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Monitoring the effects of land transformation on African clawless otters (*Aonyx capensis*) using fecal glucocorticoid metabolite concentrations as a measure of stress

Running title: Human impact-related stress in otters

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Abstract

In a time of increasing environmental change caused by anthropogenic disturbance, there is a greater need to understand animal adaptations to manmade environments. In this regard, the measurement of stress-related endocrine markers provides a useful tool to examine the impact of environmental challenges and the physiological consequences for wildlife occupying such space. The aims of the present study were to validate fecal glucocorticoid metabolite (fGCM) concentrations as a measure of stress using samples

from a male African clawless otter (*Aonyx capensis,* Schinz 1821; *n* = 1) and to compare fGCM concentrations of otters occurring in a transformed and in 2 natural areas in South Africa. From the 5 different enzyme-immunoassays (EIA) tested, a cortisol and oxoetiocholanolone (measuring 11,17 dioxoandrostanes) EIA revealed the highest response (74% and 48% increase, respectively) 30 and 24 hours after a stress event (translocation of a captive individual as part of its rehabilitation prior to release), respectively. For both EIAs, fGCM concentrations were comparable for samples collected up to 3 h post-defecation. Using the cortisol EIA for subsequent analyses, fGCM concentrations of animals from the transformed area (*n* = 20; mean [± SD]: 0.468 [± 0.539] µg/g dry weight [DW]) were significantly higher (*p* = 0.013) than those from otters in the natural areas (*n* = 17; 0.242 [± 0.226] µg/g DW). These preliminary results suggest that African clawless otters may have increased adrenocortical activity that could be due to conditions linked to living in a transformed environment.

Key words: African clawless otter, fecal glucocorticoid metabolites, non-invasive hormone measurement, stress, urbanization

INTRODUCTION

Land use change (land transformation) is the most important factor contributing to habitat degradation, alteration and fragmentation globally (Marzluff & Ewing 2001). In addition, land transformation promotes biotic homogenization because transformed habitats are constructed to meet the requirements of humans (McKinney 2006). Isolated heterogeneous habitats exist within transformed areas (Honnay *et al.* 1999; McIntyre *et al.* 2001; Gibb & Hochuli 2002), where the surrounding matrix is inhospitable to some species living within these isolated pockets (Öckinger *et al.* 2012; Freeman *et al.* 2018).

Food availability is a key factor contributing to population size and dynamics (Chapman *et al.* 2015) and transformed areas may offer benefits such as increased food resources, shelter and, in some instances, decreased or altered predation, which some species are able to exploit (Bonier *et al.* 2006; McKinney 2006). However, species living in urban environments are faced with selective pressures such as increased human activity, noise

and toxin levels (Bonier *et al.* 2006). As a result, species roaming in transformed areas tend to have elevated stress-related hormone levels compared to those living in more natural environments (Fokidis *et al*. 2009; Scheun *et al.* 2015; McLennan *et al.* 2019).

There are many definitions of stress. Herein, stress is defined as a general syndrome occurring in response to any stimulus (stressors) that threatens or appears to threaten the homeostasis of an individual (Selye 1936; Wielebnowski 2003). When confronted with a stressor, an animal will rely on different biological systems (e.g. behavioral, autonomic nervous, neuroendocrine and immune) to prompt a stress response to cope with the stressor and restore homeostasis (Moberg & Mench 2000). The stress response includes the activation of the hypothalamic–pituitary–adrenocortical (HPA) axis and the sympatho-adrenomedullary system, which is sensitive to environmental stressors (Cavigelli *et al.* 2005). In addition, the HPA axis responds to factors such as alteration in diet (Kalliokoski 2012) or reproductive status, circadian rhythms and aging of an animal (Cavigelli *et al.* 2005). HPA axis activation results in an increase in the secretion of glucocorticoids by the adrenal glands (Palme 2019). Catecholamines are secreted rapidly in response to (short-term) stressors (Palme *et al.* 2005), while glucocorticoids (GCs) are secreted in response to acute and long-term stressors. The secretion of these hormones in response to perceiving a stressor stimulates rapid changes in the physiology of the animal (e.g. increased heart rate and shift in energy balance) to allow it to respond and ideally cope with the stressor (e.g. initiate fight or flight response) (Wielebnowski 2003). However, if stressor(s) are perceived over a prolonged period, this may decrease individual fitness through immunosuppression and atrophy of tissues (Möstl & Palme 2002). At population level, a prolonged stress response can decrease reproductive capacity, increase disease susceptibility and impair growth (von der Ohe & Servheen 2002; Wielebnowski 2003).

Measuring concentrations of GC or its metabolites in matrices such as feces instead of blood is a non-invasive approach. Especially when working with free-roaming wildlife, feces can often be collected more easily and safely, and animals are usually not disturbed during the procedure and, thus, sampling can be regarded as feedback-free (Touma & Palme 2005). Therefore, measuring GC metabolites in feces gives reliable

information on the welfare status of animals (Lane 2006). There are species-specific and sex-specific differences in the types of GC metabolites found in feces (Wasser *et al.* 2000; Touma *et al.* 2003; Palme *et al*. 2005) and multiple criteria should be taken into account when applying any non-invasive approach for measurement of glucocorticoid metabolites from feces in a species being investigated for the first time (Ganswindt *et al.* 2012). To account for these differences, an assay measuring fecal glucocorticoid metabolite (fGCM) concentrations must first be established through analytical, physiological and/or biological validation (Touma & Palme 2005). In short, assay reliability is examined by stimulating a stress response in an animal and measuring fGCM concentrations before and after the stress event, and suitable assays will show a related peak in fGCM concentrations (Palme 2019). GCs are metabolized prior to excretion and resulting fGCM can be even further metabolized by bacterial enzymes post-defecation (Palme 2005). Furthermore, activation of the HPA axis responds to the circadian rhythm in most vertebrates (Cavigelli *et al.* 2005) and, consequently, diurnal variations of fGCM have been reported (Touma & Palme 2005, Ganswindt *et al.* 2012) Thus, the time-dependent stability of GC metabolites post-defecation (Crossey *et al.* 2018) and the effect of the circadian rhythm of circulating GCs (Ganswindt *et al.* 2012) must be investigated to warrant the comparability between different samples.

In a time of increasing environmental change caused by the increase in the human population, thus increasing demand for land use change, studies on the biology of taxa which exploit a wide range of habitats and how animals adapt to transformed areas are becoming increasingly important (Bateman & Fleming 2012). African clawless otters (*Aonyx capensis,* Schinz 1821) display a broad distribution encompassing a variety of habitats and climate regimes, with a distribution range stretching from East Africa to South Africa (excluding the Congo basin) (Somers & Nel 2013). The species responds behaviorally to the abundance and behavior of their prey (Somers & Nel 2004; Nel & Somers 2007), and is able to utilize resources provided by anthropogenic landscapes (Ponsonby & Schwaibold 2019). Therefore, African clawless otters are a useful model species for studying phenotypic adaptability and potential fitness costs in the face of increasing anthropogenic pressure.

The aim of the present study was to evaluate and compare fGCM concentrations as a measure of stress in African clawless otters occurring in natural and transformed areas. As a prerequisite, the study also aimed to establish a non-invasive approach for measuring fGCM concentrations in a male African clawless otter. More specifically, this part of the study aimed at: (i) determining stress-related physiological responses in African clawless otter feces by monitoring a transport event as a form of biological validation (n = 1); (ii) investigating the potential influence of time of day (circadian rhythm) on fGCM excretion; and (iii) examining the changes in fGCM concentrations over time after defecation. We hypothesized that the otters occurring in the transformed area will have higher fGCM concentrations compared to the natural areas due to factors linked to anthropogenic disturbance.

MATERIALS AND METHODS

Study sites and sample collection

Fecal samples were collected from free-ranging otters at Cobham Nature Reserve (n = 13 collected between 8 October and 1 November 2018), at Verloren Vallei Nature Reserve (n = 4 collected between 28 June and 19 August 2018) and at Millstream Farm (n = 20 collected between 26 June and 18 August 2018) (Fig. 1).

Cobham Nature Reserve (29°41′58.8″S, 29°24′50.3″E) is situated in the Southern Maloti-Drakensberg Park, KwaZulu Natal, South Africa. The size of the reserve is 520 km^2 with 2 main vegetation zones, Alti-montane and Afro-montane grassland. Samples were collected along the Polela River. The area has an annual average rainfall of 800 mm and temperatures range between −15°C and 35°C (SAWS 2018). The 58.91 km^2 Verloren Vallei Nature Reserve (25°19′10.9″S, 30°07′38.8″E) in Mpumalanga, South Africa, is situated in the Highveld montane grassland and contains several permanent wetlands. The reserve has an annual mean rainfall of 664 mm and temperatures range between −13°C and 21.9°C (SAWS 2018). Both nature reserves (Cobham and Verloren Vallei) have had little anthropogenic transformation in their landscapes and were, therefore, chosen as areas that are mostly ecologically intact and characterized by very low levels of direct anthropogenic disturbance.

The third study site, Millstream Farm (25°27′07.3″S 30°05′30.7″E), is situated in the Mpumalanga Highveld, South Africa. Millstream Farm offers accommodation on-site and recreational fly-fishing in 8 dams and 13 weirs along the Witpoort River (all stocked with rainbow trout, *Oncorhynchus mykiss*). While offering suitable habitat for otters in terms of the vegetation cover, and water and food availability, this area is also characterized by increased human presence (permanent inhabitants and visiting anglers). The farm is located 21 km from Verloren Vallei Nature Reserve and is characterized as experiencing similar climatic conditions.

African clawless otter feces were identified based on the shape, size and characteristic odor (Stuart & Stuart 2000). Samples were collected from comparatively large fecal material (diameter >2 cm) because these samples were likely from adult otters (Stuart & Stuart 2000). The freshness of a sample was estimated based on moisture content, temperature and anal jelly texture. In addition, to avoid collecting older samples, samples were not collected during wet weather conditions (e.g. rain and fog). While wearing gloves, approximately half of the fresh fecal material was thoroughly mixed and placed in labeled small plastic containers and put on ice immediately. Subsequently, samples were frozen within 1 h of collection and kept frozen until further processing and analysis at the Endocrine Research Laboratory, University of Pretoria, South Africa. The study was performed under the approval of the University of Pretoria Animal Use and Care Committee (Project number EC012-18) and the University of KwaZulu Natal Animal Research Ethics Committee (Project number AREC/033/018).

Sampling during animal translocation

Fecal samples were collected from 1 captive adult male otter between 8 June and 8 August 2018. A mobile cage (2 m × 1 m × 1 m) within its enclosure was baited with fish and the otter entered voluntarily. The otter was translocated in the mobile cage within a vehicle from the Wild4Life wildlife rehabilitation center to Millvale Golf Estate, both located in Rustenburg, South Africa (61 km apart) (Fig. 1). In both areas, the otter was housed in an enclosure (5 m × 3 m × 3 m) that included a pool and foraging pans and was fed fish or chicken once a day. In addition, the rehabilitation

program at Millvale Golf Estate included a once a week foraging event for the otter in the nearby lake. Samples that were voided during the day (07:00–18:00 hours) were collected within 1 h of defecation (n = 55), whereas samples voided at night (00:00–06:00 hours) were collected in the morning (06:00 hours) (n = 10). In total, 65 fecal samples were collected (28 before and 37 after translocation).

Time-dependent alteration in fecal glucocorticoid metabolite concentration post-defecation and influence of defecation time

To evaluate alteration in fGCM concentration post-defecation in samples when frozen at different times, 10 fresh samples collected before the translocation were thoroughly mixed and immediately divided into 27 subsamples. Subsamples were exposed to air and direct sunlight at room temperature (21.7°C–34.5°C). Subsequently, 3 subsamples (triplicates) were frozen at −20°C after 0, 1, 3, 6, 12, 24, 48 and 72 h and 1 week of exposure.

Samples collected from the captive otter during the morning (07:00–09:00 hours; n = 10 and evening (16:00–17:30 hours; n = 10) were used to explore the influence of time of defecation on fGCM concentrations.

Fecal sample processing and steroid analysis

Fecal samples were lyophilized, pulverized and sieved through a mesh to remove existing indigestible material (Fieß *et al*. 1999). A weighed amount (0.10–0.11 g) of fecal powder was extracted by adding 3 mL of 80% ethanol in water and vortexed for 15 min. Thereafter, samples were centrifuged at 1500 *g* for 10 min. The supernatant formed was transferred into microcentrifuge tubes and stored at −20°C for further analysis.

A subset of 14 samples from the translocation event (samples 72 h prior [n = 3] and up to 5 days after; n = 11) was measured for fecal glucocorticoid metabolite (fGCM) concentrations using 5 enzyme immunoassays (EIA): (i) an 11β-hydroxyetiocholanolone EIA (detecting fGCM with a 5β,3α,11β-diol structure); (ii) an 11-oxoetiocholanolone I EIA (detecting 11,17-dioxoandrostanes); (iii) an 11-

oxoetiocholanolone II EIA (detecting fGCM with a 5β-3α-ol-11-one structure); (iv) a 5α-pregnane-3β,11β,21-triol-20-one EIA (detecting fGCM with a 5α-3β, 11β-diol structure); and (v) a cortisol EIA. Detailed assay characteristics, including full descriptions of the assay components and cross-reactivities, have been provided by Palme and Möstl (1997) for the 11-oxoetiocholanolone I and cortisol EIA, by Möstl *et al.* (2002) for the 11-oxoetiocholanolone II EIA, by Touma *et al.* (2003) for the 5α-pregnane-3β,11β,21-triol-20-one EIA, and by Frigerio *et al.* (2004) for the 11β-hydroxyetiocholanolone EIA. The sensitivities of the EIA were 0.6 ng/g dry weight (DW) (cortisol, 11-oxoetiocholanolone I and 11-oxoetiocholanolone II), 1.2 ng/g DW (11β-hydroxyetiocholanolone) and 2.4 ng/g DW (5α-pregnane-3β,11β,21-triol-20-one), respectively. Intra-assay coefficients of variation (CV) of high-concentration and low-concentration controls were 9.56% and 12.56% (cortisol), 8.33% and 8.65% (11-oxoetiocholanolone I), 6.41% and 10.30% (11-oxoetiocholanolone II), 6.67% and 13.18% (11β-hydroxyetiocholanolone), and 10.20% and 10.42% (5α-pregnane-3β,11β,21-triol-20-one), respectively. Subsequently, only the cortisol and 11-oxoetiocholanolone I EIA were used for analyzing the fecal extracts derived from the time-dependent alteration in the fGCM concentration post-defecation experiment (see Results section). Finally, the influence of defecation time and free-ranging otter sample fGCM concentrations were determined using the cortisol EIA. The serial dilutions of fecal extracts gave displacement curves that were parallel to the respective standard curves, the relative variation of the slope of the trend lines was < 5% for the cortisol EIA, and < 3% for the 11-oxoetiocholanolone I EIA. Inter-assay coefficients of variation of high-concentration and low-concentration controls were 5.96% and 6.90% for the cortisol, and 5.65% and 6.11% for the 11-oxoetiocholanolone I EIA, respectively. All steroid concentrations are expressed per mass of fecal DW matter.

Data analysis

All statistical analysis was conducted using the program R, with the use of the R Studio interface (R Core Team 2016). Suitable EIA were identified based on the increase in fGCM concentration following translocation by calculating the percentage response based on the highest fGCM peak post-translocation over the median baseline fGCM concentration calculated from samples collected over 72 h before the translocation (*n* =

3). To determine a time-dependent alteration in fGCM concentration post-defecation, fGCM concentrations for each subsample were expressed as percentages based on the triplicate mean value of subsamples exposed for 0 h (representing 100%) for the respective EIA. The fGCM concentration percentages from 11-oxoetiocholanolone I EIA were normally distributed. However, fGCM concentration percentages derived from the cortisol EIA had a positive skewed distribution and large differences in variance of the data observed and, thus, were $\log_{10}$-transformed prior to statistical analysis. A repeated-measures one-way analysis of variance (ANOVA) was used to test the difference between fGCM concentration at each time point in relation to $t = 0$ for each EIA. Pair-wise *t*-tests, with a Bonferroni correction, were conducted post-hoc to determine which time treatments were significantly different from $t = 0$. A student's *t*-test was also used to test the differences in fGCM concentrations between samples collected in the morning and evening, as well as for differences in fGCM concentrations between study areas (these data were also $\log_{10}$-transformed).

RESULTS

Biological validation

From the 5 different EIA tested, the cortisol and 11-oxoetiocholanolone I EIA revealed the highest transport-related response (74% and 48%, respectively). Peak fGCM levels were found 30 h (second sample collected post-transport) and 24 h (first sample collected post-transport) after transport for the 2 EIA, respectively. The remaining 3 EIA tested showed only a marginal to no elevation in fGCM concentrations a day after transport (Table 1, Fig. 2).

Time-dependent alteration of fecal glucocorticoid metabolite concentrations post-defecation

There was a significant change in the alteration of fGCM concentrations post-defecation over time for the cortisol EIA ($F_{8,18} = 21.089$, $n = 27$, $p < 0.001$) (Fig. 3). The cortisol EIA showed a significant ($P = 0.009$), 269%, increase in fGCM concentrations after 12 h (3.101 ± 0.057 µg/g DW). Concentrations peaked at a 505% increase and had high variability after 24 h ($P = 0.001$, 4.251 ± 1.491 µg/g DW). Although not statistically

significant (P = 1.000), there was already a 38% increase in fGCM concentration after 6 h post-defecation (1.159 ± 0.136 µg/g DW).

There was also a significant change in the alteration of fGCM concentrations post-defecation over time for the 11-oxoetiocholanolone I EIA ($F_{8,18}$ = 8.567, n = 27, P < 0.001) (Fig. 3). At 48 h, there was an 82% decrease in fGCM concentrations measured by 11-oxoetiocholanolone I EIA, which was statistically significant (P = 0.018, 0.213 ± 0.124 µg/g DW). Although not statistically significant (P = 1.000), fGCM concentrations had already decreased by 38% after 12-h post-defecation (1.150 ± 0.545 µg/g DW), with higher variability in fGCM concentrations between triplicates already at 6 h (SD = 48%).

Influence of defecation times

There was no significant difference ($F_{1,18}$ = 0.059, n = 20, P = 0.811) in fGCM concentrations between fresh samples collected during the morning (mean ± SD = 0.704 ± 0.133 µg/g DW) and evening (mean ± SD = 0.660 ± 0.125 µg/g DW).

Fecal glucocorticoid metabolite concentrations of African clawless otters in natural and transformed areas

There was no significant difference ($F_{1,15}$ = 1.433, n = 17, P = 0.250) between fGCM concentrations of otters from the 2 nature reserves; Cobham (n = 13, mean ± SD = 0.211 ± 0.202 µg/g DW) and Verloren Vallei (n = 4, mean ± SD = 0.346 ± 0.301 µg/g DW). Thus, the data from the 2 study sites were combined (herein referred to as "natural areas") for subsequent data analysis. Fecal samples collected from otters at Millstream ("transformed area," n = 20, mean ± SD = 0.468 ± 0.539 µg/g DW) had significantly higher ($F_{1,35}$ = 6.809, n = 37, P = 0.013) fGCM concentrations compared to those collected from animals in the natural areas (n = 17, mean ± SD = 0.242 ± 0.226 µg/g DW) (Fig. 4).

DISCUSSION

The results reported here firstly illustrate how a translocation event was successfully used as a biological stressor for examining the suitability of enzyme-immunoassays for measuring fGCM concentrations in 1 male African clawless otter. The study further shows that an EIA utilizing an antibody against cortisol-3-CMO can be used to reliably measure fGCM concentrations in an adult male African clawless otter, and that the time of day for fecal sample collection may not affect fGCM concentrations. Furthermore, limited changes in fGCM concentrations were evident in samples collected within a 3-h post-defecation window but became significantly different at time periods exceeding 6 h post-defecation. When interpreted conservatively, this result suggests that samples should be collected within 3 h of defecation to ensure reliable measurement of fGCM. Finally, fGCM concentrations of African clawless otters were significantly higher in a transformed area (Millstream farm) characterized by anthropogenic disturbance when compared to natural areas (Verloren Vallei Nature Reserve and Cobham Nature Reserve) with minimal human presence. This indicated that factors related to human-dominated landscapes could inflict stress on African clawless otters, reflected by an increase in their adrenocortical activity. Alternatively, differences in fCGM concentrations could be due to metabolic or dietary differences among habitats, which might change concentrations in fGCM but not circulating cortisol concentrations (Rangel-Negrin et al. 2009).

The validation of EIA for reliably monitoring adrenocortical activity in mammals using alterations in fGCM concentrations is often achieved by performing an adrenocorticotropic hormone stimulation test (ACTH challenge) (Touma & Palme 2005; Ganswindt *et al.* 2012; Scheun *et al*. 2015; Webster *et al*. 2018; Jepsen *et al*. 2019). In addition to pharmacologically stimulating GC production, studies increasingly include biological validations such as individual separation (Ganswindt *et al*. 2012; Jepsen *et al*. 2019), reproductive suppression (Laver *et al.* 2012) or translocations (Goymann *et al.* 1999; Franceschini *et al.* 2008). Such presumed stressors, specifically translocation, are highly likely to induce a stress response (Dickens *et al.* 2010) and are, therefore, good opportunistic events for biologically validating a monitoring system like EIAs (Goymann *et al*. 1999; Rothschild *et al*. 2008; Dickens *et al*. 2010). Ideally, both a

pharmacological and biological validation should be conducted. However, as highlighted by Palme (2019), the ACTH challenge requires permission to conduct animal experimentation, which is often not granted due to regulations regarding endangered species and/or lack of access to captive individuals. The results of this study affirm the usefulness of translocations as an alternative to an ACTH challenge for EIA validation. Application of high-pressure liquid chromatography would be a helpful addition to assay validation to assess possible sex-related differences in the composition of fGCM excretion as well as potential co-measurement of androgen metabolites with fGCM assays tested.

The tested cortisol and 11-oxoetiocholanolone I EIAs showed the highest transport-related response 30 h and 24 h after the translocation, respectively. Similarly, Zalewski (2011) used an EIA with an antibody against cortisol to monitor stress responses of North American river otter (*Lontra canadensis,* Schreber 1776*)* to ecological factors such as habitat quality. In other African mustelids, EIAs measuring 5β,3α,11β-diol structures or 11,17-dioxoandrostanes were used to determine changes in fGCM concentrations in meerkats, *Suricata suricatta* (Schreber 1776; Goncalves *et al.* 2016) or banded mongooses, *Mungos mungo* (Gmelin 1788; Laver *et al.* 2012). These reports highlight the variation in the suitability of EIAs for monitoring alterations in fGCM concentration within the Mustelidae family and underline the importance of validating EIAs when applying fGCM monitoring to a species for the first time.

Using the cortisol EIA, the alterations in fGCM concentrations seen post-defecation were characterized by a distinct temporary increase in fGCM concentrations. This resembled the pattern reported for African wild dogs, *Lycaon pictus* (Temminck 1820; Crossey *et al.* 2018) and African buffalo, *Syncerus caffer* (Sparrman 1779), using the 11,17 DOA EIA (Ganswindt *et al.* 2012). In contrast, the 11-oxoetiocholanolone I EIA alteration showed a decrease in fGCM concentration similar to that of the banded mongoose (Laver *et al.* 2012) and African elephants, *Loxodonta africana* (Blumenbach 1797; Webber *et al.* 2018). The observed differences in the alteration in African clawless otter fGCM concentration post-defecation are possibly due to bacterial conversion. Bacterial conversion alters the composition of immunoreactive compounds

within a sample, which then could cross-react differently with the antibodies used in the respective applied EIA(s) (Washburn & Millspaugh 2002; Lexen *et al.* 2008). However, further research similar to that of Lexen *et al.* (2008), including samples from more individuals, is necessary to better understand the effects of potential alteration in immunoreactive compound composition for fGCM monitoring in African clawless otters.

Apart from the differences in the alteration of fGCM concentrations seen over time when using the cortisol and 11-oxoetiocholanolone I EIA, there was comparatively higher variation between triplicates at 6–72 h post-defecation compared to respective steroid levels of sample triplicates taken at the other sampling points. There are at least 2 possible reasons for the variation within triplicates: (i) sample material was not sufficiently mixed when conducting the experiment; or (ii) there were different rates of bacterial conversion between triplicates as a consequence of varying environmental conditions due to sample placement (Webber *et al.* 2018; Webster *et al.* 2018). The first reason is a more likely cause for the 24-h triplicates in particular because high variance in fGCM concentrations can be seen when analyzing respective samples with both the cortisol and 11-oxoetiocholanolone I EIA. For triplicates taken at 6 and 12 h post-defecation, substantial variation was only evident when samples were analyzed with the 11-oxoetiocholanolone I EIA, whereas triplicates taken at 48 and 72 h post-defecation revealed substantial variation only when using the cortisol EIA for analysis. These EIA specific differences might be a result of different rates of bacterial conversion between triplicate samples, as variation in environmental conditions such as sunlight, temperature and humidity are likely to affect the duration of bacterial enzyme activity, and, consequently, might affect immunoreactive compound composition within a sample (Webber *et al.* 2018; Webster *et al.* 2018). Because the exposure to certain environmental variables like sunlight might not have been fully standardized in this study, the higher variation found in some triplicates could be indicative of varying bacterial enzyme sensitivity between these samples, resulting in quantitative differences in respective immunoreactive compound compositions relevant only for the one or the other EIA. However, additional investigations exploring the effect of environmental conditions and time on fGCM concentrations in African clawless otters are necessary.

Although fGCM concentrations were stable (as indicated by no evidence for statistically significant differences) for up to 6 h post-defecation when measured with the cortisol EIA, an overall difference in fGCM concentrations of 38% was recorded when comparing respective steroid levels between 3 and 6 h. It is important to note a possible biological significance associated with such a change: for example, McLennan *et al*. (2019) found a 32% difference in fGCM concentrations between subadult and adult chimpanzees (*Pan troglodytes;* Blumenbach 1799*)*. Thus, taking potential biological meaningful alterations in fGCM concentrations into account, respective hormone metabolite levels of African clawless otters can be regarded as comparable when feces as a hormone matrix were collected within 3 h post-defecation and fGCM concentrations were then determined with the described cortisol EIA. Due to the predominant nocturnal behavior of free-ranging African clawless otters, collecting samples that are fewer than 3 h old (fresh) requires collecting from latrines as early as possible. To identify those samples, collectors should look out for anal jelly and water vapor when collecting material (Majelantle 2018, personal observation). Alternatively, because the results suggest that time of day potentially does not affect fGCM concentration, all fecal material present at a known latrine could be marked (e.g. with glitter as done by Guertin *et al*. [2010]). Thereafter, the site should be checked every 3 h, thereby ensuring all newly deposited samples (without glitter) are sufficiently fresh.

A notable limitation of our study is that our samples were limited to 1 male captive otter. Thus, confounding factors such as sex (Webster *et al*. 2018), age (McLennan et al. 2019), reproductive state (Wolf *et al*. 2018), and diet (Kalliokoski *et al.* 2012) could not be accounted for. Moreover, the results on the potential influence of circadian rhythm on fGCM concentrations are further limited by possible differences in captive and wild African clawless otter daily activity patterns. Monitoring of additional captive otters would be needed to validate the fGCM assay more comprehensively.

African clawless otters in the transformed (anthropogenically disturbed) area were characterized by higher stress-related biomarkers compared to the otters monitored in natural areas. The difference in fGCM concentrations found is unlikely due to study

design, chosen study sites, or season. Verloren Vallei Reserve and Millstream samples were collected within the same timeframe and within the same area, and, thus, the otters encountered the same broad environmental conditions. While Cobham samples were collected at a different location and time, the similarity between overall fGCM concentrations of these samples and those from Verloren Vallei support our approach to include data from Cobham in the study and combine the 2 datasets under the label "Natural areas." However, because field samples were collected from unknown adult individuals, sex and reproductive status could not be accounted for (a limitation applicable to all study sites). The possibility of some bias due to the small sample sizes cannot be excluded, particularly at Verloren Vallei. Unfortunately, small sample sizes were expected, due to the shy and elusive behavior of African clawless otters and the fresh sample requirement. Yet, samples from Millstream farm had considerably higher fGCM concentrations than both natural sites (analyzed separately as well as combined). Thus, we consider it likely that the disturbances associated with the transformed environment are responsible for elevated fGCM levels in African clawless otters. The significantly higher fGCM concentrations in transformed landscapes compared to natural areas is consistent with results reported for other species inhabiting both types of habitats. Examples of similar responses include studies on chimpanzees (McLennan *et al.* 2019), African lesser bushbabies, *Galago moholi* (Smith 1836; Scheun *et al.* 2015), European blackbirds, *Turdus merula*, and Northern mockingbirds, *Mimus polyglottos* (Linnaeus 1758; Fokidis *et al.* 2009).

Based on the findings of the monitored translocation, an acute stress response seems to reflect in otter feces approximately 30–48 h after the stress event. The samples were collected from each study site for a minimum of 25 days, which reduces the likelihood of collecting samples primarily after an acute stress response. Possible stressors we have identified on this site include human activity, predators (including encounters with humans and their pets), food availability, and intraspecific and interspecific competition. Because there is very limited direct contact with humans in Millstream Farm (Majelantle 2018, personal observation) due to African clawless otter nocturnal behavior, we consider human presence an unlikely stressor. Limited food availability and resulting low food intake are associated with increased glucocorticoid levels: for

example, in ring-tailed lemurs (*Lemur catta;* Linnaeus 1758), Pride (2005) found a direct negative relationship between food intake and glucocorticoid levels. Otters on Millstream Farm are known to forage on the stocked rainbow trout and, therefore, we consider food likely to be relatively abundant there. Conversely, increased food availability and differential food intake could result in higher fGCM concentrations due to higher levels of circulating GCs linked to differential metabolism (Kalliokoski *et al.* 2012). In addition, there is evidence that excessive body mass can lead to prolonged activation of the HPA axis and glucocorticoid secretion in humans (Björntorp & Rosmond 2000). Similar results were reported for African lesser bushbabies, where animals living in transformed landscapes have higher body mass index and concomitant increased levels of glucocorticoids compared to animals living in natural areas (Scheun *et al.* 2015). Further research is, thus, absolutely required to find out more about the possible stressors, including behavior, food intake and related factors such as weight, body mass index and agility rate in the different landscapes.

CONCLUSION

African clawless otters occurring in a transformed area appeared to show relatively higher stress-related biomarkers within the study period, possibly due to anthropogenic disturbance. The ability to reliably validate adrenocortical function through fGCM monitoring in a male African clawless otter provides a promising non-invasive tool to further investigate endocrine responses to putative stressful circumstances in this species in both captive and free-ranging settings.

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Figure 1 Representation of spatial locations where African clawless otter fresh fecal samples were collected from 8 June 2018 to 1 November 2018.

Figure 2 Median percentage change of African clawless otter fecal glucocorticoid metabolite (fGCM) concentrations (µg/g DW) in relation to baseline. The red line represents the translocation event and the dotted line indicates the median baseline fGCM concentration before the translocation.

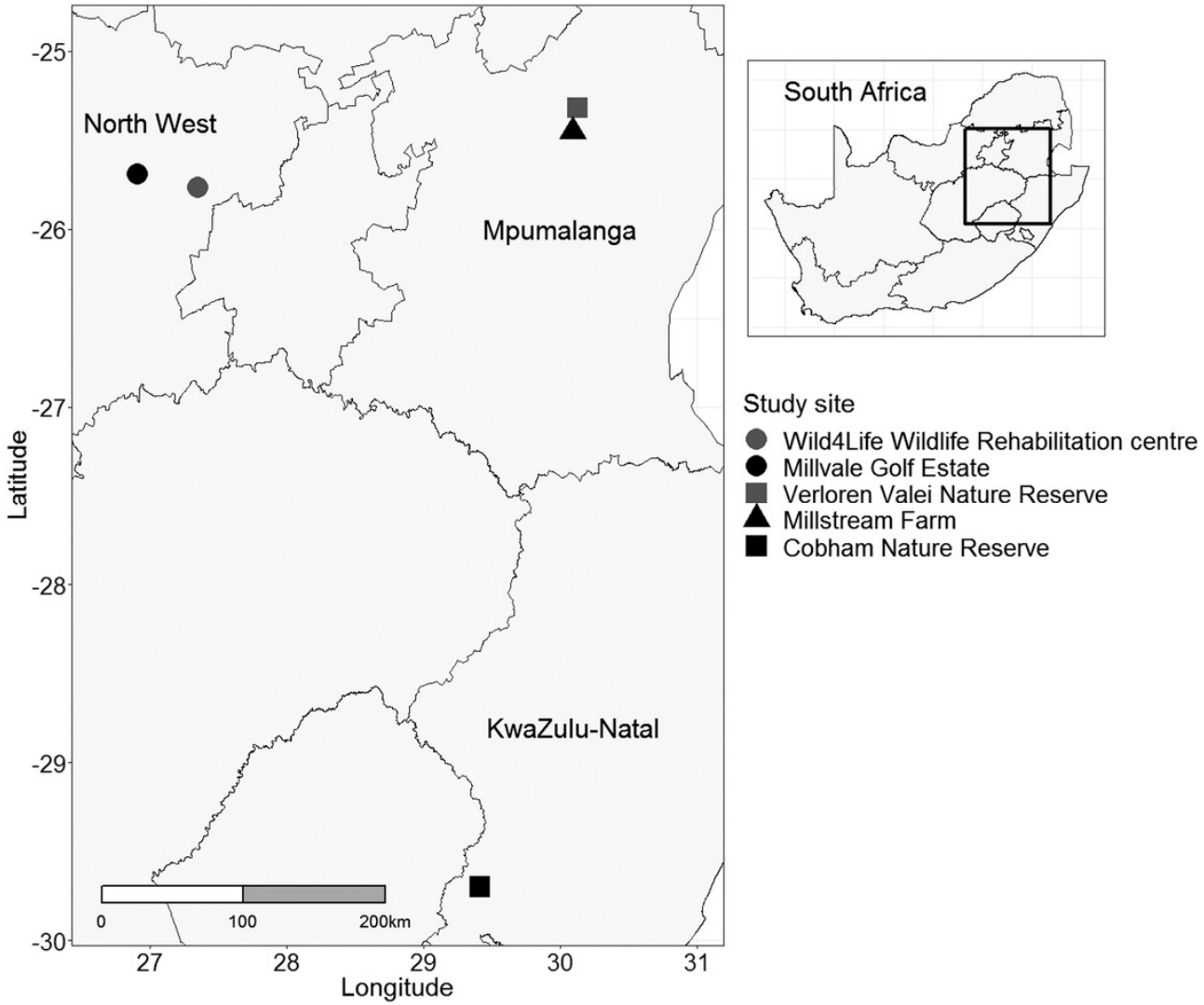
Figure 3 Time-dependent change in African clawless otter fecal glucocorticoid metabolite (fGCM) concentrations (µg/g DW) post-defecation (0–168 h). The points represent mean fGCM concentrations (µg/g DW), and lines above and below show the mean ± SD. Different superscripts indicate statistically significant differences ($P < 0.05$) between time points.

Figure 4 Comparison of fecal glucocorticoid metabolite (fGCM) concentrations of otters from natural areas and the transformed area. Bar represents mean fGCM concentrations (μg/g DW), and lines above and below indicate the mean ± SD. Statistically significant difference ($P < 0.05$) is represented by * and *n* denotes the number of samples.

Table 1 Summary of fGCM concentrations (μg/g DW) before (up to 72 h) and after (24–30 h) translocation of a male African clawless otter determined with 5 different EIAs

EIA	Cortisol†	11-oxoetiocholanolone I†	11-oxoetiocholanolone II	11β-hydroxyetiocholanolone	5α-pregnane-3β,11β,21-triol-20-one
Baseline median (μg/g DW)	0.878†	0.821†	0.357	0.595	0.178
Post-translocation peak (μg/g DW)	1.536†	1.216†	0.197	0.632	0.208
Response (%)	174†	148†	55	106	116
Hours after translocation	30†	24†	30	24	24

†EIAs selected for subsequent analysis. DW, dry weight; EIA, enzyme-immunoassay; fGCM, fecal glucocorticoid metabolite.



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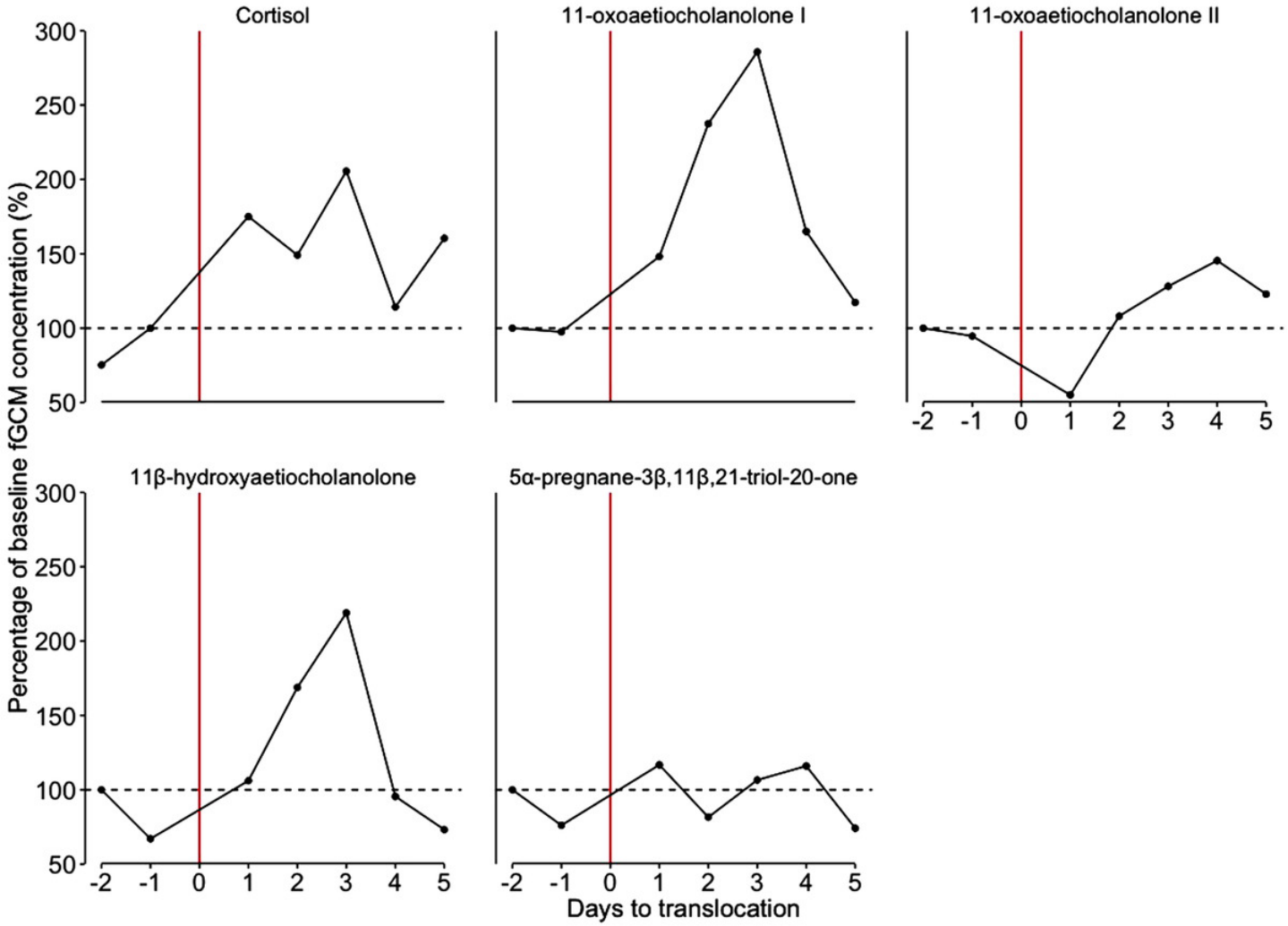

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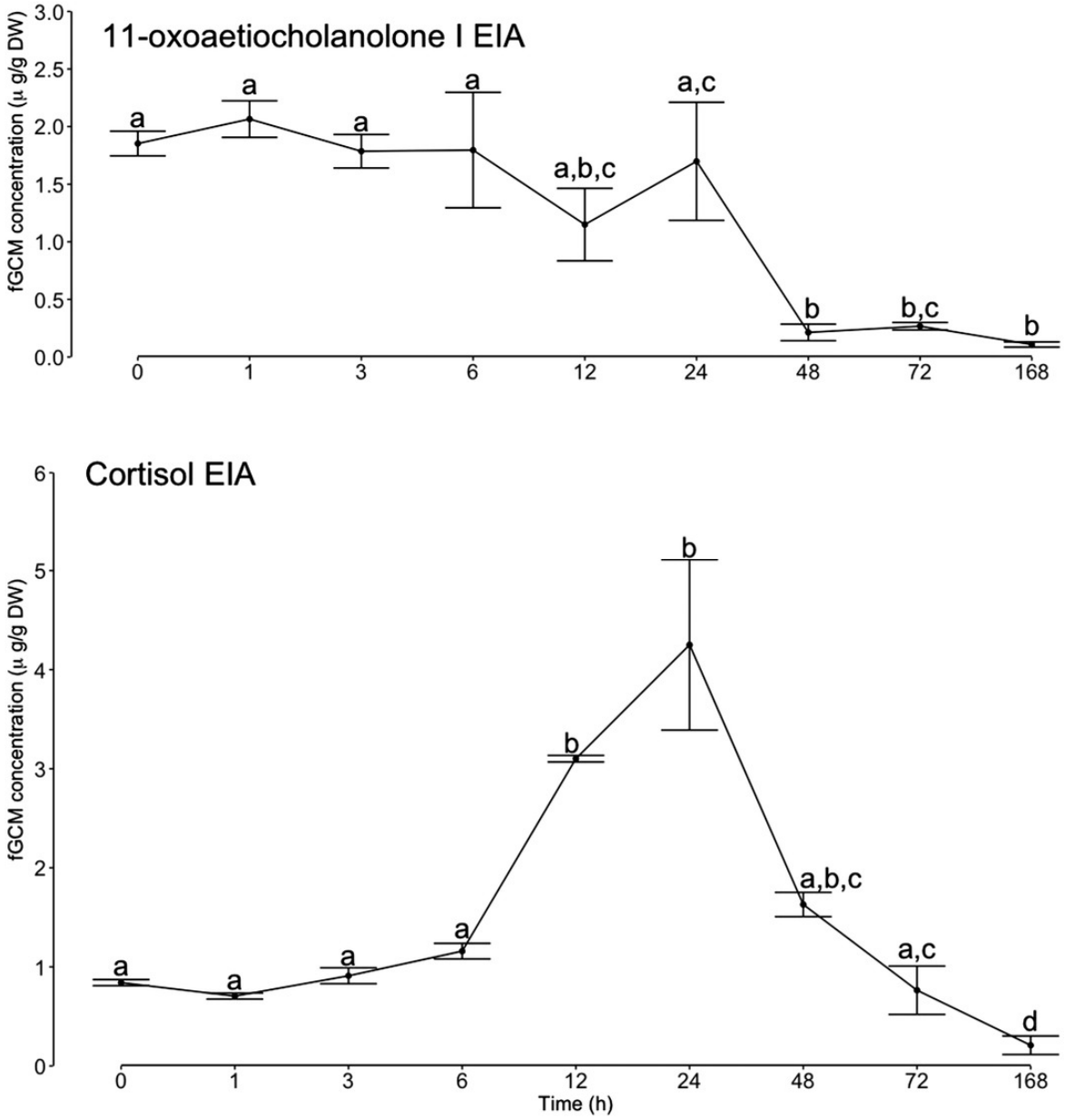
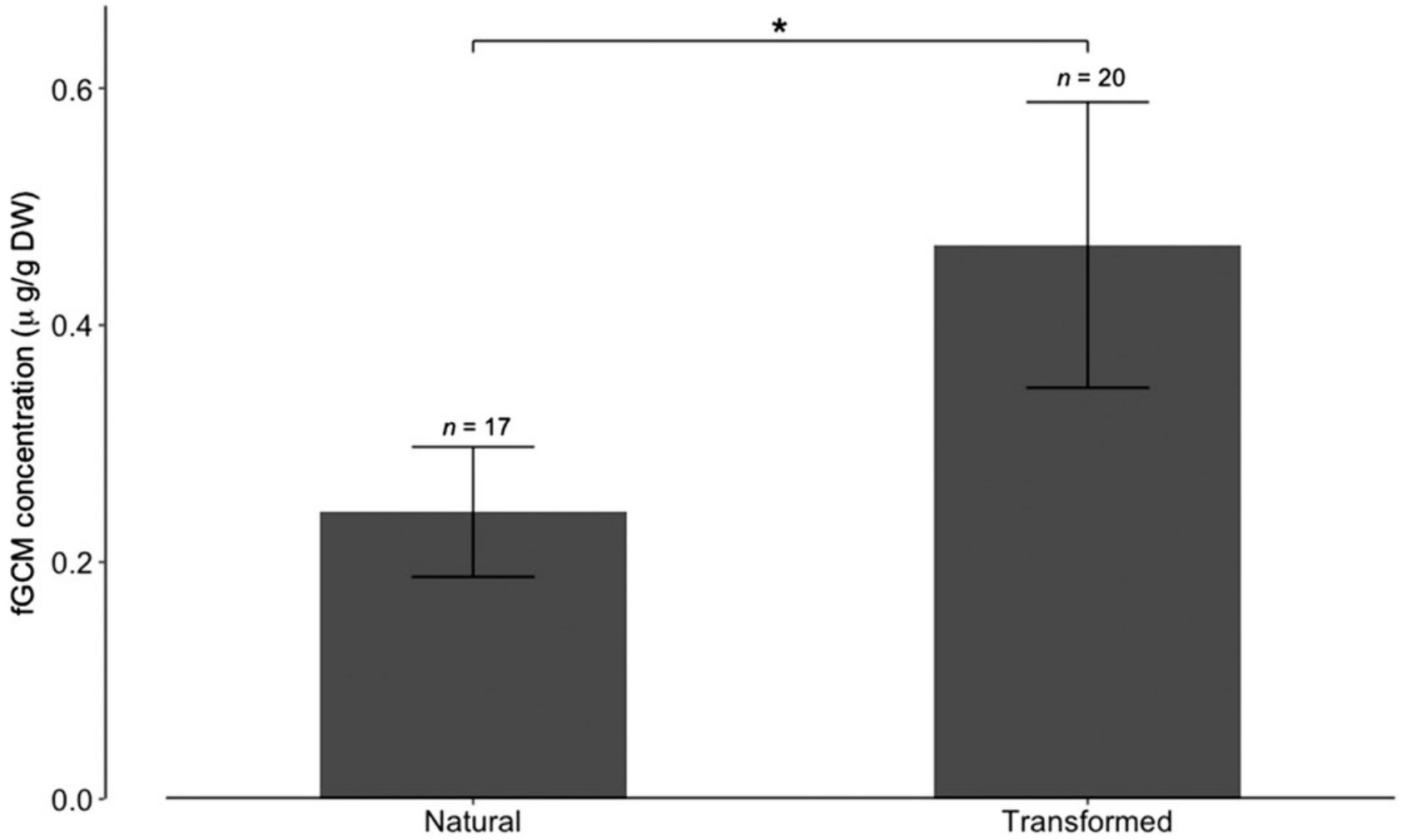


Figure 3. Time-dependent change in African clawless otter fecal glucocorticoid metabolite (fGCM) concentrations (μg/g DW) post-defecation (0–168 h). The points represent mean fGCM concentrations (μg/g DW), and lines above and below show the mean ± SD. Different superscripts indicate statistically significant differences ($P < 0.05$) between time points.



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