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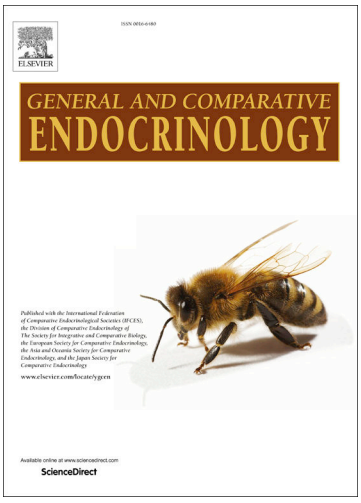


Research paper

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# Simultaneous investigation of urinary and faecal glucocorticoid metabolite concentrations reveals short- versus long-term drivers of HPA-axis activity in a wild primate (*Papio ursinus*)

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## Abstract

Glucocorticoids (GCs), a class of steroid hormones released through activation of the hypothalamic-pituitary-adrenal (HPA) axis, perform many vital functions essential for survival, including orchestrating an organism's response to stressors by modulating physiological and behavioural responses. Assessing changes and variation in GC metabolites from faecal or urine samples allows for the non-invasive monitoring of HPA-axis activity across vertebrates. The time lag of hormone excretion differs between these sample matrices, which has implications for their suitability for studying effects of different temporal nature on HPA-axis activity. However, simultaneous

comparisons of predictors of faecal and urinary GC metabolites (fGCs and uGCs, respectively) are lacking. To address this gap, we employ frequent non-invasive sampling to investigate correlates of fGCs and uGCs in wild chacma baboons (*Papio ursinus*) (n=17), including long-term (dominance rank, season, and female reproductive state) and short-term (time of day, and daily weather conditions) factors. Correlated with increasing day length, fGCs gradually decreased from winter to summer. No seasonal effect on uGCs was found but 'rain days' were associated with increased uGCs. Pregnant females had significantly higher fGCs compared to cycling and lactating females, whereas uGCs were not statistically different across reproductive states. A circadian effect was observed in uGCs but not in fGCs. Dominance rank did not affect either fGCs or uGCs. Our study highlights the difference in inherent fluctuation between uGCs and fGCs and its potential consequences for HPA-axis activity monitoring. While uGCs offer the opportunity to study short-term effects, they undergo more pronounced fluctuations, reducing their ability to capture long-term effects. Given the increasing use of urine for biological monitoring, knowledge of this potential limitation is crucial. Where possible, uGCs and fGCs should be monitored in tandem to obtain a comprehensive understanding of short- and long-term drivers of HPA-axis activity.

**Keywords**: HPA-axis activity; Non-invasive sampling; Glucocorticoid metabolites; Faeces; Urine; Excretion time lags

## 1. Introduction

To survive, organisms need to maintain homeostasis, which refers to the stability in body temperature, pH and glucose levels, and oxygen tension (McEwen and Wingfield, 2003). Homeostasis is maintained through various physiological mediators, including hormones (Romero, 2002; Romero et al., 2009). During different life history stages or when changes in the physical or social environment perturb these vital processes (i.e. "stressors" as defined by Nelson, 2005), physiological mediators

operate to reinstate and maintain these internal systems within the optimal ranges for bodily functions (McEwen and Wingfield, 2003). Among the primary physiological mediators in maintaining homeostasis are glucocorticoids (GCs) (Romero, 2002; Sapolsky et al., 2000). Along with a suite of other hormones, these steroid hormones are released from the adrenal cortex into the bloodstream, when the hypothalamic-pituitary-adrenal (HPA) axis is activated in response to a stressor (Sapolsky et al., 2000). GCs promote the mobilisation of energy needed to respond to the stressor and suspend processes that are not essential for immediate survival (Sapolsky et al., 2000), allowing the organism to cope and return to homeostasis (Romero and Wingfield, 2015; Sapolsky et al., 2000).

Biomedical research has shown that prolonged activation of the HPA-axis can have negative consequences as the physiological mediator itself (e.g., GCs) begins to disrupt normal bodily functions (Kirby et al., 2009; Korte et al., 2005; Sapolsky, 2002; Spencer et al., 2010). However, studies linking increased GCs to negative fitness outcomes (the "Cort-Fitness Hypothesis") in the wild are inconclusive (Bonier et al., 2009) and recent reviews highlight the need to move away from the portrayal of GCs as detrimental "stress hormones" (Beehner and Bergman, 2017; Boonstra, 2013; MacDougall-Shackleton et al., 2019). Nevertheless, measuring relative changes in GCs remains an informative and widely used tool for tracking the physiological challenges faced by an organism (see e.g., Beehner and Bergman, 2017; MacDougall-Shackleton et al., 2019; McEwen & Wingfield, 2003; Romero, 2002). To compare the physiological stress-response between populations (MacLarnon et al., 2015) or within subjects over time (Tkaczynski et al., 2019; Young et al., 2019), the "demonstrated reactive scope" (DRS) measure and its coefficient of variation (DRScv) provide a scale-free index of the range and variation in GCs (MacLarnon et al., 2015; based on the Reactive Scope Model: Romero et al., 2009).

Assessing HPA-axis activity, which is driven by cumulative and interactive stressors (McEwen and Wingfield, 2003), introduces methodological challenges. While blood samples have traditionally been used to assess circulating GCs (see Hodges et al., 2010; Sheriff et al., 2011 for reviews), capture and restraint itself can cause a stress response which can interfere with the measurement of the

stressors of interest and may logistically limit the opportunity for repeated sampling, particularly in wild animals (Palme, 2019; Sheriff et al., 2011; Touma and Palme, 2005). Furthermore, GC production is inherently dynamic with peaks and troughs (Sapolsky et al., 2000) and blood samples only reflect a point-in-time, which may not be suitable when investigating long-term effects on HPA-axis activity (Creel, 2001; Touma and Palme, 2005). Non-invasive sampling of excreta in wild animals offers a solution to the aforementioned issues (Touma and Palme, 2005) and has made it possible to monitor hormone levels without disrupting the study animals (Palme, 2019; Sheriff et al., 2011).

Faecal GC metabolites (fGCs) have been used to study HPA-axis activity across a wide range of wild animals (reviewed by Palme et al., 2005), whereas the use of urinary GC metabolites (uGCs) has largely been used in captive/semi-free ranging settings (Brown et al., 2010; Carlstead et al., 1992; Saltz and White, 1991; Smith and French, 1997). In the wild, uGCs, to our knowledge, have been measured only in non-human primates (e.g., *Pan troglodytes*: Muller et al., 2007; Muller and Wrangham et al., 2004; Emery Thompson et al., 2010; Tkaczynski et al., 2020; Wessling et al., 2018; Wittig et al., 2015; *Pan paniscus*: Surbeck et al., 2012; Tkaczynski et al., 2020; *Lophocebus albigena*: Jaimez et al., 2012; *Macaca fascicularis*: van Schaik et al., 1991; *Macaca assamensis*: Touitou et al., 2021; *Cercopithecus ascanius*: Aronsen et al., 2015; Jaeggi et al., 2018; *Piliocolobus rufomitratus*: Aronsen et al., 2015; *Gorilla gorilla beringei*: Robbins and Czekala, 1997) and in dwarf mongooses (*Helogale parvula*: Creel et al., 1992). However, with the development and implementation of new urine collection techniques (Salivettes: e.g., Danish et al., 2015; Fürtbauer et al., 2020; filter paper: e.g., Mouri and Shimizu, 2021; Shideler et al., 1995) and an emerging interest in various urinary biomarkers (Behringer et al., 2017; Crockford et al., 2013; Deschner et al., 2020; Emery Thompson and Knott, 2008; Fürtbauer et al., 2020; Heistermann and Higham, 2015; Higham et al., 2020; Preis et al., 2018; Sadoughi et al., 2021), we can expect an increase in the use of uGCs in future studies of HPA-axis functioning in wild animals. It is therefore necessary to investigate how measuring uGCs compares as a tool for measuring HPA-axis activity, relative to the more frequently used fGCs (Palme, 2005), and to understand the potential limitations and advantages of using faeces or urine.

A primary consideration of which matrix to use is that GC excretion lag times differ between faeces and urine (Heistermann, 2010; Touma and Palme, 2005), which spans the time between GC production, metabolism and excretion which, in the case of faeces, also includes gut passage time (Palme, 2019). Time-lags are species-specific (see e.g., Bahr et al., 2000; Heistermann, 2010; Schatz and Palme, 2001), but generally fGCs capture the cumulative secretion of GCs over longer time periods (many hours/days: Behringer and Deschner, 2017), while uGCs represent shorter time-windows (a few hours: Behringer and Deschner, 2017; Heistermann, 2010). The differences in the temporal nature of the stressor can thus determine whether faecal or urine samples are appropriate measures of HPA-axis activity. For instance, fGCs might be more useful for studying longer-term effects (Touma and Palme, 2005; Wielebnowski and Watters, 2007), such as environmental factors and female reproductive state (Gesquiere et al., 2008; Weingrill et al., 2004). In contrast, uGCs offer the opportunity to study shorter-term effects on HPA-axis activity induced, for example, by acute stressors, such as singular aggressive interactions (Smith and French, 1997; Wittig et al., 2015; Wittig et al., 2016) or translocation to novel environments (Morrow et al., 2000; Rooney et al., 2007).

Simultaneous measurements of fGCs and uGCs have been conducted in laboratory and captive settings to determine excretion routes and time lags in a range of species (Bahr et al., 2000; Chen et al., 2017; Fanson et al., 2013; Freeman et al., 2018; Hirschenhauser et al., 2012; Medger et al., 2018; Medger et al., 2020; Palme et al., 1996; Paramastri et al., 2007; Schatz and Palme, 2001; Scheun et al., 2018; Teskey-Gerstl et al., 2000). However, to our knowledge, a direct comparison of the predictors of variation in fGCs and uGCs has not been published (but see: Emery Thompson, 2005; for a direct comparison of faecal and urinary reproductive hormones in wild chimpanzees). The use of both sample matrices in independent studies on the same species has shown that changes in food availability and/or intake, dominance rank, and female reproductive state are reflected in variation in both fGCs (*Pan troglodytes*: Markham et al., 2014; McLennan et al., 2019; Murray et al., 2018; *Macaca assamensis*: Berghänel et al., 2016; Fürtbauer et al., 2014) and uGCs (*Pan troglodytes*: Muller et al., 2021; Muller and Wrangham, 2004; Emery Thompson et al., 2010; Wessling et al., 2018; *Macaca*

*assamensis*: Touitou et al., 2021). These studies confirm that both methods provide biologically relevant information on HPA-axis activity in response to similar stressors. However, given that uGCs and fGCs were not quantified simultaneously, it remains inconclusive whether they would have been affected in the same way by the same predictors.

Direct comparisons between independent studies are often precluded as the focus of any given study will dictate what data is collected, analysed and presented. For instance, categories or definitions of female reproductive state often differ between studies (e.g., *Pan troglodytes*, fGCs: Murray et al., 2018; uGCs: Emery Thompson et al., 2010; *Macaca assamensis*, fGCs: Fürtbauer et al., 2014; uGCs: Touitou et al., 2021). Moreover, direct comparisons of studies using uGCs and fGCs may not be advisable. Differences in social or ecological contexts between studies may influence the intensity of the predictor, and hence associated HPA-axis activity (e.g., see discussions in Markham et al., 2014; Muller et al., 2021), making it difficult to disentangle whether any observed differences in predictors are linked to changes in biological context or the sample matrix used. Finally, it is worth noting that simultaneous measurements of faecal and urinary measures of reproductive hormones (ovarian steroids: Emery Thompson, 2005; testosterone: Ganswindt et al., 2002) have been shown to correlate, but do not necessarily produce the same results when linked to known predictors (e.g., conception cycles *Pan troglodytes*: Emery Thompson, 2005; musth *Loxodonta africana*: Ganswindt et al., 2002). These differences may be linked to methodological factors (e.g., uneven representation of reproductive categories for each sample type; see Emery Thompson, 2005), however, potential effects of sample matrix are not discussed. To establish whether variation in fGCs and uGCs is driven by the same predictors, and thus can be used interchangeably, a simultaneous investigation is needed.

In the present study, we simultaneously investigate correlates of fGCs and uGCs in wild chacma baboons on the Cape Peninsula, Western Cape, South Africa. Predictors of HPA-axis activity have been robustly documented in long-term field studies of baboons, revealing the effects of reproductive state (Engh et al., 2006; Gesquiere et al., 2008; Weingrill et al., 2004), dominance rank (Bergman et al., 2005; Gesquiere et al., 2011; Levy et al., 2020) and environmental predictors

(Gesquiere et al., 2008; Weingrill et al., 2004) on fGCs. However, there are currently no studies on drivers of variation in uGCs in wild baboons. Here, we consider female reproductive state, social and environmental predictors of GC variation. For fGCs, we predicted i) a seasonal decline in fGCs from winter to summer (here July to November), which has previously been found for baboons in the Western Cape and was linked to warmer temperatures, lower rainfall, and increased day length (Chowdhury et al., 2021; Weingrill et al., 2004), ii) higher fGCs in pregnant compared to cycling and lactating females (Engh et al., 2006; Gesquiere et al., 2008), and iii) no rank effect (Beehner et al., 2005; Engh et al., 2006; Weingrill et al., 2004). Rank effects have been shown for males (Gesquiere et al., 2011), but because our study focussed on females (n=19 females, n=2 males) in which rank-effects appear to be detectable in long-term studies only (Levy et al., 2020), we did not expect rank effects. For uGCs, we predicted iv) elevated uGCs in the morning ("cortisol awakening response": Fries et al., 2009) with a gradual decline throughout the day (e.g., Czekala et al., 1994; Muller and Lipson, 2003). Regarding environmental effects, we predicted v) a seasonal decline in uGCs (as predicted for fGCs) and also consider potential acute effects of sporadic rain days. Furthermore, as for fGCs, we predicted vi) higher uGCs in pregnant females compared to cycling and lactating females (French et al., 2004; Smith and French, 1997; Touitou et al., 2021; Ziegler et al., 1995) and vii) no rank effect on uGCs. Because the objective of our analyses is to present a robust, but not necessarily exhaustive, comparison of predictors of uGCs and fGCs, we do not consider acute stressors such as agonistic interactions (see e.g., Wittig et al., 2015) or anthropogenic stressors (e.g., Muehlenbein et al., 2012), but rather focus on predictors for which we have continuous data which can be time-matched to all uGCs and fGCs measurements, thereby maximising our sample size.

**Methods**

*2.1. Study site and troop*

The study was conducted on a troop of wild chacma baboons consisting of approximately 50 individuals (n=21 adults) whose home range was predominantly in Table Mountain National Park and included a residential suburb of Da Gama Park, on the Cape Peninsula, Western Cape, South Africa (-34.15562°N, 18.39858°E) (for more details see: Bracken et al., 2021; Fürtbauer et al., 2020). Baboon diets on the Cape Peninsula vary seasonally, comprising of predominantly annually flowering plants during winter, and more indigenous fynbos and alien plants during summer (Van Doorn et al., 2010; Davidge, 1978). In some troops, including ours, consumption of anthropogenic foods also increases during winter (Chowdhury et al., 2021; Van Doorn et al., 2010). Sampling occurred between July and November 2018 (23/07/2018 - 24/11/2018), comprising late winter, spring and early summer months (Fürtbauer et al., 2020). Faecal and urine samples were collected from n=17 adult baboons of known identity (n=15 females, n=2 males). Between 25/07/18 - 02/08/18 adult baboons were fitted with tracking collars as part of a larger project, and faecal samples collected during collaring were used for biological validation of our fGC metabolite assay (Hämäläinen et al., 2014; Suedkamp Wells et al., 2003; Terio et al., 1999; Wasserman et al., 2013; see Supplementary Material S1.1). Work was approved by Swansea University's Ethics Committee (IP-1314-5) and local authorities (Cape Nature, permit number: CN44-59-6527; SANparks, permit number: CRC/2018-2019/008 – 2018/V1; CITES, sample export permit: 208683). Weather data was provided by South African Weather Service (SAWS) (https://www.weathersa.co.za), from the Slangkop weather station, approximately 7 km from the field site (Climate number: 0004549-2, -34.1480°N, 18.3190°E).

*2.2. Sample collection and processing*

*2.2.1 Faecal samples*

N=603 faecal samples were collected (mean±SD samples per individual: 35±11; n=17) opportunistically throughout the day and stored in 40ml screw-top polypropylene containers. To

achieve even distribution of hormones, large faecal samples were homogenised before being sub-sampled (Palme, 2005). The containers were labelled in the field (date, time, individual ID, sub-sample or whole-sample, and potential contamination with urine). Samples were kept on ice packs throughout the day and were frozen at -20°C at the research house until further processing. To remove moisture, faecal samples were freeze-dried at -45°C for a minimum of 24 hours (using a New Brunswick, New Brunswick Scientific Co., Inc., New Jersey USA and Scanvac CoolSafe, LaboGene ApS freeze-dryer) at the University of Cape Town. Samples were stored at -20°C until ambient shipment to the endocrinology laboratory at Swansea University. For hormone extraction, faecal samples were pulverised using a mortar and pestle and sifted to remove vegetation and seeds (Keay et al., 2006). A sub-sample of 0.09-0.12 g (mean±SD: 0.103±0.012 g, n=603) was extracted with 80% watery methanol (Palme et al., 2013). Faecal suspensions were vortexed (1500 rpm, 10 min) in a Hercuvan TT-2500-VM Multi-Tube Vortex Mixer and centrifuged (3000 rpm, 10 min) in a VWR Mega Start 1.6 centrifuge. Subsequently, the supernatant was decanted into two safelock Eppendorf tubes and stored at -20°C. Prior to hormone analysis, 0.25 ml of the faecal extract were transferred to microcentrifuge tubes and evaporated at -38°C under a stream of nitrogen. The dry tubes were sent at ambient temperature to the endocrinology laboratory of the German Primate Centre, Göttingen, Germany for hormone analysis.

*2.2.2 Urine samples*

N=385 urine samples (not diluted with rainwater) were collected (mean±SD samples per individual: 23±4; n=17) opportunistically throughout the day. Depending on the substrate, urine samples were either collected with synthetic Salivettes (Starstedt Salivette Cortisol code blue, order number 51.1534.500; n=193 (Danish et al., 2015; Fürtbauer et al., 2020) or by pipetting directly into 2ml safelock Eppendorf tubes (n=192). Urine samples were predominantly collected from rocks and other hard surfaces (n=350 samples, 91%), from soil/plants (n=9) or surface type was not recorded (n=22).

Urine samples were kept on ice packs during the day and samples collected with Salivettes were centrifuged (3000 RPM, 5 min) and transferred to 2ml safelock Eppendorf tubes after return from the field. All urine samples were stored at -20°C at the research house within a maximum of 12 hours of collection. Samples were shipped to Swansea University on dry ice at the end of the field season and were stored frozen at -20°C until analysis.

*2.3. Hormone analyses*

*2.3.1 Faecal samples*

Faecal extracts were analysed by an enzyme immunoassay for the measurement of immunoreactive 11β-hydroxyetiocholanolone, a major faecal metabolite of cortisol in primates (Heistermann et al., 2006). The assay has been previously used successfully to track adrenocortical activity in numerous wild-living primate species of all major taxa (e.g., Hämäläinen et al., 2014; Kunz et al., 2021; McLennan et al., 2019; Rimbach et al., 2013; Rudolph et al., 2020; Young et al., 2014), including species of the baboon clade (Carrera et al., 2020; Kalbitzer et al., 2015). Prior to analysis, the dried faecal extracts were reconstituted in 0.25 ml of 80% watery methanol by sonication in a water bath for 5 minutes, followed by 30 seconds of vortexing (Mausbach et al., 2017; Shutt et al., 2012). Initially, all samples were assayed in a 1:50 dilution in assay buffer. If samples had concentrations above the highest standard curve concentration, they were re-assayed in a higher dilution (up to 1:300). The assay was carried out as described in detail by Heistermann et al. (2006). Sensitivity of the assay at 90% binding was 12 pg/ml. Inter-assay coefficients of variation, determined from repeated measurements of high- and low-level quality controls run on each assay plate (n=19) were 8.7% (high) and 11.0% (low). Intra-assay coefficients of variation using the same quality controls were 5.6% (high; n=16) and 7.9% (low; n=16). All faecal hormone data are expressed as ng/g faecal dry weight.

*2.3.2 Urine samples*

Urinary cortisol concentrations (uGCs) were quantified using a commercial enzyme-linked immunosorbent cortisol assay kit (ELISA; IBL, Hamburg, Germany; Cat.-no.: RE52241) according to the manufacturer's instructions. The majority of samples were measured undiluted (n=343 samples) and samples with concentrations above the highest standard curve value (>200 ng/ml) were diluted with Standard 0 (1:2-1:10; n= 42 samples) and re-analysed. Sensitivity of the assay was 0.22 ng/ml. High- and low-level quality controls were run in duplicates on each plate (n=13), and inter-assay coefficients of variation were 9.3% (high) and 7.3% (low). Intra-assay coefficients of variation of quality controls were 7.3% (high; n=20) and 7.0% (low; n=20), according to manufacturer. uGC concentrations were corrected for specific gravity (SG; measured using a manual handheld refractometer), i.e., the density of urine relative to the density of distilled water, using the following formula: $uGC_{SG} = uGC_{ng/ml}*(SG_{pop}-1)/(SG-1)$, where $SG_{pop}$ is the mean SG value for the population (1.020) (see e.g., Fürtbauer et al., 2020; Miller et al., 2004). SG corrections offers advantages over creatinine corrections in terms of robustness to factors related to unfavourable environmental conditions of sample collection, processing and storage, including contamination of urine with faeces, storage of samples at higher temperatures and freeze and thaw cycles (Anestis et al., 2009; Higham et al., 2020 for a direct comparison of the two indexing measures in response to various sample treatment issues) and where urine samples are highly diluted (Miller et al., 2004). SG corrected uGC values are reported and are expressed in ng/ml.

*2.4. Demonstrated reactive scope*

To assess and compare the scale-free variation in fGCs and uGCs, the coefficient of variation of the demonstrated reactive scope (DRScv; MacLarnon et al., 2015) was calculated for each individual baboon, using the following formula which corrects for sample size (Tkaczynski et al., 2019):

$$\text{DRScv} = \left(1 + \frac{1}{4n}\right) \times \frac{\text{standard deviation (GC)}}{\text{mean (GC)}} \times 100$$

The DRScv was calculated using all samples collected throughout the study period for fGCs and uGCs separately (Table 1). Samples collected on trapping days (between 25/07/2018 – 02/08/2018: same day for uGCs; + 2-day delay for fGCs) were not included as this does not represent natural variation in fGCs and uGCs. Based on results from LMM3 (see below), urine contaminated faecal samples were also excluded, resulting in n=380 urine and n=498 faecal samples being used for the DRScv calculations.

*2.5. Female reproductive state*

Assessment of female reproductive state has been described previously (Fürtbauer et al., 2020). In brief, n=3 females transitioned from pregnancy to lactation and n=1 was in early lactation (infant between 0-6 months) throughout the study period. For the remaining n=11 females for whom reproductive state was uncertain, faecal samples (n=336, mean±SD=30.5±10.6 samples per female) were analysed for progesterone metabolites (Fürtbauer et al., 2020). This analysis confirmed that n=4 females were acyclic (with infants of > 6 months), n=1 female was pregnant, n=6 females were cycling, of which n=2 females conceived during the study. New infants were recorded around estimated parturition dates for pregnant females (pers. comm. with Human Wildlife Solutions).

To establish biologically relevant categories of reproductive state, we investigated potential differences in uGCs and fGCs between females in early (±first 3 months) and late (±last 3 months) pregnancy (Weingrill et al., 2004) and between females in early lactation (with infants with black, natal coat; between 0-6 months) and late lactation (with infants with brown coat; between 6 to 12 months) (Altmann et al., 1977). Late gestation is often associated with increased levels of plasma GCs (Keller-Wood and Wood, 2001), as reflected in fGCs (Cavigelli, 1999; Nguyen et al., 2008) and uGCs (Smith

and French, 1997; Ziegler et al., 1995) in non-human primates. For lactation, the categorisation may differ based on whether the underlying stressor is hypothesised to be energetic (e.g., Emery Thompson et al., 2010; Foerster et al., 2012) or psychosocial (e.g., Engh et al., 2006; Weingrill et al., 2004). If increased GCs associated with lactation are driven by increased energetic demands, lactation should be defined as the physiological state in which females are acyclic and still producing milk (hormonal definition, using progesterone criteria described above). If increased GCs are linked to the risk of infanticide, the definition should be based on whether the female is still caring for offspring vulnerable to infanticide (social definition). In our study n=2 females were cyclic but also had infants that were targeted/injured by the beta male (pers. obs.), thereby falling into both the hormonal and social definition. In this paper, we present results using the hormonal definition (for results using the social definition see Supplementary Material Table S6; findings are comparable).

*2.6. Dominance rank*

Assessment of female dominance rank has been described previously (Fürtbauer et al., 2020). In brief, rank was calculated based on the outcome of dyadic agonistic interactions (displacements, chases, aggressive displays, n=634) collected ad libitum over 78 days of group follows (Bracken et al., 2021; Fürtbauer et al., 2020). Analyses were run using the packages 'AniDom' (Farine and Sánchez-Tójar, 2017) and 'Compete' (Curley, 2019) in R Studio (version 1.2.5033). The hierarchy was determined to be very steep, repeatable and highly linear. In baboon troops, all adult males outrank all adult females (Engh et al., 2009; Silk et al., 2004). For the two males, rank was calculated based on the outcome of aggressive interactions (Bracken et al., 2021). Ranks were standardized between 0 and 1 (with 0 being the lowest and 1 the highest).

*2.7. Statistical analyses*

All analyses were run in R Studio (version 1.2.5033). Linear mixed effect models (LMMs) were fitted using the package 'lmerTest' (Kuznetsova et al., 2017), to investigate the effect of various predictors on fGCs and uGCs. In all LMMs, baboon ID and date of collection were included as random effects to control for individual differences in fGCs and uGCs and uneven sampling on different days (Schielzeth and Forstmeier, 2009). In all LMMs, fGCs and uGCs were log-transformed to meet normality criteria (assessed visually with QQ-plots; R function *qqnorm*). To test whether full models were significantly different from null models, likelihood ratio tests were used (R function *anova*). To obtain the conditional and marginal r-squared values, which denote the variance explained by the full model (with random terms) and the variance explained by the fixed effects in the model respectively, the function *r2_nakagawa* (R package 'performance') was used (Nakagawa and Schielzeth, 2013). N=53 faecal samples and N=5 urine samples were collected during the collaring period (See Biological validation in Supplementary Material S1.1), which would constitute an unnatural stressor that could interfere with detecting the effect of the natural baseline predictors. Therefore, all analyses (LMM2 – LMM6) were also run with a reduced dataset not including fGCs from the trapping period (+ 2 days; Wasser et al., 2000; Wasser et al., 1994) and uGCs on the day of trapping, respectively. These models obtained comparable results (Supplementary Material, Tables S9-S11). Finally, to rule out that uneven sample sizes (fGCs: n=603; uGCs: n=385) may have biased our results, we sub-sampled the fGCs dataset to n=385 samples and re-ran the main analyses (LMM3 and LLM4), which obtained comparable results (Supplementary Material, Table S7-S8).

*2.8. Predictors of fGCs*

The initial model of fGC predictors (n=603 samples, n=17 individuals; LMM2, Table S3 in Supplementary Material) included: month (July - November), AM/PM (before/after 12PM, to account for potential effects of circadian cortisol rhythms; Coe and Levine, 1995; Fries et al., 2009), sex (male, female, to control for potential sex-differences in baseline GCs; Touma and Palme, 2005) and urine

contamination (n=55 samples; see Palme, 2005; excluding these 55 samples revealed comparable results for all models), as categorical fixed effects, and standardized dominance rank (between 0-1; Fürtbauer et al., 2020) as continuous fixed effect (M1 and M2 were assigned the same standardised rank as F1 and F2 respectively, to avoid covariation between sex and rank).

To investigate responses to daily weather parameters, the main model was run with the same dataset (n=603 samples, n=17; LMM3), where rain, minimum temperature (°C) and day length (minutes between sunrise and sunset, obtained from www.timeanddate.com) from 2 days prior to sample collection were included as fixed effects (see Biological validation in Supplementary Material S1.1 and previous studies using the same time-lag in baboons: Bergman et al., 2005; MacLarnon et al., 2015). Minimum temperature was used given that 'cold stress' is more likely than 'heat stress' at this time of year and in this Mediterranean climate (Chowdhury et al., 2021; Weingrill et al., 2004). Rain day was included as a categorical effect (yes/no; between 07:00 and 19:00). It rained on n=34 days out of n=125 days and mostly over short periods of 1-3 days (>80%), with a median 3 (range=0-19) 'dry days' in between. Based on the results from LMM4, a 2-level factor "pregnant" and "not pregnant" (including lactating and cycling females, and males) was included as a fixed effect. Weather conditions (temperature, day length and rainfall) differed between months (Supplementary Material Fig. S2). To test for collinearity between climatic predictor variables (minimum temperature, day length and rain), we calculated Variance Inflation Factors (VIFs) (R package 'usdm'). No issues of collinearity were found with maximum VIF=1.5 (excluding factors with VIF > 3 is considered a stringent approach to rule out collinearity; Zuur et al., 2010).

To test for the effects of female reproductive state on fGCs, we ran a model (LMM4) including data for females only (n=505 samples, n=15 individuals). We included the same predictors as in LMM3 (see above) and added reproductive state (cycling, pregnant, lactating) as a categorical fixed effect.

*2.9. Predictors of uGCs*

The initial model of uGC predictors (n=385 samples, n=17 individuals; LMM5, Table S3 in Supplementary Material) tested for effects of month (July - November), sample collection time (to account for effects of circadian cortisol rhythms in uGCs: Czekala et al., 1994; Muller and Lipson, 2003), sample collection method (pipette versus Salivette; shown to affect hormone measurements: Fürtbauer et al., 2020), sex (male/female), and standardized dominance rank. To investigate responses to daily weather parameters, the main model was run using the same dataset (n=385 samples, n=17 individuals; LMM6), including minimum daily temperature (°C) and total day length on the day of sample collection (assuming ±4.5 hour time-lag: Wasser et al., 1994) as continuous fixed effects, and rain during the daytime (yes/no) as a categorical fixed effect (see Predictors of fGCs for details on weather data), controlling for sample collection time and method. To test for the effect of female reproductive state on uGCs, we ran a model including female samples only (n= 338 samples, n=15 individuals; LMM7). We included standardized dominance rank, collection method, time of collection, and reproductive state (cycling, pregnant, lactating) as fixed effects.

**Table 1:** Details on study animals, including rank, median, range and sample size (n) for fCs (ng/g) and SG-corrected uGCs (ng/ml). DRScv calculated by SD/mean*100 and corrected for sample size (see Tkaczynski et al., 2019). Samples were collected between 23/07/2018 and 24/11/2018.

| ID | Rank | Median fGC (ng/g) (range; n) | Corrected DRScv | Median uGC (ng/ml) (range; n) | Corrected DRScv |
|---|---|---|---|---|---|
| **M1** | 1 | 462.0 (735.8; 37) | 32.0 | 48.0 (1002.4; 21) | 213.6 |
| **M2** | 2 | 477.4 (727.4; 48) | 34.3 | 83.1 (510.8; 25) | 101.5 |
| **F1** | 1 | 486.9 (598.6; 29) | 29.2 | 56.1 (183.7; 21) | 67.4 |
| **F2** | 2 | 293.9 (637.3; 51) | 35.9 | 81.3 (282.6; 21) | 81.8 |
| **F4** | 4 | 398.3 (645.9; 26) | 37.4 | 97.8 (358.6; 19) | 78.9 |
| **F5** | 5 | 326.0 (403.8: 20) | 29.4 | 37.4 (210.4; 24) | 93.7 |
| **F6** | 6 | 487.0 (803.8; 31) | 40.2 | 74.2 (320.5; 17) | 86.6 |
| **F7** | 7 | 370.7 (775.1; 26) | 42.3 | 23.7 (81.0; 19) | 78.2 |
| **F9** | 9 | 625.9 (712.6; 36) | 32.0 | 47.2 (184.7; 25) | 82.1 |
| **F10** | 10 | 310.6 (817.2; 33) | 50.0 | 55.9 (725.8; 31) | 137.9 |
| **F13** | 13 | 597.8 (935.6; 14) | 48.4 | 117.2 (349.9; 22) | 65.4 |
| **F14** | 14 | 461.5 (778.6; 25) | 33.9 | 103.1 (237.2; 22) | 55.8 |
| **F15** | 15 | 420.8 (1071.8; 24) | 49.0 | 23.7 (48.4; 16) | 59.1 |
| **F16** | 16 | 511.8 (721.8; 16) | 34.1 | 52.7 (215.5; 25) | 94.2 |
| **F17** | 17 | 259.5 (702.6; 27) | 51.2 | 38.0 (120.2; 22) | 68.8 |
| **F18** | 18 | 270.1 (321.1; 33) | 32.3 | 32.1 (79.3; 25) | 53.9 |
| **F19** | 19 | 325.4 (490.0; 22) | 31.9 | 78.7 (165.0; 25) | 62.3 |
| **Mean** | | | **37.8** | | **87.1** |

*samples collected on trapping days (between 25/07/2018 – 02/08/2018: same day for uGCs; + 2-day delay for fGCs) were not included in order to sample only natural variation in uGCs/fGCs without the effect of an unnatural stressor. Based on results from LMM3, urine contaminated faecal samples (n=55) were also excluded in these calculations, resulting in n=380 urine samples; n=498 faecal samples.

## Results

### *3.1 Predictors of fGCs*

fGCs ranged from 79.7 to 2400.0 ng/g (n= 603 samples), with a mean DRScv of 37.8% (Table 1). Our initial model (LMM2) confirmed monthly changes in fGCs, suggesting a gradual decrease from winter to summer months (Fig. 1a; Supplementary Material: Table S3). The main model (LMM3) was significantly different from the null model ($\chi2 = 114.29$, $p<0.001$). Baboon ID was highly significant, indicating consistent individual differences in fGCs ($p<0.001$). Dominance rank and sex had no significant effect on fGCs (Table 2). fGCs did not differ significantly between samples collected in the AM or PM (Table 2; Fig. 2b). fGCs were significantly negatively correlated with day length (Table 2; Fig. 1). Minimum temperature and rain days did not predict fGCs (Table 2). fGCs were significantly higher in pregnant females compared to cycling and lactating females (Table 3, Fig. 3; LMM4: full versus null model: $\chi2 =110.64$, $p<0.001$). No significant differences in fGCs were found between cycling and lactating females (estimate±se = 0.07±0.09, t-value=0.772, $p=0.447$; Fig. 3). Note that no statistically significant differences were found between early and late pregnancy (Supplementary Material; Table S4; Fig. S3) or between lactating females during early or late stage of lactation (Supplementary Material: Table S5; Fig. S3). Finally, we found that urine-contaminated faecal samples had significantly higher fGCs (Table 2; Table 3).

*3.2 Predictors of uGCs*

uGCs ranged from 2.58 to 1007.36 ng/ml (n= 385 samples), with a mean DRScv of 87.1% (Table 1). Month was not a significant predictor for uGCs (LMM5; Fig. 1a; Supplementary Material: Table S3). The main model (LMM6) was significantly different from the null model ($\chi2 = 69.394$, $p<0.001$). Baboon ID was highly significant, indicating consistent individual differences in uGCs ($p<0.001$). Time of sample collection had a significant effect on uGCs (with a decline from morning to evening; Table 2, Fig. 2a). uGCs were significantly lower in samples collected with Salivettes compared to pipettes (Table 2; sampling methods were balanced across months and reproductive states) and uGCs were significantly higher on rain days (Table 2). Sex and rank did not predict uGCs (LMM6, Table 2). Female

reproductive state did not predict uGCs (LMM7, Table 3; Fig. 3) and no statistically significant differences were found between early and late pregnancy (Supplementary Material: Table S4; Fig. S3) or between lactating females in the early or late stage of lactation (Supplementary Material: Table S5; Fig. S3).

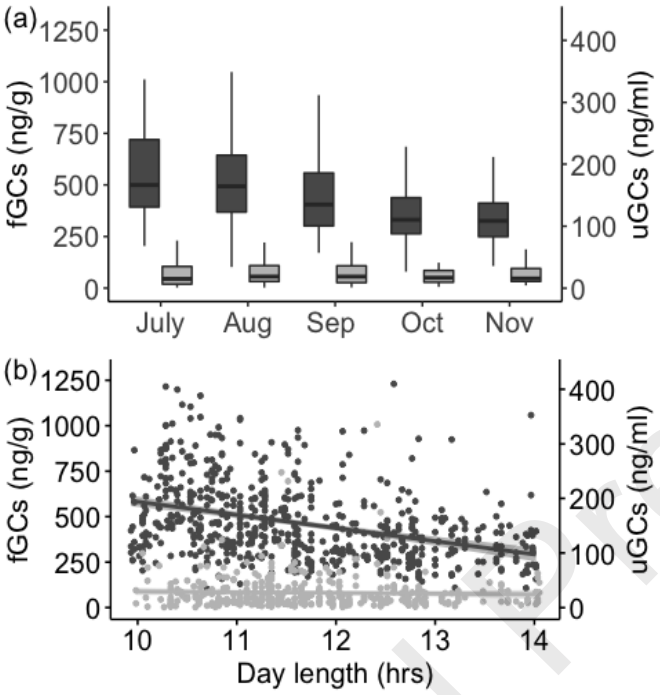


**Figure 1: (a)** Monthly variation in fGCs (n=603 samples) and SG-corrected uGCs (n=385 samples) in n=17 wild chacma baboons. Note that non-transformed data are shown. Boxes (dark grey = fGCs, light grey = uGCs) show interquartile ranges, lines denote median values and whiskers denote maximum and minimum values. **(b)** Variation in fGCs (dark grey) and SG-corrected uGCs (light grey) in relation to day length. Dots present real datapoints, the solid line is the linear regression line (fGCs/uGCs ~ Day length) with 90% confidence intervals. Four datapoints not shown for uGCs (>500 ng/ml, n=2 collected in September, n=2 collected in October). Two data points not shown for fGCs (>1500 ng/g, n=1 collected in July, n=1 collected in August).

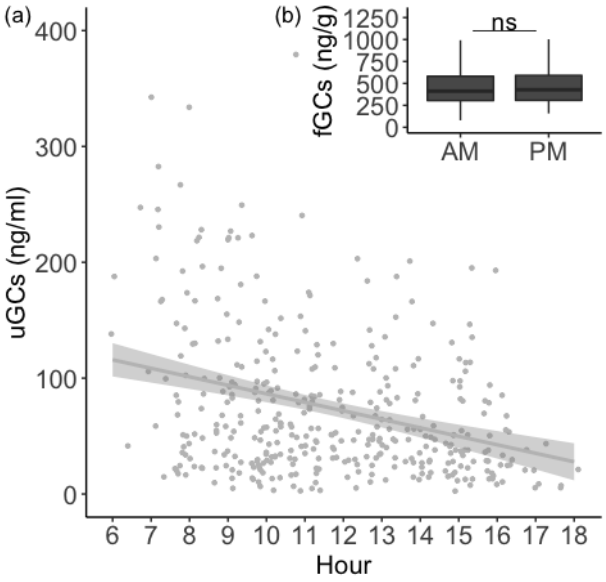


**Figure 2**: **(a)** Effect of sample collection hour on SG-corrected uGCs (n=385). Note that four data points are not shown (>500 ng/ml; collected before 8:00h) and one data point (372 ng/ml in the 14$^{th}$ hour eclipsed by panel (b)). Dots present real datapoints, the dark grey line is the linear regression line (uGC ~ Time) with 90% confidence intervals in grey. **(b)** Non-significant effect of AM/PM on fGCs (n=603) Note that two outliers are not shown (>1500 ng/g; n=1 PM, n=1 AM). Boxes show interquartile ranges, lines denote median values and whiskers denote maximum and minimum values.

**Table 2**: Effects of sex, dominance rank, day length, minimum temperature, rain day (Y/N) on fGCs (LMM3; n=603 samples) and SG-corrected uGCs (LMM6; n=385 samples) in n=17 wild chacma baboons. Sample collection time (AM/PM) and urine contamination were controlled for in LMM3. Sample collection time (hour of the day) and collection method (Salivette or pipette) were controlled for in LMM6. Significant effects are in bold. The fixed effects in LMM3 explain 27.5 % of the variance in fGCs, the full LMM3 (with random terms) explains 54.9 % of the variance. The fixed effects in LMM6 explain 14.5 % of the variance in uGCs, the full LMM6 (with random terms) explains 43.8 % of the variance.

| **Predictor variable** | **Estimate** | **SE** | **T** | ***p*** |
|---|---|---|---|---|
| *fGCs (LMM3)* | | | | |
| **Sex (male)** | 0.180 | 0.143 | 1.252 | 0.224 |
| **Dominance rank** | 0.141 | 0.150 | 0.943 | 0.360 |
| **Day length (hrs)** | -0.169 | 0.021 | -8.184 | **<0.001** |
| **Minimum Temperature** | 0.010 | 0.011 | 0.897 | 0.372 |
| **Rain day (Y)** | -0.025 | 0.047 | -0.525 | 0.601 |
| **Reproductive state (Pregnant)** | 0.428 | 0.061 | 7.032 | **<0.001** |
| **AM/PM (PM)** | 0.050 | 0.029 | 1.716 | 0.087 |
| **Urine contamination** | 0.128 | 0.047 | 2.729 | **0.007** |
| *uGCs (LMM6)* | | | | |
| **Sex (male)** | -0.230 | 0.438 | -0.525 | 0.608 |
| **Dominance rank** | 0.421 | 0.411 | 1.023 | 0.324 |
| **Day length (hrs)** | -0.046 | 0.060 | -0.770 | 0.443 |
| **Minimum Temperature** | 0.017 | 0.027 | 0.625 | 0.534 |
| **Rain day (Y)** | 0.233 | 0.107 | 2.171 | **0.034** |
| **Collection time** | -0.126 | 0.015 | -8.265 | **<0.001** |
| **Method (Salivette)** | -0.226 | 0.084 | -2.705 | **0.007** |

**Table 3**: Effects of dominance rank and reproductive state on fGCs (LMM4; n=507 samples) and SG-corrected uGCs (LMM7; n=338 samples) in n=15 wild female chacma baboons. Sample collection time (AM/PM) and urine contamination were controlled for in LMM4. Sample collection time (hour of the day) and collection method (Salivette or pipette) were controlled for in LMM7. Significant effects are in bold. The fixed effects in LMM4 explain 29 % of the variance in fGCs, the full LMM4 (with random terms) explains 54 % of the variance. The fixed effects in LMM7 explain 12.4 % of the variance in uGCs, the full LMM7 (with random terms) explains 48.1 % of the variance.

| **Predictor variable** | **Estimate** | **SE** | **T** | ***p*** |
|---|---|---|---|---|
| *fGCs (LMM4)* | | | | |
| **Dominance rank** | 0.127 | 0.152 | 0.836 | 0.423 |
| **Reproductive state (Lactating)*** | -0.463 | 0.074 | -6.272 | **<0.001** |
| **Reproductive state (Cycling)*** | -0.393 | 0.081 | -4.843 | **<0.001** |
| **Day length (hrs)** | -0.173 | 0.019 | -9.321 | **<0.001** |
| **AM/PM (PM)** | 0.047 | 0.032 | 1.467 | 0.143 |
| **Urine contamination** | 0.118 | 0.049 | 2.421 | **0.016** |
| *uGCs (LMM7)* | | | | |
| **Dominance rank** | 0.467 | 0.466 | 1.002 | 0.341 |
| **Reproductive state (Lactating)*** | -0.147 | 0.208 | -0.705 | 0.483 |
| **Reproductive state (Cycling)*** | -0.063 | 0.217 | -0.292 | 0.771 |
| **Rain day (Y)** | 0.249 | 0.112 | 2.221 | **0.031** |
| **Collection time** | -0.115 | 0.016 | -7.044 | **<0.001** |
| **Method (Salivette)** | -0.223 | 0.085 | -2.607 | **0.010** |

*Reference category: Pregnant

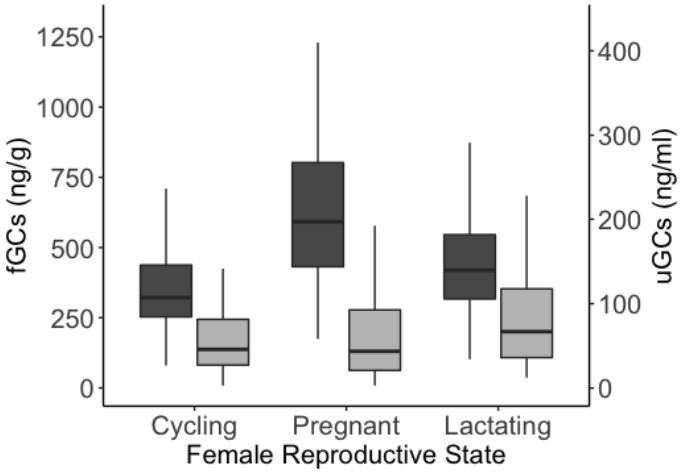


**Figure 3**: Effect of female reproductive state on fGCs (n=507 samples) and SG-corrected uGCs (n=338 samples) in n=15 wild chacma baboons. "Pregnant" and "Lactating" include early/late stages of pregnancy and lactation, respectively (see Fig. S3 for detailed female reproductive state). Note that non-transformed data are shown. Boxes (dark grey = fGCs, light grey = uGCs) show interquartile ranges, lines denote median, whiskers denote maximum and minimum values. Outliers are not shown.

**4. Discussion**

Choosing an appropriate sample type for endocrinological studies requires consideration of i) the target hormone(s), ii) the time-frame of the research question (e.g., short- versus long-term hormonal activity), iii) the route and potential time-lag of hormone excretion, and iv) the feasibility of sample collection (Cook, 2012; Heistermann, 2010; Sheriff et al., 2011). In the wild, non-invasive sampling of faeces and (to a much smaller extent) urine, has allowed endocrinologists to investigate predictors of variation in fGCs and uGCs in a wide range of species (Creel, 2005; Palme, 2019; Sheriff et al., 2011). It is well established that, depending on the sample matrix, different time lags need to be accounted for when linking GCs and their metabolites in urine and faeces to predictors of interest (Heistermann, 2010; Touma and Palme, 2005). However, the suitability of the sample type to investigate predictors with different temporal profiles, although acknowledged in several review papers (Behringer and Deschner, 2017; Creel, 2001; Heistermann, 2010), has not been explicitly tested in a comparative

study, and therefore the relative merits of the either approach was previously only assessed indirectly. To address this gap, we investigated the effects of long-term, gradually changing factors (seasonal changes, reproductive state) and short-term, acute factors (daily weather parameters; circadian rhythm) on variation in uGCs and fGCs simultaneously in a wild primate. Below, we discuss our findings and implications for investigating stressors of different temporal nature in future research, focussing on the use of urine for GC measurements. uGCs may become increasingly used as they can be quantified alongside other biomarkers (Behringer et al., 2017; Crockford et al., 2013; Deschner et al., 2020; Emery Thompson and Knott, 2008; Fürtbauer et al., 2020; Heistermann and Higham, 2015; Higham et al., 2020; Preis et al., 2018; Sadoughi et al., 2021) and open new research avenues regarding short-term changes in HPA-axis activity (Wittig et al., 2015; Wittig et al., 2016).

Our results confirm previously established predictors of fGCs in chacma baboons. First, and in line with findings reported for Western Cape (Weingrill et al., 2004), and other wild baboon populations (Engh et al., 2006; Gesquiere et al., 2008), we find increased fGCs in pregnant compared to cycling and lactating females (Fig. 3). Increased GCs are expected during pregnancy, and have been ascribed to the interaction between elevated levels of oestrogen and the HPA-axis (Coe et al., 1986; Pepe et al., 1982), as well as the release of corticotropin-releasing hormone which can further increase HPA-axis activity (McLean and Smith, 1999). Second, as previously reported for baboons in the Western Cape (Chowdhury et al., 2021; Weingrill et al., 2004), we find a strong negative relationship between day length and fGCs (Fig. 1), possibly pointing towards the physiological consequences of compressed time budgets. Chacma baboons in the Western Cape experience four hours of difference in day length between summer and winter (Hill et al., 2003), with shorter days imposing a temporal bottleneck on key activities (i.e. feeding, moving, resting and grooming: Dunbar et al., 2009; Hill et al., 2003; van Doorn et al., 2010). This bottleneck results in reduced time resting and socializing, which could explain heightened fGCs (Chowdhury et al., 2021; Weingrill et al., 2004). Seasonal variation in food availability/quality may translate to changes in HPA-axis activity (e.g., Gesquiere et al., 2008; Sapolsky, 1986). Generally, Cape baboon diets differ between winter and summer (Davidge, 1978;

Lewis & O'Riain, 2017; Van Doorn et al., 2010) and in our troop, we observed an increase in undigested seeds in faecal samples from winter to summer (pers. obs.). However, urinary C-peptide levels during the same study period did not suggest winter to be more energetically challenging (Fürtbauer et al., 2020). Additionally, diet composition may affect faecal bulk and the metabolism of hormones (Goymann et al., 2012), but expressing fGCs in dry weight (present study) controls for most of this variation (Wasser et al., 1993). While we cannot test the effect of diet on fGCs as systematic data was not collected, the similarity in findings from baboon troops living at the same southerly latitude but with differences in habitat and diet (details for: 'De Hoop'; Hill, 1999; 'Tokai'; Chowdhury, 2018), points towards the temporal constraint imposed by day length as an important driver of seasonal changes in fGCs (see above). Finally, we found higher fGCs in urine-contaminated faecal samples, which has been assumed but, to date, not demonstrated (Palme, 2005; Palme et al., 2013).

In contrast to fGCs, simultaneously measured uGCs were unrelated to female reproductive state (Fig. 3) and day length (Fig. 1). This is likely due to the larger inherent fluctuation of uGCs, which we discuss in detail below. As expected, we found a diurnal effect on uGCs with highest concentrations in the morning and a gradual decline throughout the day (Fig. 2a; 'cortisol awakening response': Fries et al., 2009), which was not expected nor detected in fGCs (Fig. 2b), as baboons are large-bodied animals with long gut passage times (see reviews by: Behringer and Deschner, 2017; Heistermann, 2010). Moreover, rain days had a weak positive effect on uGCs. Unlike studies where rainfall is used as a proxy for food availability (Foley et al., 2001; Pereira et al., 2006; Pride, 2005), here rainfall was investigated on a daily basis as a potential acute thermoregulatory stressor (e.g., rainfall may compound cold temperatures as has been proposed in baboons and other primates, Chowdhury et al., 2021; Foerster et al., 2012) or a time constraint stressor (similar to day length; see above) as activities such as feeding or grooming are interrupted due to sheltering (Hanya et al., 2018; Majolo et al., 2013). Finally, we found that uGCs measures were lower in samples collected using Salivettes compared to those collected by direct pipetting, which could be due to absorption of steroid hormones by the synthetic swab (see Discussion in Fürtbauer et al., 2020).

Taken together, our results show that while uGCs and fGCs are both used to measure HPA-axis activity (see examples listed in introduction), they do not necessarily convey the same information. While this is to be expected, our study forms the first direct comparison demonstrating that fGCs correlate with states or gradually changing (e.g., reproductive state or incremental increases in day length) long-term predictors, whereas uGCs reflect circadian patterns and sporadic, short-term predictors (e.g., rainfall). This difference is likely due to their respective time lags to excretion, which affect the inherent fluctuation of GCs in each sample type (Behringer and Deschner, 2017). To quantify this fluctuation in uGCs and fGCs, we used the coefficient of variation of the Demonstrated Reactive Scope (DRScv) (MacLarnon et al., 2015; Tkaczynski et al., 2019), showing that variation in uGCs was on average twice as high as variation in fGCs over the course of the study period (Table 1). This supports the notion that, in comparison to uGCs, fGCs provide a more cumulative measure of GC production, less influenced by episodic fluctuations (Touma and Palme, 2005), making it a more suitable measure of baseline and long-term HPA-axis activity. uGCs are excreted with shorter-time lags and more closely resemble the pattern of blood GCs (as has been demonstrated in a range of farm animals: Hay et al., 2000; Higashiyama et al., 2009; Mormède et al., 2007), making them more suitable for measuring the effect of acute stressors (Fig. 4). Note that while the DRScv provides a valuable tool for assessing variability in uGCs and fGCs independently, our results strongly suggest that they are matrix-dependant, and thus, should not be used to compare across sample types, as proposed previously (MacLarnon et al., 2015).

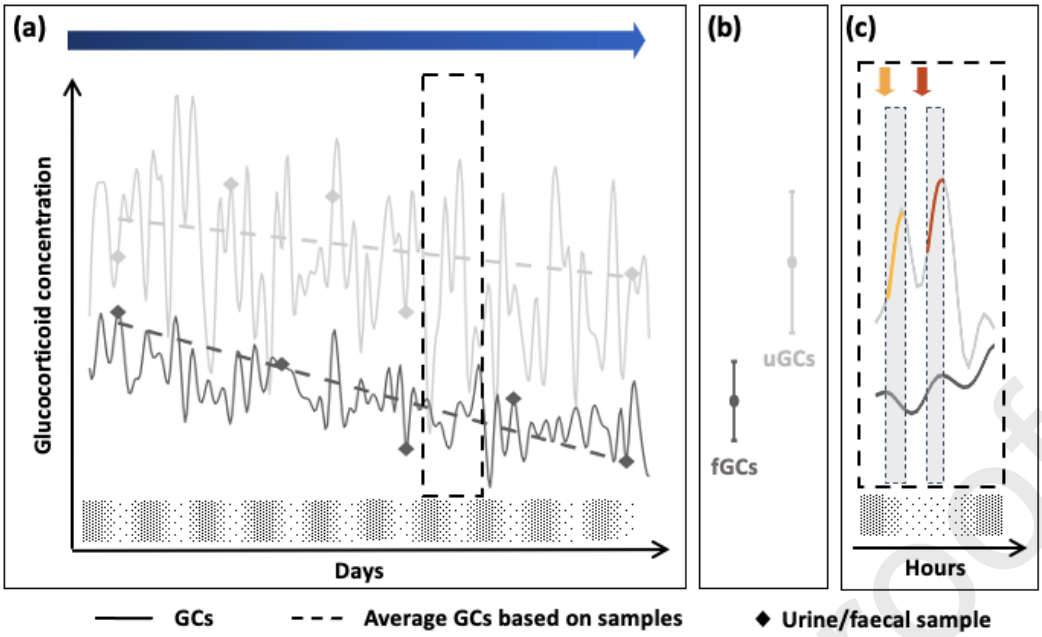


**Figure 4**: Schematic representation of hypothetical HPA-axis activity measured through faecal GC metabolites (fGCs; dark grey solid lines) and urinary GCs (uGCs; light grey solid lines); diamonds represent hypothetically collected samples and dashed lines represent average uGCs/fGCs calculated based on these samples. Time periods are denoted by the bottom band (density of dots denote night to day cycles). Plotted values (solid lines) were randomly generated in R (function "rnorm") within the constraints of the observed variation (DRScv) for fGCs/uGCs throughout the study period (see Table 1). To simulate the circadian peak in uGCs, values were set at 60% the mean value at the onset of the day (based on AM uGCs being 60% higher than the mean uGCs; Fig. 2a); **(a)** over a 10-day period a long-term stressor (blue horizontal arrow) can be linked to an average decrease (dashed line) in fGCs even when samples are collected ad libitum, as fGCs reflect cumulative HPA-axis activity, limiting the effect of daily fluctuations. Conversely, the effect of the long-term stressor is not significant for uGCs due to larger daily fluctuations. **(b)** Mean and standard deviation for fGCs and uGCs across 10 days (summary measures used to calculate the DRScv; Table 1) **(c)** within a single day, uGCs fluctuate across hours; grey boxes represent ±4.5 hr time-windows (based on excretion lag for uGCs; Wasser et al. 1994), this means circadian patterns (yellow vertical arrow) and short-term stressors (brown vertical arrow) are captured over the course of hours, while they are not reflected in fGCs which (in baboons) are excreted with a time lag of ±2 days.

The apparent differences between predictors of variation in uGCs and fGCs raise the question: can we use sample matrices with excretion lags that do not correspond to the temporal profile (i.e., short- versus long-term) of the stressor being investigated? For fGCs, the answer seems relatively straight-forward as it is likely a matter of the severity of the stressor of interest: if a short-term stressor is severe enough, even a 'muted' fGCs profile will reflect the stressor (with a species-specific time lag; Heistermann, 2010; Palme, 2019). This has been clearly demonstrated in a number of biological validation studies, where fGCs increase after capture and/or handling (Hämäläinen et al., 2014; Terio et al., 1999; Wasserman et al., 2013) or after ACTH injections which induce short-term rises in plasma cortisol (Carrera et al., 2020; Schatz and Palme, 2001; Terio et al., 1999). Acute but less severe stressors as well as circadian patterns in HPA-axis activity, on the other hand, are less likely to be reflected in fGCs, particularly in large-bodied mammals with low defecation rates relative to small-bodied animals (Rimbach et al., 2013) where the pooled endocrine activity would not provide the adequate "resolution" to capture smaller peaks (Heistermann, 2010).

Whether uGCs can reflect long-term HPA-axis activity is a qualitatively different question; can a long-term predictor be detected despite the 'noise' introduced by short-term stressors? First, the duration of the study period may play a role. Longer study periods benefit from samples being temporally spread out which may permit the detection of overall HPA-axis activity patterns correlated with long-term stressors despite daily fluctuations in uGCs. Our study period was relatively short (20 weeks) compared to studies on uGCs in other primates (between 1 and 20 years: Emery Thompson et al., 2010; Muller et al., 2021; Touitou et al., 2021; Wessling et al., 2018), which report variation in uGCs correlated with what can be considered long-term predictors of HPA-axis activity, e.g., dominance rank (Muller et al., 2021), seasonal changes in fruit availability (Muller and Wrangham, 2004), and female reproductive state (Emery Thompson et al., 2010; Touitou et al., 2021). For example, high uGCs during late stages of pregnancy have been noted in wild (Touitou et al., 2021) and captive primates (Smith and French, 1997; Ziegler et al., 1995) including baboons (French et al., 2004). In our study, the increase in uGCs between "early" and "late" pregnancy was marginally insignificant (Supplementary

Material: Fig. S3; Table S4). This pattern could potentially have become apparent with larger sample sizes across stages of pregnancy.

Second, sampling frequency may be important if uGCs are to be used as a measure of long-term HPA-axis activity. Low sample sizes will decrease the confidence around the mean generally (Ross, 2020), but this may be particularly pertinent for a sample matrix with larger inherent fluctuation like uGCs. Studies with low sampling frequencies for uGCs face the risk of overall patterns (which are informative for long-term predictors) becoming masked if analytical or methodological precautions (see below) are not taken to reduce the impact of samples which reflect uGC values at range extremities. This could have played a role in our study despite a relatively high sampling frequency compared to similar studies in the wild (e.g., Emery Thompson et al., 2010; Muller et al., 2021; Touitou et al., 2021; Wessling et al., 2018) (total n=385 urine samples across 20 weeks; see Table 1). If sampling frequency is low, or more generally, to reduce the effect of circadian patterns and acute stressors on uGCs measurements, various steps can be taken. Statistically, calculating uGCs averages, e.g., on a monthly basis (Muller et al., 2007; Muller and Wrangham, 2004) may provide a less 'noisy' estimate of uGCs, making the detection of long-term predictors more likely (but this will reduce the sample size for statistical analysis and the mean will again be subject to sample size effects; Ross, 2020). To control for circadian patterns, studies can restrict their analyses to a specific time-frame (e.g., <10:00 AM Muller and Wrangham, 2004), control for time of urine sample excretion (Muller et al., 2021; Touitou et al., 2021; Wessling et al., 2018; present study) or correct uGC values for excretion time (Emery Thompson et al., 2020; Emery Thompson et al., 2010). However, as shown in the present study (controlled for time of sample excretion), this may not be sufficient to capture long-term effects on uGCs.

Finally, some long-term stressors may be reflected in uGCs depending on their severity or co-occurrence with short-term stressors. For instance, in the same chimpanzee study population, uGCs increased during a period of low fruit availability in one (Muller and Wrangham, 2004) but not in another study period (Muller et al., 2021). It was suggested that food deprivation may need to fall

below a critical threshold to be reflected in increased uGCs (Muller et al., 2021). Additionally, uGC measurements may reflect long-term predictors if they are associated with acute/day-to-day stressors. For instance, in male chimpanzees, dominance rank is positively associated with uGCs (Muller et al., 2021; Muller and Wrangham, 2004), which is mediated by aggressive interactions (Muller et al., 2021). Similarly, parous females have increased uGCs, which is linked to increased male harassment during this reproductive state (Emery Thompson et al., 2010). In these cases, uGCs correlate with long-term predictors (dominance rank, reproductive state), and may capture the effect of the psychological stressor or metabolic cost of anticipated or actual aggressive interactions, which constitute acute stressors. Indeed, in a study on male chimpanzees, the effect of dominance rank was only apparent in afternoon uGCs (after a day of social interactions), suggesting that acute stressors can reveal long-term predictors (Muller and Wrangham, 2004). In contrast, superimposed acute stressors which are not part of predictable life history or environmental predictors (McEwen and Wingfield, 2003), may contribute to fluctuations and overall variation in uGCs, potentially decreasing the likelihood of detecting long-term effects. This may be particularly relevant for animals exposed to unpredictable anthropogenic stressors, an increasingly studied aspect in wildlife endocrinology (Hammond et al., 2019; Kaisin et al., 2021; Kleist et al., 2018; Van Meter et al., 2009).

## 5. Conclusions

The present study, to our knowledge, provides the first simultaneous investigation into the predictors of variation in fGCs and uGCs, offering empirical evidence for the long-standing assumption that GCs in sample matrices with different excretion time-lags reflect predictors with different temporal profiles. Our findings highlight the difference in inherent fluctuation between uGCs and fGCs and its potential associated consequences for investigating short- versus long-term effects on HPA-axis activity. While uGCs offer the opportunity to study short-term effects, they undergo more pronounced fluctuations, and thus, may be limited in their ability to capture long-term effects. Given the increasing

use of urine for biological monitoring, knowledge of this potential limitation will be crucial to consider, in particular in studies of shorter duration or where sampling frequency is low. Where logistically and financially possible, uGCs and fGCs should be monitored in tandem to obtain a comprehensive understanding of short- and long-term drivers of HPA-axis activity.

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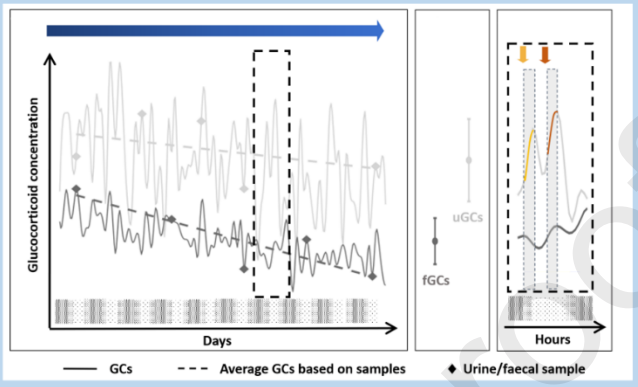
**Simultaneous investigation of urinary and faecal glucocorticoid metabolite concentrations reveals short- versus long-term drivers of HPA-axis activity in a wild primate (*Papio ursinus*)**

Faecal and urinary glucocorticoid metabolites (fGCs and uGCs) are widely used to investigate HPA-axis activity in wild animals

GC excretion lag times differ between urine and faeces, leading to larger inherent fluctuations in uGCs...




...making fGCs more suitable for investigating long-term effects, and uGCs for short-term effects

fGCs were predicted by day length and reproductive state...

...and uGCs were predicted by rain and time of day

**Conclusion: In tandem, uGCs and fGCs provide a more comprehensive picture of HPA-axis activity**

**CRediT authorship contribution statement:**

**Charlotte Christensen:** Conceptualization, Methodology, Formal analysis, Investigation, Data Curation, Writing – Original Draft, Visualization, Project administration **Anna M. Bracken**: Formal analysis, Investigation, Writing – Review & Editing **M. Justin O'Riain:** Resources, Project administration, Writing – Review & Editing **Michael Heistermann:** Investigation, Resources, Writing – Review & Editing **Andrew J. King:** Resources, Project administration, Writing – Review & Editing **Ines Fürtbauer:** Conceptualization, Methodology, Investigation, Resources, Data Curation, Writing – Review & Editing, Visualization, Supervision, Project administration, Funding acquisition.

**Highlights**

- First simultaneous analysis of uGCs and fGCs to study short- and long-term effects 2
- Matrices differ in inherent fluctuation with implications for HPA-axis monitoring 3
- uGCs fluctuate more, reducing their ability to capture long-term effects 4
- In tandem, uGCs and fGCs offer a comprehensive understanding of HPA-axis activity 5