism and possible route of excretion, as previous studies demonstrated (Touma and Palme 2005). Moreover, previous studies reported individual differences in peaks of fGCM concentrations, based on different individual perceptions of the biological stressor, reproductive status and health conditions (Yoshimura et al. 2003, Kudielka and Kirschbaum 2005, Reeder and Kramer 2005). Further, diet composition, due to the alteration of the gastrointestinal microbiota, can also influence glucocorticoid metabolism (von der Ohe and Servheen 2002, Sheriff et al. 2011). Therefore, different assays can be optimal to detect adrenocortical activity for the two sexes within the same species, as demonstrated, for example, for goats *Capra aegagrus hircus* (Kleinsasser et al. 2010).

Our study observed a maximum rises of fGCM concentrations post-ACTH administration 15–24 h post injection. This is in line with previous studies on species of similar or smaller size mammals, like the African lesser bushbaby (*Galago moholi*; 14–18 h) and banded mongoose (*Mungos mungo*; 24 h) (Laver et al. 2012, Scheun et al. 2015). The fGCM concentrations in the stability post-defecation experiment did not change until 8 h post-defecation when analysed with the cortisol and 11β-hydroxyaetiocholanolone EIA, respectively. After that, the cortisol EIA showed a continuous increase in fGCM concentrations until 24 h post deposition, whereas the 11β-hydroxyaetiocholanolone EIA showed a constant decrease in fGCM concentrations. An increase in fGCM concentrations following excretion has been found in domestic livestock (Möstl et al. 1999) and brown bear *Ursus arctos* (Dalerum et al. 2020), while a decrease was found in banded mongoose (Laver et al. 2012), wild crested

macaque (*Macaca nigra*, Gholib et al. 2018), brown hyaena (*Hyaena brunnea*, Hulsman et al. 2011) and African elephant (Webber et al. 2018). A previous study on sheep (*Ovis aries*, Lexen et al. 2008), demonstrated that the depicted trend in which fGCM concentrations change can depend on the EIA used, as shown in the current study. In addition, Webber et al. (2018) speculated that glucocorticoid metabolites characterized by a 5β-3α-ol-11-one structure are a common output of bacterial alteration in mammal faeces, as in the case for the African elephant. A more in-depth examination of the glucocorticoid metabolite composition and abundance in rock hyrax faeces could thus be beneficial to better explain the pattern of fGCM concentrations found post-defecation.

We examined seasonal changes in fGCM concentrations of wild populations of rock hyrax living at the PBG, in relation to different levels of human numbers. Observing tolerance to human proximity was a prerequisite. Absolute numbers of humans were generally higher in late spring, presumably due to warmer climatic conditions, as, generally, tourism tends to increase when climatic conditions are more favourable (de Freitas 2003). We found higher variability in visitor numbers in section 1, likely due to the number of recreational activities offered in the area, section 2 in winter months due to an increase in hiking activity and section 3 in late spring due to the onset of maintenance work within the area. These findings underline the suitability of camera trapping as an adequate tool to estimate the activity of people in urban green areas, as previous studies on human presence in protected areas demonstrated (Campbell 2006, Buxton et al. 2018, Nickel et al. 2020).

FID varied between animals of different sections, with a decreasing trend with increasing people numbers, which indicates different levels of habituation across the rock hyrax population at the PBG. These findings are in line with research conducted by Mbise et al. (2020), which demonstrated a reduction in FID for rock hyrax living around dwellings in Serengeti National Park, Tanzania, compared to those living away from human activities. Further confirmation of a decrease in FID due to habituation to humans came from studies on 180 species of birds, 16 mammal species and 16 species of lizards (McGowan et al. 2014, Williams et al. 2014, Brubaker and Coss 2015, Samia et al. 2015). Those findings, including the current study, are in line with the optimal escape theory, affirming that the longer the FID the greatest the risk perceived by an individual. Individuals, therefore, tend to modify the flee distance to correspond with the risk level, ultimately optimizing energy costs (Ydenberg and Dill 1986). Furthermore, Lima and Bednekoff (1999) postulated that animals might reduce anti-predatory behaviour in response to the increment of a constant high-risk event; thus, study animals from section 1 may have regulated their anti-predatory behaviour in response to a constant human presence. Different variables have been determined as affecting FID, such as the position of the observed subject and its distance from the shelter (Bonenfant and Kramer 1996), mutual gaze between the animal and the observer (Cooper William 1997), external temperature and quality of food for feeding animals (Sreekar and Quader 2013). One or more of these variables could explain some of the FID variation measured in our study. The results revealed no seasonal differences in FID for animals from the same section, which is in line with a previous study on urban Eurasian red squirrels (*Sciurus vulgaris*, Uchida et al. 2016) in Hokkaido, Japan. However, anti-predatory behaviour can vary seasonally in relation to habitat modifications, animal conditions and resource availability (Sih et al. 2011), as demonstrated for rural Eurasian red squirrels showing lower FID in autumn compared to rural Eurasian red squirrels in spring (Uchida et al. 2016). Therefore, the absence of seasonal variation in anti-predator behaviour in urban areas might reflect an adaptation to greater food availability or the absence of predators (Shochat et al. 2006, Sih et al. 2011). Although there was no significant difference in season-related FID in the same sections, the results indicated higher variability in section 3 in late spring, which could be due to increased human activity around the colony den during that time, as mentioned above, possibly explaining the significance of the interaction term season-section. Higher anthropogenic activity registered in the area might have caused an increase in vigilance and flee behaviour, utilized as anti-predatory responses to a perceived disturbance (Tablado and Jenni 2017). A study on sika deer (*Cervus nippon*, Borkowski 2001) reported similar FID variability where flight distance variability was higher when tourism presence in the Tanzawa Mountains, Japan, became more persistent.

Overall fGCM concentrations of rock hyraxes were significantly higher in late spring than winter, while differences between the sections were not related to variations in adrenocortical activity. Seasonal variations in fGCM concentrations occur in different wild vertebrates as a response to environmental and social changes, reproductive activity or pathologies related to ageing, depending on the species (Sapolsky 1987, 1992, Smith and Thomson 1991, Romero 2002). For instance, fGCM concentrations in squirrel monkeys (*Saimiri sciureus*, Coe and Levine 1995, Schiml et al. 1996) are higher during the breeding season, while banded mongooses (Laver et al. 2020) have seasonal fGCM increases, correlated mainly with feeding constraints and secondly with reproduction. Rock hyrax living at the PBG are less affected by seasonal food shortages than their counterparts surviving outside the urban landscape, with a range of edible plant species available throughout the year. However, anthropogenic food sources are rare (Linette Ferreira, pers. comm., Interpretation Officer, PBG). The species is an opportunistically selective feeder (Skinner and Chimimba 2005, Naylor 2015, Wiid and Butler 2015), which is in accordance with the feeding behaviour we observed. Further research on seasonal food availability and hyrax feeding preferences at the PBG would be necessary to investigate possible correlations with fluctuation in fGCM concentrations noted in the present study.

Rock hyraxes are synchronized breeders, and the mating season varies in different geographical locations (from February to August), being related to photoperiod (Millar 1971, Skinner and Chimimba 2005). During the current

study, we recognised at the PBG, several young rock hyraxes, distinguishable by comparing body sizes (Fairall 1980), during early November (late spring), probably born between late September and early October 2019. Although sampling was not done during the mating season, there was no evidence that seasonal variations in fGCM concentrations could be related to the reproductive activity period. Lactation can still represent a cause of physiological alteration in the study of females (Clutton-Brock et al. 1989, Hayssen 1993).

Although anthropogenic activity is often a driver for a physiological stress response (Creel et al. 2002, Szott et al. 2020), it is important to note that habituation to different disturbances can vary among species (Ellenberg et al. 2006). For example, previous results on Alaskan brown bears *Ursus arctos horribilis*, living at Katmai National Park and Preserve, Alaska, United States, showed no significant correlation between anthropogenic activity and fGCM concentration variations (von der Ohe et al. 2004). Conversely, wildcats *Felis silvestris* in Natural Park Montes do Invernadeiro, Spain, showed increased fGCM concentrations in areas characterized by a higher number of humans (Piñeiro et al. 2012). However, anthropogenic presence and their activities, like habitat alterations, can elicit changes in fGCM output (Lucas et al. 2006). Therefore, the high variability in fGCM concentrations found in animals from our third study section might be related to their habitat's physical rearrangement and higher human numbers registered in the area. More in-depth research could be conducted, attempting to provide new information on the species' ability to respond to different environmental stimuli, and investigating the impact of reproductive activity on fGCM concentrations.

The established method, along with the information gathered on sampling methodology, allows detailed insight into the physiological responses of rock hyraxes to anthropogenic activities and their ability to adapt to human presence and changing environmental conditions. Further studies on rock hyrax responses to recreational activities, habitat manipulation and season would be necessary to examine the species' adaptive abilities (behavioural and physiological plasticity) and the repercussions that such stimuli have on its fitness. This could help conservationists and wildlife managers to manage urban and wild populations of rock hyrax and possibly other hyrax species.

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Data availability statement

Data are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.7h44j0zvk> (Carlin et al. 2021).

Supporting information

The supporting information associated with this article is available from the online version.

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