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Assessing the effect of roads on impala (*Aepyceros melampus*) stress levels using faecal glucocorticoid metabolites

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Preface

The background for this study is the imminent threat of global species decline, which I believe deserve immediate attention to enable us to preserve the Earth's biosphere and ensure that future generations will inherit a functioning planet. It is in direct association with the principles of the Convention on Biological Diversity (CBD) of the United Nations, which aims to conserve biological diversity, ensure sustainable use and an equal sharing of our resources.

This Master thesis was conducted at the Department of Biology at the Norwegian University of Science and Technology (NTNU). It was supervised by Professor Eivin Røskaft (NTNU) in collaboration with scientists from Tanzania Wildlife Research Institute (TAWIRI). Field work and part of the laboratory procedures were carried out in the Serengeti National Park, Tanzania, and the EIA analysis was conducted by the lab of Dr Rupert Palme, University of Veterinary Medicine, Austria.

There are so many fine individuals who deserve commendation for their part in this study. I would in particular like to direct a warm thank you to my supervisor Prof. Eivin Røskaft for placing his trust in me and sending me off to Africa after only a few meetings. He has been a great supervisor and provided me with exactly what I needed when I needed it. The people of Tanzania deserve a special thanks, first and foremost Yasinta and Juma for staying with me and never complaining, even when there were more biting tsetse flies around us than I thought could ever be possible. Thanks to Dr Robert Fyumagwa, Brian Harris of Grumeti, Elias, Grayson, Maulidi, Noel and the rest of the staff at the Serengeti Wildlife Research Centre for helping me with everything from permissions and advice to freezer space, and picking me up in the middle of nowhere when my car broke down. I would also like to thank all my friends in the Lion, Hyena, Grass and Cheetah house for sharing food or beers after a week's hard work, the welcoming Frankfurt people and the park staff of Serena lodge that let the "muzungo" play volleyball with them after work. Thanks to Dan, Daniel and Jeff for great laughs and talks about our research and making feel at home in Serengeti, and finally my parents for being supportive all the way. For help with the write-up I would like to thank Thomas K., Prof. Christophe Pelabon and Tomas H. for valuable professional advice, and the other master students who gave me helpful advice and a pat on the back in times of need. Jon, Torgeir, Ivan, AC, Elida, Kristine and the rest; you were a great support and it would have been a lot harder doing this alone. Thanks to my friends Eivin, Knut and Fredrik for dropping by and checking on me, and to the rest of you that helped me or just enhanced my master project experience that I have not included here – you know who you are – thank you. Finally I would like to thank my co-supervisor Prof. Claus Bech for having an open door and a smile every time I stopped by – you are an amazing man – and Craig Jackson and Dr Rupert Palme for scientific advice and proof-reading. To all of you, I am deeply grateful for you sharing your time and your presence with me in my life, I will always treasure it.

Now, let’s go!

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Even Tvede Lunde

Abstract

*Loss of habitat is the main cause of species extinction, which today is 100 - 1000 times faster than the background extinction rate before the presence of mankind. One cause is that natural areas are being increasingly perturbed by roads, which degrades and fragments the habitat. In Tanzania, a proposed road through the Serengeti National Park (SNP) has caused international debate on whether it will cause irreversible damage to the ecosystem. I have assessed the current effect of roads and traffic on wildlife in the Serengeti, by using the impala (*Aepyceros melampus*) as a model species. I measured how faecal glucocorticoid metabolites (FGM), an indicator of stress levels, were affected by different road categories with different levels of traffic. Other stress related variables such as distance to the road, vegetation type and group size were accounted for. 196 faecal samples from 165 groups were collected over a 2 month-period from June – August 2012. FGM were measured using an enzyme immunoassay (EIA) validated for ruminants. I found that FGM levels were significantly increased near major roads with elevated levels of traffic compared to minor roads and side roads with less traffic in the Serengeti ecosystem. Predators, illegal hunting and social stress might also affect FGM levels in the impala, but these variables could not explain the variation observed. These results show for the first time that a large African mammal exhibit significant physiological stress in relation to roads. I argue that roads and traffic have the potential to cause major physiological stress in mammals, which in turn can affect the fitness of the organism. Care must be taken before implementing roads in protected areas, as increased disturbance can have severe consequences for the ecosystem if keystone species are affected. Future studies should assess the levels of stress wildlife can tolerate without it affecting fitness. Management plans for natural areas should include the possibility for increased physiological stress in animals living near major infrastructures and be proactive to ensure population and ecosystem viability.*

Sammendrag

*Tap av habitat er hovedårsaken til utryddelse av arter, som i dag er 100 - 1000 ganger raskere enn bakgrunnsutryddelsen før menneskets tilstedeværelse. En årsak er at naturområder i økende grad blir påvirket av veier, som kan fragmentere og ødelegge habitatet. I Tanzania har en planlagt vei gjennom Serengeti Nasjonalpark skapt internasjonal debatt om hvorledes denne vil forårsake uopprettelig skade på økosystemet. Jeg har vurdert dagens effekt av veier på dyrs stressfysiologi i Serengeti Nasjonalpark ved å bruke impala (*Aepyceros melampus*) som modell, for å kunne estimere effekten av økt forstyrrelse fra veier. Jeg målte hvordan glukokortikoid metabolitter fra avføring (FGM) hos impala var påvirket av veityper med ulik størrelse og trafikkmengde. FGM er en indikator på stress, og jeg korrigerte for andre stress relaterte variabler (som avstand til vei, vegetasjonstype flokkstørrelse, m.m.). 196 avføringsprøver fra 165 grupper ble innsamlet over en 2-måneders periode juni – august 2012. FGM ble målt ved hjelp av et "enzyme immunoassay" (EIA) som på forhånd var validert for drøvtyggere. Jeg fant ut at FGM var signifikant høyere nær hovedveier (med mer trafikk) sammenliknet med mindre veier og side-veier (med mindre trafikk) i forskjellige deler av Serengeti økosystemet. I tillegg til veier kan også predatorer, ulovlig jakt og sosialt stress forårsake stress hos impala, men ingen av disse variablene kunne forklare variasjonen i de målte FGM nivåene. For første gang viser vi nå at et stort afrikansk pattedyr viser signfikant økte stressnivåer i forhold til veier med økt trafikk, i forhold til mindre veier med mindre trafikk. Veier og trafikk har potensiale til å forårsake økning i fysiologisk stress hos pattedyr, som videre kan påvirke reproduksjon og overlevelse hos arten. Det manes derfor til forsiktighet og at føre-var-prinsippet benyttes før nye veier bygges i naturlige habitater fordi økt forstyrrelse kan ha alvorlige konsekvenser for økosystemet, spesielt dersom nøkkel-arter påvirkes. Fremtidige studier anbefales å fokusere på hvor høyt stressnivå dyreliv kan tåle uten at det går ut over overlevelse og reproduksjon. Forvaltningsplaner for naturvernområder bør inkludere muligheter for økt fysiologisk stress hos arter som lever nær stor infrastruktur og være proaktive for å forsikre livskraftige populasjoner og økosystemer.*

Table of contents

Introduction

Anthropogenic extinctions and conservation biology

It is estimated that 99% of current extinctions are linked to human activities (Primack 2010), and human population growth is increasing exponentially along with species decline. This is due to encroachment into natural areas, overharvesting and pollution; the main driving force behind extinctions being habitat destruction (Primack 2010). The current extinction rate is estimated to be between 100 and 1000 times greater than the background extinction rate, and the cause is human activities (Rockstrom et al. 2009). With our continued increase in the human population, the importance of combating anthropogenic extinctions is greater than ever. Conservation biology is a multi-field crisis discipline that aims to preserve species and prevent further extinctions (Primack 2010). Coined in the 1980's, it is an important discipline in combating the current species decline. Conservation biology aims to document the global biodiversity, investigate the human impact on species, genetic variation and ecosystems, and develop approaches to prevent species extinctions and restore biological communities (Primack 2010). It represents several sciences, including anthropology, ecology, physiology, genetics and more (Primack 2010).

Conservation physiology

Conservation physiology lies under the umbrella discipline of conservation biology and is an emerging field which aims to use physiological tools and knowledge to obtain information on how anthropogenic changes affect organisms. It has been defined as “the study of physiological responses of organisms to human alteration of the environment that might cause or contribute to population declines” (Wikelski & Cooke 2006). Conservation physiology goes beyond pattern descriptions and provides a mechanistic understanding of what causes conservation problems, and is therefore an important tool in conservation biology (Wikelski & Cooke 2006). The physiological mechanisms can be metabolic, hormonal, nutritional, thermal and immune relationships (Wikelski & Cooke 2006). Physiological tools have previously proven successful in addressing conservation issues related to both plants and animals. Pywell et al. (2003) used physiological traits to assess the performance of plants in ecological restoration, Ruxton and Schaefer (2012) described how plant and animal physiology affects seed dispersal interactions and Tingvold et al. (2013) showed how African elephants (*Loxodonta africana*) have lower stress hormone levels inside protected parks compared to individuals outside. Conservation physiology can thus be used to understand and potentially solve a wide variety of conservation related problems.

Stress

Regarding animal welfare and conservation, the need for measuring stress in animals is growing as human-animal interactions are increasing. Anthropogenic stressors can take the form of climate change, hunting, tourism, traffic and more. Species vary greatly in how they cope with anthropogenic environmental change, and population decline can culminate in the extinction of unaccustomed species. In areas designated to protect animals, it is important to assess the impact of human activities on the animals, thus identifying stressors can be of major importance for preserving biodiversity. The management plan should include these results to ensure the protection of the species. Methods for measuring stress include behavioural observation, bio-telemetric methods and measuring faecal glucocorticoid metabolites (FGM). Measuring FGM is increasingly used by conservation physiologists and can provide accurate levels of glucocorticoids (GC) in a non-invasive manner (Palme et al. 2005; Sheriff et al. 2011). Because vertebrates share a similar physiological pathway involved in the stress response, FGM can be used to assess stress levels in birds (Wasser et al. 1997), reptiles (Rittenhouse et al. 2005) and mammals (Creel et al. 2002; Thiel et al. 2011; Tingvold et al. 2013).

The stress response

In an animal's stress response, the steroid hormones cortisone, cortisol or corticosterone are secreted from the adrenal gland (Hill et al. 2008). These hormones are collectively called glucocorticoids, due to their ability to raise blood glucose and because they are excreted from the adrenal cortex. The adrenal gland is part of the hypothalamus-pituitary-adrenal (HPA) axis, in which the secretions from one endocrine gland triggers the release of hormones from subsequent glands (Hill et al.

2008). The HPA axis works together with the autonomic nervous system in order to elicit a stress response to a threat. The mammalian stress response pathway starts with the central and autonomic nervous system sending impulses that causes the hypothalamus to release corticotrophin-releasing hormones (CRH) into the hypothalamo-hypophysial portal system. This reaches the anterior pituitary, which then secretes adrenocorticotropic hormone (ACTH). ACTH is in turn carried to the adrenal cortex, where it triggers the release of GC (Hill et al. 2008). GC stimulate the release of glucose into the blood, and also the catabolism of fats and proteins. At the same time, feeding, digestion and reproduction are inhibited.

Stressors

In animals, stress is defined as an environmental stimulus that causes an imbalance in the homeostasis of the organism. The resultant change in the behaviour or physiology of the animal is known as the stress response (Möstl & Palme 2002). The duration of a stressor can vary greatly, from short term, acute stressors like predator attacks to long term, chronic stressors like drought or possibly road traffic. An acute stressor causes an immediate physiological stress response in the animal, like the attack of a predator triggering the fight or flight behavioural response and subsequent rise in GC levels (Sapolsky et al. 2000). Acute stress responses are important and normal in healthy animals, where the animals' defence systems are primed and the body is prepared for the change of situation. A chronic stressor however, causes an elevated stress response over time. This stress results in sustained increases in GC levels, and may reduce fitness and increase pathology of the animal (Sapolsky et al. 2000; Sheriff et al. 2011). GC levels can be measured through blood, saliva, urine and faecal samples (Palme et al. 1999). Blood and saliva samples require handling of the animal, which can be stressful for the animal and dangerous to perform, and urine samples can be difficult to obtain. The use of FGM to measure GC levels eliminates the need to restrain the animal, and circumvents the risk of handler induced stress. In addition, due to the delay between the stress response and the measurement, FGM are not as affected by the pulsatile increases in GC seen in blood samples, which can result in high point readings (Palme et al. 1999). By accounting for the lag time of metabolism, measuring FGM can therefore be an important tool for assessing stress in animals. The lag time for measuring FGM in faeces is dependent on the intestinal passage time of the animal, and thus highly dependent on species. Faecal steroid hormones of ruminants (including cows and sheep) have been reported to peak 12h after appearing in the plasma (Möstl et al. 2002; Möstl & Palme 2002; Palme et al. 1996). It can therefore be expected that other ruminants exhibit a lag in peak FGM levels after being exposed to physiological stress, and thus be an accurate predictor of stress. It has previously been shown that FGM levels can predict mortality in ring-tailed lemurs (*Lemus catta*) (Pride 2005). This can be attributed to the fact that the increased immune functioning observed after exposure to an acute stressor is actually depressed after subsequent intermittent exposures to a stressful stimulus (Bartolomucci et al. 2005). When exposed to a stressor that becomes chronic, the negative long-term effect of elevated GC levels can result in fitness decrease or pathology of the animal (Busch & Hayward 2009; Sapolsky et al. 2000). Using FGM as a measure of fitness can therefore be useful in assessing the impact of anthropogenic stressors of wildlife.

Roads

Natural areas are being increasingly perturbed by roads. In the U.S.A, more than 6 million kilometres (km) of public road has emerged the last 400 years, carrying an estimated 200 million cars (Forman & Deblinger 2000). It has been estimated that one-fifth of the entire U.S.A. land area may be directly affected by the road system (Forman & Deblinger 2000). The impacts of roads on wildlife have been studied since the 1970s, and the effects on movement, mortalities and behaviour have been assessed in a comprehensive review by Coffin (2007). Behavioural effects of roads and traffic on animals include 1) the avoidance of noise (sounds, lights, and chemical emissions), 2) the avoidance of the road surface, and 3) the avoidance of vehicles on the road, e.g. "moving out of the way" (Jaeger et al. 2005). Previous studies have shown that the resilience to roads varies between species and animal groups. A review by Kaseloo (2004) showed that several bird species across the United States and the Netherlands experienced negative effects proportional to the amount of traffic up to three km from the road. Lapwing (*Vanellus vanellus*) and black-tailed godwit (*Limosa limosa*) showed avoidance of roads, while seven other grassland species showed reduced breeding densities correlated with traffic increase. Woodland species showed similar results, with 26 out of 43 species showed population declines in effect distances that increased with the amount of traffic. Kaseloo (2004) proposed that the deleterious sound level threshold may be as low as 50 dBa (A-weighted decibels). However, some species take advantage of the new habitat provided near roads. For species not present in forest habitat and not affected by noise, the change in vegetation near roads can provide new habitat to these species (Ferris 1979). Kaseloo (2004) lists several examples of how large mammals can avoid roads, and possible reasons include noise and hunting avoidance. However, few studies have investigated the direct effect of roads on FGM levels of animals, although a few exist. Wasser et al. (1997) showed that male northern spotted owl (*Strix occidentalis caurina*) showed elevated FGM levels close to logging roads and forest harvest areas. Creel et al. (2002) demonstrated how elk (*Cervus elaphus*) and wolves (*Canis lupus*) showed increased FGM levels in relation to snow mobile activity. A study on elk by Millspaugh et al. (2001) showed a positive correlation between FGM levels and road use during summer. FGM levels have been linked to mortality (Pride 2005), and elevated stress caused by roads can therefore possibly lead to population decline. Jaeger et al. (2005) proposed that road and traffic can affect animal populations detrimentally in four ways: 1) By decreasing habitat size and quality, 2) by increasing mortality due to road kills, 3) by preventing access to resources on the other side and 4) splitting animal populations into smaller, more vulnerable fractions. Habitat quality can be difficult to assess, because animals may still be present in a habitat although its quality is decreased. A way of assessing whether habitat quality is decreased in relation to roads is to measure the FGM levels of the animals near roads. This can give us an important clue to how populations that appear unaffected by roads are responding to this infringement in their habitat.

The Serengeti road

A road in a protected area of recent controversy is the planning of a two-lane stretch through the northern part of Serengeti National Park (SNP), Tanzania. Apart from being of inherently intrinsic value, containing important endangered species and acting as a carbon sink (Dobson et al. 2010), the SNP contributes significantly to Tanzania’s gross national product with more than 6 million U.S. dollars annually in entrance fees alone (Kaltenborn et al. 2008). It is therefore drawing massive attention whether the new road going 50 km through the northern part of the park will cause irreversible damage to the ecosystem, by increasing road kills and blockage of migration movements (Dobson et al. 2010). Dobson et al. (2010) argue that the road will cause commercial traffic to run night and day, and that the subsequent need for fencing following road kills will halt the wildebeest (*Connochaetes taurinus*) and zebra (*Equus quagga*) migration. In addition to this, the authors argue that the road will split newly re-established populations of African wild dogs (*Lycaon pictus*) and black rhinoceros (*Diceros bicornis*), preventing gene flow between populations. However, there are also arguments to why these claims may be over exaggerated, and that the road may divert some of the increasing traffic from existing roads which experience annual increases in traffic (Fyumagwa et al. 2013; Homewood et al. 2010). It is therefore an important issue to assess the effects of roads and traffic on wildlife in the Serengeti, to ensure that the animals will not suffer from the proposed road project. Whereas the effects of the proposed road can only be estimated, the effects of the current roads in the Serengeti can be quantified. On simple and useful method is to quantify the road categories and levels of traffic and correlate it with stress hormones exhibited by the animals near the park roads, by measuring FGM levels. If stress levels are high, it can be a sign that roads are already making an impact on wildlife in the Serengeti. Relating this information to the knowledge of how chronic stress can decrease survival and reproduction in animals, this can have a negative impact on the protection of species in the SNP. If animals in the park are predicted to suffer from the new road or traffic increases on current roads, this should be included in the management plan for the area. To assess potential negative effects from the proposed road on large mammals, I used the impala (*Aepyceros melampus*) as a model species to assess its FGM levels in relation to roads and traffic. The impala is distributed in local populations over large areas of the park, is relatively place bound and faecal samples can be easily obtained from the animals and analyzed by existing methods validated for ruminants. I hypothesized that the impala would show elevated FGM levels near main roads that enabled high amounts of traffic, and lower FGM levels on smaller, less used roads.

Methods

The study was conducted between 11.06.2012 and 07.08.2012 in the Serengeti ecosystem, Tanzania.

Study species

The impala is a semi-large antelope resident in savannah woodland in the sub-Saharan region (Averbeck 2002). It is sexually dimorphic, with full-grown males reaching a weight of 75 kg and females a weight of 50 kg. It is an edge species, and prefers light woodland with little undergrowth and medium to low grass length. The diet consists of fresh grasses and leaves, and to some extent feeding on dry leaves. Being a grazer and browser, the impala can adapt to various environments while maintaining its food supply (Averbeck 2002). During the rut (May – July) it resides in herds of one male with a harem of females, or bachelor groups consisting of sub-dominant males that eventually will break out and try to establish their own territories (Murray 1981). Females are sexually mature at 1 year old and normally give birth to a single offspring 4 – 6 months after the rutting period, while males are sexually mature at 4 years of age (Averbeck 2002). Only males have horns, which develop at the age of three months. These males are chased out of the herd by the territorial male as they grow older, and form bachelor groups (Averbeck 2002). The age of the animals can be categorized as calf (< 3 months with no horns), sub-adult (males have horns and both sexes have not reached full size) and adult (males have horns and both sexes have reached full size). The group size can range between one single male and 200 individuals, and females can have home ranges with a radius of only a few hundred meters (Murray 1981). Although home range size can vary with season, geographical location and extend several km, the centre of activity is often confined to a small area (Averbeck 2002). The residential nature of the impala makes it a good model species to quantify the effect of roads and traffic, where it can be used as an indicator of how animals in protected areas respond to roads. Using FGM to quantify their levels of stress, this can be used to predict the effects of increased disturbance from the proposed road or existing roads.

Study area

The location of the SNP is east of Lake Victoria and south of the Masai Mara National Reserve in Kenya. The study was done inside the SNP (14 763 km²) and the adjoining areas of Grumeti (Game Reserve, ca. 400 km²), Ikorongo (Game Reserve, ca. 600 km²), and Loliondo (Controlled Area, ca. 4000 km²) (Setsaas et al. 2007). The areas within SNP were divided into Central, North and West (fig. 1).

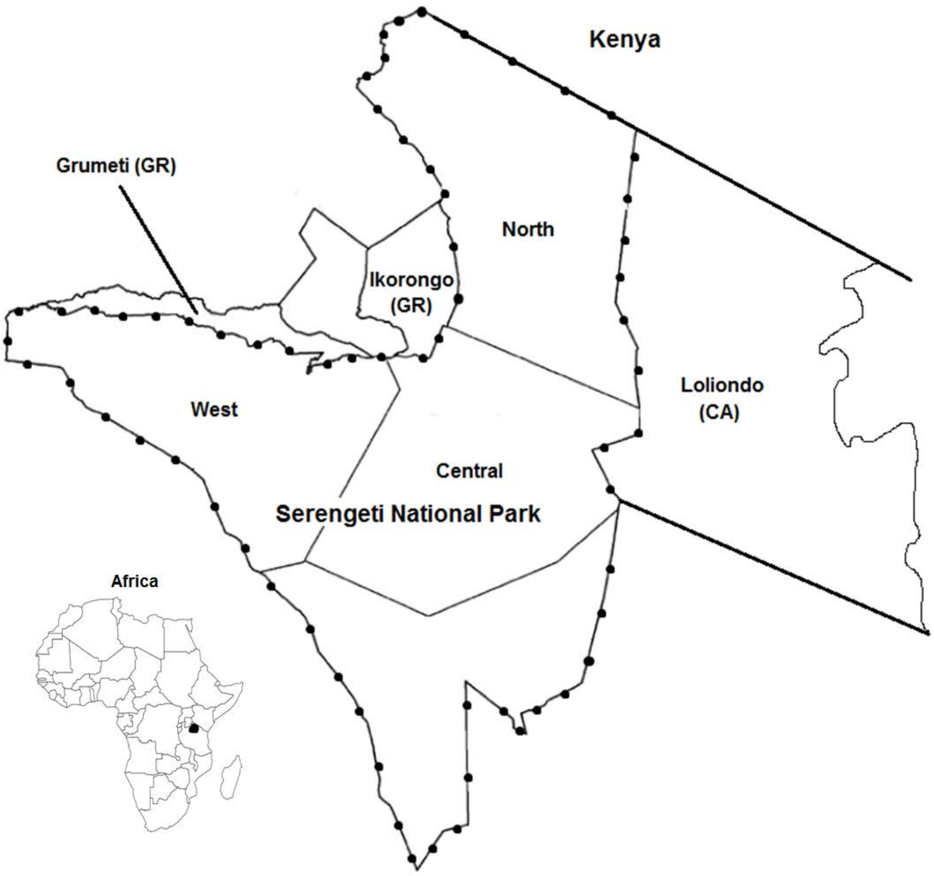

Figure 1 *Map of SNP with the adjoining areas. The SNP is a protected area and is divided into parts on the map. The study was done in the North, Centre and West area inside the park, and in Grumeti, Ikorongo and Loliondo outside the park. Dots indicate park borders. GR = Game Reserve, CA = Controlled Area. Map modified from Setsaas et al. (2007).*

Road categories

I divided the different roads into three main categories. Category 1 (main road) was defined as a 2-lane road that is listed as a major road on the map, is made of gravel and also used frequently by all types of vehicles (trucks, safari jeeps and privately owned). These roads were found in Central and West. Vehicles travelling at speed (60-100 km/ h) are frequently observed on category 1 roads, increasing road kills as these are correlated with traffic speed (Case 1978, fig. 2).

Figure 2 *The Ikoma road in the Central area. A category 1 road has two lanes of compacted gravel enabling vehicles to travel at speed. The killed worth hog (*Phacochoerus africanus*) is attributed to a road kill, given the structure of damage observed and the absence of predators. (Photo: E. T. Lunde)*

Category 2 (minor road) was defined as a gravel-made road that can sometimes be listed as a main road, but carries less commercial traffic and vehicles at high speed. The only main road on the map that was classified as a category 2 is the B144 road going from Central to North (fig. 2). A typical category 2 road is pictured below (fig. 3).

Figure 3 *The Sopa lodge road in central is a category 2 road. A road of this category is narrower than a category 1 road, and is less frequented by vehicles. The size is limiting for traffic speed and therefore road kills. (Photo: E. T. Lunde)*

Category 3 (side road) is a road less frequently used by traffic than main and minor roads, is rarely used by trucks and other non-tourist cars and grass is often prevalent in the middle of the road. These roads were normally scenic routes for safari vehicles or led to campsites. Travelling at speed on side roads is difficult due to the size and quality of the road (fig. 4).

Figure 4 *A road of category 3 is less used than category 1 and 2, often enabling grass to settle. Safari vehicles were normally the only vehicles observed here (if any vehicles at all were observed). It is also comparatively smaller than category 1 and 2. The picture is taken in the North area where the proposed road is planned. (Photo: E. T. Lunde).*

Group identification

I travelled by car following fixed transect routes, which included the road categories described above. When an impala group was observed, the vehicle was stopped. Groups were located and studied from the road, at a distance where the animals did not react to the vehicle I observed from. This distance to the road could vary from 0 – 300 meters, depending on the area sampled. If the animals were close to the road, I stopped the vehicle at a distance of >75 m from the group, to avoid it changing behaviour. If the visibility did not allow observation at from this point, the distance was decreased, but not so much that it initiated a flight response. The flight response and flight distance of impala can be attributed to predator avoidance and is described in Setsaas et al. (2007). I recorded data on the distance between the road and the nearest animal by a rangefinder (Leica Rangemaster 900), road category, the number of cars, grass quality and group size. Grass quality was estimated as green (fresh), green-brown (mix of fresh and dried grass) and brown (dried grass). Foliage quality of trees was not recorded due to difficulties with estimating foliage quality. I recorded the data in ten-minute time slots for up a total of one hour, depending on time of defecation and if the group moved away. This enabled me to record parameters that I could later quantify without compromising the quality of the faeces, which was my main objective.

Faecal sample collection

A study on the use of FGM in conservation biology (Millspaugh & Washburn 2004) advised that faecal samples should be obtained within a few hours to preserve true FGM values, but to avoid degradation of faeces by insects I collected the sample within 25 minutes from defecation. In order to do the collection quickly, I developed a simple yet effective method to reliably link the sample to the animal, which can be of interest to other scientists collecting faecal samples. When an animal defecated, I identified the age and sex of the animal, measured the distance to the animal and took a photo. A person then walked to the place of defecation, and this distance was measured to make sure it was the exact same location as the studied animal had occupied (fig. 5).

Figure 5 *A method developed for obtaining the correct faeces within the subscribed time period. Distance to the animal was recorded with a range finder along with a photo, and matched with the distance to the person locating the faeces. This enables the correct faeces to be collected within a short time period. (Photo: E. T. Lunde)*

This made it possible to accurately obtain the sex and age for each animal from which a faecal sample was obtained and importantly, ensure that the sample was not older than the prescribed time limit. After collection I placed the sample(s) on ice in a cooler box. Pellets from the whole defecation were collected to minimize individual variation, and 1 – 3 animals were sampled from each group. Only adult males and females were sampled, and samples contaminated by urine were not collected as the hormone metabolites in urine can bias FGM interpretation.

Lab processing

The samples were transported back to the research station and placed in a freezer. All samples were processed within two months after collection. For lab processing, I thawed the samples, and then mixed pellets from each sample by hand to account for within-sample variation that can bias assay interpretation (Millspaugh & Washburn 2003). I then transferred 0.5 grams of mixed feces and 5 ml 80% ethanol to a centrifuge tube (Nunc ®, 10 ml). I homogenized the mix (Omni µH) and centrifuged the samples for 20 minutes at 4000 rpm (Unico Powerspin ™ LX). I transferred 1 ml supernatant from each sample to a 2 ml microtube, and let the samples dry at room temperature. This method was used with the available equipment after conferring with Dr Rupert Palme (personal communication). In total, 196 faecal samples from 166 groups were processed. The dried samples were transported back to the Norwegian University of Science and Technology (NTNU), Trondheim, Norway, and placed in a freezer at -20˚C.

Detection of FGM

The hormone analysis of the samples was done eight months after collection. The analysis of samples was conducted in the lab of Dr Rupert Palme, Department of Biomedical Sciences/ Biochemistry, University of Veterinary Medicine, Vienna, Austria. An 11-oxoaetiocholanolone enzyme immunoassay (EIA) described by Möstl et al. (2002) that was found to be suitable to measure FGM in several ruminants (Corlatti et al. 2012; Huber et al. 2003; Kleinsasser et al. 2010) was used. Details on the EIA including cross-reactions can be found in Möstl et al. (2002).

Statistical analysis

The statistical analysis was done using *R* (R Development Core Team 2011). FGM was log transformed to comply with the normality assumption of a linear model. I estimated the average number of cars for each road category by dividing the number of cars counted during the observation period for a group with the number of minutes observed, to obtain cars per minute. This gave a rough estimate of traffic for each group to give an indication of traffic levels within each road category. Since trucks produce at least 2 times more noise than smaller cars, I did a pre-analysis where each truck counted as 2 cars, to get an estimate of the effect. It did have a weak effect, so I included it in my analyses. Within some groups, I had obtained a faecal sample from more than one animal. To avoid pseudoreplication, I used the average FGM level from these groups in my analysis, after first checking that FGM did not differ significantly between sexes. To test the interaction effects of road category with the other explanatory variables, I created a linear model with FGM as the dependent variable and the interaction between the independent variables road category, cars per minute, park area, grass quality, distance to the road and group size. I looked at biologically relevant interaction effects between all of these, and removed non significant interactions and variables to obtain the most parsimonious model.

Results

Distribution of data

FGM levels were recorded from a total of 165 groups, and ranged from 35 to 3520 ng FGM/ g faeces (ng g^{-1}) with a mean of 383 and a median of 210 ng g^{-1}. The groups sampled in each area were observed to be separate from other groups, with no particular area or road category showing signs of animals competing for space. In some cases, the male in a harem group was observed to confront bachelor groups and chase them away from his immediate territory. The majority of groups in my two-month study period stayed in the area they were observed for the entire study period, indicating a limited centre of activity within their home range and subsequent proximity to the road. The pre-analysis on sex showed that the high and low FGM values were equally spread among sex, and FGM values were not significantly affected by this (ANOVA, $p = 0.75$). The mean number of cars per minute for each of the three different road categories was calculated to be 0.28, 0.12 and 0.04 cars for road category 1, 2 and 3, respectively. This confirmed that road category 1 appeared to carry more traffic than categories 2 and 3. The average number of cars per minute varied significantly between the three road categories (Kruskal-Wallis one-way ANOVA, chi-squared = 55.8, df = 2, $p < 0.0001$). A Tukey HSD test was applied to test which groups were significantly different from the others. The difference was significant between road category 1 and 2 ($p < 0.0001$) and 1 and 3 ($p < 0.0001$), but not between 2 and 3 ($p = 0.09$, fig. 6).

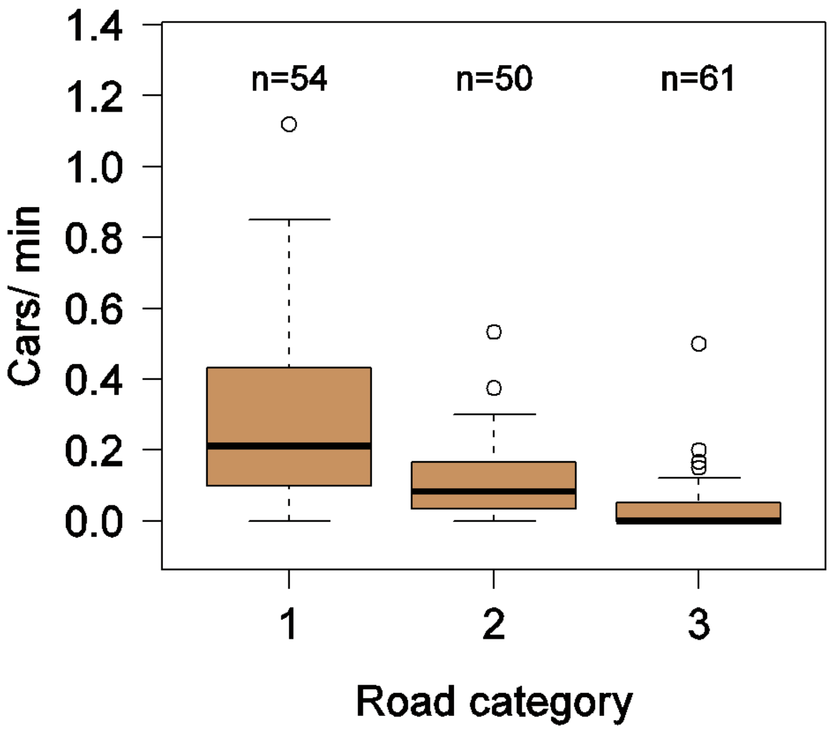

Figure 6 *Number of cars per minute in relation to road category. (1=Main road, 2=Semi-main road, 3=Side road.) Bold line: Median. Box: 50% of the data. Whiskers: 25% of upper and lower values. Circles represent outlier(s), "n" represents number of samples.*

In addition to the difference in traffic levels, cars were observed to drive at higher speeds on road category 1, and a conservative estimate placed several vehicles at >80 km/ h. This was attributed to the size and quality of the road which enabled the vehicles to travel faster.

Interaction effects

In the full model distance to the road, cars per minute and group size had an insignificant effect on FGM levels ($p = 0.14$, $p = 0.22$ and $p = 0.26$, respectively) with no biologically relevant interaction effects, and were therefore removed. I obtained the most parsimonious model with FGM as the response variable and road category, park area and grass quality as predictor variables. This model showed that road category, park area and grass quality significantly affected FGM, without any significant interaction effects (Table 1). The model explained 25% of the variation in FGM levels (Regression, df = 155, adjusted $r^2 = 0.25$).

Table 1. *The most parsimonious model showing the effects of the different variables on FGM levels.*

Analysis of variance			
Response: FGM	df	F-value	p-value
Road category	2	18.6	<0.0001
Park area	5	3.4	0.006
Grass quality	2	4.2	0.017

Effect of road categories

As seen in table 1, road category had a significant effect on FGM levels. The FGM levels were the highest in road category 1 and the lowest in category 3, with mean FGM levels of 653, 266 and 233 ng g^{-1} for road category 1, 2 and 3, respectively. A post hoc analysis using a Tukey HSD test showed that FGM differed significantly between road category 1 and 2, road category 1 and 3, but not between road category 2 and 3 ($p < 0.0001$, $p < 0.0001$ and $p = 0.85$, respectively, fig. 7).

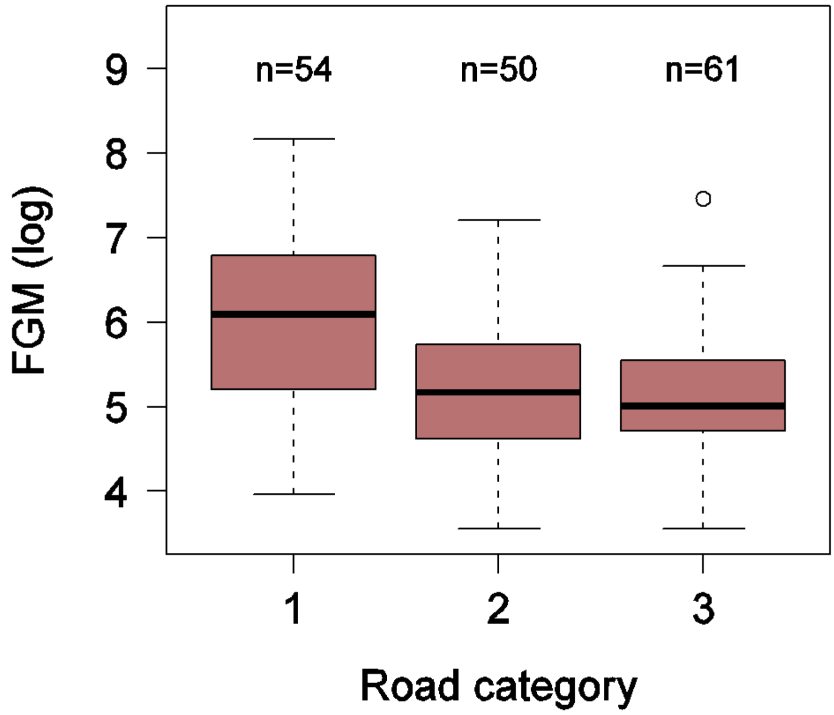

***Figure 7** FGM levels in relation to different road categories. (Bold line: Median. Box: 50% of the data. Whiskers: 25% of upper and lower values. Circles represent outlier(s). "n" represents sample size. See fig. 6 for road categories).*

Effect of park area

FGM levels were also significantly different in the various areas of the park (Table 1, fig 8a). This was expected because all park areas did not contain all road categories, and had different levels of traffic. The mean FGM levels for the different areas were: Central: 463 ng g^{-1}, Grumeti: 361 ng g^{-1}, Ikorongo: 241 ng g^{-1}, Loliondo: 86 ng $g^{-}1$, North: 171 ng g^{-1}, West: 512 ng g^{-1}. A Tukey HSD test was performed to see which areas differed significantly in their FGM levels. Of a total of 15 comparisons, four were significant. The significant difference was found between the following four areas: Central – Loliondo ($p = 0.002$), Central – North ($p = 0.0001$), West – Loliondo ($p = 0.003$), West – North ($p = 0.0004$, fig. 8a).

Cars per minute differed significantly between different areas of the park (Kruskal-Wallis chi-squared = 33.4, df = 6, p = <0.0001). The average number of cars per minute in each area was: Central: 0.23, Grumeti: 0.11, Ikorongo: 0.00, Loliondo: 0.05, North: 0.10, West: 0.09 (fig. 8b). A Tukey HSD test was performed to see which areas differed significantly in their mean amount of traffic. Of a total of 15 comparisons, three were significant. The significant difference was found between the following three areas: Central – Ikorongo (p = 0.03), Central – West (p = 0.0006), 13), central – north (p = 0.004, fig. 8b).

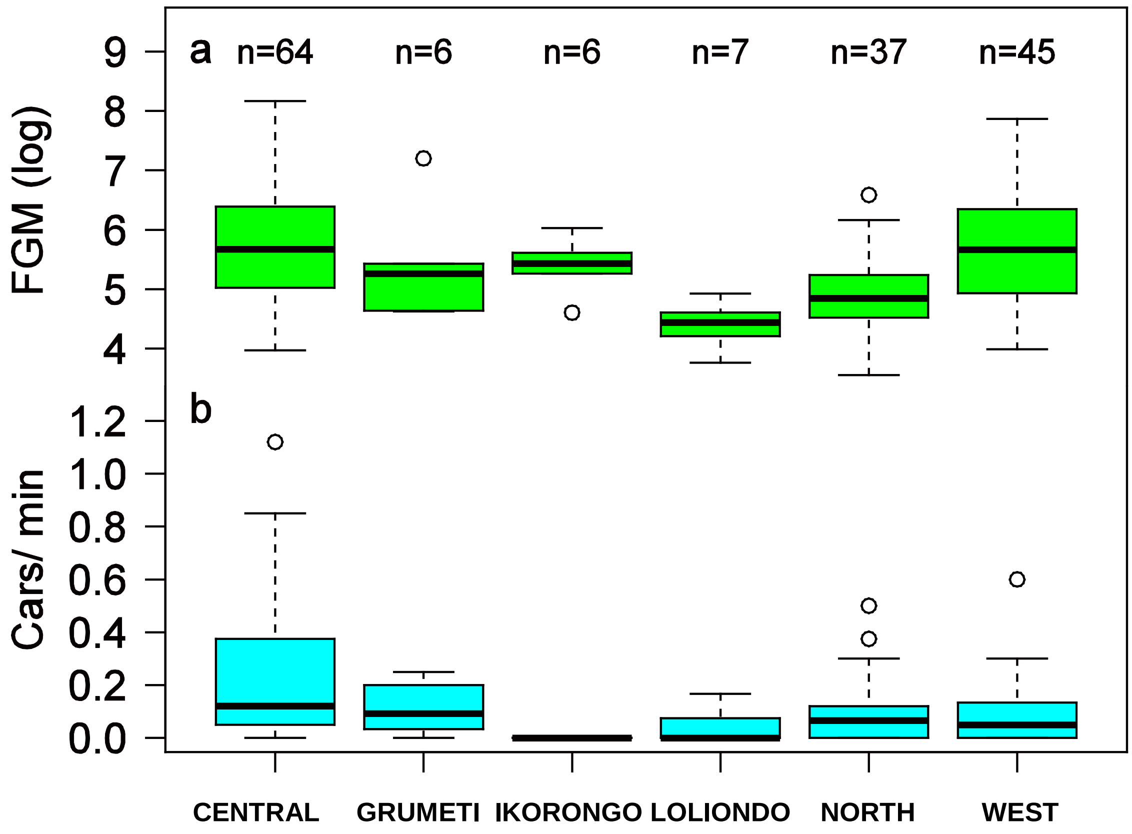

Figure 8 a) *FGM levels varied significantly between areas.* ***b)*** *Cars per minute varied significantly between park areas. Bold line: Median. Box: 50% of the data. Whiskers: 25% of upper and lower values. Circles represent outlier(s), "n" represents number of samples.*

Effect of grass quality

There was a difference in FGM levels as the grass quality changed from fresh, green grass to dry, brown grass. FGM levels differed significantly between grass qualities (p = 0.017). A post-hoc test (Tukey HSD) showed that FGM levels were significantly lower in areas with grass quality 1 than grass quality 3 (p = 0.018), but not between qualities 1 and 2 (p = 0.074) or between 2 and 3 (p = 0.253, fig. 9).

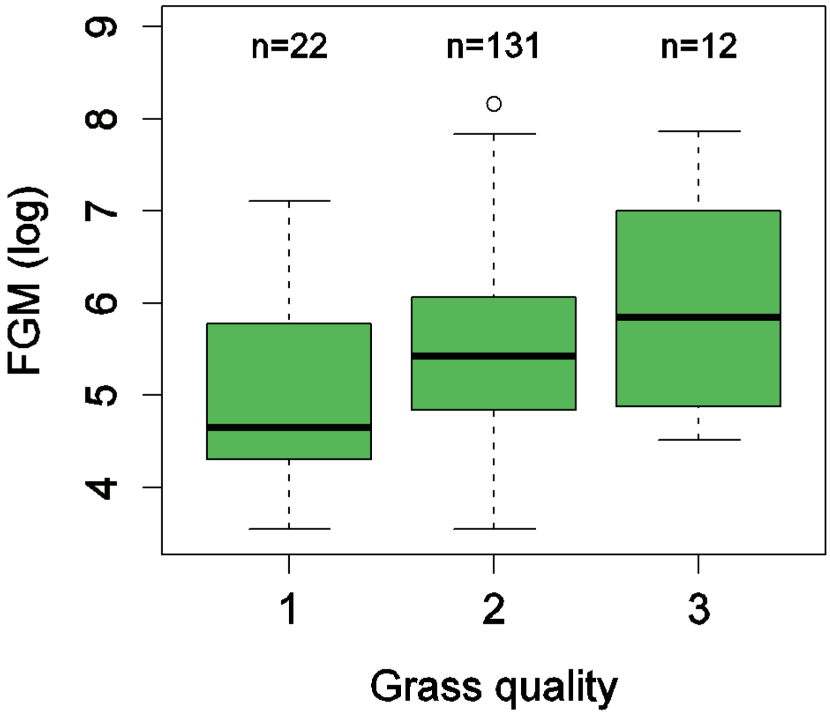

Figure 9 *The grass quality was dominated by green brown grass and was evenly distributed among the different road categories. 1 = Green grass, 2 = Green-brown grass, 3 = Brown grass. Bold line: Median. Box: 50% of the data. Whiskers: 25% of upper and lower values. Circles represent outlier(s), "n" represents number of samples.*

Because of the significant effect of grass quality on FGM levels, I did an analysis to assess the distribution of grass qualities between road categories. Green, green-brown and brown grass qualities was found in all road categories, with no statistically significant distribution between these (Fisher's Exact Test, p = 0.26, Table 2).

Table 2 *The grass qualities were found near all road categories. Bold numbers represent road categories, italicized numbers represent sample size of the different* *grass qualities within each road category.*

	Road category		
Grass quality	**1**	**2**	**3**
1 (green)	*n = 8*	*n = 6*	*n = 8*
2 (green-brown)	*n = 43*	*n = 43*	*n = 45*
3 (brown)	*n = 3*	*n = 1*	*n = 8*

Effect of distance to the road

Although distance to the road did not significantly affect FGM levels, there was a tendency for a negative relationship between the two variables and it is included due to its biological relevance. Most of the groups sampled occurred near the road, and only some (n = 7) occurred > 200 m from the road (fig. 10).

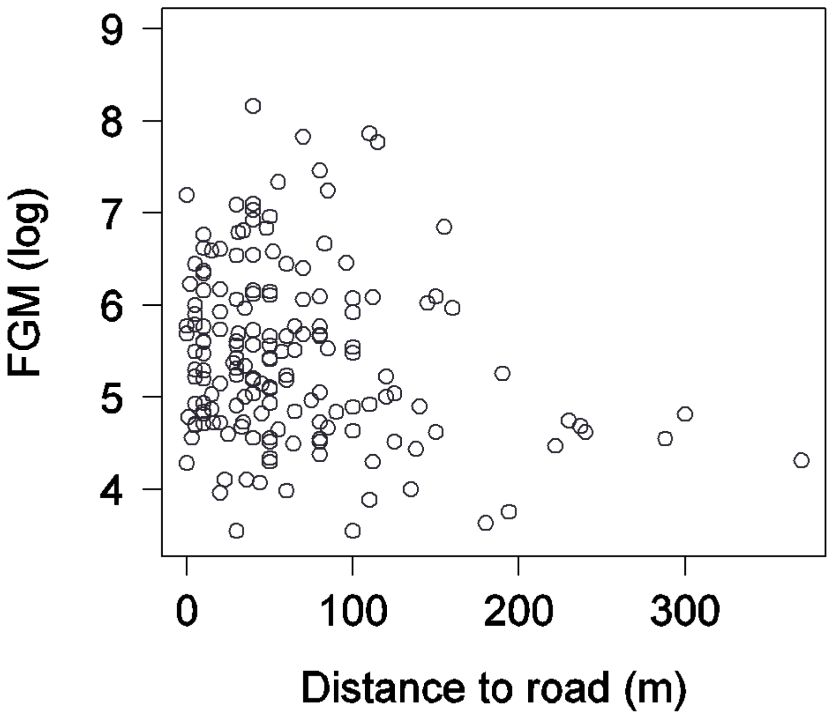

Figure 10 *FGM levels showed a tendency to decrease as the animals' distance to the road increased, although no statistical significance was detected.*

Discussion

Road effect

This study examined the effect of roads on faecal cortisol metabolite levels of the impala. Main roads were found to both have more vehicles and higher speed due to the properties of the road. My hypothesis was that the impala would show elevated stress levels near main roads with increased levels of traffic, and this was confirmed with the impala near road category 1 exhibiting significantly higher FGM levels than impala near the less trafficked, smaller roads of categories 2 and 3. This indicates that in the SNP, animals are affected by the current levels of human disturbance. Findings that stress resulting from motorized vehicles chronically elevates FGM levels in mammals have previously been shown in a study by Creel et al. (2002). The authors compared FGM levels of elk and wolves in relation to snowmobile activity, and found that both species showed significant increases in FGM levels when snowmobile activity was high. This supports the findings of Millspaugh et al. (2001) correlating FGM of elk with levels of vehicle activity. The fact that impala show elevated levels of stress near main roads can be attributed to the level of vehicle activity, but also the speed and subsequent noise which was found to be higher on road category 1. In addition to the effect of motorized vehicles, a number of studies have looked at the effect of tourism on FGM levels in animals. Zwijacz-Kozica et al. (2013) showed that FGM levels of the chamois (*Rupicapra rupicapra tatrica*) increased significantly with the number of tourists in Tatra National Park, Poland. Thiel et al. (2008) showed how wood grouse (*Tetrao urogallus*) altered their habitat preference to undisturbed areas when ski tourism was high, and that FGM levels were significantly higher in areas with high or medium tourism intensity. Arlettaz et al. (2007) and Thiel et al. (2011) demonstrated that the elevation in FGM levels found in relation to human disturbance is not limited to a particular area. The authors of the first study assessed the FGM levels of both experimentally stressed black grouse (*Tetrao tetrix*) and populations of black grouse near different levels of winter sport intensity. In both cases the FGM levels of black grouse was significantly elevated. The authors in the second study examined FGM levels of populations of wood grouse in Switzerland and Germany in relation to areas with recreational activity during winter, and found that in homogenous forests, populations displayed increased FGM levels in areas near recreational activity. This further supports that the results of this study are indeed a result of the effects of anthropogenic activity. This is of concern because roads are increasing worldwide and in particular for the SNP where the proposed road is a main road enabling more vehicles and higher speed. Although the speed limit for the park is 50km/ h, the results of this study indicate that vehicles on the main roads in the park travel faster. The effect of main roads appears to be negative on the animals stress levels. The effects of chronic stress have been attributed to increased mortality by influencing reproduction, growth and survival (Pride 2005; Sapolsky et al. 2000), and the results here can therefore give valuable information on how animals may respond to increased anthropogenic activity.

In addition to the observed effect of road category on FGM levels, there was also a significant difference in FGM levels in the different areas of the park. Although cars per minute followed the same pattern as FGM levels for several areas, cars per minute is a rough estimate and should be considered with caution, although supported by Creel et al. (2002) and Millspaugh et al. (2001). However, it is interesting to note the significant differences in FGM levels between the different areas. The highest FGM levels are found in the areas that contain road category 1, Central and West.

Poaching

It is possible that other factors than the road influence FGM levels in these areas. One factor that can potentially induce stress in animals is poaching. Poaching has previously been known to occur in the Grumeti, Ikorongo and Western part of SNP, where the majority of poachers are located (Holmern et al. 2004; Kidegesho 2010; Metzger et al. 2007; Setsaas et al. 2007). Poaching in West could therefore possibly explain the increased FGM levels in these areas. Grumeti showed decreased FGM levels in this area compared to West and Central, although there was not a statistically significant difference. FGM levels in Ikorongo where higher that in Grumeti, but not significantly different from other areas. A larger sample size for Grumeti and Ikorongo could have shed more light on these relationships. Depending on how animals react to poachers, it is therefore a possibility that poaching influence FGM levels. African elephants have shown increased FGM levels in areas with higher poaching risk (Gobush et al. 2008; Tingvold et al. 2013). However, poaching is unlikely to be the only factor causing stress, because West and Central have the highest FGM levels, while poaching is linked to the areas on the western side of the park (Holmern et al. 2004; Metzger et al. 2007). Poaching is not believed to occur in Central due the greater distance from the park borders (Metzger et al. 2007), and the activity from tourism and park staff is also high in this area which can make it harder for poachers to operate unnoticed. The elevated FGM levels near roads in Central can therefore be explained by the roads and their level of disturbance in this area. Leblond et al. (2013) found that caribou (*Rangifer tarandus caribou*) were increasingly avoidant of roads as the road size, traffic intensity and active construction sites increased, highlighting the effect of roads on large herbivores. Furthermore, it is proposed that poachers enter the park areas through the entire western side of the park, including the North area (Metzger et al. 2007). North had low FGM levels compared to West, which does not indicate that the animals are stressed by poachers in this area. It is therefore possible that there is an interaction between vehicles and poaching in the West, further increasing FGM levels.

Predators

Predators in the SNP are not believed to cause stress in animals in these areas, because they are widely distributed over large areas of SNP and the effect would have increased FGM levels of impala in all park areas. In addition, a study on the effect of predators on FGM levels in elk (*Cervus elaphus*) showed no significant increase in FGM levels with predators (Creel et al. 2009). Animals can thus elicit a strong behavioural effect in relation to predators without showing elevated FGM levels, or show little behavioural response to motorized vehicles but increasing FGM levels significantly (Creel et al. 2002; Creel et al. 2009).

Social stress

Stress of social nature, can cause animals to show a stronger and longer lasting stress response equivalent to chronic stress (Bartolomucci et al. 2005). However, stress of social nature is unlikely to explain the variation in FGM because animals in the Northern part of the park exhibited low levels of FGM, while animals in the Ikoma, West and Central area showed comparatively higher levels of FGM.

Distance to the road

FGM levels showed a tendency to decrease as the animals distance to the road increased. Although this was statistically insignificant, it may further support our conclusions if the animals stayed within a narrow centre of activity. This may further indicate that roads do elevate FGM levels in the impala, and is supported by the results of Thiel et al. (2011) who found that FGM levels of wood grouse decreased as the distance to the recreation areas increased. However, due to the unknown movements of the group it is not possible to know whether this result is by chance or not. A separate study on impala assessing this in the SNP or other areas incorporating group movements (e.g. using Global Positioning System (GPS) tracking) with FGM levels in relation to roads is encouraged.

Grass quality

Apart from park area, the only variable consistent with FGM levels not attributed to roads was grass quality. Although the sample size for green grass and brown grass were somewhat small, there is a clear trend. Stress hormones of ungulates have previously been demonstrated to be higher during the drought than the wet season (Chinnadurai et al. 2009), but my results indicate that forage quality within the dry season impacts FGM levels as well. The grass quality was evenly distributed with no grass quality being statistically significantly more prominent in one road category, and is therefore not confounding the FCM levels observed in relation to road category. However, the significant effect of grass quality on FCM levels highlights the importance of recording feed quality as a possible confounding factor on stress levels.

Sex

There was no relationship between sex and the FGM levels of the animals. Since the sample sizes were relatively high for both sexes, this indicates that this represents a true estimate of the populations in general. There has been the concern that the 11-oxoaetiocholanolone EIA used for FGM detection in ruminants can detect androgens of placental origin in cows, and should thus be used with care for pregnant animals at the end of their term (Creel et al. 2009; Möstl et al. 2002). It is not known if this applies to the impala, but the sampling was done well outside of calving season. In addition, the high number of females sampled and subsequent absence of strong outliers should provide a representative mean.

Effects of increased stress levels

Based on the results of this study the variable which best explains the difference in FGM levels between groups is road category. Given the significant relationship of road category and cars per minute this is consistent with the findings of Creel et al. (2002) and Millspaugh et al. (2001), where vehicle activity increased FGM levels in elk and wolves. Because plasma concentrations of GC can vary over orders of magnitude (Pride 2005), it cannot be inferred that the observed results in this study equals decreased individual fitness or predicts population decline. However, the observed FGM levels showed substantial differences in relation to larger roads where current traffic levels were not very high, compared to traffic on highways which can amount to more than 50 000 vehicles per day (Kaseloo 2004). It is particularly interesting to note that there is very little difference in FGM levels between road category 2 and 3. In addition to being of smaller size and having less traffic, these roads were observed to have cars travelling at slower speed than road category 1, especially than the Ikoma road in Central. This can indicate that there is a threshold for when roads increase sustained GC levels in animals, and this threshold appears to be when road size and traffic intensity increase to what was observed in road category 1. The possible negative impacts of chronically elevated GC levels on immune functioning, feeding, reproduction and survival are well known (Sapolsky et al. 2000), and the results of this study indicate that animals are indeed affected by roads. A baseline threshold for detrimental GC levels in the impala is yet to be established, but this study gives very important information of how animals in the SNP can be expected to react to a larger road with increased traffic. However, because the road is not yet built, size and traffic is not yet known. In addition, traffic in the Serengeti is increasing with 10.5% per annum, increasing the disturbance from the current roads (Fyumagwa et al. 2013). It is therefore argued that these roads will increase disturbance on animal populations if not relieved by the proposed highway (Fyumagwa et al. 2013). It is not the aim of this study to argue for or against roads, but the results of this study provide information that can prove useful in the decision making. They indicate that the current main roads in the SNP are affecting the animals, but not affecting fitness to a degree which results in population decline. However, if disturbance is significantly increased, it may cause stress resulting in a change in the physiology or behaviour of the animals. This can result in decreased fitness near the road or the avoidance of the road up to a distance of 5 km, which has been shown to be the response of caribou to high disturbance roads (Leblond et al. 2013). Mountain goats (*Oreamos americanus*) have been shown to hesitate crossing roads when hearing a truck changing gears 1 km away (Singer 1978), while bird densities can decrease significantly within 3 km of a road (Kaseloo 2004). To avoid this in the SNP, the road disturbance should be maintained at current levels and this should also apply to the proposed road. Although no other study has assessed the specific effect of roads on FGM levels in mammals, studies previously discussed have shown that both snowmobile activity, vehicle intensity and tourism directly affect GC levels in animals (Creel et al. 2002; Millspaugh et al. 2001; Thiel et al. 2008; Thiel et al. 2011; Zwijacz-Kozica et al. 2013) This is consistent with the results of this study, and a road with high disturbance is likely to cause a substantial increase in GC levels of species inhabiting the area, resulting in the subsequent behavioural response to avoid the area. This has been demonstrated in a recent study by Strasser and Heath (2013), who showed that the reproductive output of the American kestrel (*Falco sparverius*) was significantly negatively affected by large, busy roads and developed areas. If animals are unable to move to a new habitat, chronically elevated GC levels from road disturbance can decrease the fitness of the populations near the road (Sapolsky et al. 2000). The SNP has already limited habitat for several species due to poaching along the park borders which is causing negative population increases in these areas (Holmern et al. 2004; Metzger et al. 2007; Metzger et al. 2010). Although the new road can possibly reduce poverty and therefore reduce poaching (Fyumagwa et al. 2013), subsequent road disturbance should be limited to avoid the possible negative effects described above. It is therefore advised to be conservative before introducing *any* new stressors within the SNP. In particular, when Metzger et al. (2007) proposed the suitability of habitat for translocation of the critically endangered black rhinoceros (*Diceros bicornis*), they described an area on both sides of the proposed highway. It would be proactive to maintain such areas stress free due to the importance of conserving a species risking extinction. Due to the observed physiological response of the impala, the results found in this study acts as an early warning system of how ungulates in the SNP may respond to a larger and busier road through the park, or increased disturbance from current roads. They indicate that roads through the SNP can, if their disturbance is significantly increased, be detrimental for resident populations of ungulates. Ungulates, including wildebeest, are keystone species in the Serengeti ecosystem, affecting predator populations and plant, insect and bird diversity (Sinclair et al. 2007). A decline or redistribution of keystone species following avoidance of roads can therefore result in severe ecological perturbations in the Serengeti ecosystem. It is therefore important that the proposed road, and roads in the SNP and wildlife areas in general, stays within limits that are conservative and have a maximum size, traffic intensity and speed limit. This may, if the area allows it, implicate that alternative roads circumventing the park can be a solution, as suggested by Dobson et al. (2010). However, the value of increased socioeconomic benefits (Fyumagwa et al. 2013) should be balanced with the value of the ecosystem, to promote a win-win situation for both the human community and the ecosystem. Future studies are advised to assess the levels of stress wildlife can tolerate without it affecting fitness. Until this has been established, it is advisable to follow the precautionary principle and make sure that new and existing roads in the SNP will not substantially exceed the disturbance levels of the current roads, to avoid damaging an ecosystem of which there is only one.

Conclusion

This study examined the effects of roads on FGM levels in the impala, and showed that animals near major roads have significantly higher FGM levels than animals in the less trafficked minor roads and side roads. The results are important for several reasons. 1) It demonstrates for the first time that a large African mammal shows increased FGM levels in relation to a road category which is larger and carries more traffic than other road categories in the same area 2) It provides advice needed, as a predictor to how animals may react to increased road disturbance in the SNP, and other areas in general, if traffic levels are not managed. 3) It highlights the importance of limiting human activity in areas that are too small for the animals to respond to changes by altering their behaviour. This is particularly important for conserving species like the black rhinoceros in the SNP, which has the most suitable habitat in the area where the road is planned.

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