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The relationship between physical injury, body condition and stress-related hormone concentrations in free-ranging giraffes

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A physiological stress response can be triggered by a variety of intrinsic and extrinsic stimuli, but minimal information is available about the physiological stress response related to pain in wildlife. Recently established techniques now allow the non-invasive measurement of faecal glucocorticoid metabolite (fGCM) concentrations to monitor the physiological stress response in giraffe. We examined the consequences of injury of various severities and loss of body condition in relation to glucocorticoid output in free-ranging giraffes. Body condition (BC) was visually estimated based on the amount of fat and muscle covering the bones, using a species-specific scoring system (one – emaciated to eight – obese). An adapted animal trauma triage scoring system was also applied to evaluate the severity of injuries observed. Individual fGCM concentrations were determined to assess stress-related glucocorticoid output using an enzyme immunoassay technique, and compared with assigned BC and injury scoring. Significantly elevated fGCM levels were found in injured individuals that showed wounds with deep tissue involvement and/or poor BC, but not in individuals that showed superficial wounds. Responsible for the observed changes in fGCM levels may be a combination of differences in the severity of the injuries and the subsequent degree of pain associated with it, the influence of the stress response on the energetic condition, and the duration of the injuries. The results of this study are somewhat limited due to the small sample size, and therefore the effect of food intake on the body conditions cannot be controlled for. However, euthanasia is a common management tool used to prevent unnecessary suffering, nevertheless, especially in wild animals the severity of an injury and the associated pain perceived may be difficult to assess. Combining an assessment of BC and analysis of individual stress-hormone levels can help improve health assessments in free-ranging giraffes and thus assist management decisions.

When confronted with a stressor, animals respond through multiple mechanisms, such as physiological and behavioural alterations to cope with the challenge. An almost immediate physiological response to a stressor is an increase in catecholamine (adrenaline and noradrenaline) secretion. This rapid response causes changes in respiratory rate, cardiovascular tone and blood flow to the muscles, and also increases blood glucose levels, which then delivers the needed energy to facilitate the 'fight-or-flight' response (Nelson 2011). In addition, the hypothalamic–pituitary-axis (HPA) is activated and glucocorticoids (GC) will be released to help restore homeostasis. The major role of GC is the provision of energy through increased gluconeogenesis, protein and fat metabolism, and a decreased sensitivity to insulin (Möstl and Palme 2002, Touma and Palme 2005, Sheriff et al. 2011). This response is delayed over the course of minutes, and in some instances up to an hour (Sapolsky et al. 2000). All these reactions appear to deal with the perceived stressor and to restore homeostasis within the body (Sapolsky 2002, McEwen and Wingfield 2003).

A stress response can be triggered by internal (e.g. hypoglycaemia or anoxia), as well as external (e.g. heat, exercise) stimuli (Reeder and Kramer 2005) and is regulated by negative feedback at various points in the HPA axis, which down regulates the production of GC (Reeder and Kramer 2005). The stress response can therefore be separated into three phases: the alarm phase (activation of the sympathetic nervous system), the resistance phase (activation of the HPA axis), and the exhaustion phase (Selye 1978). The latter possessing deleterious effects of long-term increases in GC concentrations (e.g. muscle and bone atrophy, impaired immune system or poor wound healing) due to severe acute or chronic stressors, which prevent the HPA axis to reach a recovery phase (Boonstra et al. 1998, Hardy et al. 2005,

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Sheriff et al. 2009). Furthermore, changes in GC concentrations can be used as an indicator of the energetic condition of an individual, as GC are involved in lipid metabolism and increase during periods of energy intake shortages (Kitaysky et al. 2001, Reeder and Kramer 2005). In Steller sea lions *Eumetopias jubatus* for example a decrease in body mass during food shortage was associated with an increase in GC levels (Jeanniard du Dot et al. 2009).

Glucocorticoids also interact in various ways with the immune axis, for example the pain reception and inflammatory processes are suppressed (Sapolsky 2002, Reeder and Kramer 2005). Pain perception is a very subjective experience and difficult to measure. Even in humans the reaction to a stimulus varies between and even within individuals, as the need to balance between the benefits of responding or not to a stimulus has to be taken into consideration (Bateson 1991). Pain can graduate from a tolerable low level towards an excruciatingly high one (Bateson 1991). Respective studies often focus on production animals, as they undergo painful procedures like castration or beak-trimming (reviewed by Rutherford 2002). Thus, much less is known about the pain responses in wildlife, although wildlife research often includes invasive human interference, like branding or mounting of tracking devices. In Steller sea lions the consequences of the implantation of an intra-abdominal tracking device on their behaviour have been evaluated (Walker et al. 2009). However, the consequences of naturally occurring potentially painful circumstances such as physical injuries, on the adrenal stress response is not well documented, mainly due to the reduced chances in encountering these incidents in wildlife (Ganswindt et al. 2010). However, in elephants for example, an increase in GC concentrations during times of physical foot injuries has been documented (Ganswindt et al. 2010).

Giraffes show a variety of skin disorders, including lumpy skin disease (LSD), giraffe ear disease (GED), and giraffe skin disease (GSD). LSD is a viral disorder, common in ungulates (Hunter and Wallace 2001). however, the epidemiology of GED and GSD still remains unknown. With the ongoing giraffe population decline, now listed as Vulnerable in the Red List of Threatened Species (IUCN 2017), it is important to better understand the factors affecting giraffe welfare. As a key prerequisite, previous studies have demonstrated that non-invasive monitoring of stress hormone levels in the form of faecal glucocorticoid metabolites (fGCM) is feasible in giraffes (Bashaw et al. 2016, Wolf et al. 2018).

In our study, we examined the consequences of injury of various levels of severity and loss of body condition on glucocorticoid output in free-ranging giraffes. The revealed information will help to better assess the impact of recorded injuries and related loss in body condition on overall well-being of giraffe in the wild and thus could assist when taking important management decisions in view of welfare, health and conservation efforts.

Material and methods

Study area and animals

A giraffe population at Mbuluzi Game Reserve (MGR) in northeast Swaziland was observed opportunistically and faecal samples for hormone monitoring collected over a six-week period between April and May 2017. Observations and sample collection took place in an area of approximately 23.5 km^2 of Lowveld acacia savannah. An unmanaged population of approximately 60 giraffes is kept at MGR, which is physically divided into two sections, the north and south. The northern section has an estimated population of 35 individuals in an area of approximately 16 km^2. The southern section has an estimated population of 25 individuals in an area of approximately 7.5 km^2.

Body condition and injury scoring

Body conditions (BC) of observed giraffes were visually estimated using an already established species-specific eight-scale scoring system (one – emaciated to eight – obese) (EAZA 2006, Table 1). In addition, an adapted animal trauma triage (ATT) scoring system was applied to evaluate the severity of injuries detected in the observed animals (Rockar et al. 1994). Briefly, this scoring system gives a maximum of three points for each of six physiological criteria. As this was a complete non-invasive study, only two of the six criteria were used: skin condition and orthopaedic state (Table 2), resulting in a theoretical maximal score of six points for an individual, with high scores indicating conditions that are more serious. Finally, giraffes were assigned to one of three different categories depending on their individual BC and ATT scores. Category one contains individuals with a BC score of 5 or 6 and an ATT score of 0 (healthy individuals; Fig. 1A). Category two contains giraffes with a BC score of 5 or 6 and an ATT score of 1 or 2 (giraffes in good BC but with superficial wounds; Fig. 1C–D). Category three contains animals with a BC score of 1 to 4 and/or an ATT score $\geq$2 (giraffes with poor BC; Fig. 1B and/or wounds that present deep tissue involvement; Fig. 1E–G).

Faecal sample collection

Eighty-six faecal samples from 41 individuals were collected and analysed for faecal glucocorticoid metabolite (fGCM) concentrations. To minimize alteration in fGCM concentration post-defecation (Möstl and Palme 2002, Heistermann 2010) , faeces were collected within 20 min post-defecation, placed on ice immediately, and frozen within six h. All collected materials were kept frozen until reaching the Endocrine Research Laboratory, University of Pretoria, South Africa, for further processing.

Faecal steroid extraction and analysis

Hormone extraction followed already established protocols, with faeces being freeze-dried, pulverized, and sieved through a thin metal strainer in order to remove fibrous material (Fieß et al. 1999). Subsequently, 0.10–0.11 g of faecal powder was vortexed for 15 min with 80% ethanol in water (3 ml). The suspension was centrifuged for 10 min at 1500 g and the supernatant aliquoted and stored at –20°C until analysis (Seeber et al. 2013, Wolf et al. 2018).

Steroid extracts were measured for faecal glucocorticoid metabolite (fGCM) concentrations using an established enzyme immunoassay (EIA) for fGCM monitoring in male and female giraffe (Bashaw et al. 2016, Wolf et al. 2018).

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Table 1. Body condition score (BCS) system for giraffes (EAZA 2006).

BCS	Visual indicators for body condition score estimation
1	Emaciated. Ribs and spine of scapula may be visible. Muscle wasting has occurred.
2	Poor condition. Cervical vertebrae are visible. Protruding spine. Distinct hollows cranial to hipbones. Crest of illium and outline of scapula are visible. Thin legs. Hips appear sunken and shoulders are slim.
3	Hipbones prominent. Definite outline of spine. Sacrum and first two cervical vertebrae visible. Chest may appear sunken.
4	Tailhead is noticeable and point of hipbone is visible. Slight hollow in centre of chest. Sufficient muscling in hindquarters, shoulders, and neck.
5	Back and hips rise smoothly to top line with no visible outline or denting along backbone. Some fat around tailhead. Point of hipbone just visible.
6	Good condition. Back is level and wide. Hipbone not visible. Smooth chest. Visible thickening in lower neck.
7	Overweight. Slight crease along backbone. Smooth chest and thick neck.
8	Obese. Definite crease along backbone. Tailhead no longer clearly visible. Soft fat along tailhead. Thick neck.

The EIA uses an antibody against 11-oxoaetiocholanolone (detecting fGCMs with a 5β-3α-ol-11-one structure; Möstl et al. 2002). The assay procedure followed established protocols (Ganswindt et al. 2002). Sensitivity of the assay at 90% binding was 1.2 ng g^{-1} faecal dry weight (DW). Serial dilutions of faecal extracts gave displacement curves that were parallel to the respective standard curve of the EIA. Inter-assay coefficients of variation as determined by repeated measurement of high- and low-value quality controls was 10.4% and 13.2%. The coefficient for intra-assay variance also determined by repeated measurement of high- and low-value quality controls was 3.3% and 5.6%. All hormone analyses were performed at the Endocrine Research Laboratory, University of Pretoria, South Africa.

Statistical analysis

Differences in individual median fGCM levels of giraffes, assigned to the three different categories, were compared using Kruskal–Wallis (KW) one way analysis of variance on ranks (ANOVA). For post hoc pairwise analysis a Wilcoxon rank sum test was used, with an adjustment of the α level using Bonferroni correction (Holm 1979). All statistical analyses were done using R ver. 3.0.2 (< www.r-project.org>).

Results

Of the 41 individual giraffes monitored, 73% (n = 30) of the individuals were categorised as healthy individuals (category 1), 15% (n = 6) of the giraffes were assigned to category 2, and 12% (n = 5) of the animals were assigned to category 3. The overall median fGCM concentration for healthy individuals (category 1) was 2.18 μg g^{-1} DW and ranged between 0.65 and 4.55 μg g^{-1} DW. Category 2 giraffes showed an overall median fGCM concentration of 2.00 μg g^{-1} DW (range 0.55–3.57 μg g^{-1} DW), and the overall median fGCM concentration for giraffes assigned to category 3 was 5.76 μg g^{-1} DW (range 3.29–55.22 μg g^{-1} DW). Individual median fGCM concentrations were significantly different between categories (KW $\chi^2 = 11.62$, df = 2, $p = 0.003$; Fig. 2), with significantly higher fGCM concentrations seen in giraffes assigned to category 3 ($W = 4$, $p = 0.003$ against healthy giraffes and $W = 1$, $p = 0.026$ against category 2 giraffes). Individual median fGCM concentrations of healthy giraffes and those with superficial wounds (category 2) did not differ statistically ($W = 100.5$, $p = 1.000$).

Discussion

The HPA axis is activated when an individual encounters an acute or chronic stressful situation, with 'pain' being one such trigger. For example in horses, higher GC concentrations have been measured in animals that have undergone surgery, or had to be treated for colic (Merl et al. 2000). However, pain is difficult to measure, even in humans, as it is often a very subjective experience, and its assessment is even more difficult in animals, due to species-specific variation in pain threshold (Bateson 1991, Rutherford 2002). Some species may show specific pain related behaviours (e.g. abnormal lying positions in calves; Molony et al. 1995), but, wild animals rarely show signs of weakness in order to avoid predator attention (Walker et al. 2009). Common methods to assess pain in animals include measurements of general body functions (e.g. food intake and weight gain),

Table 2. Animal trauma triage (ATT) scoring system. Fx = fracture.

Grade	Skin condition	Orthopaedic state
0	Abrasion, laceration: none or partial thickness	Weight bearing in 3 or 4 limbs, no palpable fracture or joint laxity
1	Abrasion, laceration: full thickness, no deep tissue involvement	Closed limb/rib or any mandibular fx. Single joint laxity/luxation including sacroiliac joint Pelvic fx with unilateral intact SI-ilium-acetabullum. Single limb open/closed fx at or below carpus/tarsus.
2	Abrasion, laceration: full thickness, deep tissue involvement, and arteries, nerves, muscles intact	Single long bone open fx above carpus/tarsus with cortical bone preserved. Non-mandibular skull fx.
3	Abrasion, laceration: full thickness, deep tissue involvement, artery, nerve, muscle compromised.	Vertebral body fx/luxation except coccygeal. Multiple long bone open fx above tarsus/carpus Single long bone open fx above tarsus/carpus with loss of cortical bone

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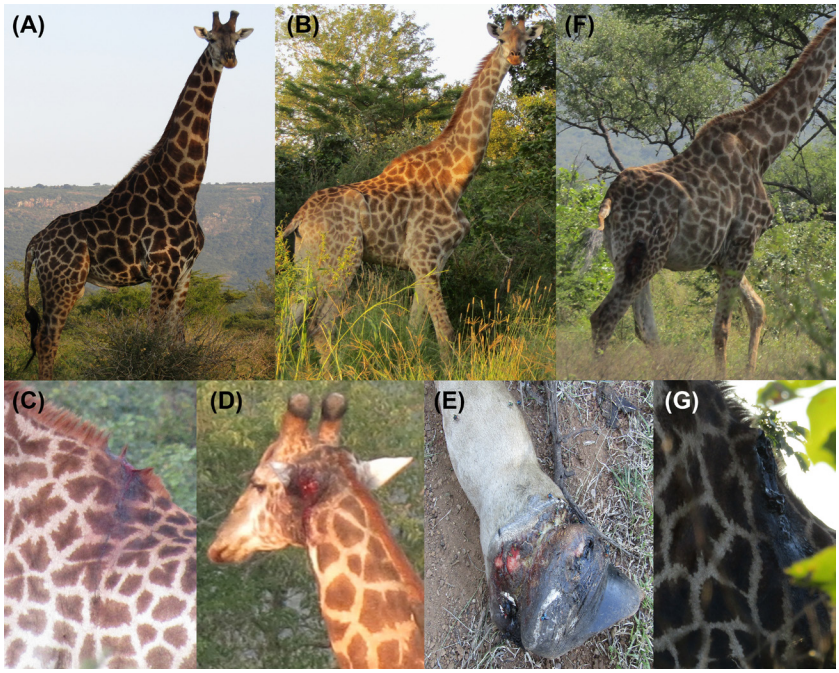


Figure 1. Giraffes in good (A), or poor (B) body condition (B, C). Giraffes with presumed superficial wounds reaching epidermis and dermis (C, D), a distinct injury of the right front foot below carpus (E), presumed deep wounds with muscle involvement (F, G). Photos: Dr G. Benavides, Mbuluzi Game Reserve.

behavioural changes (e.g. vocalisations), and physiological reactions (e.g. changes in heart rate) (Weary et al. 2006, Hellyer 2010). Measuring GC levels non-invasively can therefore provide an indication into the internal state of an animal and thus help to assess if an individual is encountering pain or discomfort without causing additional stress due to capture and handling. In our study, we used faecal samples to compare fGCM levels in free-ranging giraffes with and without injuries and found elevated fGCM levels in individuals that showed wounds with deep tissue involvement and / or poor body condition (category 3), but interestingly not in individuals that showed superficial wounds (category 2). Further, the highest fGCM concentration was determined in an individual with lacerations presumably resulting from a crocodile attack, supporting the view that severe pain leads to an increase in GC release (Sapolsky 2002). A similar pattern can be seen in elephants, where it has been shown that severe and fatal injuries lead to an up to 10-fold increase in fGCM levels (Ganswindt et al. 2005).

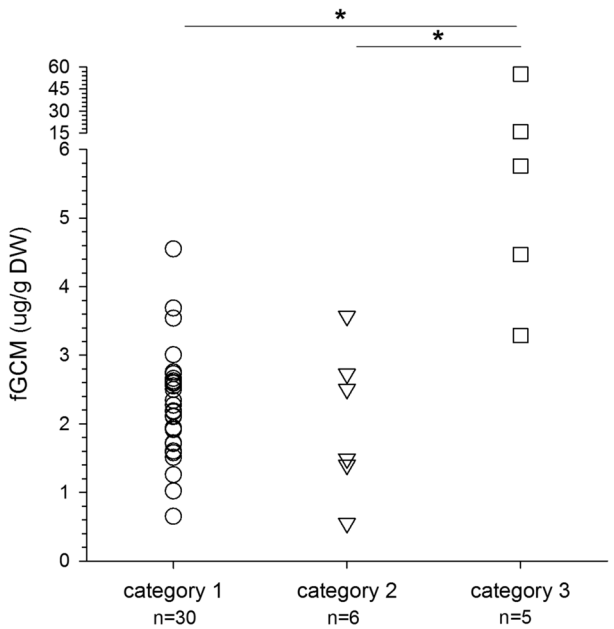


Figure 2. Individual median fGCM levels of healthy giraffes (category 1), of giraffes with superficial wounds (category 2), and animals with wounds that present deep tissue involvement/poor BC (category 3). Significant differences between categories are indicated with asterisks.

The majority of individuals assigned to category 2 showed one or two skin lacerations frequently with myiasis. This type of wound in free-ranging wildlife is normally attributed to an initial puncture by ticks and enlarged by oxpeckers, and frequently infested by fly larvae (Weeks 2000, Oberem and Oberem 2011). Giraffes show a variety of skin disorders, including lumpy skin disease (LSD), giraffe ear disease (GED), and giraffe skin disease (GSD). LSD is a viral disorder, common in ungulates (Hunter and Wallace 2001). GED causes hyperkeratosis, ulceration and discoloration on the outer ear with a possible involvement of yellow and red-billed oxpeckers (*Buphagus* sp.) (Lyaruu 2010, Muneza et al. 2016). GSD causes lesions and manifests as chronic and severe scabs, wrinkled skin, encrustations and dry or oozing blood on the legs, shoulders, or necks of giraffes (Epaphras et al. 2012, Lee and Bond 2016). It has been suggested that nematode worms, with possible secondary infections of fungi and bacteria (Lyaruu 2010, Karimuribo et al. 2011, Bond et al. 2016) cause GED and GSD, however, the epidemiology of these two diseases remains unknown.

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Only one individual showed possible signs of GSD on the lower neck and two others showed signs of GED, based on gross pathological features described in the literature. These more superficial wounds may not have been perceived as a stressor in these individuals, subsequently resulting in fGCM levels comparable to non-injured individuals.

Elevated GC levels in animals with lower BC have been observed in a number of studies on birds and mammals (Kitaysky et al. 1999, Cabezas et al. 2007, Pokharel et al. 2017). When comparing individual baseline levels in two colonies of black-legged kittiwakes *Rissa tridactyla* for example, higher levels have been found in the colony living in a food scarce environment compared to food-rich environment (Kitaysky et al. 1999). In a study on Steller sea lions, the loss of body mass was associated with an increase in GC (Jeanniard du Dot et al. 2009). In our study, three individuals assigned to category 3 not only showed severe foot and leg injuries, but also had a lower BC scoring. The lower BC could be caused due to reduced mobility and subsequent reduced food intake leading to an increase in GC levels, a similar scenario has been described for elephants with foot injuries (Ganswindt et al. 2010). Alternatively, it is also possible that the increased GC levels due to the injuries lead to a reduced BC due to an enhanced energy demand as gluconeogenesis is activated in stressful situations (Sapolsky 2002). Further studies are needed to entangle which of the two proposed scenarios are responsible for the increased GC, or if it is a combination of reduced energy intake during times of increased energy demands.

The severity, but also the duration of an injury can influence the distinctiveness of an adrenal response (Voigtländer et al. 2006). This has been shown in elephants suffering from enduring foot injuries, where higher fGCM concentrations were found in the individual afflicted by the longer lasting injury (Ganswindt et al. 2010). In our study, the individual with the persistent injury (approx. one year, pers. comm.) did not show higher fGCM levels compared to individuals with a short to medium term injury. A possible explanation could be the adaptation of the HPA axis towards a long-term stressor. Although initially helpful to cope with a stressful situation, prolonged elevation of GC levels may cause changes in physiology and behaviour of an individual, which can have deleterious implications for survival and well-being (Herman 2013). The individual may have adapted to the consequences of the injury (e.g. decreased mobility), with a subsequent decrease in GC levels over time. Individual differences in GC levels also need to be taken into considerations, as the perceived pain varies between individuals (Bateson 1991).

In conclusion, we found differences in fGCM concentrations in individual giraffes assigned to different categories of trauma and body conditions. The possible response observed may result from a combination of differences in the severity of the injuries and the subsequent degree of pain associated with it, the influence of the stress response on the energetic condition, as well as the duration of the injuries. The results of our study are limited to a small sample size and although the data was collected during the end of the rainy season when food resources can be expected to be adequate, we cannot control for the effects of food access on the body condition of the individuals. A more detailed study over a longer time would be needed to evaluate the individual food intake and the effects that the injuries have on the mobility.

Euthanasia is a common management tool used to prevent unnecessary suffering, but especially in wild animals the severity of an injury and the associated pain perceived could be difficult to assess. Combining an assessment of BC and analysis of individual glucocorticoid levels may help to improve health assessments in free-ranging giraffes and thus assist management decisions.

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