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Research article

Triiodothyronine (T3) levels fluctuate in response to ambient temperature rather than nutritional status in a wild tropical ungulate

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Animals can employ a range of physiological mechanisms in response to unpredictable changes within their environment, such as changes in food availability and human disturbances. For example, impala exhibit higher faecal glucocorticoid metabolite (FGM) levels—indicative of physiological stress—in response to low food quality and higher human disturbance. In this study, we measured faecal triiodothyronine (T3) metabolite (FTM) levels in 446 wild impala from 2016 to 2018 to test the hypothesis that environmental and human disturbances would affect their physiological status. We also validated a faecal thyroid hormone assay. T3 levels mainly regulate metabolic rate and drive thermoregulation—increasing with colder temperatures. We predicted that individuals would have lower FTM levels, indicative of poor physiological status, (i) when food quality was poor, (ii) when ambient temperature (T_a) was high, (iii) in areas of high human disturbance (due to food competition with livestock) and (iv) when FGM levels were high. Interestingly, we found that T_a was the most important predictor of FTM—FTM levels decreased by 70% from lowest to highest T_a—and food quality and human disturbance only influenced FTM levels when T_a was accounted for. FTM levels also tended to increase with increasing FGM levels, opposite our predictions. Our results suggest that food quality and availability may only partially influence FTM levels and that fluctuations in T_a are a significant driver of FTM levels in a wild tropical ungulate. Given that thyroid hormones are primarily responsible for regulating metabolic rate, they may be better indicators of how wild animals metabolically and energetically respond to environmental factors and only indicate poor nutritional status in extreme cases.

Key words: glucocorticoid, impala, Serengeti, stress, thyroid hormones, validation

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Introduction

Anthropogenic disturbances influence wild animal populations both through direct human–wildlife interactions and through human- and climate-induced changes in food availability (Midgley and Bond, 2015; Segan *et al.*, 2016). For example, in East Africa, climate change is severely altering weather patterns and reducing forage quality (Pettorelli *et al.*, 2005; Boko *et al.*, 2007; Niang *et al.*, 2014). Wildlife populations have already declined dramatically in the region, particularly in areas with high human disturbance (Ogutu *et al.*, 2009; Veldhuis *et al.*, 2019). While some animals may cope with and adapt to these changes via behavioural and physiological mechanisms (Sih *et al.*, 2011), many populations face extinction. One of the primary physiological indicators used to understand the impact of environmental stressors on animal populations are glucocorticoid hormones (GCs; also termed 'physiological stress hormones'), given their direct relationship to individual fitness (Breuner *et al.*, 2008; Bonier *et al.*, 2009; Sheriff *et al.*, 2009; Dantzer *et al.*, 2014). For example, high GCs were found to predict lower survival rates in both ring-tailed lemurs (*Lemur catta*; Pride, 2005), vervet monkeys (*Chlorocebus pygerythrus*, Young *et al.*, 2019) and cliff swallows (*Petrochelidon pyrrhonota*; Breuner *et al.*, 2008; but see Brown *et al.*, 2005). Thus, understanding the physiological response of animals may provide key insights into how environmental stressors can alter individual fitness and population dynamics. Here, we investigated how environmental fluctuations and human disturbance alter thyroid hormone levels—a different, yet equally important and understudied hormone—in a free-living tropical ungulate in the Serengeti ecosystem.

Thyroid hormones (THs) are produced as the end product of the activation of the hypothalamic–pituitary–thyroidal (HPT) axis (reviewed in Behringer *et al.*, 2018). Their main function relates to the regulation of metabolic rates, and as such they are important in metabolically active tissues such as brown adipose tissue and skeletal muscles (López *et al.*, 2013). Studies have shown that when animals are food-deprived, such as during starvation or hibernation, thyroid hormone levels are significantly lower and this acts as a mechanism to reduce metabolic rate and save energy (Behringer *et al.*, 2018). For example, yellow-breasted capuchins (*Sapajus xanthosternos*) were found to have lower FTM levels when energy intake was low (Schaebs *et al.*, 2016), and TH levels in American black bears (*Ursus americanus*) decreased considerably and consistently during hibernation (Azizi *et al.*, 1979). THs are also critically important for thermoregulation (Silva, 2006) and usually increase in response to colder ambient temperatures (Behringer *et al.*, 2018). This has been found in several ungulates, including rams (*Ovis* spp.), burros (*Equus asinus*) and llamas (*Lama glama*; Brooks *et al.*, 1962; El-Nouty *et al.*, 1978). There is significant interaction (i.e. cross-talk) between THs and GCs, with increasing levels of GC generally decreasing THs levels (Charmandari *et al.*, 2005). Studies have also shown that low levels of THs are associated with reduced reproductive success (Torre *et al.*, 2014) and increased longevity (Bowers *et al.*, 2013). THs have therefore been proposed as a good indicator of the physiological status of an animal related to their overall fitness potential, with higher levels associated with better condition and higher fitness (Behringer *et al.*, 2018). However, most studies on thyroid hormone physiology in mammals have been done in captivity, and studies on wild populations are lacking. Studying how wild mammals regulate thyroid hormone concentrations to cope with natural stressors in complex environments is important to fully understand the function of thyroid hormones. To our knowledge, this is the first study investigating thyroid hormone physiology in a wild African ungulate (Behringer *et al.*, 2018).

In this study, we used a wild impala (*Aepyceros melampus*) population in the Serengeti ecosystem to investigate the influence of environmental fluctuations and human disturbance on TH levels. Impala are an ideal species for this study given their small home ranges (typically between 5 and 10 km^2; Averbeck *et al.*, 2010), high local abundance (Ford *et al.*, 2014) and non-migratory behaviour (Jarman, 1979). This allows us to compare individuals experiencing vastly different, spatially and temporally explicit variation in environmental factors and human disturbance. Working in the same ecosystem, we previously found that impala had greater GC levels when food quality was low (Hunninck *et al.*, 2020a). Similarly, wildebeest (*Connochaetes taurinus*) GC levels decreased considerably in response to new plant growth (Stabach *et al.*, 2015). Impala have also been shown to be sensitive to heat stress and fluctuations in ambient temperature (Cain *et al.*, 2006; Shrestha *et al.*, 2014). Furthermore, studies have shown that animals often have higher GC levels when in areas with higher human disturbances (Creel *et al.*, 2002; Ahlering *et al.*, 2011; Hunninck *et al.*, 2017), including impala (Lunde *et al.*, 2016; Hunninck *et al.*, 2020a). While human disturbance may directly influence GC levels in wild animals, it may also indirectly influence GC levels by reducing access to high quality forage. In the Serengeti ecosystem, Masai pastoralists keep their cattle in semi-permanent enclosures ('boma') at night, from which they are shepherded to nutritious pastures during the day. Livestock herding has been shown to result in competition between wildlife and livestock (Prins, 2000; Young *et al.*, 2005), and livestock grazing significantly decreases the available forage near such bomas (Riginos *et al.*, 2012). Because THs respond strongly to vegetation changes but not to direct human disturbance, and GC levels respond to both vegetation changes and direct human disturbance (Hunninck *et al.*, 2020a), studying variations in both hormone levels concurrently may provide important insights to how animals are impacted differently by food availability and human disturbance (Behringer *et al.*, 2018). For example, theoretically, high GC and high TH levels would indicate a physiological response to human disturbance rather than nutritional deficits.

We measured faecal thyroid hormone metabolite (FTM) levels to test the hypothesis that changes in food quality and anthropogenic disturbances would affect the physiolog-

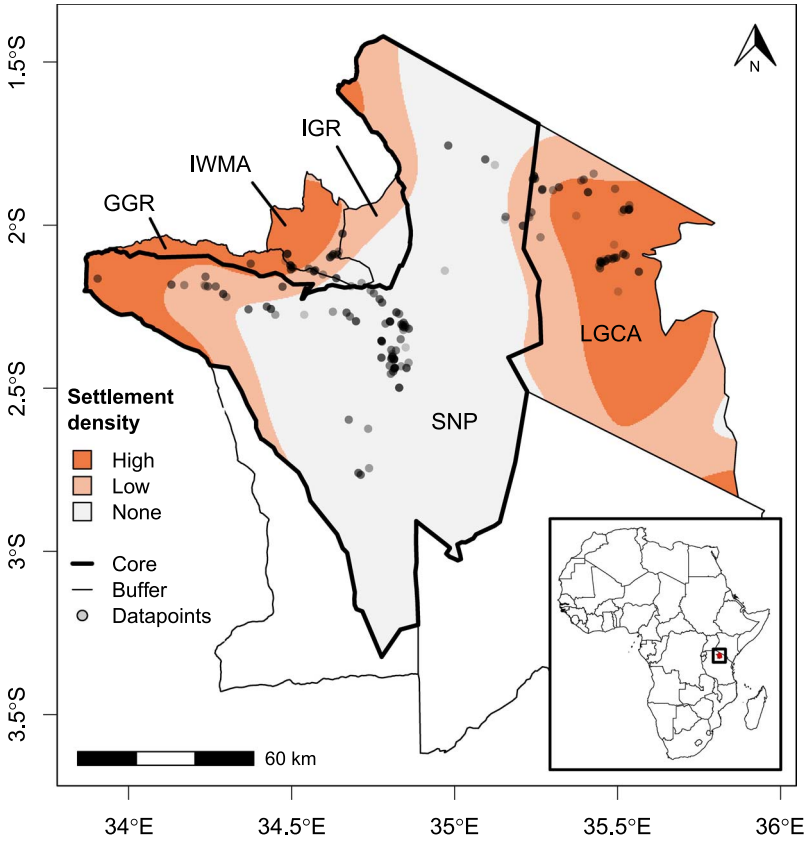

Figure 1: Map of the study area. The Serengeti ecosystem, including the Serengeti National Park (SNP) as the core protected area, and adjacent partially protected buffer areas Grumeti Game Reserve (GGR), Ikona Wildlife Management Area (IWMA), Ikorongo Game Reserve (IGR) and Loliondo Game Controlled Area (LGCA). Settlement density (categorized) is based on a kernel density estimation of settlement data from an aerial census (TAWIRI, 2016). Data points ($N = 446$) are shown as grey circles; overlapping points are darker. Inset shows location of study area on the African continent

ical status of wild impala living in the Serengeti ecosystem. Specifically, we predicted that individuals would have low FTM levels (i) when food quality was poor, (ii) when T_a was high, as THs drive thermoregulation, (iii) in areas of high boma density, because higher boma density results in higher foraging competition and decreased access to high quality forage, and (iv) when FGM levels were high. We have previously shown that FGM levels increased with decreasing food quality (Hunninck *et al.*, 2020a). Low FTM levels would indicate a poor physiological status of impala and possibly reduced fitness (Behringer *et al.*, 2018). Prior to our use for this study, we also provide the first validation of this method for use in wild impala.

Methods

Study system and species

The Serengeti ecosystem (± 25 000 km^2) is characterized by strong geographic and seasonal variation in rainfall, varying from 450 mm rainfall in the southeast to over 1400 mm in the north (Ogutu *et al.*, 2014). Mean ± SD daily minimum and maximum temperatures across the ecosystem are 16 ± 2.69 to 28 ± 2.40°C, respectively (range = 9–36°C, $N = 6253$ days; Seronera weather station; data courtesy of TAWIRI). The ecosystem consists of a core area, Serengeti National Park (SNP), and six adjacent buffer areas that have varying levels of protection status (Fig. 1). Our study area was limited to SNP and the buffer area Loliondo Game Controlled Area (LGCA) to the east, which, contrary to SNP, allows the building of temporary and permanent settlement, agricultural activities, regulated hunting and traditional livestock herding, i.e. pastoralism (Fig. 1).

Impala are a medium-sized, gregarious ungulate common in Eastern and Southern Africa (IUCN SSC Antelope Specialist Group, 2016). They are usually found in distinct groups: female herds (consisting of a territorial male, females and juveniles), bachelor herds (consisting of adult and sub-adult non-territorial males) and solitary males. Impala are mixed feeders, preferring high-quality forage (i.e. nutritious, more palatable green grasses) when available, but including higher quantities of low-quality forage (i.e. browse) into their diet

when grassy vegetation becomes dry (Jarman and Jarman, 1973; Wronski, 2002; Codron *et al*., 2007). Impala are constricted to relatively small home ranges throughout the year, typically between 5 and 10 km^2 (Averbeck, 2001). Though impala can be locally abundant (in the Serengeti ecosystem estimate ± SE = 74,837 ± 9,106; TAWIRI, 2010), their specific habitat requirements—preferring the edge of open savanna and open woodland—result in a patchy distribution (Ford *et al*., 2014).

Collection of faecal samples

We collected 446 faecal samples from individual impala (393 female, 53 male) over 3 years (2016, 2017 and 2018) in both wet and dry seasons (Fig. 1). We only collected faecal samples from adult individuals that were visually assessed to have a good body condition (e.g. no injuries, not obviously pregnant, no ribs showing). No sample was collected if it was contaminated by urine (Sheriff et al., 2011). When a suitable individual was seen defecating, a picture was taken and the distance to the individual was recorded with a range finder (typically between 20 and 100 m). When the herd had moved on, one person walked towards the sample with the range finder and measured the distance back to the car to until this was equal to the sample distance measured earlier; the sample location was also checked against the picture that was taken of the impala. This method allowed us to easily identify the specific sample (Lunde et al., 2016; Hunninck et al., 2020a, b). The sample was not collected when two or more samples were close to each other (within 1 m). For each sample, we recorded the sex of the individual, and the herd size and type (family or bachelor herd), and we noted the GPS location and the time of collection. We prevented pseudo-replication (i.e. sampling the same individual more than once) by not sampling one individual or one group more than once—though sometimes more than one individual from one group was sampled—and by avoiding areas where previous samples were taken. Pregnancy, even in early stages, can affect an animal's TH circulation (Behringer et al., 2018), yet it is impossible to determine early pregnancy with observational methods (Cain et al., 2012). A potential method to determine pregnancy could be found in estimating faecal sex hormone metabolites such as progesterone metabolites (Isobe et al., 2005; Pereira et al., 2006); unfortunately, the limited funds of this project did not allow this additional analysis. However, impala in the tropical Serengeti ecosystem do not have a defined calving season (Jarman, 1974; Sinclair et al., 2000) and so any possible unexplained variation that early pregnancy might add to the data is expected to be uniformly distributed among samples. We could not absolutely ensure a lack of resampling of specific individuals between years, however, as there are ~75,000 impala in the study system, the chances of pseudo-replications among years that would affect our results are extremely low.

After the samples were collected, they were immediately placed on ice (<45 min, median = 25 min) and, within 12 h of defecation (median = 4 h), stored at −20°C until further analysis (<3 years). Collection time did not affect FTM concentrations. This collection interval and time on ice is short, especially for field studies, and studies have shown that thyroid hormone metabolite concentrations in faeces stay stable—i.e. are not affected by bacterial degradation—within a similar time frame: 2 weeks stored at 4°C, and 8 h stored at 25°C (Gesquiere *et al*., 2018). This is similar to several steroid hormones (Washburn and Millspaugh, 2002; Palme *et al*., 2013; Palme, 2019).

Analyses of faecal samples and validation of FTM radioimmunoassay

FTM concentrations reflect the biologically active thyroid hormone triiodothyronine plasma levels (Behringer *et al*., 2018), and importantly, sample collection is completely non-invasive. Additionally, since FTMs are an integrative measure of plasma THs, representing an average value over the previous 24–48 h in impala (Chizzola *et al*., 2018), rather than a point value of thyroid hormone levels, FTM concentrations offer a much better insight into an animal's long-term physiological status (Behringer *et al*., 2018).

Hormone extraction

The hormone extraction method was based on (Palme, 2005) and is detailed in (Hunninck *et al*., 2020a, b). Briefly, faecal samples were defrosted at room temperature (30 min) and homogenized. Wet faces (0.5 ± 0.01 g) were mixed with 5 ml of 80% methanol, vortexed (1 min), then centrifuged (20 min; 2500*g*), and lastly, 0.5 ml of the supernatant was removed. We used a fume hood for up to 48 h to evaporate the extract and subsequently sealed and stored the tubes at −20°C until further analysis.

Thyroid hormone analysis

To determine FTM levels, we used ^{125}I Total Triiodothyronine (T3) radioimmunoassay (RIA) kits (Catalogue No. 06B254216, MP Biomedicals, Costa Mesa, CA). This kit has been validated in several species before, such as baboons (*Papio* spp.; Gesquiere *et al*., 2018), steller sea lions (*Eumetopias jubatus*; Keech *et al*., 2010) and several ungulates, including caribou (*Rangifer tarandus*) and moose (*Alces alces*; Wasser *et al*., 2010). We based our laboratory protocol on Gesquiere *et al*. (2018): we adhered to the manufacturer's protocol, with the exception that we halved the volume of all reagents, to assure detectable levels of FTM. This yielded the best results when previously analyzing FTM concentration in baboons (Gesquiere *et al*., 2018).

In order to correctly interpret the variations in hormone concentrations, it is essential to validate the method used to measure hormone metabolite concentrations in faeces in one's study species (Touma and Palme, 2005; Dantzer *et al*., 2014; Palme, 2019). Here, we conducted both an analytical validation, through the use of standard assay validations of

parallelism and precision, and a biological validation, by comparing seasonal differences (as has been done before, e.g. Chinnadurai *et al*., 2009; Gesquiere *et al*., 2018).

Parallelism analysis tests whether the assay maintains linearity under dilution (Andreasson *et al*., 2015). Precision was determined via intra-assay variation calculated by averaging coefficients of variation of all samples—which were run in duplicate, and via inter-assay variation calculated by calculating the coefficient of variation of measured FTM of one standard between assays. Cross-reactivity of the primary antibody, as reported by the manufacturer, is 100% with L-triiodothyronine (T3), 0.18% with L-thyroxine (T4), 0.44% with 3,5-diiodothyronine (T2), 0.01% with 3,3′,5′-triiodothyronine (rT3) and <0.01% for 3,5-diiodotyrosine, phenylbutazone, sodium salicylate, diphenylhydantoin and dicumerol. To control for potential batch effects between days of hormone extractions or RIA analyses, we randomly selected a subset of the 446 samples for each batch of analysis.

Glucocorticoid hormone analysis

FGM levels were analyzed using a group-specific 11-oxoetiocholanolone enzyme immunoassay (EIA), first described by Möstl *et al*. (2002), which measures metabolites with a 5β-3α-ol-11-one structure. This EIA has been specifically validated for and used previously in impala by Chizzola *et al*. (2018) and (Hunninck *et al*., 2020a, b).

Environmental variables

Vegetation and temperature data were retrieved from the online Application for Extracting and Exploring Analysis Ready Samples (*AppEEARS*), courtesy of NASA (https://lpdaacsvc.cr.usgs.gov/appeears/). We collected data on normalized difference vegetation index (NDVI—a proxy for forage quality; MOD13Q1 & MYD13Q1 MODIS/Terra & Aqua; temporal resolution [TR] = every 8 days, spatial resolution [SR] = 250 m; Didan, 2015), percent woody cover (WC; MOD44B MODIS/Terra; TR = yearly, SR = 250 m; Dimiceli *et al*., 2015) and land surface temperature (LST; MOD11A1 MODIS/Terra; TR = daily, SR = 1000 m; Wan Z., 2015). Using the pixel reliability dataset that accompanies the NDVI dataset, pixels containing clouds were filtered out, and data were adjusted to account for empty data points using a Savitzky–Golay smoothing filter. In order to obtain an NDVI value specific to each of our faecal samples with regards to location and time of collection, we extracted the closest value in space and time (Hunninck *et al*., 2020a). Similarly, to obtain a better representation of relevant variation in ambient temperature (T_a), we calculated the average T_a over a 7-day period prior to sample collection for each sample, specific to its location, which made sure we had no empty values in our data. We could not use weather station data to estimate temperature as there is only one weather station in the study system (located in Seronera 100 km from our furthest sampling sites); therefore, its use may not be particularly accurate for any given location, nor provide any spatial variation; furthermore, this data was only available until 2015. Impala have small home ranges (5–10 km^2; Averbeck *et al*., 2010; Jarman, 1974) and limited movement—impala equipped with a GPS collar moved a median ± SD distance of 767 ± 7140 m in 1 week (N = 212000; *unpublished data, not part of this study*) away from their initial location. Therefore, our data (NDVI and T_a) provide a reasonable representation of the environment utilized by the sampled impala over the past week (Hunninck *et al*., 2020a).

Care should be taken with analyzing remotely sensed NDVI measurements. First, due to differing vegetation cover and structure, NDVI estimates should only be compared within the same habitat, and not between different ecosystems (Pettorelli *et al*., 2005). Impala generally prefer semi-open savanna with limited woody cover (Jarman and Jarman, 1973; Ford *et al*., 2014). We assessed differences in woody cover among sample locations using remotely sensed data on woody cover (MOD44B MODIS/Terra; Dimiceli *et al*., 2015) and found cover percentage at sample locations was low (mean ± SD = 5 ± 3.2%, N = 446). Therefore, although NDVI presents a proxy for overall greenness of land cover (e.g. both grass and woody cover), at our sample locations, variation in NDVI is mostly due to changes in grassy vegetation. Further, since grassy vegetation is considerably more palatable than woody browse and therefore preferred by impala (Jarman and Jarman, 1973; Codron *et al*., 2007), NDVI represents an unbiased proxy for forage quality for impala.

We performed several analyses to validate the use of LST as a proxy for T_a in our system. Firstly, though LST is significantly affected by variation in land cover (i.e. certain surfaces heat up more than other), as mentioned before, the proportion of woody cover in the areas where we collected samples was low. Therefore, variation in land cover between sampling locations did not affect our LST measurements. Secondly, we examined whether our LST measure adequately represented ambient temperature by correlating our weekly average LST estimates between 2012 and 2015 of the specific location around (i.e. specific pixel) the Seronera weather station, with concurrent weekly averages of maximum air temperature measured at the Seronera weather station (located in central SNP). As this correlation was 0.67 (N = 166), we believe LST can sufficiently approximate a relevant measure of T_a. Although direct solar radiation (a main driver of LST) represents a significant part of the heat load but is to an extent mitigate by animals seeking shade, since LST correlates strongly with T_a (representing air temperature) and woody cover was low (representing limited shade) at sample sites, this should not affect the interpretation of our results. Lastly, since NDVI measurements are used in the calculation of LST, we correlated NDVI and LST measures from all cells in the study system on one day (r = −0.50) and calculated the difference in LST given an NDVI of 0 and 1 (i.e. min and max values of NDVI) while keeping surface reflectance constant (the only other input variable to calculate LST). This resulted in only a 0.01°C difference in LST, showing that NDVI has a negligible impact on LST estimation. We transformed the LST values

Table 1: Model estimates from the mixed-effect regression model, explaining the variation in faecal total triiodothyronine (T3) metabolite (FTM) levels in impala

Model: *log(FTM) ~ NDVI + Temperature + log(FGM) + Boma density + (1|Group)*					
Fixed effects	**Scaled est. ± SE**	**df**	***t* value**	***P* value**	
(Intercept)	6.75 ± 0.05	113.88	12	<0.001	***
NDVI	−0.14 ± 0.04	114.58	−3.43	<0.001	***
Temperature	−0.28 ± 0.04	108.3	−6.79	<0.001	***
log(FGM)	0.05 ± 0.03	414.48	1.85	0.065	.
Boma density—low	−0.07 ± 0.08	115.14	−0.81	0.422	
Boma density—high	−0.23 ± 0.09	108.93	−2.59	0.011	*
Random effects	**Variance**	**Std. dev.**			
Group	0.09	0.29			
Residual	0.22	0.47			

Scaled estimates and their standard error (SE) represent relative effect sizes of predictors, allowing comparison among predictors within a model. Reference level for predictor Boma is 'None'. See text for further details. Significance codes: $P < 0.001$ ***; 0.001–0.01 **; 0.01–0.05 *; 0.05–0.1.

based on maximum air temperatures from Seronera to better represent the T_a range experienced by impala. Taken together, these analyses show that LST is a useful and relevant proxy for T_a in our study system and that NDVI and LST are practically independent and can be used as separate variables in regression models (Hunninck *et al.*, 2020a).

Human disturbance proxy

Boma density was based on the Tanzanian Wildlife Institute data on settlement locations, which included bomas (i.e. used by pastoralists to protect their livestock), thatch roof huts and iron sheet huts/houses in and around most of the Serengeti ecosystem (Fig. 1; TAWIRI, 2016). We applied a Kernel density estimation (KDE) to the data and obtained a specific settlement density score for each faecal sample. Due to a high number of samples with very low settlement density scores, we categorized the data into three quantile based categories: None ($N = 224$; mean boma density per 10 km^2 [BD] ≈ 0.2), low ($N = 110$; BD ≈ 3) and high ($N = 112$; BD = 9). Data for relevant human disturbance proxies with high temporal and spatial resolution are particularly difficult to come by, and this dataset presents the most recent and accurate data on settlement locations in our study area. Furthermore, interannual spatial variation in settlement density is unlikely to have changed dramatically. Therefore, we believe that this proxy still presents a highly relevant insight in the spatially explicit patterns of human disturbance.

Statistical analyses

We used a multiple linear mixed-effect regression model (*lmer* function of the *lme4* package v.1.1-17; Bates *et al.*, 2015) with FTM as the response variable. The response variable was log-transformed to obtain normal distribution of model residuals. We included (i) NDVI (i.e. proxy for forage quality in the landscape), (ii) T_a (i.e. based on LST), (iii) boma density (i.e. proxy for human and livestock disturbance) and (iv) FGM as predictor variables. We included group ID (i.e. individual herds) as a random effect to control for differences among groups; differences among sampling locations did not explain any additional variation and was therefore excluded from the model. Similarly to previous studies in impala (Lunde *et al.*, 2016), FTM levels were not different between the sexes (est. −0.05, $t = -0.61$, $P = 0.542$), and including sex did not improve the model (ANOVA; $P = 0.671$). Group size (median ± SD = 34 ± 24.5; range = 4–124) similarly did not affect FTM levels (est. −0.0005, $t = -0.282$, $P = 0.778$), and including this variable did not improve the model (ANOVA; $P = 0.371$). Therefore, these variables were excluded from the model. Results of the model are presented in Table 1. Model estimates were standardized (*z*-score: centred around the mean; scaled by dividing by the standard deviation; *scale()* command in R); scaled estimates represent relative effect sizes of predictors, allowing comparison among predictors within a model.

Residuals were visually checked for normality and heteroskedasticity, and multicollinearity was assessed with a generalized variation inflation factor (GVIF) analysis, which is a measure of the harm done by collinearity among predictors (Fox and Weisberg, 2011). No heteroskedasticity was found, and residuals were normally distributed; GVIF values corrected for the degrees of freedom (GVIF^[1/(2*df)]) were all lower than 1.23 (*vif* function of the *car* package v.3.0-0 in R (Fox and Weisberg, 2011)), which is well below the conservative threshold of 3 (Zuur *et al.*, 2010). There was no temporal autocorrelation (ANOVA; model without autocorrelation, LR = 136.38, $P < 0.001$), which was assessed by comparing the final model with and without an autocorrelation

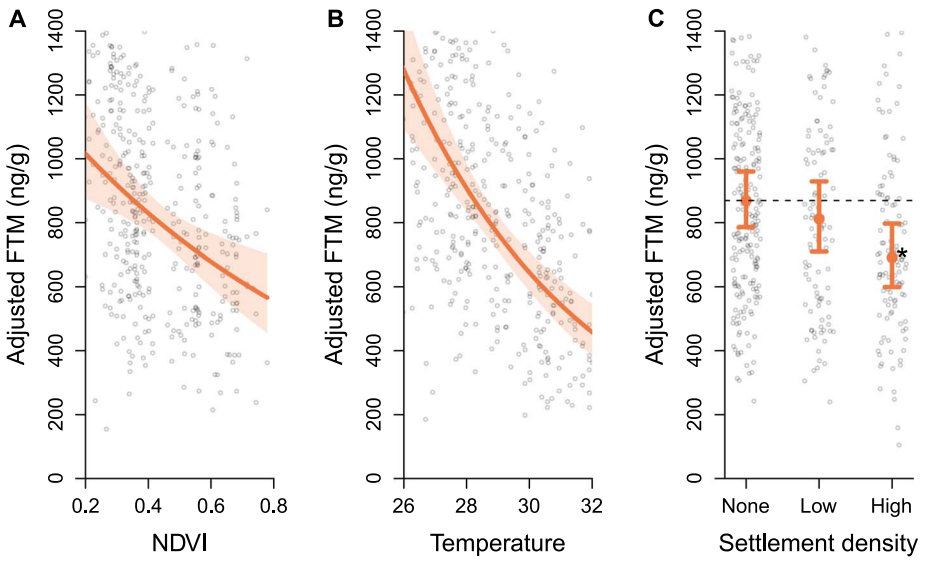

Figure 2: Model effects on impala faecal total triiodothyronine (T3) metabolite (FTM) levels. The effect (red line) of (**A**) NDVI (Normalized Difference Vegetation Index; proxy for forage quality), (**B**) Ambient temperature (°C; based on LST estimation) and (**C**) Settlement density (categorized based on kernel density estimate of settlement locations; TAWIRI, 2016) on impala FTM levels. Adjusted response values are represented as points; 95% confidence interval is the shaded red area or the error bars. On panel C, star denotes significant difference from reference level (dashed line; $P < 0.05$)

structure based on date of sampling (functions *corAR1* within *lme*; package *nlme* v.3.1-140; Pinheiro *et al.*, 2020). Model fit was also assessed by calculating marginal (i.e. variation explained by fixed predictors only) and conditional (i.e. variation explained by both fixed and random effects) R^2 values (Nakagawa and Schielzeth, 2013). All statistical analyses were performed in the statistical program *R*, v.3.5.0 (RCoreTeam, 2018), using RStudio v.1.1.453 (RStudio, 2016).

Results

Validation of FTM assay

Serial dilutions of faecal FTM extracts resulted in displacement curves that were parallel to the standard T3 curve (quotient of slopes = 0.97). Inter-assay variation was 5%, and average intra-assay variation was 4% ± 2 (mean ± SD, $N = 499$).

FTM concentrations were significantly different between seasons, such that FTM levels in the wet (December–January) season were 42% lower than in the dry season (May–June–July; est. = −0.55, $t = -7.86$, $P < 0.001$).

Model results

We found that impala had significantly higher FTM levels in areas with lower NDVI values, such that mean FTM levels increased by 44% from 566 ng/g (95% confidence interval (CI) = 456–703 ng/g) at the highest NDVI values to 1018 ng/g (CI = 876–1183 ng/g) at the lowest NDVI values (Table 1; Fig. 2A). FTM levels increased significantly with decreasing T_a, such that mean FTM levels increased by 70% from 414 ng/g (CI = 338–507 ng/g) at the highest T_a, to 1358 ng/g (CI = 1147–1609 ng/g) at the lowest T_a (Table 1; Fig. 2B). Mean FTM levels where significantly higher (20%) in areas without the presence of bomas (869 ng/g; CI = 786–960 ng/g) compared to areas with high boma density (691 ng/g; CI = 599–797 ng/g; Table 1; Fig. 2C). FTM levels tended to increase with increasing FGM levels, such that mean FTM levels increased by 28% from 674 ng/g (CI = 562–808 ng/g) at the lowest FGM levels to 935 ng/g (CI = 772–1133 ng/g) at the highest FGM levels (Table 1; Fig. 3).

Interestingly, we found that T_a was the most influential predictor in the final model (Table 1), having an effect size approximately twice that of NDVI (Table 1). The final model explained 40% of the variation in impala FTM levels (*conditional* R^2); fixed effects (i.e. *marginal* R^2) explained 16% of the variation (Nakagawa and Schielzeth, 2013).

Discussion

We investigated the relationship between FTM level and changes in environment and anthropogenic disturbances in wild impala. We hypothesized that FTM, a proxy for nutritional status, would be influenced mainly by variation in food quality. However, we instead found that T_a was the most important predictor of FTM levels, more so than food quality, human disturbance and FGM levels. Furthermore, we found that FTM levels decreased with increased food quality,

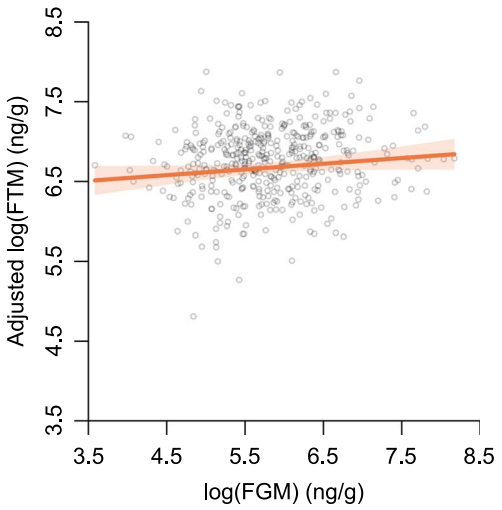

Figure 3: Model effect of faecal glucocorticoid metabolite (FGM) levels on impala faecal total triiodothyronine (T3) metabolite (FTM) levels. The correlation (red line) between impala FGM and FTM levels. Adjusted response values are represented as points; 95% confidence interval is the shaded red area

opposite to our prediction. This implies that thermoregulation and not food quality foremost drives FTM levels in free-living impala.

The effects of food quality on FTM levels

We found that FTM levels decreased with increasing NDVI, a measure for food quality (rather than availability) in the landscape. THs have been studied extensively in relation to the nutritional status of animals, and many studies have found that FTM levels decrease significantly when animals experience low food availability, such as during starvation or fasting (Behringer *et al.*, 2018). For example, howler monkeys (*Alouatta palliata*) were found to have lower FTM levels when energy intake was low (Dias *et al.*, 2017), and European black bears (*Ursus arctus arctus*) were found to have significantly lower thyroid hormone levels both during times of reduced food and during hibernation (Tomasi *et al.*, 1998). In these situations, low FTM values are indicative of an energy saving mechanism, as this would result in a slower metabolism. In our study, we found the opposite, that impala had low FTM levels when food quality was high. This could be explained by two non-exclusive mechanisms.

First, impala may employ an energy saving mechanism when food quality is high. Although impala, like all tropical ungulates, have little subcutaneous fat reserves compared to their temperate and arctic relatives (Ledger, 1963), impala store energy reserves (i.e. kidney fat and bone marrow) during the wet season when food quality is high (Smith, 1970; Sinclair and Duncan, 1972; Dunham and Murray, 1982). Impala have been shown to mobilize these reserves during energetically challenging situations such as pregnancy or rut to maintain energy balance (Dunham and Murray, 1982). Having low levels of THs while food quality is high could increase their ability to store energy.

Second, impala may need to increase circulating TH levels when food quality (but not availability) is low in response to necessary increases in time spent foraging and food intake. Many tropical ungulates increase their food intake when food quality is low (Jarman and Jarman, 1973). For example, Owen-Smith (1997) found that in the dry season, kudu (*Tragelaphus strepsiceros*) maintained their nutritional condition by increasing their time spent foraging and food intake, thereby compensating for the lack of forage quality by increasing forage quantity intake. Impala similarly increase time spent foraging (Jarman and Jarman, 1973; Wronski, 2002; Oliver *et al.*, 2007), and increase their rumen volume to accommodate the increased forage intake during the dry season (Lane *et al.*, 2014). The increase in FTM levels we found at low NDVI could be associated with increased metabolic rate to accommodate the increased digestion needed to deal with the higher quantities of low-quality forage. It has been shown previously that T3 levels increase consistently when animals consume more food (Dauncey, 1990). This increased cost of digestion is potentially exacerbated as impala spend proportionally less time ruminating—thereby decreasing mechanical digestion—when forage quality is low, probably to maximize food intake (Jarman and Jarman, 1973). Interestingly, Kong *et al.* (2004) found that T3 directly stimulated appetite, acting at the level of the hypothalamus, thus providing an additional hypothesis that elevated thyroid hormone levels may also stimulate increased foraging when food quality is low.

Therefore, FTM levels might not reflect nutritional state *per se*, but rather be an adaptive response to changing nutritional value of their food: being lower when food quality is high as an energy saving mechanism, but upregulating THs, and thus metabolic rate (and possible stimulating increases in foraging), when food quality is low to accommodate for increased food intake.

Although we did not find any evidence that lower forage quality resulted in lower FTM levels as would be expected in poor condition individuals, we did not sample any individuals that were visibly in poor body condition (e.g. ribs showing). Mortality from starvation is rare in the wild, as such individuals usually die of diseases or predation (Sinclair and Duncan, 1972). We do not rule out the possibility that low FTM levels may reflect nutritional deficiencies, particularly in animals where reduced food quality is not accompanied by an increase in food intake, possibly FTM levels reflect nutrient deficiencies only in extreme cases (i.e. when they are starving), though future studies are required to further test this.

The effects of T_a on FTM levels

We found that T_a was the most influential predictor of FTM levels, whereby FTM levels increased as T_a decreased. THs are

crucially important in thermogenesis and thermoregulation in homeotherms—increasing in concentration in response to cold temperatures (Silva, 2006). For example, Barbary macaques (*Macaca sylvanus*) were found to have significantly higher TH levels when T_a decreased (Cristóbal-Azkarate *et al.*, 2016), and burros (*Equus asinus*) and llamas (*Lama glama*) similarly increased TH levels in response to cold temperatures (El-Nouty *et al.*, 1978).

Although we expected a negative relationship between FTM levels and T_a, we did not expect this to be the main driver of FTM variation in a tropical ungulate. Like all mammals, tropical ungulates are sensitive to fluctuations in T_a, however, unlike their Arctic counterparts, tropical ungulates experience extreme heat, not cold (Ledger, 1963; Mitchell *et al.*, 2018). Indeed, impala have been shown to be sensitive to heat and show signs of heat stress at T_a between 35 and 50°C, while extreme heat can negatively affect their diurnal activity (Maloiy and Hopcraft, 1971; Mills *et al.*, 1986; Shrestha *et al.*, 2014). As such, tropical ungulates lack subcutaneous fat that would presumably make coping with the extreme heat even more difficult (Ledger, 1963; Owen-Smith, 1997). Because of the lack of subcutaneous fat reserves as a thermal insulator, however, impala are vulnerable to even modest decreases in T_a (Shrestha *et al.*, 2014) and must likely rely on physiological responses to reductions in temperature, such as increases in their TH levels and metabolic rate (Silva, 2006). This explains the sensitivity and importance of T_a in driving changes in FTM that we found.

The effects of human disturbance on FTM levels

FTM levels were lower in areas with highest boma density. This effect takes into account the other predictors in the multiple regression model; i.e. NDVI (i.e. forage quality) and the other predictors are kept constant. Therefore, FTM levels are lower in high boma density, regardless of forage quality. Increasing boma density results in reduced access to forage for wildlife because of foraging competition with livestock (Riginos *et al.*, 2012). Evidence for competition between livestock and wildlife has been found before (Prins, 2000), for example, zebra (*Equus* spp.) were found to be 46% more abundant in areas where cattle was excluded (Young *et al.*, 2005). Therefore, our results suggest that even with similar forage quality, impala cannot acquire the same quantity of forage in areas with high boma density, hence reducing their FTM levels.

Our results further suggest that human disturbance may influence impala by reducing their access to forage through increased competition with livestock. While impala generally increase forage intake in areas where food quality is poor, human disturbance may limit this behavioural response, and thus the reduction in FTM in relation to boma density may act as an energy saving mechanism. Thus, taken with our previous results it seems that impala reduce FTM levels when food quality is naturally high and also have reduced FTM levels when overall energy availability (food quantity) is limiting. Both may act as energy-saving mechanisms; however, the driver is qualitatively different, the former being proactive, the latter being a reactive response.

The relation between FTM and FGM levels

FTM levels tended to be higher with higher FGM levels. We predicted that individuals with low FTM levels, indicative of poor physiological status, would have greater FGM levels. Laboratory-based studies have shown that the activation of the hypothalamic–pituitary–adrenal (HPA) axis—responsible for the production of GCs—decreased TH levels in the body (Charmandari *et al.*, 2005). Yet, few studies in wild animals have investigated both THs and GCs, and the relationship between THs and GCs is still unclear in free-living animals. In a declining population of free-living Hawaiian monk seals (*Neomonachus schauinslandi*) FGM levels were positively correlated with FTM levels, perhaps indicating that the seals required a high metabolic rate (i.e. high FTM) to acquire prey or that prey may be stressful to acquire, hence eliciting high FGM levels (Gobush *et al.*, 2014). Similarly, there was a positive relationship between FTM and FGM in wild moose (*Alces alces*), with high FGM levels perhaps due to increased exposure to predators when moose increased foraging, thus increasing FTM levels (Jesmer *et al.*, 2017). If animals increase their foraging behaviour and food intake when food quality is low, as impala do (Wronski, 2002), they may also increase their TH levels (Dauncey, 1990; Kong *et al.*, 2004). Increased foraging behaviour may result in greater GC levels if it exposes animals to greater threats (e.g. risk of predation; Clinchy *et al.*, 2013; Sheriff *et al.*, 2020) or if animals require greater energy mobilization due to the increased foraging activity (Birt-Friesen *et al.*, 1989; Jesmer *et al.*, 2017). These two mechanisms could explain both our result and those mentioned above.

Furthermore, as the digestion of food is an energetically costly process (McBride and Kelly, 1990), an increase in GC (and hence FGM) levels might be required to supply the required energy (Young, 1977; Exton, 1979). Supporting this hypothesis, we found that FGM levels were highest with low NDVI, i.e. when impala consumed the highest quantity of forage (Hunninck *et al.*, 2020a). Clearly, much work is needed to better understand the relationship between GCs and THs in wild animals.

Additionally, the positive relationship between FTM and FGM levels could also be due to the importance of THs for thermoregulation. When ambient temperatures drop, mammals must generate heat through thermogenesis to maintain homeothermy, an energetically costly process mainly regulated by THs (Silva, 2006). Since GCs are critically important for energy mobilization and gluconeogenesis (Young, 1977; Exton, 1979), an increase in GCs, resulting in increased energy availability, might be necessary to facilitate the energy requirements for thermoregulation. For example, Shipley

et al. (2019) found that FGM levels in ruffed grouse increased as ambient temperature decreased. Thus, as THs are primarily responsible for thermoregulation, this could in part explain the positive correlation between FTM and FGM levels found here and in other studies.

Confounds of diet

When studying hormone (metabolite) concentrations in a matrix, care should be taken with the interpretation of the estimates, as the variation in concentrations can be affected by other sources than physiological changes. Indeed, Dantzer *et al.* (2011) found that fibre content of a diet can significantly affect faecal hormone metabolites concentrations. The mechanisms by which this could happen are unclear, and studies have found that increasing fibre content can increase (Dantzer *et al.*, 2011), decrease (Wasser *et al.*, 1993) or not affect faecal hormone metabolites (Von Der Ohe *et al.*, 2004). Although impala are mixed feeders that include more browse in their diet during the dry season, the average fibre content of the diet in the dry and wet seasons are similar (e.g. 30 vs 23%, Dunham, 1980; 42 vs 36%, Meissner *et al.*, 1996). This difference is small enough that fibre content probably did not significantly affect our FTM or FGM estimates.

Though we performed an analytical and biological validation of the method to accurately measure FTM concentrations, we were not able to perform a physiological validation within the scope of this study. Such a validation could be conducted using a thyroid-stimulating hormone (TSH) challenge on captive impala, though this has rarely been done (Behringer *et al.*, 2018; but see, for example, Keech *et al.*, 2010). Additionally, though sampling of individuals in poor condition could have yielded a particularly interesting biological validation, it was too difficult to find and sample such individuals, probably due to the high predation of impala—which have many natural predators—in poor condition.

Conclusion

Understanding how environmental factors drive FTM levels in wild animals is important given the link between thyroid hormones and fitness (Behringer *et al.*, 2018). Contrary to previous findings, we found that FTM levels increased, rather than decreased, with lower food quality and suggest this may be due to increased food intake seen in impala in response to lower food quality. We found that ambient temperature was the most important factor driving FTM levels in wild impala, increasing as temperatures decreased. We suggest that thermoregulation may be a critical aspect of TH regulation and that THs may only provide an indication of nutritional status in extreme cases (e.g. starvation). As expected, we found lower FTM levels in impala living in areas with high boma density, likely due to competition with livestock; impala may not be able to compensate for the reduction in access to high-quality food by increasing foraging activity due to human activity and in such cases may need to lower THs. This suggestion is supported by our previous findings that impala have greater FGM levels in areas with higher boma density but that such an effect is negated in areas with high food quality (Hunninck *et al.*, 2020a). Overall, we propose that in wild animals, TH levels should be considered as a metabolic or energetic regulator and that variation in TH levels should be interpreted in relation to the energetic needs and state of an animal, rather than a strict indicator of nutritional status of an individual. Clearly, more studies on wild populations are needed to elucidate how THs facilitate energy regulation in response to rapid environmental change and human disturbances.

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