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Vigilance behaviour and its endocrine correlates in Plains zebra (*Equus burchelli*) living in a predator-free landscape

By

Clasina Elizabeth Fourie

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Note on text: Each chapter is formatted in the style of the journal to which it will be submitted.

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Summary

Group size affects individual and collective vigilance levels of prey species. As individual vigilance decreases with increasing group size, the indirect risk of predation to each individual and the group as a whole will also decrease (dilution and many-eyes effect) which may have a decreasing effect on stress responses on group level as well. Where predation risk is low, other factors like group size might influence stress-related glucocorticoid output in prey species.

I test the relationship between group size and individual and collective vigilance levels in a plains zebra (*Equus burchelli*) population living in an environment with low adult predation risk due to the absence of lions (*Panthera leo*) in the Dinokeng Game Reserve (DGR). I also test for an effect of season (wet vs. dry) on these levels. Vigilance levels are expected to be higher in summer than winter, due to a seasonal influence on susceptibility to predation or the ease of predator detection. Finally, I test if the presence of foals in a band influences individual and collective vigilance levels. Mothers are expected to increase their vigilance levels since foals are most susceptible to predation by smaller predators in the reserve. I also aimed to specifically investigate how group size affects adrenocortical endocrine activity in male and female individuals of plains zebra bands living in an environment with low predation risk. With an increase in group size, and individual vigilance expecting to decrease, the indirect risk of predation to each individual and the group as a whole will also decrease (due to both dilution and many-eyes effect) which may decrease stress on group level as well. Affiliative social interactions between females could also have the effect of improving fitness and reducing stress levels in this species. We therefore evaluated the reliability of different enzyme-immunoassays

(EIA) for monitoring glucocorticoid metabolite levels in plains zebra faeces by conducting an adrenocorticotropic hormone (ACTH) challenge test and associated translocation event.

Individual vigilance decreased with group size, confirming a classic group size effect; while collective vigilance remained constant meaning individuals could reduce their own scanning and rely on scanning by other group members. Individual vigilance differs between seasons, with higher levels recorded in summer, but no seasonal effect is found on rate of scanning and collective vigilance. Interestingly, bands with foals have a lower level of individual vigilance than bands without foals, which may be due to added nutritional stress on mothers during lactation. A constant collective vigilance level means mothers may be able to increase foraging time without increasing predation risk.

I successfully identified two group-specific EIAs as suitable for assessing adrenocortical endocrine activity in male and female plains zebra, but gender-specific differences in response to ACTH administration and translocation were detected. In winter, bands of free-ranging plains zebra differ significantly in terms of faecal glucocorticoid metabolite (FGM) output, but due to the fact that the bands tested also differed in size as well as composition, the potential band size effect were analysed for males and females separately. Males in bands of greater size exhibit higher FGM levels than males in small bands; explained by the females' need for protection, resource competition and reproduction pressure. FGM levels in females did not differ between bands of different sizes, indicating that potential changes in vigilance behaviour per se might not alter FGM output in a low-predation risk environment.

This study confirms firstly, the classic group size effect on vigilance in plains zebra in this low predation risk environment and secondly, that group size and sex should be considered when looking at FGM levels in this social species.

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List of abbreviations

ACTH: Adrenocorticotropic hormone

AUCC: Animal Use Care Committee

DGR: Dinokeng Game Reserve

EIA: Enzyme immunoassay

FGM: Faecal glucocorticoid metabolite

GC: Glucocorticoid

GCM: Glucocorticoid metabolite

HPA: Hypothalamic-pituitary-adrenal

HPLC: High-pressure liquid chromatography

NZG: National Zoological Gardens

PSFH: Predator-sensitive food hypothesis

RDH: Resource dispersion hypothesis

RIA: Radio immunoassay

SE: Standard error

UP: University of Pretoria

CHAPTER 1

General Introduction

1.1 Predation-risk effects in prey species

Predation can affect prey numbers through direct killing or prey behaviour through risk effects, with the latter even sometimes having a more pronounced effect on prey species than the act of predation (Creel and Christianson 2008; Valeix et al. 2009). Risk effects in prey species can include changes in spatial redistribution, the selection of specific habitat types (Creel et al. 2005), activity patterns (Sih and McCarthy 2002), anti-predator vigilance (Hunter and Skinner 1998), foraging time (Lima and Bednekoff 1999a), movement patterns (Sih and McCarthy 2002) and group sizes (Valeix et al. 2009) mostly as a result of predator avoidance behaviour. Prey uses a range of behavioural and morphological defences to avoid predation (Lima 1998; Preisser and Bolnick 2008).

The predation risk allocation hypothesis of Lima and Bednekoff (1999a) suggests that prey that feed under temporal variation in the risk of predation must optimally allocate anti-predator behaviour across varying levels of risk, with these behaviours not acting independently of each other. The predator-sensitive food hypothesis (PSFH) suggests that behavioural responses to predation risk can constrain foraging activity or efficiency (Creel et al. 2009), and as food and predation limit populations, animals take greater risks to obtain more food and, in the process, may be killed (Sinclair and Arcese 1995). Therefore, a balance must be sought to minimize the joint risk of predation and starvation (because of food scarcity), because both limit population size (Sinclair and Arcese 1995). Two of the most obvious changes in behaviour of prey in

response to predation risk are changes in vigilance levels and group formation and the interaction between the two (Winnie and Creel 2007). The response of prey species to risk of predation can also be costly in terms of survival, growth rates and reproduction (Preisser and Bolnick 2008; Creel and Christianson 2008; Creel et al. 2009). For instance, in elk (*Cervus elaphus*) the risk of being preyed on by wolves (*Canis lupus*) negatively correlates with faecal progesterone levels and reproduction rates in females (Christianson and Creel 2008).

In the following two sections, the focus will be on two of the most important anti-predatory defence mechanisms employed by prey species, group-living and vigilance.

1.2 Group-living in prey species

The aggregation of animals in groups is a common sight, especially in gregarious ungulates inhabiting African savannahs (Thaker et al. 2010). The resource dispersion hypothesis (RDH) suggests that where resources are heterogeneously distributed, several individuals can share these resources in a common area and satisfy their needs without incurring any cost (Johnson et al. 2002). This means that a territory might contain enough resources to satisfy both the needs of the original residents and additional individuals and costs will only be incurred when the requirements of the additional individuals are not met (Johnson et al. 2002).

Sociality, or group-living, should evolve when the benefits exceed the costs, which can vary between species and habitats (Krause and Ruxton 2002; Silk 2007). Living in groups reduces predation risk through increased protection and detection, increases the chance of finding food, water and mating partners and facilitates reproduction (Childress and Lung 2003; Fernandez et

al. 2003; Thaker et al. 2010). Individuals living in a group benefit from the 'many eyes effect', with a greater number of animals being able to scan the surroundings while feeding (collective vigilance), and also the 'dilution effect' where large group sizes lower the risk of a specific individual being preyed on (Lima 1995; Beauchamp 2008; Fortin et al. 2009; Valeix et al. 2009). Also, when approached by a predator, a group of animals that scatter may be able to confuse the predator (confusion effect) (Goodale and Ruxton 2010). After predators have been detected by a group of prey, they may be forced to abandon attacks (Krause and Ruxton 2002). The position and behaviour of a group can act as a potential source of information on the locality of food for group members (Krause and Ruxton 2002). Social integration in mammals increases reproductive success (feral horses (*Equus caballus*); Cameron et al. 2009) and reduces cortisol levels (baboons (*Papio hamadryas ursinus*); Silk 2007), the secretion of which is often used as a hormonal measure of stress in wild animals (Millspaugh and Washburn 2004). Fryxell et al. (2007) also provide some evidence that social group formation, particularly in populations of wildebeest (*Connochaetes taurinus*), contribute to the stability of predator-prey interactions and also reduce kill rates by lions (*Panthera leo*) in the Serengeti ecosystem.

However, animals that aggregate in groups are disadvantaged by an increase in parasite transmission, competition for resources and partners and the increased probability of being detected by predators (Rubenstein 1978; Krause and Ruxton 2002; Neuhaus and Ruckstuhl 2002; Thaker et al. 2010). Certain social behaviours may have evolved to minimise these disadvantages like for instance social grooming, which can reduce parasite transmission and also well-developed communication systems to limit competition (Rubenstein 1978). In some prey species (e.g. Alaskan moose (*Alces alces*) group size is negatively correlated with foraging efficiency which can reduce fitness (Molvar and Bowyer 1994).

These costs and benefits but also other factors like the level of predation risk, habitat conditions (open or forested habitats), season and reproduction influence the size, composition and distribution of groups (Krause and Ruxton 2002; Thaker et al. 2010). For individuals to maximise fitness, an optimal group size should exist (Krause and Ruxton 2002). As group size increase group-members will deplete food patches more rapidly. This may lead to an increase in the distance that groups must travel for resources and increased energy output (Silk 2007). When prey aggregate in large groups, stalking predators are more disadvantaged than ambush predators, as stalking predators often target individual prey or smaller groups (Thaker et al. 2010). Prey inhabiting open habitats will be more easily detected and thus will form larger herds to lessen the probability of capture, with those living in forest habitats forming smaller groups to reduce their chances of being detected and captured (Pays et al. 2007; Fortin et al. 2009; Thaker et al. 2010). With more females present in a group, the riskiest time for predation will be during late gestation, as females will have higher energetic needs and will apply riskier tactics while feeding (Molinari-Jobin et al. 2004; Thaker et al. 2010). With more males present in a group the riskiest time will be during the mating season, as males will spend more time competing for the acquisition and defence of territories as well as for access to females (FitzGibbon 1990; Thaker et al. 2010).

Group living and group size has an effect on individual and collective vigilance levels in a range of bird and mammal species (Lima 1995; Treves 2000; Beauchamp 2008). In some species, support is shown for a negative relationship between time spent being vigilant and the size of the group (the group-size effect), and a positive relationship between collective vigilance and group size (Pays et al. 2007; Michelena and Deneubourg 2011).

When part of a group, each member should coordinate their scans in a non-overlapping way as to avoid raising their head when another group member is already vigilant (Fernandez et al. 2003; Pays et al. 2007). To succeed in this, each individual must then monitor the behaviour of the other group members (Fernandez et al. 2003). Group members are dependent on the numerous other animals surrounding them to be vigilant when they are not (Hunter and Skinner 1998). Through this process an animal can devote more time to foraging without increasing its personal risk of being preyed upon (Hunter and Skinner 1998; Beauchamp 2008). However, this may not always hold in high risk situations, and it may be better to be the first to detect predators as discussed by Lima and Bednekoff (1999b).

In Defassa waterbuck (*Kobus ellipsiprymnus defassa*) members of a group reduce the time spent being vigilant while increasing time spent feeding without this affecting their chances of detecting a predator. Their vigilance behaviour is not coordinated and can overlap between group members. Thus, in this species collective vigilance emerges through the synchronisation of vigilance bouts, which also increases with group size (Pays et al. 2007). In blesbok (*Damaliscus pygargus phillipsi*) and impala (*Aepyceros melampus*), foraging or vigilance drives behavioural changes over different group sizes, but other behaviours (i.e. walking, grooming and social interactions) can also be the driving force behind changes in foraging and vigilance rates in these two species (Dalerum et al. 2008). Thus, relationships between group size, foraging and vigilance can be highly variable between species (Dalerum et al. 2008).

To remain as part of a group, individuals must synchronise their resting, foraging and moving activities with the other members (Fortin et al. 2009). Differences in behaviours of males and females that live in the same group are evident in the activity budgets of Rocky Mountain

bighorn sheep (*Ovis canadensis*), the feeding behaviour of Alpine ibex (*Capra ibex*), and the synchronisation of behaviour of African buffalo (*Syncerus caffer)* (Ruckstuhl and Neuhaus 2002). All of these species have a high degree of sexual dimorphism that may decrease their ability to live in groups and increase the cost of synchrony (Neuhaus and Ruckstuhl 2002). When group-living animals have similar body sizes and similar needs, the synchrony of their activities will be less costly. In plains zebra (*Equus burchelli*), Neuhaus and Ruckstuhl (2002) found no difference in activity budgets of males and females, which may be a requirement for or a consequence of group stability.

1.3 Vigilance as a key behavioural trait in prey species

Vigilance is the act of an individual raising its head and visually scanning the surroundings while feeding (Treves 2000; Pays et al. 2007; Beauchamp 2008). The risk of being preyed upon mainly shapes animal vigilance (Hunter and Skinner 1998; Treves 2000), and together with group-size, may also be influenced by competition, risk and aggression in the group (Treves 2000; Lung and Childress 2007; Pays et al. 2007; Beauchamp 2008; Shi et al. 2010). Through the process of scanning, an animal can also receive signals given by other wary animals or associates when they detect threats, but it may not be very reliable (Treves 2000). For the individual, there is a trade-off between increasing its vigilance and lessening time spent feeding, sleeping, grooming and fighting (Treves 2000). Past research has shown that individual vigilance is mainly influenced by predation risk, but also herd size, intra-group competition and surveillance, body size, sex, distance to cover, nearest-neighbour distance and position in the herd (Childress and Lung 2003; Cameron and du Toit 2005).

Benefits of grouping are evident in situations where high as well as low predation pressure is present (Hunter and Skinner 1998). The benefit of the 'group-size effect' for individual vigilance can be best explained by 1) the 'many-eyes' hypothesis or detection effect, 2) the 'safety in numbers' hypothesis or dilution effect and 3) the 'scramble competition' hypothesis (Lima 1995; Beauchamp 2003; Cameron and du Toit 2005; Beauchamp 2008; Valeix et al. 2009). The first two imply that with a greater number of individuals (or eyes) present in a group, the risk of predation to each individual and level of individual vigilance will decrease (Bednekoff and Ritter 1994; Lima 1995; Treves 2000; Beauchamp 2008), but it also depends on the way in which predators target their prey (Beauchamp 2003). The 'scramble competition' hypothesis exists as an alternative to this, stating that a reduction in vigilance levels with increasing group size may be due to an increase in scramble competition for limited resources (Beauchamp 2003; Cameron and du Toit 2005; Li et al. 2009).

The 'group-size effect' is evident in various gregarious ungulates like zebra, impala, springbok (*Antidorcas marsupialis*) wildebeest and white-tailed deer (*Odocoileus virginianus*) (Scheel 1993; Burger and Gochfeld 1994; Hunter and Skinner 1998; Burger et al. 2000; Clayton 2010), but interestingly enough this effect is lacking in primates, giraffes (*Giraffa camelopardalis*) and elk (Treves 2000; Luandre et al. 2001; Cameron and du Toit 2005). The 'group-size effect' is also evident in feral goats (*Capra hircus*), but with the exception of this population having been predator-free for almost 170 years. This means that the loss of predators does not necessarily lead to a loss of group-size effects and this behaviour may have some other function that is sufficient to maintain it (Shi et al. 2010). Vigilance in this species may serve the purpose of monitoring conspecifics as well as other competitor sympatric species in the vicinity (Shi et al. 2010).

Many other factors besides within-group interaction and predation risk can confound the group-size effect on vigilance including food density and quality, distance from cover, habitat obstructions and visibility, edge effects, sex, age, breeding status and time of day (Shi et al. 2010). In numerous studies of mammalian taxa differences exist between vigilance behaviour of males and females. In plains zebra, male presence has a pronounced effect on female vigilance levels (Simpson et al. 2011). Higher vigilance rates in males versus females have been detected in ungulate species such as plains zebra (41 % vs. 12 %, Simpson et al. 2011), African buffalo, wildebeest, springbok, and waterbuck (*Kobus ellipsiprymnus*), but seasonal differences occur in male and female giraffe (Burger and Gochfeld 1994; Burger et al. 2000; Pays and Jarman 2008). In contrast to this female kangaroos (*Macropus giganteus*) and elk spend more time being vigilant than the opposite sex (Childress and Lung 2003; Pays and Jarman 2008).

Two hypotheses related to foraging and breeding strategies may explain the difference in the amount of time that males and females spent being vigilant (Pays and Jarman 2008; Li et al. 2009). Firstly, intra-specific competition between males (territoriality) in gregarious species forces them to scan their surroundings to assess the presence and activities of rivals, but this can also happen when they compete for access to females during the breeding season (Cameron and du Toit 2005; Pays and Jarman 2008; Li et al. 2009). Vigilance levels of female elk is affected by predation risk and herd size outside the breeding season, but male elk vigilance levels particularly increase in the mating season when the focus is shifted from feeding to mating in the midst of increasing predation risk (Lung and Childress 2007). In African ungulates, vigilance levels in males and females may also change with breeding status, with adult males more at risk of being preyed upon in the breeding season and adult females during the gestation period (Thaker et al. 2010).

Alternatively, as seen in kangaroos, it can be that the different sexes in sexually dimorphic species have differing energy requirements, thus larger males would need more time to forage than smaller females (Pays and Jarman 2008). In elk, male vigilance is not as much influenced by predation risk than female vigilance, which can also be explained by the high degree of sexual dimorphism in body mass (Lung and Childress 2007).

Linked to the influence of sex on vigilance levels is the parental status of an individual. It is expected that lactating females or female individuals with young will have higher vigilance rates than non-lactating females or females without young, with vigilance serving the purpose of protecting young from predators or preventing them from straying too far (Burger and Gochfeld 1994; Hunter and Skinner 1998). This is evident in species like African elephant (*Loxodonta africana*), zebra, buffalo, waterbuck, impala, wildebeest and elk (Burger and Gochfeld 1994; Hunter and Skinner 1998; Childress and Lung 2003; Lung and Childress 2007). However, mothers also have to trade-off their energetic constraints against the risk of offspring predation. Mothers may spend more time rearing their young which increases nutritional stress and lead to females increasing their feeding activity to compensate for this (White and Berger 2001). By doing this, individual vigilance in females will decrease. Mountain goat (*Oreomnos americanus*) females with young do not show a higher investment in vigilance, possibly due to energetic constraints (Hamel and Côté 2008).

Although it has not been documented well, variation in vigilance between seasons (summer vs. winter) is evident in ungulates e.g. goitered gazelle (*Gazella subgutturosa;* Xia et al. 2011) and giraffe (Ginnett and Demment 1997). Resource availability varies between seasons, with resources being scarcer in winter months. Animals need to increase their feeding activity and

decrease their vigilance levels to compensate for this (Thaker et al. 2010). This variation may also be due to visibility and the ability of prey to detect predators increasing (winter) or decreasing (summer) due to varying vegetation height (Burger et al. 2000). Variation in vigilance levels is evident between the breeding and non-breeding season in elk (Luandré et al. 2001; Lung and Childress 2007). During the breeding season more time is allocated to reproductive activity and less time is spent on anti-predator behaviour like vigilance (Luandré et al. 2001).

Diurnal variations in scan rates have been documented in various prey species. Scan rates of plains zebra are highest on moonlit nights and cloudy days and much lower during moonless nights and sunny days (Scheel 1993). It also varies through the day, with higher vigilance during midday than during the morning or evening (Stecker and Olsen 1999). Springbok have higher vigilance rates at night, but it is also higher later in the morning than during the afternoon (Bednekoff and Ritter 1994).

In species with social hierarchies, intra-specific aggression among males may determine dominance rank (Lung and Childress 2007). A study focusing on the correlation between dominance rank and behaviour in a herd of zebra found that adult zebra individuals have the highest vigilance levels and it decreases with decreasing dominance rank (Clayton 2010). Stallions display vigilance behaviour more frequently than other individuals and the frequency of behaviours like resting, feeding and movement also correlate positively with dominance rank (Clayton 2010). In giraffes, the largest bulls are the most dominant in the group and have easy access to females (Cameron and du Toit 2005). Mature giraffe bulls are aggressive to one another and they will be continually aware and vigilant to avoid same-sex aggression from conspecifics (Cameron and du Toit 2005).

The within-group surveillance hypothesis suggests that vigilance can also function to monitor the behaviour of conspecifics (Cameron and du Toit 2005). This vigilance activity is useful to increase knowledge of resources, to follow potential mates and to reduce intra-group aggression and risk (Cameron and du Toit 2005; Lung and Childress 2007) in the presence or absence of predation risk. An increase in group size may shift the balance from anti-predator vigilance to social monitoring vigilance (group surveillance) (Cameron and du Toit 2005; Lung and Childress 2007). Males in a group may be more vigilant than females because males compete for females (Cameron and du Toit 2005). A recent study on the various factors affecting vigilance in elk found that variation in female vigilance can be predicted by the anti-predatory hypothesis and variation in male vigilance by the social monitoring hypothesis (Lung and Childress 2007). Vigilance behaviour in giraffes is influenced by sociality, with these mammals devoting a large portion of their vigilance towards conspecifics (Cameron and du Toit 2005). As mentioned above, dominant giraffe males have direct access to females, and this has the effect of other bulls using vigilance scanning to avoid aggressive interactions with the same sex (Cameron and du Toit 2005). Thus it may be assumed that as the presence of adult males increase, intra-group aggression and risk will also increase leading to a higher level of social vigilance (Pays and Jarman 2008). In feral goats (*Capra hircus*) living in a predator-free environment, vigilance may serve the purpose of monitoring conspecifics and other competitor sympatric species (Shi et al. 2010).

1.4 Endocrine indicators of stress

Stress is a challenging concept to define. For example, Morgan and Tromborg (2007) define it as intrinsic or extrinsic demands that exceed an individual's resources for responding to those

demands, but it can also be seen as a state in which homeostasis is disrupted (Reeder and Kramer 2005). This condition is comprised of multiple stages involving the sympathetic nervous system and a class of steroid hormones named glucocorticoids (Franceschini et al. 2008). The stress axis forms part of normal day-to-day activities like walking, locomotion, exploratory behaviour, increased appetite and food-seeking behaviour (Boonstra 2005). Stress can affect metabolism, reproduction and growth by disrupting the hormonal control of these important life systems, and the secreted glucocorticoids can also suppress the immune-response (Matteri et al. 2000).

In this section the following key factors of this concept will be discussed: the sources of stress, the stress response, the endocrine indicators of stress, the ways in which the response can be measured, and some factors specific to this study that can influence these measurements and results.

1.4.1 The sources of stress

Physical stressors can be from internal or external sources including hypoxia, hypoglycaemia, heat or cold, exercise or injury or other noxious stimuli (Reeder and Kramer 2005). Psychological stressors have an effect on emotions and can include fear, anger, anxiety or frustration (Reeder and Kramer 2005). Difficult conditions may not lead to a stress response except when the conditions are unpredictable or uncontrollable (Creel 2001; Creel et al. 2009). Anthropogenic stressors can negatively affect behavioural patterns and species biology, but detailed impacts are not known in and between ungulate species (Stankowich 2008). The predation stress hypothesis suggests that when an animal is exposed to predators, stress hormone levels will be elevated, suppressing reproduction, and indirectly reducing survival and

reproduction through the functioning of the immune and digestive system being affected (Creel et al. 2009). Ecological (i.e. seasons, resource availability and habitat) and social (group size, reproduction, aggression) pressures also influence the adrenocortical response and the secretion of stress hormones (Millspaugh and Washburn 2004; Schwarzenberger 2007).

1.4.2 The stress response

Romero (2004) defines the stress response of an animal as the specific physiological, hormonal and behavioural responses exhibited by healthy individuals. The hormonal response is linked to the degree of control an animal has over a specific stressor (Romero 2004). The stress response can be divided into three stages namely the identification of a stressor, the biological protection against the stressor and the consequences of the stress response (Moberg 2000). The central nervous system identifies a potential threat to homeostasis followed by the development of biological protection which can include some or all of the following: a behavioural response, nervous system response, neuro-endocrine response or immune response (Moberg 2000). The first response to a stressor is a behavioural response, where an animal may avoid the stressor by moving away from the threat, e.g. an animal may seek shade if its body temperature becomes elevated (Moberg 2000). The nervous system response affects heart rate, blood pressure and gastrointestinal activity for a short duration of time, and may not have a significant impact on the long-term welfare of an animal (Moberg 2000). The activation of the hypothalamic-pituitary-adrenal (HPA) axis is the most well known response to stress (Matteri et al. 2000). It mediates neuro-endocrine responses by secreting hormones which regulates and affects immunity, reproduction, metabolism and behaviour over a long period of time (Matteri et al. 2000; Moberg 2000; Sapolsky et al. 2000). During the response, energy is used and it can vary depending on

the environmental conditions and the life-history stage of an animal (Reeder and Kramer 2005). The immune system is also one of the major defence systems that respond to a stressor. The change in biological function occurring during a stress response may suppress immune competence, rendering the animal susceptible to pathogens that may be present in the environment (Blecha 2000). Factors like early experience, genetics, age, social relationships and human-animal interactions can all influence the way an animal responds to stress (Moberg 2000).

Short-term stress responses help an animal to cope with adverse environmental conditions, but chronic stress responses can be reflected in elevated glucocorticoid metabolite (GCM) levels and decreased health (Franceschini et al. 2008; Morgan and Tromborg 2007). Acute and chronic stress can lead to distress in an animal (Moberg 2000). Adaptive behavioural responses related to orientation, alarm and increased vigilance levels are associated with acute, short-term stressors (Morgan and Tromborg 2007). These responses can either interrupt vitally important biological events or redirect biological resources away from other biological functions (Moberg 2000). Behaviourally, chronic, long-term stress reduces reproductive fitness and increases abnormal behaviour, behavioural inhibition, vigilance and aggression (Morgan and Tromborg 2007).

1.4.3 Glucocorticoids as endocrine indicators of stress

It is difficult to measure stress in animals, but behavioural measures and endocrine and immunological techniques can be used (Millspaugh and Washburn 2004). The hormones adrenaline or noradrenaline are the indicators of acute stress responses and in the case of long-term stress responses, glucocorticoids are the endocrinological indicator (Millspaugh and Washburn 2004). During the stress response an increased amount of adrenocorticotropic

hormone (ACTH) is released, which results in elevated glucocorticoid levels (Möstl et al. 1999). GCM (i.e., cortisol and corticosterone) secretion is used as a hormonal measure of stress in wild and captive animals (Millspaugh and Washburn 2004). The type of GCM secreted differs between species (Palme et al. 2005), but cortisol is primarily secreted in humans, primates, carnivores and ungulates and corticosterone in rodents, birds and reptiles (Matteri et al 2000; Touma and Palme 2005). Glucocorticoids mobilise energy reserves that are needed to cope with challenges in an environment (Huber et al. 2003). Glucocorticoids exhibit vast circadian and circannual rhythms to regulate energy balances in specific environments (Reeder and Kramer 2005) and it may affect behavioural patterns (Möstl and Palme 2002). The severity of the stressor influences the amount of glucocorticoids secreted (Reeder and Kramer 2005).

The HPA–axis releases glucocorticoids in the form of cortisol or corticosterone in the bloodstream, which are transported to the liver and kidneys. In the liver, cortisol undergoes metabolism and conjugation and one part is excreted as urine from the kidneys. The rest is transported as bile to the gut and after deconjugation some is transported back to the blood via the enterohepatic cycle, but some also undergo bacterial metabolism and is excreted in the faeces (See Figure 1) (Chinnadurai 2006; Möstl and Palme 2002). The main route of excretion of glucocorticoids (either via urine or faeces) differs vastly between species and sex (Palme et al. 2005).

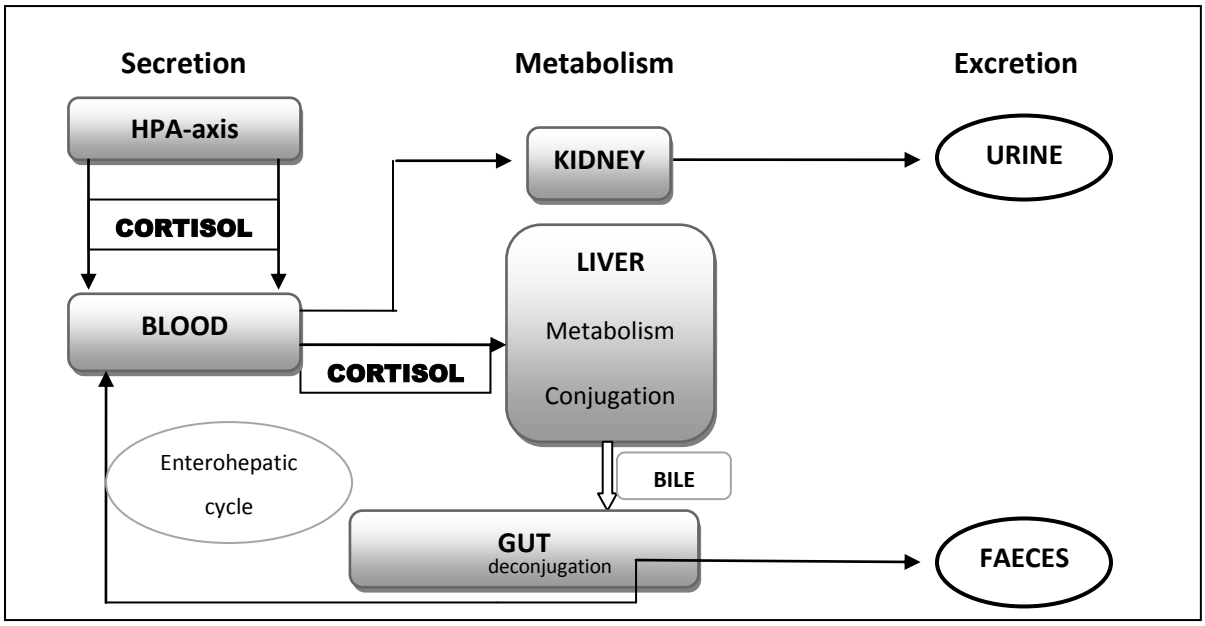

Figure 1: The secretion, metabolism and excretion of glucocorticoids.

1.4.4 GCM monitoring

Glucocorticoid concentrations can be measured in an invasive or non-invasive way. Invasive methods include the collection of blood and saliva from the animal. The well-being of the animal, the ease of collection, the need for anaesthetic and the safety of the investigator needs to be considered before using this method (Sheriff et al. 2011). When collecting blood the animal needs to be captured, handled and restrained, maybe anaesthetized and the sample collected with minimum stress (Sheriff et al. 2011). The stress associated with animal-handling during sample collection can elevate glucocorticoid levels, and alter the results, but the upside of this is that the state of the animal can be seen at that instant (Möstl and Palme 2002; Sheriff et al. 2011; Touma and Palme 2005).

In contrast, non-invasive faecal hormone metabolite assays are particularly useful because animals are not disturbed in their natural behaviour and it is an accurate means of assessing

chronic stress without capture-induced increases in glucocorticoids levels being measured (Franceschini et al. 2008; Millspaugh and Washburn 2004). A range of techniques for non-invasive faecal steroid analysis have been developed and used in research on various mammal, bird, reptile, amphibian and fish species (Schwarzenberger 2007). This research has been done on captive, free-ranging, domestic and laboratory species of all sizes (Schwarzenberger 2007; Touma and Palme 2005). In recent years, non-invasive measures have been used to evaluate stress associated with dominance, aggression or human disturbance (Monfort 2003). Through this technique long-term studies on individual animals are a possibility without many problems arising (Millspaugh and Washburn 2003). Faecal corticosteroid assays are accurate in reflecting endogenous changes in adrenal activity (Monfort 2003), but reproductive status can also be examined (Millspaugh and Washburn 2004). Faecal steroid analysis can be applied in long-term studies and can be combined with other parameters, such as behaviour or reproduction to better understand the endocrinology of a certain species (Schwarzenberger 2007). Various questions relating to stress and animal welfare, reproductive physiology, behavioural ecology, conservation biology and biomedical research have been investigated through the use of non-invasive techniques (Touma and Palme 2005).

Radio-immunoassays (RIA) and enzyme-immunoassays (EIA) are used to measure cortisol and glucocorticoid metabolite concentrations in samples collected non-invasively (Hodges et al. 2010). The concept of RIA is based on the principle of competitive binding of an antibody to its antigen, and the antigen is also "labelled" with radioactivity (Nelson 2011). An EIA also works on the same principle, but radioactive tags are not required, and the antibody is tagged with an enzyme that changes the optical density (colour) of a substrate molecule (Nelson 2011).

However, the non-invasive measurements and assays must be biologically and physiologically validated for the specific species and matrix to ensure proper quantification of the glucocorticoid metabolites (GCM's) ((Schwarzenberger et al. 1996; Sheriff et al. 2011). For this purpose and to ensure that the species-specific range of stress hormone metabolites is detectable by the assay(s) used, an adrenocorticotropic hormone (ACTH) challenge test can be performed. When ACTH is administered to a mammal it stimulates the release of glucocorticoid metabolites. After glucocorticoid levels peak in the plasma their concentration return to baseline values (Wasser et al. 2000; Keay et al. 2006). This rise and fall is reflected in the faeces after the glucocorticoids are metabolised and excreted (Keay et al. 2006). The ACTH challenge test is a functionality test for this part in the physiological stress response reaction by demonstrating the cause-and-effect relationship between physiological changes and the excretion of hormone metabolites (Monfort 2003; Hodges et al. 2010).

Various factors need to be considered regarding non-invasive faecal steroid analyses. Before analysis, the specific sample storage methods must be validated, because environmental conditions may influence concentrations of GCMs samples (Touma and Palme 2005). Other important factors to consider before and during analyses are the stability of faecal metabolites, the gut transit time of the focal animal, environmental conditions, diurnal and seasonal variations, as well as species type, gender and diet (Millspaugh and Washburn 2003; Touma and Palme 2005; Schwarzenberger 2007).

1.4.5 Group living, sex and GCM measurements

Animals living in groups are advantaged by cooperation and social support which may also have the added benefit of decreasing allostatic load, or the cumulative physiological burden exerted on the body of an animal while it adjusts to factors like season, environmental change or reproductive status (Goymann and Wingfield 2004). The social environment of animals and the presence of a bonding partner can act in a positive way by reducing neuroendocrine responses in stressful situations (Sachser et al. 1998; Wittig et al. 2008). Affiliative social interactions have immediate benefits to individuals such as reduced heart rate in horses and reduced stress in guinea pigs (*Cavia porcellus*) and baboons (Hennessy et al. 2008; Wittig et al. 2008; Cameron et al. 2009). In female macaques (*Macaca* spp.) elevated stress responses are dampened by the presence of a preferred grooming partner and in female chacma baboons (*Papio hamadryas ursinus)* individuals form bonds with other females after the loss of a companion, which has the effect of reducing stress (Engh et al. 2006). Male Guinea pigs form small harems of females in the wild, and social bonds are evident between the male and females in such a harem (Hennessy et al. 2008). In this social environment, where monogamous species form attachment-like bonds, the male partner reduces the plasma cortisol response in females (Hennessy et al. 2008). Females that are socially integrated with other females will benefit from reduced harassment which may have the effect of reducing stress levels (Cameron et al. 2009).

In contrast, social group-living animals may experience social conflict which can increase allostatic load, and in the past it has been thought that this load will especially result in higher baseline glucocorticoid concentrations in subordinate individuals (Goymann and Wingfield 2004). Additional studies, however, show that in cooperative social species with high

reproductive skew it is rather the dominant individuals that feel the pressure of higher allostatic load (Creel 2001; Goymann and Wingfield 2004). Thus, the relative allostatic load of social status can be used to predict whether dominant or subordinate individuals will express higher or lower concentrations of glucocorticoids, but other factors like sex, group size and species type can also affect this (De Vries et al. 2003; Goymann and Wingfield 2004). In male spotted hyenas (*Crocuta crocuta*) individuals in bigger clans have higher levels of faecal corticosteroids, probably because social interactions are becoming more complicated with an increase in group size (Goymann et al. 2003). The "group-size effect" on vigilance levels has been intensively studied in a range of mammalian prey species (Treves 2000; Beauchamp 2008). This effect indicates a negative relationship between group size and the time animals spends being vigilant. As individual vigilance decreases with increasing group size, the indirect risk of predation to each individual and the group as a whole will also decrease (dilution and many-eyes effect) (Treves 2000), which may have the effect of decreasing stress on group level as well.

In terms of sex, females of most mammal species have higher basal glucocorticoids and they respond more vigorously to stress (Touma and Palme 2005). The gestation and lactation periods are also more expensive in terms of their metabolic needs (Reeder and Kramer 2005; Touma and Palme 2005). Clear differences in baseline GCM levels, the reactivity of the HPA axis to stressors and the metabolism of glucocorticoids exist between the sexes (Touma and Palme 2005; Monclús et al. 2006). Examples of species where higher faecal GCM levels were found in females include the domestic dog (*Canis lupis familiaris*), African wild dog (*Lycaon pictus)*, domestic cat (*Felis catus*) and cheetah (*Acinonyx jubatus*) (Touma and Palme 2005). In contrast to this, higher faecal GCM levels have been found in male rats (*Rattus rattus*), mice (*Mus musculus*), sea lions (*Eumetopias jubatus*), chickens (*Gallus gallus*) and rabbits (*Oryctolagus*

cuniculus) with no difference between the sexes in the wolf, black rhinoceros (*Diceros bicornis*) and red deer (Touma et al. 2003; Touma and Palme 2005; Monclús et al. 2006). Differences also exist between the sexes in the amount of metabolites being excreted via urine or faeces.

Sometimes when predators are in the vicinity of prey species, their presence and odours will lead to immediate, short-term increases in glucocorticoid levels of the prey (Creel et al. 2009). A direct encounter with a predator should cause a rapid anti-predatory reaction, associated with a strong stress pulse (Ylonen et al. 2006). In bank voles (*Clethrionomys glareolus*), weasel odour elevates faecal corticosteroid concentrations over a short time period, but it is not costly in terms of longer-lasting stress (Ylonen et al. 2006) and in European rabbits, fox (*Vulpes vulpes*) odour resulted in an increase in faecal corticosteroid metabolite concentrations (Monclús et al. 2006). In snowshoe hares, sub-lethal effects of predation stress through increased glucocorticoid concentrations resulted in reduced reproduction (*Lepus americanus)* (Sheriff et al. 2009).

1.5 The Plains zebra

Plains zebra are large-bodied (~ 250 kg), diurnal, grazing ungulates and inhabit mesic habitats like grasslands and savannah woodlands close to water, with many wild populations being seasonally migratory (Hack et al. 2002; Blom 2009; Rubenstein 2010). The plains zebra is seen as one of Africa's most common, adaptable and successful grazers (Neuhaus and Ruckstuhl 2002). Their present distribution range is shrinking, and in South Africa naturally occurring populations of plains zebra can only be found in larger, state-owned nature reserves in Limpopo Province and Mpumalanga (i.e. Kruger National Park) and in north-eastern Kwazulu-Natal, but

also in small protected areas, private game reserves and on game farms (Bowland et al. 2001; Skinner and Chimimba 2005).

Plains zebra live in long-lasting, stable social groups (bands) with a single breeding stallion and one to six adult mares and offspring of both sexes up to the age of 2-3 years (Hack et al. 2002; Pluháček et al. 2006; Rubenstein 2010). The average size of bands correlates with the environmental conditions as well as the level of predation the band is subjected to (Skinner and Chimimba 2005). The social system of this species has a unique structure because females in bands are unrelated (Neuhaus and Ruckstuhl 2002). These bands are not territorial and home ranges can overlap (Grubb 1981). Females and males of this species can live together in these bands for a number of years (Neuhaus and Ruckstuhl 2002). A rank hierarchy with a consistent filing order exists which is based on the time of joining (Estes 1991). This social structure, based on long-term associations between unrelated individuals, is unusual among ungulates, shared only by other equids such as wild horses (Linklater 2000; Linklater et al. 2000; Linklater and Cameron 2009). These non-territorial, one-male harem systems are especially uncommon in mammalian social systems and just a few other mammal species exhibit this type of social organisation (e.g. gorilla (*Gorilla gorilla*) and bush pig (*Potamochoerus porcus*); Estes 1991). Where these equids find themselves in stressful situations, their stable social environment and membership in a band may decrease the negative effect of stress (Cameron et al. 2009).

Simpson et al. (2011) found that in "nursery groups' males can either be present or absent but female membership remains stable regardless of this. The forming of such strong social bonds between females may be an adaptive strategy, where affiliative social interactions are beneficial to individuals by reducing heart rate and stress in various species (reviewed in Cameron et al.

2009). The social integration in mares can act to reduce harassment, and increase reproductive success (Cameron et al. 2009). Thus, stable social relationships in these animals are an important factor in their ecology, behaviour and overall health.

The mating system in plains zebra is polygynous, with males mating with a number of females while defending the band and its females (Sundaresan et al. 2007; Rubenstein 2010). Males that are reproductively unsuccessful, live as bachelors in loose aggregations (stallion groups), but they may switch between the two associations (Grubb 1981; Hack et al. 2002; Fischhoff et al. 2009). Bands and bachelor groups can also join each other to form unstable herds whose size changes regularly (Fischoff et al. 2007; Rubenstein 2010). In herds, females with foals band together, especially when habitat visibility decreases and predation risk increases (Rubenstein 2010). The avoidance of predators and the necessity of finding food and water can affect relationships between same-sex individuals and individuals of the opposite sex (Rubenstein 1986).

Although females can breed on a year-round basis, equids inhabiting temperate zones are more seasonal in reproduction (Asa 2002; Neuhaus and Ruckstuhl 2002). The gestation period in plains zebra is 12 months (Estes 1991) and the minimum interval between foaling episodes is 13 months (Grange and Duncan 2006). Mares cannot produce successively in the same season (Grange and Duncan 2006). A link exists between female foraging and reproduction rate, with a peak in reproduction occurring when conditions are favourable for vegetation growth i.e. in the first months of the rainy season (Estes 1991; Rubenstein 1994). Lactating as well as pregnant females are common in a group (Neuhaus and Ruckstuhl 2002). Mating and birthing periods can overlap in this species (Rubenstein 1994). Male zebras can be reproductively active throughout

the year and consequently they will persistently harass females for an opportunity to mate. This potentially adds to the stress experience by female individuals as they are trying to increase intake of food and water to ensure good body condition for lactation and the survival of themselves and their offspring (Rubenstein 1994). Females can decrease the pressure of harassment by males by associating more with high-ranking males which will allow them more freedom of movement (Rubenstein 1994). By banding together in a group with males, females increase their own net gain by increasing reproductive success (Rubenstein 1994).

By living in groups zebra may be advantaged by the increased likelihood to find food and water and the decreased risk of being preyed on, but competition over food resources and the synchronisation of their specific behaviours may be costly (Neuhaus and Ruckstuhl 2002). The formation of groups, but especially mixed-sex groups is beneficial to mating and reproduction. Through this arrangement, females can mate with males through the year which also increases the reproductive success of males (Neuhaus and Ruckstuhl 2002). Good quality males also increase female reproductive success and the chances of offspring surviving to the age of independence (Rubenstein 2010). Also, when females band together with a male this coalition will reverse the chances of new females being integrated into the group which reduces aggression (Rubenstein 1994). Large bands, with a large number of females, reduce aggression because resident females act to exclude new females as they try to join the band (Rubenstein 1994). This leads to a more peaceful co-existence and an increase in time spent foraging, which ultimately boosts reproduction rate in females (Rubenstein 1994). Thus, the formation of groups is a win-win situation for both sexes.

Lions, wild dogs, cheetahs and spotted hyenas are the main predators of plains zebra (Cillie 2004), with the latter particularly preying on foals (Skinner and Chimimba 2005). In Kruger National Park, mortality is mostly caused by lions, which mainly prey on juveniles (< 1 year old) and females, with foals being taken more often than expected (Mills and Shenk 1992). This mortality is season dependent, with most zebras killed during the late wet season, which is probably due to vegetation height decreasing visibility and obscuring approaching predators (Owen-Smith 2008). In general, adult females and young are more vulnerable than males to predation by lions (Owen-Smith 2008; Thaker et al. 2010). Zebra foals are the most vulnerable to being preyed on by large predators and the mother will hide the foal behind the other band members when in a high-risk situation (Estes 1991). Attacks by lions on zebras are more successful on solitary than group-living animals (Rubenstein 1994) because when threatened by a predator, the whole band will stay together and cooperate to protect a member that is at risk of being attacked (Estes 1991).

Females differ in size and reproductive state which influence physiological demands and together with differing distributions of food, water and safe feeding sites, also shape their time budgets and activity patterns (Rubenstein 1994). Females rarely compete while foraging, and an increase in group size and the number of females may decrease aggression (Rubenstein 1994), but also may decrease levels of vigilance because of the 'group-size effect' (Bednekoff and Ritter 1994; Lima 1995; Treves 2000; Beauchamp 2008). Environmental conditions can differ between seasons and years which will affect foal survival, adult sex ratio and population density (Hack et al. 2002).

Plains zebra is one species that may offer insight into the ways in which predation shapes the behaviour of prey (Fischhoff et al. 2007). The variation that these animals show in response to predation danger may ultimately also lead to variation in survival rates (Fischhoff et al. 2007). This variation includes the avoidance of grassland habitats and an increase in use of woodland habitat when predators are present but also changes in movement and speed at night (Fischhoff et al. 2007). In areas where predation danger is high, zebras will also form larger herds (Fischhoff et al. 2009). In the Serengeti zebra numbers are limited by low juvenile survival rates, which could be due to predation (Grange and Duncan 2006).

In contrast, in environments with low predation risk, other factors predominantly shape the behaviour of prey species. These can include changes in the environment from one season to the next which influence the amount of resources available (bottom-up processes), the reproduction status of individuals, competition within groups, group size, the social relationships between group members and disease (a top-down process) (Grange and Duncan 2006; Shi et al. 2010). In Laikipia, where large mammalian predators are not abundant, zebras are mostly limited by food resources. In different ecosystems, different factors act to regulate zebra populations (Grange and Duncan 2006).

1.6 The study site – Dinokeng Game Reserve

The newly proclaimed and developing Dinokeng Game Reserve (DGR), is situated in the North-West quadrant of the Gauteng Province of South Africa (S 25 22.693 E 28 19.257), approximately 50 kilometres north of the city of Pretoria (see Fig. 2 and 3). The game reserve is approximately 18 000 ha in size, but the greater Dinokeng area covers approximately 281 000 ha

of rural land incorporating Roodeplaat, the towns of Cullinan and Rayton and open bushveld north of the R573 roadway.

The area forms part of the savannah and grassland biomes of South Africa (Mucina and Rutherford 2006). The vegetation varies from a dense, short bushveld to a rather open tree savannah. Seven national vegetation types are found within the Dinokeng area including Sprinbokvlakte thornveld, Loskop Mountain bushveld, Central Sandy bushveld, Marikana thornveld, Norite Koppies bushveld, Gold Reef Mountain bushveld and Rand Highveld grassland. (Mucina and Rutherford 2006).

The altitude above sea-level falls from around 1550 m in the far south to as low as 950 m in the extreme north-east. Rivers are made up of smaller non-perennial streams and drainage lines, with few perennial rivers being present. The largest perennial rivers in the area are the Pienaarsriver, which flows northwards from the Roodeplaat Dam close to the western boundary and the Elandsriver which flows northwards to the Rust de Winter Dam, then eastwards towards Marble Hall. These are the two major dams in the Dinokeng area.

The climate in the area varies, becoming warmer and drier from south to north. In the southern parts, the summers are warm and moist with daily temperatures varying between 16 °C and 29 °C and the dry and cold winters sees temperatures varying between 0 °C and 18 °C. Average annual rainfall is around 620-650 mm with 85 % falling between October and March. Frost occurs on 32 of the 100 days between May and August. In the northern parts, the summer

temperatures vary between 18 °C and 31 °C and between 4 °C and 22 °C in winter. Average annual rainfall is 520-550 mm with 85 % falling between October and March.

The grassland bushveld habitat of the greater Dinokeng area supports an abundance of wildlife. Game farms and lodges in the area also own and manage their own wildlife. Approximately 700 plains zebra inhabit the reserve. In October 2011 the area was restocked with elephant (*Loxodonta africana*) and lion (*Panthera leo)*, but no large predators were present during the course of this study. Another large mammal species in the Reserve is white rhino (*Ceratotherium simum*), with the introduction of African buffalo (*Syncerus caffer*) being planned for 2012. Other predators, like leopard (*Panthera pardus*) brown hyena (*Parahyaena brunnea)* and black-backed jackal (*Canis mesomelas*) also inhabits the area, although their numbers are not known.

Figure 2: The location of Dinokeng Game Reserve in South Africa (outlined in white).

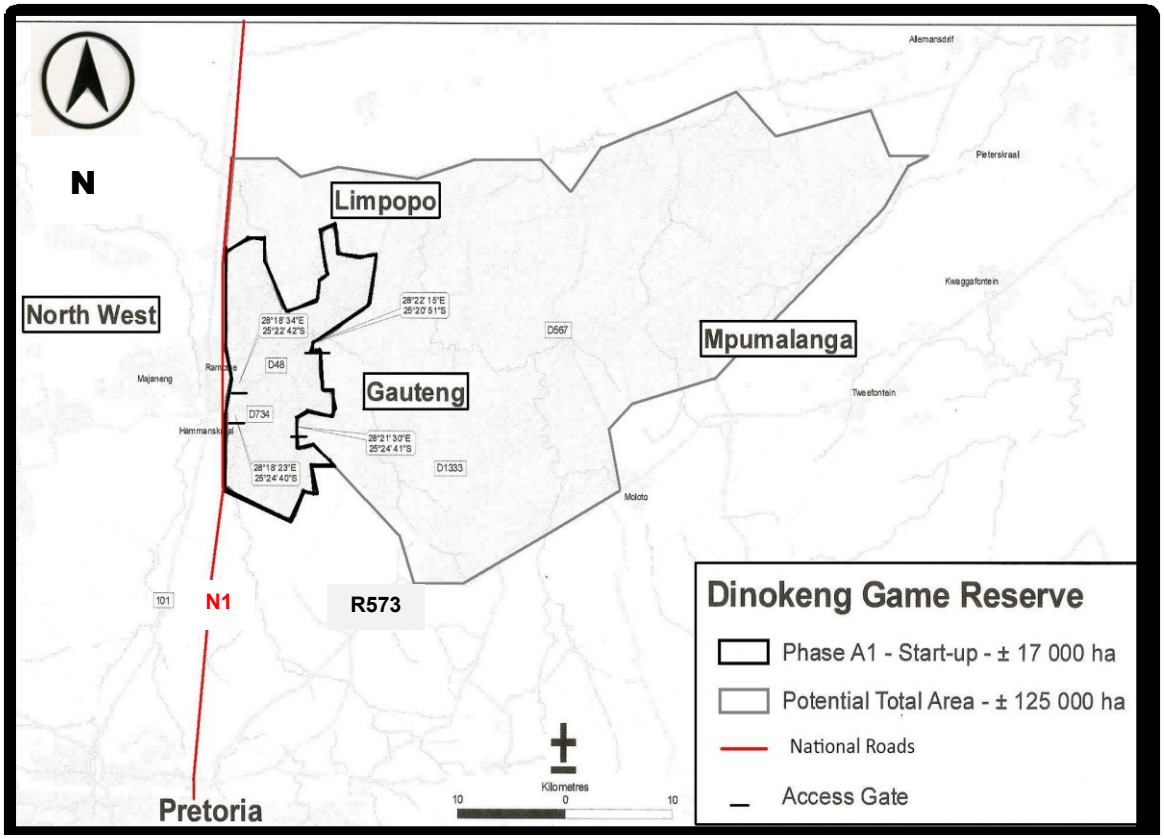

Figure 3: The location of The Dinokeng Game Reserve, showing the start-up (outlined in black) and developing phases (outlined in grey).

1.7 Objectives of the study

The overall aim of this study is to investigate the group size effect on vigilance and its endocrine correlates in a free-ranging plains zebra population living in a low predation risk environment.

The study has the following objectives and specific questions:

1) To test the group size effect and its underlying assumptions by simultaneously investigating individual and collective vigilance in plains zebra bands living in an environment with low adult predation risk.

a) What is the relationship between individual as well as collective vigilance levels and group size?

b) What is the relationship between individual and collective vigilance levels?

2) To investigate the effect of season (wet vs. dry) and the presence of foals on individual and collective vigilance levels in plains zebra bands living in an environment with low predation risk.

a) What is the difference between wet (summer) and dry (winter) seasons in terms of individual and collective vigilance levels?

b) What is the effect of the presence or absence of foals in a band on individual and collective vigilance levels in this population?

3) To investigate the impact of local environmental and social factors on adrenocortical endocrine activity in bands of plains zebra living in an environment with low predation risk, but specifically, the cumulative effect of group size and sex on faecal glucocorticoid metabolite levels in this plains zebra population.

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Chapter 2

The effect of group-size, season and foal presence on individual and collective vigilance in Plains zebra (*Equus burchelli*) with low predation risk

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2.1 Abstract

In order to respond to predation risk, prey species need to detect the presence of predators through vigilance behaviour. Predator detection relies on the individual's own levels of vigilance (individual vigilance) and on the vigilance of its group (collective vigilance). Here I investigate whether group size and season influence individual and collective vigilance in a population of plains zebra exposed to low adult predation risk. Foals are at risk from the predators present, so I test whether the presence of foals within a band influences rates of individual or collective vigilance. Individual vigilance decreased with group size, while collective vigilance remained constant. Individuals could reduce their own scanning by relying on scanning by other group members. Individual vigilance differs between seasons, but no seasonal effect is found on rate of scanning and collective vigilance. Interestingly, bands with foals have a lower level of individual vigilance than bands without foals, which may be due to added nutritional stress on mothers during lactation. Collective vigilance remained constant so mothers may increase foraging time and still detect predators. Here I confirm a classic group size effect, with individuals decreasing scanning in larger groups and collective vigilance remaining constant across group sizes.

Keywords: Plains zebra; *Equus burchelli*; group size; vigilance; seasons; foals

2.2 Introduction

Predation can either have a lethal (direct) or non-lethal (indirect) effect on prey species (Creel & Christianson 2008). Indirect (risk) effects in prey species may involve changes in spatial redistribution (Creel et al. 2005), activity patterns (Sih & McCarthy 2002), foraging time (Lima & Bednekoff 1999a), movement patterns (Sih & McCarthy 2002), anti-predator vigilance (Hunter & Skinner 1998) and group sizes (Valeix et al. 2009) due to predator detection and avoidance behaviour. These indirect effects (i.e. risk effects) may even be more profound than the direct effect of killing on prey species as these anti-predator responses can have physiological or energetic costs for prey which can alter prey dynamics (Creel & Christianson 2008). For example, since the reintroduction of wolf in the elk range in Yellowstone National Park, the proportion of female elk with calves has declined as a result of reduced reproduction rates (Creel & Christianson 2008). These changes in reproduction were associated with strong changes in female elk behaviour, habitat selection and grouping, and the sum of direct predation and risk effects on calf survival are manifested in changes in survival rates (Creel & Christianson 2008).

In order to respond to predation risk, prey species need to detect the presence of predators (Hunter & Skinner 1998). Consequently, individuals spend some of their time vigilant which aids in risk detection (Krause & Ruxton 2002). However, vigilance is costly, as time that could have been utilised for other activities (e.g. feeding) is used (Treves 2000), except when herbivores adequately match scanning with chewing events (Fortin et al. 2004). Therefore vigilance is a flexible behaviour that responds to risk factors and ecological factors, like degree of satiation and food density (Beauchamp 2009). Furthermore, predictions relating to vigilance

distinguish between the individual's own level of vigilance ('individual vigilance') and the vigilance level of the group it is a member of ('collective vigilance'; Treves 2000).

Group size has an effect on individual and collective vigilance levels in a range of prey species (Lima 1995; Treves 2000; Beauchamp 2008). This 'group-size effect' implies that with an increase in group size individual vigilance levels decline and collective vigilance increases or stays the same (Lima 1995; Treves 2000). It is hypothesised that this group size effect could result from both detection (many-eyes) and dilution effects (Dehn 1990; Beauchamp 2008). These imply that with a greater number of individuals present in a group, the risk of predation to each individual will decrease (due to dilution) and level of individual vigilance will also decrease (due to more individuals looking, or increased collective group vigilance, i.e. many eyes; Lima 1995; Treves 2000; Beauchamp 2008). A group size effect has been shown in a number of species such as impala (*Aepyceros melampus)*, springbok (*Antidorcas marsupialis*) wildebeest (*Connochaetes taurinus*), Tibetan antelope (*Pantholops hodgsoni*) and white-tailed deer (*Odocoileus virginianus*) (Scheel 1993; Burger & Gochfeld 1994; Hunter & Skinner 1998; Burger et al. 2000; Xinming et al. 2007; Clayton 2010). Interestingly, some species do not show a decline in individual vigilance with increasing group size such as primates, giraffes (*Giraffa camelopardalis*) and elk (*Cervus elaphus*) (Treves 2000; Laundré et al. 2001; Cameron & du Toit 2005). This may be because other factors can confound the group-size effect on vigilance including food density and quality (affected by seasons and rainfall), habitat obstructions and visibility, and breeding status of individuals (Shi et al. 2010).

In months of high rainfall (summer) food density and quality will increase resulting in decreased levels of feeding activity and scramble competition for resources. In summer with grass growing tall and lush, visibility will decrease, which ultimately inhibits the prey's ability to see danger approaching. This will force group members to increase individual vigilance levels as to ensure the detection of predators. Predation risk may also increase during summer as the conditions makes stalking easier and predator detection more difficult (Burger et al. 2000). Favourable conditions exist in the summer months for the birth and rearing of young. Therefore, during summer months predators will especially concentrate on juvenile ungulates, the most vulnerable members of a band (Owen-Smith 2008), and mothers may increase their own vigilance levels during this period. Vigilance levels can also be influenced by intra-group competition, risk and aggression (Hunter & Skinner 1998; Treves 2000; Lung & Childress 2006). Furthermore, individual vigilance can be influenced by the composition of groups, inter-individual differences and the position of individuals, because these factors affect how the animals judge group size and perceive the risk of predation (Treves 2000).

However, collective detection is the core assumption of the 'many-eyes' hypothesis, whereby at least one group member should be alert at all times so that at least one member is able to detect a threat (Lima 1995). This ensures that even if individual vigilance decreases with increasing group size, collective detection will increase or stay the same and a threat will still be detected. Lima & Bednekoff (1999b) also present evidence that while individual vigilance decrease, individuals may still engage in head-down vigilance, which may add to the collective detection of threats. This may enable non-vigilant animals to still detect predatory attack, monitor group mates and transfer information within the group (Lima & Bednekoff 1999b).

However, even though changes in individual vigilance have been documented in various species, (e.g. Hunter & Skinner 1998; Xinming et al. 2007) a positive relationship between group size and collective vigilance has only rarely been investigated or demonstrated (Pays et al. 2007). The assumption that individual and collective vigilance co-vary in a predictable way needs to be tested in a variety of species.

Prey species may also allocate their behavioural time budgets according to other factors besides predation risk (Hopewell et al. 2005; Xinming et al. 2007) including resource availability (bottom-up processes), nutritional requirements, reproduction status, intra and inter–group aggression, group size, social relationships between group members and disease (a top-down process) (Hunter & Skinner 1998; Treves 2000; Grange & Duncan 2006; Lung & Childress 2006; Shi et al. 2010).

The Plains zebra (*Equus burchelli*) suffers from high predation and aggregates in groups (bands) (Fischhoff et al. 2009; Thaker et al. 2010). Plains zebra form stable, long-term and non-territorial social systems (bands) (Rubenstein 2010) with these usually consisting of one male, up to six females and their offspring – an unusual structure because females in bands are unrelated (Neuhaus & Ruckstuhl 2002). Predation on foals is a particularly important determinant of population growth rates in plains zebra (Grange et al. 2004). Interestingly, in different ecosystems, different factors act to regulate zebra populations and their behaviour (Grange & Duncan 2006). Some evidence suggests that when large mammalian predators are not abundant, zebra populations and their behaviour will mostly be regulated by food resources, the social structure of bands and the presence or absence of males (Grange & Duncan 2006, Simpson et al. 2011).

In this study I test the relationship between group size and individual and collective vigilance levels in a plains zebra population living in an environment with low adult predation risk due to the absence of lions in the Reserve. I aim to test the group-size effect and underlying assumptions by simultaneously investigating individual and collective vigilance in zebra groups of varying size. I hypothesize that 1) Individual vigilance declines with increasing group size, 2) Collective vigilance stays the same or increases with increasing group size and 3) There is a positive relationship between individual and collective vigilance. I also test for an effect of season (wet vs. dry) on individual and collective vigilance levels, since this may influence susceptibility to predation, or the ease of predator detection. Finally, I test whether the presence of foals in a band influences vigilance, since foals are most susceptible to predation in the absence of lions. I hypothesize that individual and collective vigilance levels will be higher in summer than winter, and that the presence of foals in a band will lead to an increase in both individual and collective vigilance due to foals being more vulnerable to predation by smaller predators in the reserve.

2.3 Materials and Methods

2.3.1 Study Species and Site

Plains zebra are large-bodied (~ 250 kg), grazing ungulates, diurnal and inhabit mesic habitats like grasslands and savanna woodlands with many wild populations being seasonally migratory (Hack et al. 2002; Blom 2009; Rubenstein 2010). Lions (*Panthera leo*), wild dogs (*Lycaon pictus*), cheetahs (*Acinonyx jubatus*) and spotted hyenas (*Crocuta crocuta*) are the main predators of plains zebra (Cillié 2004). I studied a population of approximately 106 plains zebra in Dinokeng Game Reserve with band size ranging from two to nine.

The newly proclaimed and developing Dinokeng Game reserve (DGR) is approximately 18 000 hectares in size and is situated in the North-West quadrant of the Gauteng Province of South Africa (S 25 22.693 E 28 19.257). The area forms part of the savannah and grassland biomes of South Africa and the vegetation varies from a dense, short bushveld to a rather open tree savannah (Mucina & Rutherford 2006). Average annual rainfall is around 620-650 mm in the south and 520-550 mm in the north with 85 % falling between October and March, classified as the wet season. In October 2011 the area was restocked with elephant (*Loxodonta africana*) and lion (*Panthera leo)*, but no large predators were present during the course of this study. Other predators, like leopard (*Panthera pardus*), brown hyena (*Parahyaena brunnea)* and black-backed jackal (*Canis mesomelas*) also inhabit the area, although their numbers are not known. Leopard may prey on both adult zebra and foals (Estes 1991) and black-backed jackal may prey on foals (Walton & Joly 2003).

2.3.2 Field Methods

The main study period was during the winter dry season (April–June) and summer wet season (November-December and March) months of 2010 and 2011. Field data was collected every day from dawn to mid-morning (0600 – 1000 hours) and again from mid-afternoon to dusk (1500 – 1800 hours), the time when zebra bands are most active (Blom 2009).

I identified bands of zebra when these conformed to the usual social structure of this species with one male, females and their foals (Rubenstein 2010). Bachelor groups were not studied. The bands were identified by the number of individuals in the group (band) and the presence of young. Every individual was identified by sex and approximate age where possible. Bands were

viewed at an approximate distance from inside a vehicle to minimize viewer disturbance of the band.

To collect vigilance data from the whole band, I used a hand-held digital video camera (Sony HDR-XR100E Handycam). Once the band was in a relaxed state and feeding, and each member of the band was in the camera's field of view, the whole band was recorded for a maximum of 30 minutes. The recording was stopped when an individual or the whole band disappeared from view. When possible, the band was followed by vehicle and recording was resumed when the animals were feeding and all individuals were in view. For each observation I recorded the date, time of day, length of recording, band identity, the number of individuals in the band and their sex plus information on the weather, habitat, and other species in the vicinity. In total 61 recordings amounting to 10 hours of behavioural data were collected.

2.3.3 Video and Data Analysis

An animal was classified as vigilant when it raised its head above horizontal scanning its surroundings and did not move its feet (after Pays et al. 2007). To determine individual vigilance, focal animal sampling was used (Altmann 1974; Martin & Bateson 1993). For every individual in the band (except foals), the start and end-time of each scanning bout was recorded. To determine collective vigilance the process was repeated, but the group was treated as a unit so that the length of time that at least one individual in the band was scanning was recorded. From this, the proportion of time during the recording that each individual was vigilant was calculated plus the proportion of time of the recording during which at least one member of the group scanned the environment. The frequency of scanning for every individual was also measured (scans per minute).

2.3.4 Statistical Analysis

To calculate whether a predictive relationship exists between 1) group size and individual vigilance, 2) group size and collective vigilance and 3) individual vigilance and collective vigilance a linear regression analysis was done (Zar 1999).

To test for the influence of seasons (wet vs. dry season) as well as the presence of foals in a band on individual and collective vigilance levels and also rate of scanning a two-tailed t-test (unpaired) or Mann-Whitney rank sum test was used (Zar 1999). A two-tailed t-test (unpaired) was specifically used to compare individual and collective vigilance levels between seasons (wet vs. dry) and between bands with and without foals but also to compare the rate of scanning between seasons. To specifically compare the rate of scanning between bands with and without foals a Mann-Whitney rank sum test was used. The α (alpha) level of significance was set at 0.05. Data was analysed with the statistical software package Statistica© (Hill & Lewicki 2007) and Microsoft Excel© (Albright et al. 2008).

2.4 Results

2.4.1 Group Size Effect

Individual vigilance decreased with increasing group size (Fig. 1, $F = 7.12$ $r^2 = 0.03$ $P = 0.008$), but collective vigilance remained constant (Fig. 2, $F = 0.83$; $r^2 = 0.01$; $P = 0.36$), despite a positive relationship between individual and collective vigilance (Fig. 3, $F = 105.41$; $r^2 = 0.30$; $P < 0.001$). Individuals also scanned less often in larger groups (Fig. 4, $F = 15.796$; $r^2 = 0.06$; $P < 0.001$).

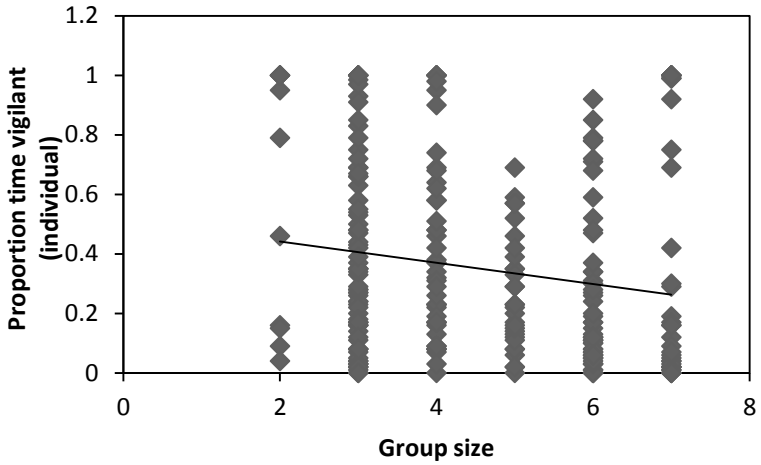

Fig. 1. The effect of group size on the proportion of time that each individual in a group is vigilant (individual vigilance). The line shows the negative correlation between individual vigilance and group size in this plains zebra population.

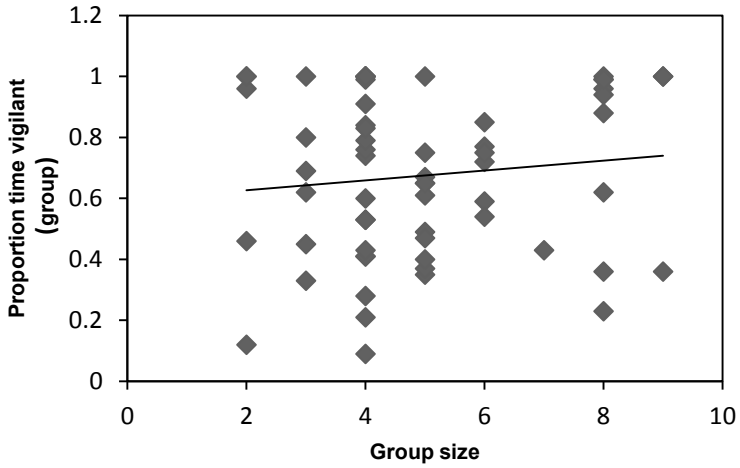

Fig. 2. The effect of group size on the proportion of total time that at least one individual in the group is vigilant (collective vigilance). The line shows the positive correlation between collective vigilance and group size in this plains zebra population.

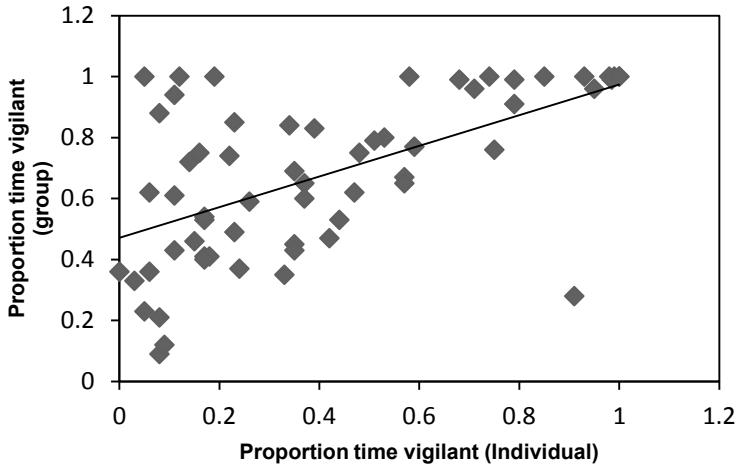

Fig. 3. The relationship between individual and collective vigilance levels is positive (line) in this plains zebra population.

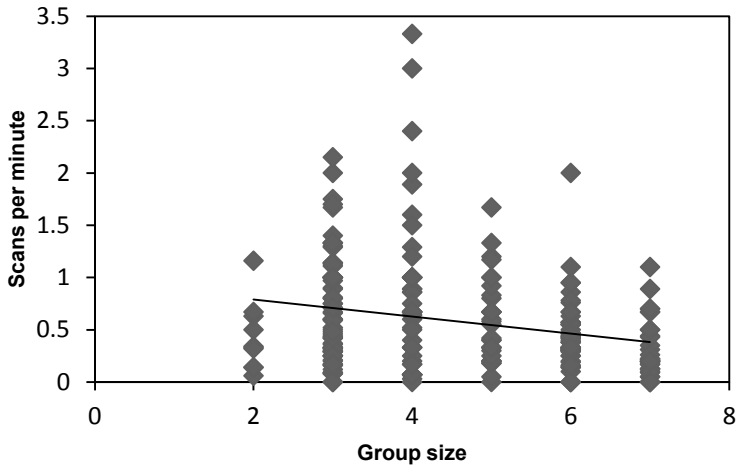

Fig. 4. The effect of group size on the rate of scanning (scans per minute) of individuals in a group. The line shows the negative correlation between these two variables in this plains zebra population.

2.4.2 Seasonal Effect

Individuals were more vigilant in the summer than in the winter (Fig. 5, *t* test: $t_{222} = 1.94$; $P = 0.05$). However, individuals did not change their rate of scanning, nor did collective vigilance change with season (scans per minute: Fig. 7, *t* test: $t_{217} = 0.16$; $P = 0.87$, collective vigilance: Fig. 6, *t* test: $t_{59} = 0.73$; $P = 0.47$).

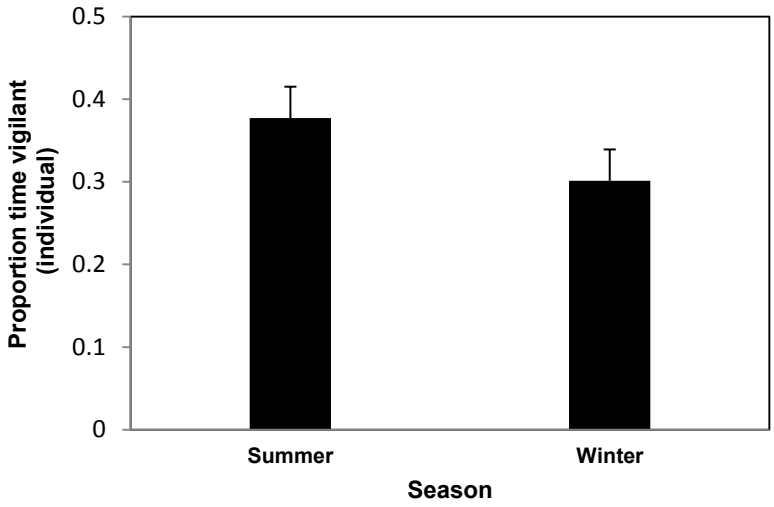

Fig. 5. Bar-plots (mean ± SE) of the difference in mean individual vigilance levels of plains zebra individuals in summer and winter.

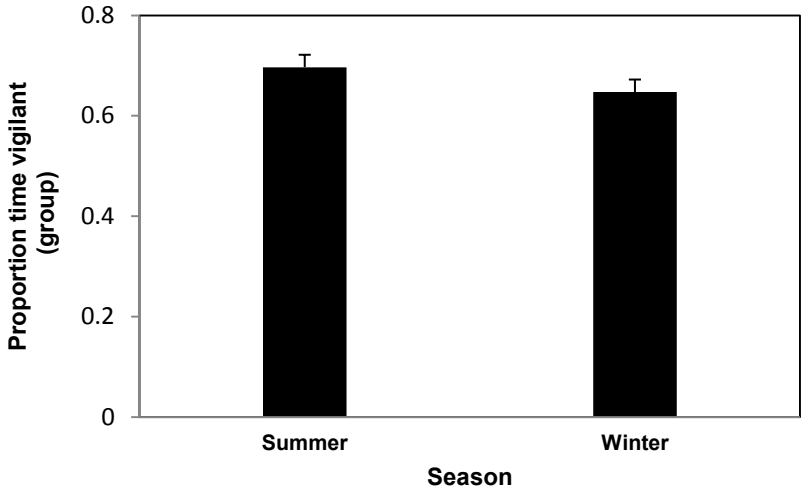

Fig. 6. Bar-plots (mean ± SE) of the difference in mean collective vigilance levels of plains zebra bands in summer and winter.

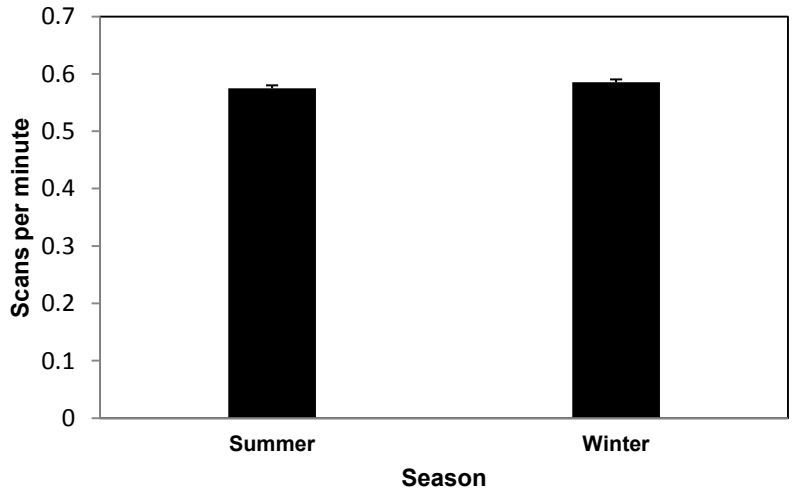

Fig. 7. Bar plots (mean ± SE) of the difference in mean rate of scanning by plains zebra individuals in summer and winter.

2.4.3 Presence or Absence of Foals

The presence or absence of foals in a band has a significant effect on individual vigilance in this population (Fig. 8, *t* test: $t_{138} = 2.09$; $P = 0.04$), with higher individual vigilance levels in bands without foals, although there was no effect of foals on collective vigilance (Fig. 9, *t* test: $t_{40} = 0.02$; $P = 0.99$). So, surprisingly, while collective vigilance remained constant, individuals reduced the amount of time they spend vigilant. There was a significant difference in rate of scanning between bands with and without foals (Fig. 10, $U = 5377.50$; $P = 0.047$), with higher rates of scanning in bands without foals.

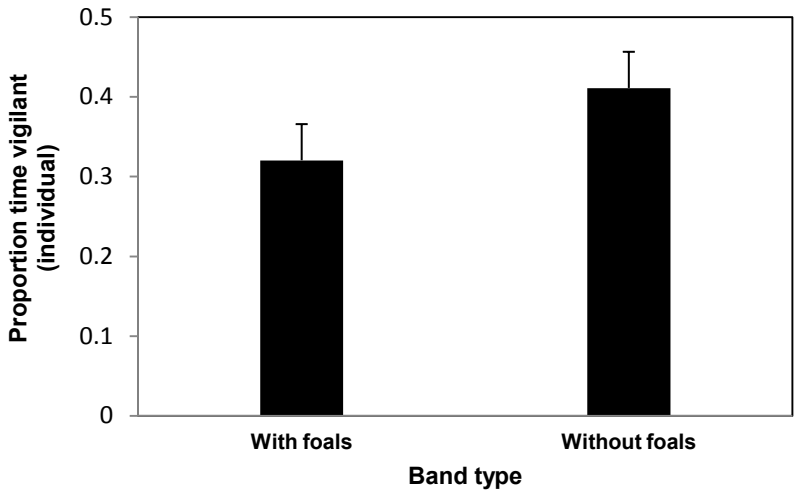

Fig. 8. Bar plots (mean ± SE) of the difference in mean of individual vigilance levels in plains zebra bands with and without foals.

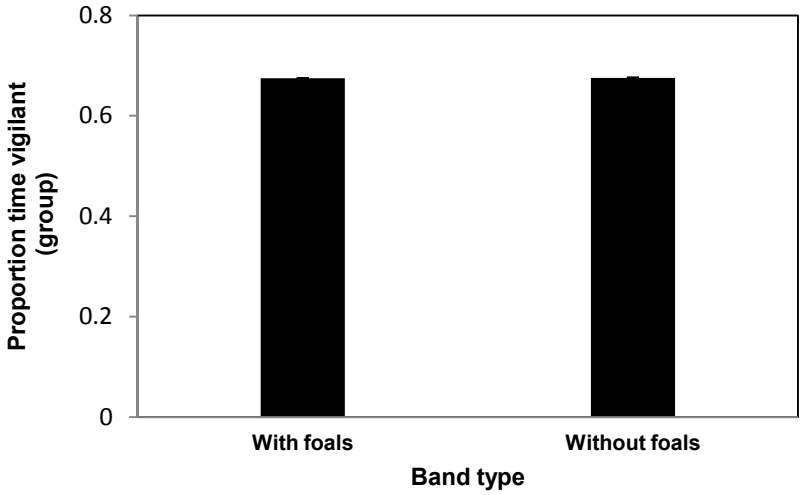

Fig. 9. Bar plots (mean ± SE) of the difference in mean of collective vigilance levels in plains zebra bands with and without foals.

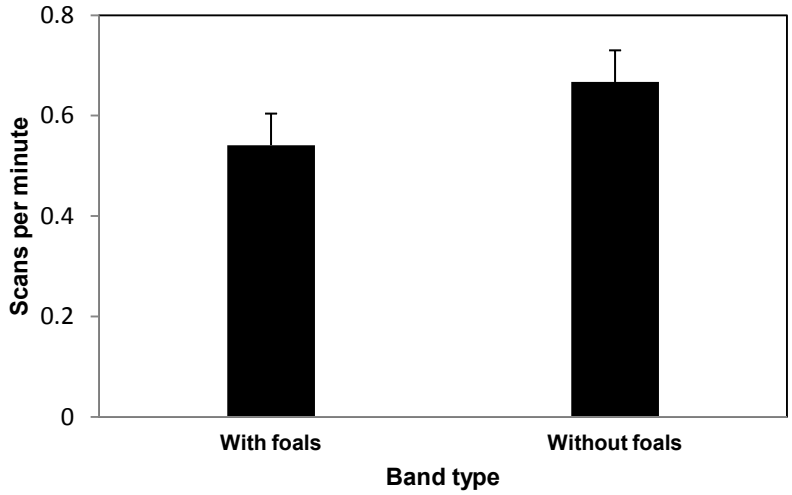

Fig. 10. Bar plots (mean ± SE) of the difference in mean of rate of scanning in plains zebra bands with and without foals.

2.5 Discussion

2.5.1 Group Living and the Group Size Effect

The effect of group size on individual vigilance levels is especially evident in this plains zebra population. The negative relationship between group size and individual vigilance as well as rate of scanning means that even with low adult predation risk, benefits of grouping, like the collective ability to detect predators, reduced levels of individual scanning and increased foraging effort, are still realised, and probably contribute to the maintenance of vigilance behaviour (Hunter & Skinner 1998; Childress & Lung 2003). This also shows that with a greater number of individuals present in a group, the risk of predation to each individual will decrease (dilution effect) and level of individual vigilance will also decrease (due to more individuals looking, or increased collective group vigilance, i.e. the many eyes effect; Lima 1995; Treves 2000; Beauchamp 2008). A similar maintenance of vigilance behaviour with low predation risk has previously been demonstrated in impala and wildebeest (Hunter & Skinner 1998).

Previous studies have shown an increase in collective vigilance with group size (e.g. kudu, *Tragelaphus strepsiceros*; Pays et al. 2012, degus, *Octodon degus*; Ebensperger et al. 2006 and Defassa waterbuck, *Kobus ellipsiprymnus defassa*; Pays et al. 2007) while I found that collective vigilance remained constant with group size in a low predation risk environment. Under both scenarios, individuals can reduce their individual vigilance without decreasing their detection ability, but then collective vigilance should not decrease. Evidence also suggests that while individual vigilance decrease with increasing group size individuals may still be engaging in head-down vigilance, therefore adding to the many-eyes effect (collective detection) (Lima and

Bednekoff 1999b). A strong group-size effect on vigilance is synonymous with habitats with high risk, but because lions, the main predator of this species, were not resident in DGR during this study, other factors, as outlined below, may have contributed to this effect and the level of risk as perceived by the population.

Some evidence suggests that when large mammalian predators are not abundant, zebra populations and their behaviour will mostly be regulated by food resources, the social structure of bands and the presence or absence of males (Grange & Duncan 2006, Simpson et al. 2011). Plains zebra social structure is different from other species where bands consist of one male, unrelated females and their offspring. In social groups with unrelated members all parties must directly benefit from grouping (Jaatinen et al. 2011). In this stable social arrangement females form strong bonds which may reduce aggression and stress and increase reproductive success and foraging time (Rubenstein 1994), possibly even more so in large groups with a large number of females. This may lead to members of large bands spending less time being vigilant and will strengthen the group-size effect on vigilance.

In this unusual environment where low predation risk prevails, vigilance may possibly serve some other purpose which is sufficient to maintain it. The within-group surveillance hypothesis suggests that social monitoring vigilance is used to monitor the behaviour of conspecifics which can be useful to increase knowledge of resources, to follow potential mates and to reduce intra-group aggression and risk (Cameron & du Toit 2005; Lung & Childress 2006) in the presence or absence of predation risk. In this study, where collective vigilance levels remained constant, individuals surrounded by band members that display high levels of vigilance may perceive a

high risk of predation, through transfer of social information, and will increase their own vigilance even if no predator is present (Lima and Bednekoff 1999b; Pays et al. 2009).

The 'scramble competition' hypothesis states that the group-size effect on individual vigilance levels may be due to an increase in scramble competition for limited resources (Beauchamp 2003; Cameron & du Toit 2005; Li et al. 2009). Thus, in larger groups, competition for resources will be high, which will reduce the time that is devoted to anti-predator scanning, also adding to the group-size effect in this population. Being a non-ruminant grazer, zebra can tolerate a range of grass of low quality, but they must obtain a higher daily food intake to meet their metabolic requirements (Thaker et al. 2010) and will therefore spend more time on feeding than on vigilance activities.

2.5.2 Seasonal Effect

There is a significant seasonal effect on individual vigilance levels in this plains zebra population, with higher mean individual vigilance levels being recorded in summer. However, there is no effect of season on collective vigilance or rate of scanning. The Dinokeng Game Reserve receives high rainfall between the months of October and March resulting in an increase in food density and quality and a possible decrease in scramble competition and feeding activity. In summer with grass growing tall and lush, visibility will decrease, which ultimately inhibits the plains zebra's ability to see danger approaching. This will force band members to increase individual vigilance levels as to ensure the detection of predators. Predation risk may also increase during summer as the conditions makes stalking easier and predator detection more difficult (Burger et al. 2000). Although reproduction in this species is

not limited to a certain season or time of the year, favourable conditions exist in the summer months for the birth and rearing of young. During summer months predators will especially concentrate on juvenile ungulates, the most vulnerable members of a band (Owen-Smith 2008), and therefore mothers may increase their own vigilance levels during this period.

In winter months rainfall decreases in the DGR and with it the availability of good quality grazing pastures which increases competition for resources between bands and band members. They feed mostly on low-quality grazing and therefore need to increase their daily food intake even more to meet their metabolic requirements (Thaker et al. 2010). Each band member will increase its feeding activity as to ensure its continued access to the best quality grazing which consequently leads to a decreased level of individual vigilance in winter in this population. Also, as a result of lower rainfall, grass will be shorter and therefore the band's ability to see approaching predators will improve. Consequently, the need of individuals to be vigilant all the time will be greatly diminished, resulting in decreased individual vigilance levels in this population.

2.5.3 Presence or Absence of Foals

The mean level of individual vigilance in bands with foals was significantly lower than in bands without foals. This is the opposite of what was predicted, as predators like leopards and black-backed jackal inhabiting DGR present a real predation risk to foals.

One possible reason for the lower individual vigilance levels in bands where foals are present may be that foals add to the group-size effect (many-eyes hypothesis), because foals are mostly members of large bands. In this study, bands without foals were in the size range of 2 and 3 and

most bands with foals were in the size range of 4 to 9. This may have affected the results due to the inverse relationship between group size and vigilance levels.

In another study on plains zebra, Neuhaus & Ruckstuhl (2002) also found that lactating females exhibited a lower rate of vigilance and higher rate of feeding when compared to non-lactating females. A second possible explanation for this is that mothers have to trade-off their energetic requirements against the risk of offspring predation. As seen in red deer (*Cervus elaphus*; Clutton-Brock et al. 1982) and bighorn sheep (*Ovis canadensis*; Ruckstuhl & Festa-Blanchet 1998) females with foals will be lactating and spending more time rearing their young which increases nutritional stress and energetic costs. Mares compensate for this by increasing their feeding activity (White & Berger 2001). By doing this, individual vigilance in females may decrease. In a study by Hamel & Côté (2008) mountain goat (*Oreomnos americanus*) females with young did not show a higher investment in vigilance possibly due to foraging decreasing the time available for this anti-predatory behaviour.

Thirdly, due to the overall low predation risk in the game reserve, the necessity of increasing vigilance in response to the presence of foals in a band is greatly diminished. Also, with the band structure of plains zebra being stable, band members are able to actively defend themselves against attacking predators (Kruuk 1972). Collective vigilance remains constant which may mean that risk effects associated with the safety of the group does not necessarily increase due to the presence of foals in a band.

To conclude, this study confirms the group-size effect in a plains zebra population exposed to low adult predation risk. Therefore, in habitats devoid of large predators other factors may

largely contribute to maintain this effect. This also means that even with low predation risk benefits of grouping, like the collective ability to detect predators, reduced levels of individual scanning and increased foraging effort are still realised and contribute to the maintenance of individual and collective vigilance. This study also shows that vigilance in prey species may serve some other purpose than protecting the group or individuals against predation, including the monitoring of group members to obtain information on the locality of resources and to minimise inter-group risk and aggression.

Individual and collective vigilance levels of prey species are affected by seasons, but in low-risk environments, this effect will mostly be shaped by resource availability, resource competition, visibility and the breeding status of the individuals in the group.

In environments with low adult predation risk, foals will be most vulnerable to being preyed on by smaller predators. However, as shown in this study, risk levels, individual vigilance and collective vigilance levels are not necessarily higher in groups with foals. This may also be due to the major part that energy requirements play in the decisions and trade-offs made by mothers regarding vigilance behaviour and the protection of their offspring.

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Chapter 3

Group size, sex and its endocrine indicators in Plains zebra (*Equus burchelli*) with low predation risk

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This chapter is prepared for submission to ***African Zoology***

3. 1 Abstract

In environments where adult predation risk is low, other stressors may influence glucocorticoid output in prey species. We aimed to specifically investigate how group size affects adrenocortical endocrine activity in male and female individuals of plains zebra bands in an environment with low predation risk. Therefore, we evaluated the reliability of different enzyme-immunoassays (EIA) for determination of faecal glucocorticoid metabolites (FGM) by assessing the biological validity of the different test-systems for monitoring adrenocortical endocrine function using an adrenocorticotropic hormone (ACTH) challenge test and associated translocation event. Overall, we successfully identified two group-specific EIAs as suitable for assessing adrenocortical endocrine activity in male and female plains zebra, but gender-specific differences in response to ACTH administration were detected. Separation from the band result in elevated FGM levels for up to 6 days. Males in larger bands exhibit higher FGM levels; probably explainable by the females' need for protection, resource competition and reproduction pressure. In contrast, FGM levels in females did not differ between bands of different sizes, indicating that potential changes in vigilance behaviour per se might not alter FGM output in a low-predation risk environment. Our study indicates that group size and sex should be considered when analysing FGM levels in plains zebra.

Key words*:* Plains zebra*,* group size, sex, ACTH challenge, enzyme-immunoassay.

3.2 Introduction

In natural environments mammal species are subjected to conditions that may disrupt homeostasis and increase their physiological stress levels (Romero 2004; Reeder & Kramer 2005). Acute, short-term stress can be beneficial by mobilizing energy reserves and changing behaviour, but long-term chronic stress can have negative implications like reduced reproduction rates and decreased immune function and health which consequently diminishes survival rates over time (Franceschini et al. 2008; Chinnadurai et al. 2009). The effect of long-term stress can be assessed by measuring adrenocortical activity through stress hormones like glucocorticoids, a class of steroid hormones which is secreted by the hypothalamic-pituitary-adrenal (HPA) axis (Möstl & Palme 2002; Franceschini et al. 2008; Chinnadurai et al. 2009). Increased glucocorticoid concentrations are used in ecological and conservation studies as indices of animal well-being in captivity and the wild, but also to monitor how potential extrinsic stressors like competitive conspecifics or predation pressure impact on species at individual and population level (Millspaugh & Washburn 2004; Sheriff et al. 2011).

Free-ranging plains zebra suffer a high risk of predation especially from lions (*Panthera leo*), but also wild dogs (*Lycaon pictus*), cheetahs (*Acinonyx jubatus*) and spotted hyenas (*Crocuta crocuta*) (Cillié 2004; Fischhoff et al. 2007). In order to respond to predation risk, plains zebra need to detect the presence of predators through vigilance behaviour (Hunter & Skinner 1998; Krause & Ruxton 2002). Living in larger groups should add to the detection of predators due to a "group-size effect", which has been studied in a range of mammalian prey species (Lima 1995; Treves 2000; Beauchamp 2008), indicating a negative relationship between group size and the time individuals spend being vigilant. As individual vigilance decreases with increasing group

size, the indirect risk of predation to each individual and the group as a whole will also decrease (due to both dilution and many-eyes effect) (Treves 2000; Beauchamp 2008) which may have an effect of decreasing stress on group level as well.

In an environment where predation risk is low, factors like group size, sex, seasons, availability of resources, reproduction and level of aggression might be predominant determinants of stress-related glucocorticoid output (Huber et al. 2003; Chinnadurai et al. 2009; Ganswindt et al. 2010; Hodges et al. 2010). Two of the major advantages of living in groups are cooperation and social support which may also have the added benefit of decreasing allostatic load (Goymann & Wingfield 2004). The social environment of animals and the presence of a bonding partner can act in a positive way by reducing neuroendocrine changes in stressful situations (Sachser et al. 1998; Wittig et al. 2008). For example, equids aggregate in bands with a stable social system in which females form bonds that may reduce aggression and stress and increase foraging time and reproductive success (Rubenstein 1994; Fischhoff et al. 2009; Thaker et al. 2010). Affiliative social interactions between females could have the effect of improving fitness and reducing stress levels in this species (Cameron et al. 2009). However, as group size increases, the competition over resources is greater (scramble competition), which may increase stress levels (Beauchamp 2003).

Faecal glucocorticoid metabolite (FGM) analysis is an established tool for monitoring responses to stressors (Touma & Palme 2005; Ganswindt et al. 2012). This non-invasive approach is particularly useful because animals are not disturbed in their natural behaviour during collection, sampling can be straightforward and frequent and it is feedback free (Monfort 2003; Millspaugh & Washburn 2004; Schwarzenberger 2007; Franceschini et al. 2008).

However, each assay must be reliably validated for the specific species and matrix to ensure proper quantification of the FGMs (Sheriff et al. 2011). For this purpose and to ensure that the species-specific range of FGMs is detectable by the assay(s) used, an adrenocorticotropic hormone (ACTH) challenge test should be performed (Touma & Palme 2005). When ACTH is administered to a mammal it stimulates the release of glucocorticoid metabolites. After glucocorticoid levels peak in the plasma, they once again return to baseline values (Wasser et al. 2000; Keay et al. 2006). This rise and fall is reflected in the faeces after glucocorticoids are metabolised and excreted (Keay et al. 2006). The ACTH challenge test is a functionality test for this part in the physiological stress response reaction by demonstrating the cause-and-effect relationship between physiological changes and excretion of hormone metabolites (Monfort 2003; Hodges et al. 2010).

Together with a physiological validation, the examination of the respective technique in terms of detecting biological meaningful alterations in the endocrine status is also important (Touma & Palme 2005; Chinnadurai et al. 2009). For such an assessment, a series of samples can be collected before and after a stressful event to demonstrate the viability of the technique in detecting biological changes in FGM levels (Touma & Palme 2005). Procedures that can influence FGM levels on a biological level include immobilization, blood collection, translocation, separation from social partners and confinement (Touma & Palme 2005).

Although adrenocortical activity has already been monitored in various species of Equidae (Schwarzenberger 2007; Franceschini et al. 2008; Flauger et al. 2010; Hodges et al. 2010), only limited information is available for monitoring stress-related physiological responses in *Equus burchelli* (plains zebra) non-invasively. Chinnadurai et al. (2009) monitored the effect of

seasons on FGM levels in plains zebra and Franceschini et al. (2008) demonstrated that, in Grevy's zebra (*Equus grevyi*), FGM levels increased due to capture, translocation and release into an unfamiliar environment. However, to our knowledge no information is currently available on the adrenocortical endocrine activity of plains zebra and the effect of group size and sex on FGM levels in a low predation risk environment.

The overall aim of this study was to investigate the impact of gender and social factors on adrenocortical endocrine activity in bands of plains zebra living in an environment with low predation risk. More specifically, this study aimed at a) Identifying a reliable EIA for determining stress-related physiological responses in plains zebra faeces by performing an ACTH challenge test and comparing FGM levels pre and post translocation as a form of biological validation and subsequently b) assessing the effect of band size and sex on FGM output in a free-ranging plains zebra population.

3.3 Materials and Methods

3.3.1 Study species and site

Plains zebra are large-bodied (~ 250 kg), diurnal, grazing ungulates, which usually inhabit mesic habitats like grasslands and savannah woodlands with many wild populations being seasonally migratory (Hack et al. 2002; Rubenstein 2010).

A wild population of plains zebra was studied at the Dinokeng Game Reserve (DGR) in Gauteng, South Africa (S 25 22.693 E 28 19.257). The area forms part of the savannah and grassland biomes of South Africa (Mucina and Rutherford 2006). The vegetation varies from a dense, short bushveld to a rather open tree savannah. The main study period was during the winter (April–June) of 2010. The resident plains zebra population (approximately 700

individuals) was exposed to low predation risk, with the main predator of the species (the lion) being absent during the course of the study. A total of 41 individuals grouped into six bands ranging in size from four to nine were monitored during the course of the study.

In September 2011, an ACTH challenge test was performed on a six year old stallion and an 11 month old mare at the National Zoological Gardens (NZG) of South Africa, Pretoria, after being removed from their band (family group). Animals were fed on a combination of lucerne and horse pellets and water was available *ad libitum*. The experiment was performed in accordance with the Animal Use and Care Committee (AUCC) of the University of Pretoria (Reference # EC058-11) and the Ethics and Scientific Committee of the National Zoological Gardens of South Africa, Pretoria (Reference # P11/10).

3.3.2 Behavioural observations

In the DGR we opportunistically searched for bands of zebra on a daily basis between 06:00 – 10:00 a.m. and 03:00 – 06:00 p.m. Bands of zebra were identified based on their social structure (Rubenstein 2010) and consisted of one male, a number of females and their offspring. When sighting a band of zebra, the number of adult individuals, the presence of young, and the sex of the individuals were recorded. In addition, vigilance behaviour was recorded as occurring when an individual raised its head and visually scanned the surroundings while feeding (Pays et al. 2007). Every session was recorded with a hand-held digital video camera. In total the 41 individuals of the six bands were observed for approximately five hours.

In the NZG, the two individuals were monitored daily from 06:00 to 18:00 throughout the experiment (14 days) using all-occurrence sampling, described by Altmann (1974) as the recording of all occurrences of certain behaviours during an observation period. Notes were

specifically made on potential stress-related behaviours and vigilance when humans approached the enclosure, faecal samples were collected and the enclosure was cleaned, but also when the individuals were feeding, resting and engaging in normal day-to-day activity. Secondly, attention was given to the social communication between the individuals and their response to being separated from each other (although visual contact was possible). Thirdly, data on noise levels and weather (cloud cover and temperature) were also collected daily.

3.3.3 Faecal sample collection

At the DGR faecal samples were collected every day from dawn to mid-morning (06:00 – 10:00) and again from mid-afternoon to dusk (15:00 – 18:00). The time and area of defecation was noted, and when the band had moved out of area the fresh sample was collected with gloves. The sample was taken from the centre of a dropping, mixed by hand (using rubber gloves), and a 5-10 g portion was placed in individual collection vials. Subsequently, the sample was put on ice and frozen within two hours following collection. In total 32 samples from 41 individuals (males and females) in 6 bands were collected during the study (see Table 1).

Table 1. The faecal sample collection regimen in the DGR.

Band	Size of bands observed	Number of males in band	Number of females in band	Samples collected		
				Total	From males	From females
1	4	1	3	7	0	7
2	9	1	8	8	8	0
3	6	1	5	3	3	0
4	6	1	5	3	2	1
5	9	1	8	6	6	0
6	7	1	6	5	0	5

At the NZG, faecal samples were collected daily from 05:30 to 18:00 from both individuals for 14 days. The first collection every morning included samples that were voided overnight. Faecal

samples were collected from each enclosure following the same protocol that was used in the DGR. In total, 90 samples were collected over the two-week period.

3.3.4 ACTH challenge test

To assure the necessary individual sample collection, the two animals (one stallion, ± 300 kg and one mare, ± 200 kg) were translocated and subsequently housed at the NZG veterinary hospital in separate, adjoining outdoor enclosures. The two plains zebra were immobilized in their normal enclosure with a combination of etorphine (mare: 4.5 mg, stallion: 7 mg; Novartis, South Africa) and azaparone (mare: 50 mg, stallion: 80 mg; Janssen-Cilag, South Africa) and transported to the enclosures mentioned above. Shortly after immobilization, 10 ml blood was collected from the jugular vein of each animal using Vacutainer® plastic tubes. 12 mg diprenorphine (Novartis, South Africa) and 50 mg naltrexone (Kyron Laboratories, South Africa) was administered intramuscularly to each animal to reverse the effects of the immobilization drugs.

After 9-10 days, both zebras were immobilized again using the same drug combination mentioned above. Approximately 10 ml blood was again collected shortly after immobilization. Subsequently, 1 IU/kg Synacthen® Depot (Novartis, South Africa) was injected intramuscularly. The animals were kept in lateral recumbency for 45 minutes after which another blood sample was collected from each animal. Diprenorphine and naltrexone was administered intramuscularly to reverse the effects of the immobilization drugs. Faecal samples and behavioural data were collected for two weeks after which the animals were transported back to their normal enclosure where they rejoined the other two individuals of the band.

3.3.5 Degradation experiment

Faecal sample collection at the NZG was not possible during the night; therefore, the degradation rate of FGM levels post-defecation was assessed to ensure reliable data interpretation.

A single fresh faecal sample was collected from an individual housed at NZG, homogenised and divided into 30 subsamples, which were stored at room temperature. Subsequently, three subsamples each were frozen at -20 °C after 0, 1, 2, 4, 6, 8, 10, 12, 16 and 24 hours. Room temperature (°C) and humidity (%) were measured throughout the experiment.

3.3.6 Sample processing and extraction

After collection, blood samples were put on ice for approximately 60 minutes until clotted, centrifuged at 4000 rpm for 5 minutes and then the serum frozen at -20 °C until assayed. Shortly before the radioimmunoassay was conducted samples were brought to room temperature.

All collected faecal samples were lyophilized for up to 72 hours, pulverised and then sifted using a mesh strainer to remove fibrous material. Approximately 110 mg of dry faecal powder was extracted with 3 ml of 80 % ethanol, vortex-mixed (15 min), and then centrifuged (1500 g for 10 min). The supernatant was decanted into micro-centrifuge tubes and stored at -20 °C until assayed.

3.3.7 Hormone analyses

To measure the amount of immunoreactive cortisol in the blood serum samples, a Coat-A-Count© Cortisol RIA (Siemens Medical Solutions Diagnostics, Unites States) was used. Plain polypropylene and antibody–coated tubes were labelled in duplicate. 25 µl of zero calibrator,

calibrator, controls and samples were pipetted into the prepared tubes. 1 ml of tracer (^{125}I Cortisol) was added to each tube and vortex-mixed. The tubes were incubated at 37 °C for 45 minutes in a water bath, thoroughly decanted, patted dry and counted for 1 minute on a gamma counter (Wallac Wizzard, Perkin Elmer). Results were given in nmol/l and the sensitivity of the assays were 5.5 nmol/l. Cross reactivity was provided in the assay instruction manual.

To measure the FGM concentrations present in the faecal samples, EIAs measuring cortisol (11ß, 17α, 21-triol-20-one), 11, 17 dioxoandrostanes (11, 17-DOA) and glucocorticoid metabolites with a 5β-3α-ol-11-one structure (3α, 11oxo-CM), were used (see Table 2). Sensitivity of the assays as well as intra and inter-assay coefficients of variation, determined by repeated measurements of pool samples, are given in Table 2. The cross-reactivity of the three antibodies are described by Palme & Möstl (1997) for cortisol and 11, 17 DOA and by Möstl et al. (2002) for 5β-3α-ol-11-one.

All assays were performed on microtiter plates according to the methods described by Ganswindt et al. (2002). In brief, 50 µl aliquots of standards, quality controls and diluted faecal extracts were pipetted in duplicate into protein-A coated microtiterplate wells. Then 50 µl of biotinylated label and antibody were added and the plates were incubated overnight at 4 °C. Following incubation the plates were washed four times and 150 µl of streptavidin-peroxidase was added to each well. Following incubation in the dark for 45 minutes while shaking, plates were washed again before 150 µl peroxidase substrate solution was added and plates were further incubated for 30-60 minutes while shaking. The reaction was terminated by adding 50 µl of $4NH_2SO_4$ and the absorbance was measured at 450 nm using an automatic plate reader. Blood

and faecal samples were prepared, processed and assayed in the Endocrine Research Laboratory, Section of Reproduction, PAS, University of Pretoria.

Table 2. Characteristics of the three EIAs used to examine glucocorticoid metabolite levels in plains zebra faeces.

	Cortisol[a]	**11-Oxo-etiocholanolone I**[a] **(11, 17 DOA)**	**11-Oxo-etiocholanolone II**[b] **(5β-3α-ol-11-one)**
Antibody	Cortisol-3-CMO[c]	5β-Androstane-11,17-dione-3-HS[c]	5β-Androstane-3α-ol-11- one-17-CMO[c]
Label	Cortisol-3- CMO[d]	5β-Androstane-11,17-dione-3-glucoronide[d]	5β-Androstane-3α-ol-11-one-17-CMO[e]
Standard	Cortisol	5ß-Androstane-3α-ol-11,17-dione	11-oxoetiocholanolone
Structure of measured metabolites	11ß, 17α,21-triol-20-one	11, 17 DOA[f]	5ß-3α-ol-11-one
Sensitivity[g]	3	3	3
Intra-assay – CV[h]	7.0 -11.60	5.2 - 7.6	6.6 -11.0
Inter-assay – CV[h]	N/A	8.7- 11.7	9.4-12.3

[a] First described by Palme & Möstl (1997)
[b] First described by Palme & Möstl (1997)
[c] First described by Möstl et al. (2002)
[d] Coupled with *N*-biotinyl-1,8-diamino-3,6-dioxaoctane
[e] Coupled with biotinyl-3,6,9-trioxaundecanediamine
[f] 11,17 – Dioxoandrostanes
[g] Given in pg/well
[h] Values represent percentage variance for high and low quality controls

3.3.8 Data analysis

To determine the hormone baseline levels of the ACTH challenge experiment the FGM concentration of all samples collected from 68 – 2 hours before ACTH administration was

averaged for each assay and each individual (t = 0; 100 % of initial hormone concentration). FGM levels of samples collected 0 - 90 hours post ACTH injection, were compared with individual and assay specific baseline levels, and expressed in % increase.

To investigate the effect of separation on FGM levels, as a form of biological validation, the mean FGM concentrations of samples collected from day 1 post separation was used as the FGM baseline levels for each assay and each individual (t = 0; 100 % of initial hormone concentration). FGM levels of samples collected from 2 to 192 hours post separation were compared with individual and assay specific baseline levels, and expressed in % increase.

To calculate the effect of degradation on FGM concentrations post defecation, hormone levels of samples frozen at 1, 2, 4, 6, 8, 10, 12, 16 and 24 hours post collection were compared with the respective hormone concentrations of samples frozen at t = 0, and the changes expressed in %.

Differences in FGM concentrations between the sexes, as well as between band sizes of males and females were examined by using a t-test or Mann-Whitney rank sum test, respectively (Zar 1999). Differences in FGM concentrations between the bands and bands of different sizes were examined by using a one-way ANOVA or Kruskal-Wallis one-way ANOVA on ranks (Zar 1999). Normality was tested with the Shapiro-Wilk test. The α-level of significance was set at 0.05. In cases of all-pairwise multiple comparison procedures, the α-level was adjusted by applying the procedure described by Holm (1979). Data was statistically analysed using SigmaPlot® statistics and graphing software, version 11.0.

3.4 Results

3.4.1 ACTH challenge test

Before ACTH administration (t = 0), cortisol values in blood were 264 nmol/l for the male and 334 nmol/l for the female. After ACTH administration (t = 45 min) measurements showed an increase in plasma cortisol levels of 28 % (339 nmol/l) in the male and 7 % (357 nmol/l) in the female.

In the male the cortisol EIA revealed a maximal increase in FGM levels of 53 % above baseline levels after 30 hours, compared to a maximal increase of 120 % and 111 % above baseline levels after 39 hours, for the 11,17 DOA and the 5β-3α-ol-11-one EIA respectively (Figure 1A). Due to the overall lower response shown, the cortisol EIA it was excluded from any further analysis. In the female the 11, 17 DOA and 5β-3α-ol-11-one EIA revealed a maximal increase of 75 % and 31 % above baseline levels after 39 hours respectively (Figure 1B).

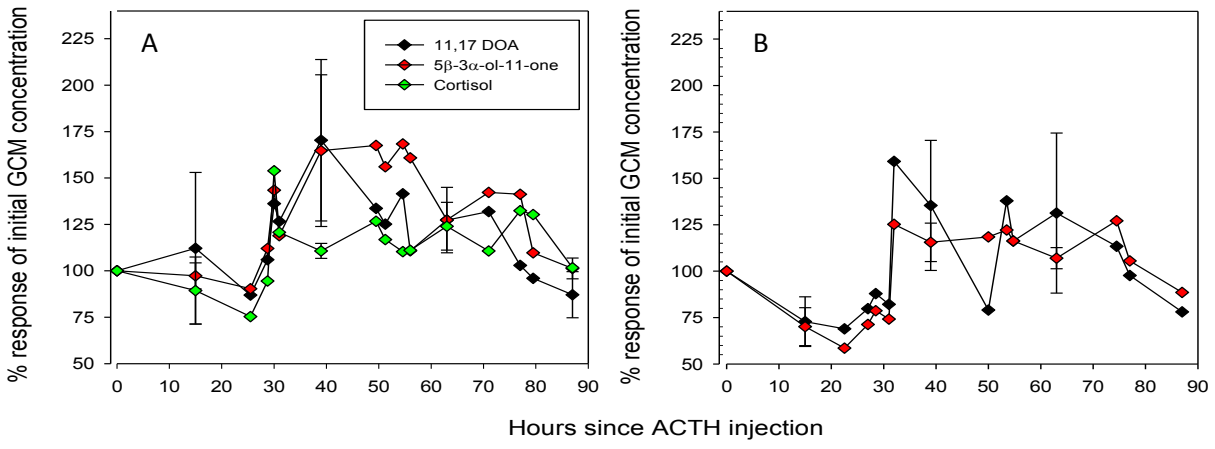

Fig. 1 Immunoreactive GCM concentrations (mean ± SE) in faeces for 90 hours following ACTH injection (A: male, B: female) expressed in % increase to hormone baseline levels. Hormone levels were assessed using a cortisol, 5β-3α-ol-11-one and 11, 17 DOA EIA for the male and a 5β- 3α-ol–11-one and 11, 17 DOA EIA for the female.

3.4.2 Separation

In the male, the 11, 17 DOA EIA revealed a clear increase in FGM levels of 107 % above baseline from 72 hours (3 days) post-separation onwards. FGM levels remained elevated and increased to peak levels of 129 % above baseline 144 hours (6 days) post separation, before marginally declining to a level of 124 % above baseline after 192 hours (8 days). In contrast, the 5β-3α-ol-11-one EIA revealed an increase in FGM levels of just 48 % above baseline 96 hours (4 days) post-separation, with levels steadily declining to 20 % above baseline after 192 hours (8 days) (Figure 2A).

In the female the 11, 17 DOA EIA revealed an increase in FGM levels of 92 % above baseline levels after 144 hours (6 days), thereafter concentrations declined to 31 % above baseline after 192 hours (8 days). The 5β-3α-ol-11-one EIA revealed an increase in FGM levels of 123 % above baseline levels after 96 hours (4 days), remained high for 2 days before declining to 82 % above baseline after 192 hours (8 days) (Figure 2B).

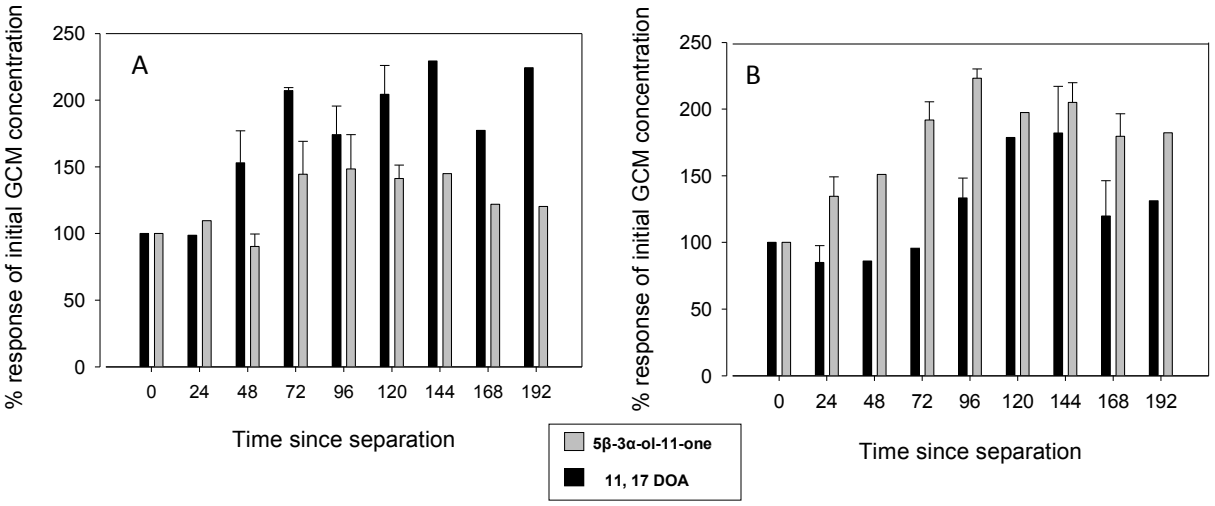

Fig. 2. Bar plots of immunoreactive GCM concentrations in faeces of a male (A) and female (B) plains zebra following separation from band. Faecal extracts were assayed using the 5β-3α-ol-11-one and 11, 17 DOA EIA. Bars represent the mean faecal GCM concentration for every point in time (hours), and error bars the respective SE.

3.4.3 Degradation experiment

The 5β-3α-ol-11-one EIA demonstrated a gradual decrease in FGM concentrations to 84 % after 12 hours and FGM concentrations continued to decrease to 63 % after 24 hours (Figure 3A).

The 11, 17 DOA EIA demonstrated an initial decrease in FGM concentration to 56 % after 2 hours, which steadily increased to 74 % after 8 hours and continued to increase to 161 % after 16 hours (Figure 3B).

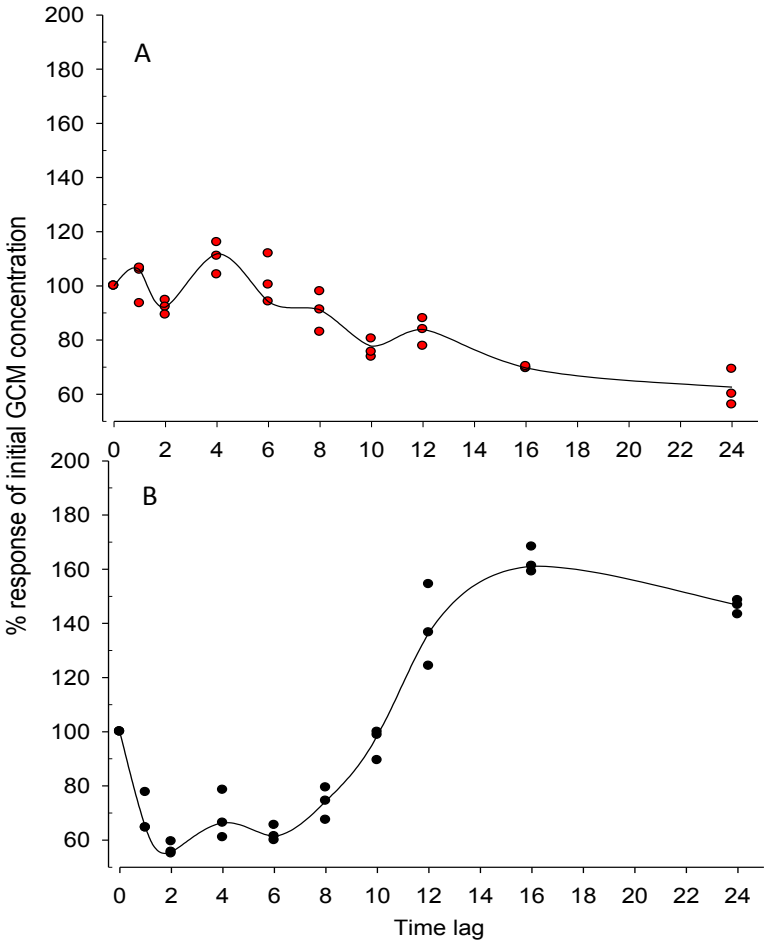

Fig. 3. Faecal GCM concentrations of plains zebra samples subjected to room temperature over a 24-hour period determined using the 5β-3α-ol-11-one (A) and 11, 17 DOA (B) EIA. Dots represent the % response value of each subsample at a specific point in time and the line the respective median of these values.

3.4.4 FGM levels and bands

Between bands, FGM levels determined using the 5β-3α-ol-11-one EIA were significantly different (F = 6.45; P = < 0.001). Post hoc analysis revealed a significant difference in FGM levels between bands 1 and 2 (t = 3.15; P = 0.048), 2 and 6 (t = 4.67; P = 0.001), 3 and 6 (t = 4.27; P = 0.003) and 4 and 6 (t = 3.18; P = 0.049).

Using the 11, 17 DOA EIA FGM levels were also significantly different between bands (H = 14.63; P = 0.012). However, the post hoc analysis revealed only a significant difference in FGM levels between bands 1 and 5 (t = 2.45; P = 0.029), 1 and 6 (t = 6.48; P = < 0.001), 2 and 6 (t = 6.56; P = < 0.001) and 5 and 6 (t = 4.79; P = 0.001).

3.4.5 FGM levels and band size

Between bands of different sizes, FGM levels determined using the 5β-3α-ol-11-one EIA were significantly different (F = 7.61; P = < 0.001). Post hoc analyses revealed a significant difference in FGM levels between bands with a size of 6 and 9 individuals (t = 4.608; P < 0.001) as well as between bands of a size of 4 and 6 zebras (t = 3.414; P = 0.010) (Figure 4A).

In contrast, no differences in FGM levels regarding band size could be found using the 11, 17 DOA EIA (F = 2.787; P = 0.059) (Figure 4B).

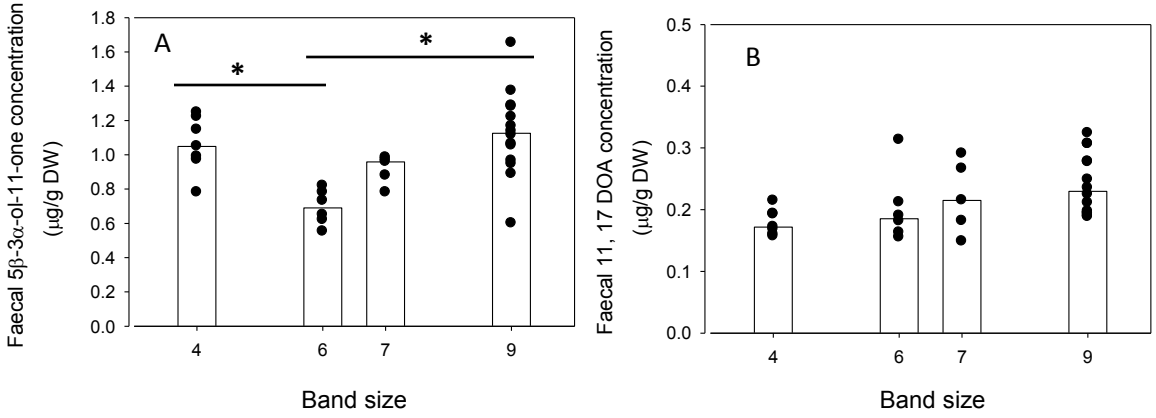

Fig. 4. Dot-bar plots of faecal 5β-3α-ol-11-one (A) and 11, 17 DOA (B) concentrations in plains zebra bands of sizes 4 to 9 animals. The symbols represent the GCM concentration of individual samples and each bar the overall median per band. Asterisks indicate statistically significant differences in faecal GCM concentrations between bands.

3.4.6 FGM levels and sex

FGM levels did not differ between the sexes using the 5β-3α-ol-11-one EIA for analysis (t = 0.510; P = 0.614) (Figure 5A).

In case of the 11, 17 DOA EIA, however, a significant difference in FGM levels was found between males and females (U = 65.0; T = 156.0; P = 0.026) (Figure 5B).

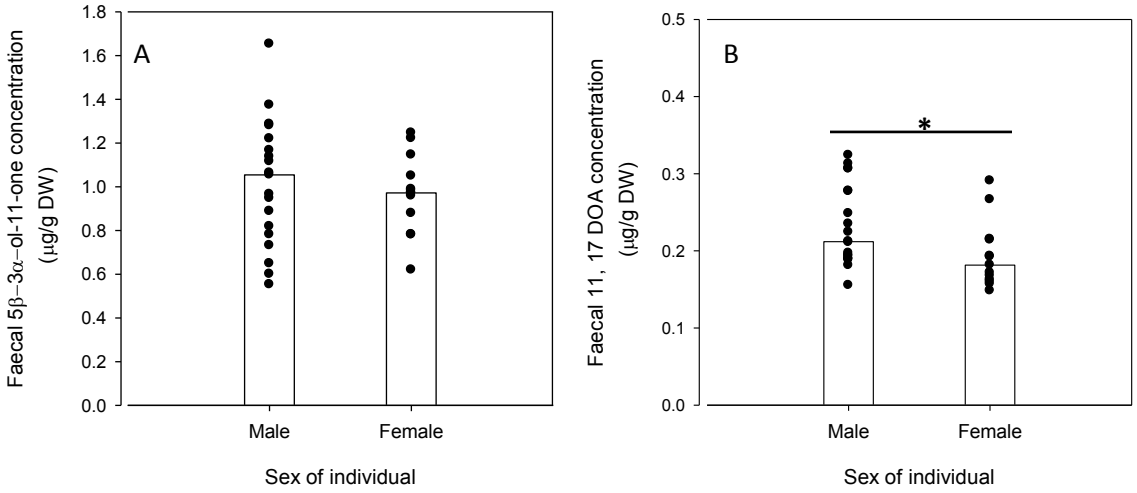

Fig. 5. Dot-bar plots of faecal 5β-3α-ol-11-one (A) and 11, 17 DOA (B) concentrations in male and female plains zebra. The dots represent individual faecal GCM concentrations and each bar the overall median of the faecal GCM concentrations for males and females respectively. Asterisks indicate statistically significant differences.

3.4.7 FGM levels vs. band size in each sex

Male

A significant difference in FGM levels between different band sizes was found using the 5β-3α-ol-11-one EIA (t = 3.605; P = 0.002) (Figure 6A), with higher FGM concentrations in the band with more members.

In contrast, the 11, 17 DOA EIA did not detect a significant difference in FGM levels between bands of different sizes (U = 21.0; T = 36.0; P = 0.211), but showed the same visual trend of higher FGM values in a band of greater size (Figure 6B).

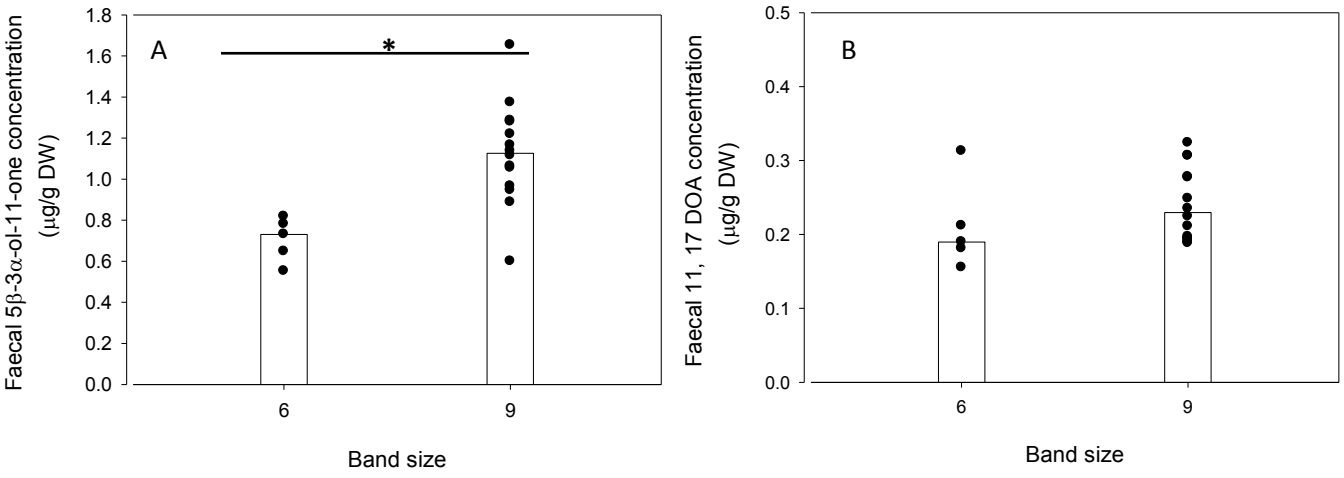

Fig. 6. Dot-bar plots of faecal 5β-3α-ol-11-one (A) and 11, 17 DOA (B) concentrations in males in bands of size 6 and 9. The dots represent the faecal GCM concentrations in samples from each band and each bar the median of the concentrations. Asterisks indicate a statistically significant difference in GCM concentrations between the band sizes.

Female

Using the 5β-3α-ol-11-one EIA for analysis, the higher FGM levels were found in the band with the fewest number of animals. Due to low sample sizes, only the bands with 4 and 7 members were used for statistical analysis. Between these two bands FGM levels were not significantly different ($t = 1.781$; $P = 0.105$) (Figure 7A).

In contrast, using the 11, 17 DOA EIA for analysis, the higher FGM levels were found in the band with the highest number of animals. However, no significant difference in FGM levels was detected between the bands with 4 and 7 members ($H = 2.001$; $P = 0.435$) (Figure 7B).

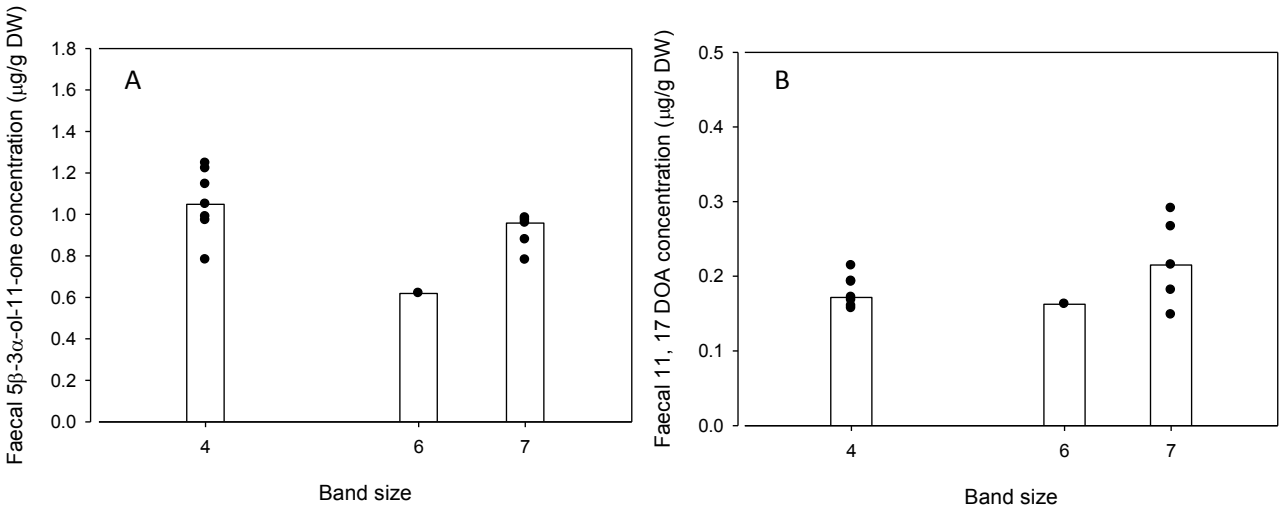

Fig. 7. Dot-bar plots of faecal 5β-3α-ol-11-one (A) and 11, 17 DOA (B) concentrations in female plains zebra in bands of size 4, 6 and 7. The dots represent the faecal GCM concentrations in samples from each band and each bar the median of the concentrations.

3.5 Discussion

3.5.1 Assay validation

Only a minimal increase in serum cortisol levels was detected in the samples collected 40 minutes post ACTH administration. A possible explanation could be that a suboptimal dose of ACTH was used for the experiment. However, similar studies on horses (*Equus caballus*), elk (*Cervus elaphus)* and elephant (*Loxodonta africana*) (Möstl et al. 1999; Wasser et al. 2000; Ganswindt et al. 2003), which detected a significant increase in serum cortisol levels when using doses of 1 IU/kg, are not supporting this explanation. Another explanation for the lag of a substantial increase in serum cortisol levels post ACTH administration may be that the "baseline" samples collected were already reflecting elevated cortisol levels because the elapsed time between darting of the zebra and immobilization (25 minutes) may have been too long as changes in plasma GC levels can be already expected after 2-5 minutes (Romero et al. 2008). Furthermore, the stallion was darted first, and this procedure may have prematurely elevated the serum cortisol levels in the mare as the animals were housed alongside one another. Additional evidence for this hypothesis comes from comparisons with baseline plasma cortisol levels in the horse (*Equus caballus*) (between 80-100 nmol/l, UP Veterinary Hormone Laboratory, personal communication), which present the evidence that the measured plasma cortisol levels preceding ACTH administration were already elevated in the two individuals. Also the translocation event and subsequent separation was reflected in elevated FGM levels, suggesting that the two animals were not relaxed even shortly before ACTH administration, and therefore added to the effect on plasma cortisol levels.

The substantial increase found in FGM levels after ACTH administration confirms the reliability of the ACTH challenge conducted. The revealed peak elevations in FGM levels of up to 120 % above baseline post ACTH administration are comparable to 140 % above baseline post ACTH administration as measured in Grevy's zebra (Franceschini et al. 2008), although different test-systems were used. Two (the 11, 17 DOA and 5β-3α-ol-11-one) of the three EIAs tested revealed a peak in FGM levels post ACTH administration. The cortisol EIA, in contrast, did not reveal the same results and therefore seems not ideal to detect stress-related changes in FGM levels in this species. The two group-specific EIAs have also been successfully validated to detect stress-related physiological responses in other Equidae (Möstl et al. 1999; Flauger et al. 2010) and mammal species (Möstl et al. 2002; Ganswindt et al. 2010; Hulsman et al. 2011) underlining the advantage of these EIAs in being applicable to a wider range of species (Hodges et al. 2010). In this study, peak FGM levels were detected 39 hours after ACTH administration which is comparable to the findings of Franceschini and colleagues (2008) who revealed peak FGM elevations around 50 hours post administration in a female Grevy's zebra.

Various potential stressors, such as noise (from surrounding inner-city roads and neighbourhoods), human (researcher) proximity, restricted movement, lack of retreat space, and social isolation were continuously recognised during the experiment and could have potentially increased baseline FGM concentrations (reviewed in Morgan & Tromborg 2007). In both animals, stress-related behaviours as described by Harewood & McGowan (2005), like pawing, vocalization, and pacing as well as aggressive and nervous behaviour were witnessed up to 8 days after separation. This seems to be reflected in prolonged elevations in FGM levels in both animals after separation over this period which indicates that both individuals have not yet acclimatized to the new environment. Unnatural social configurations, like solitary confinement

of social species, can result in disruptions of species-specific behaviour, physiological issues and chronic stress (Price & Stoinski 2007; Bayazit 2009). Plains zebra are gregarious and aggregate in bands with an inherent stable social system and strong bonds between females (Rubenstein 1994). Also, affiliative social interactions between females are beneficial for individuals and are reflected in a reduction in heart rate and enhancement of fitness through reduced stress levels (Cameron et al. 2009). Therefore, the separation of the young female from the security of the band, the other females (including its mother) and the male (separated by the enclosure wall), will increase its FGM levels. The mating system of plains zebra forces females and males into mixed-sex bands (one male, females and offspring), and the male need to stay close to the females to maximise his reproductive success but also to defend his band of females from other stallions (Neuhaus & Ruckstuhl 2002). Therefore one can understand that the unnatural separation of the stallion from its native band increases its stress levels and FGM levels. Our findings correlate with an increase in FGM concentrations over 5 weeks in Grevy's zebra (*Equus grevyi*) after being captured, translocated and held in pens before being released into a new habitat (Franceschini et al. 2008).

However, the two EIAs used for monitoring the effect of separation on FGM levels revealed not only the expected increase in FGM levels, but also sex-specific differences in terms of detection of group-specific metabolites. Whereas the 11, 17 DOA EIA detects higher FGM levels compared to the 5β-3α-ol-11-one EIA in the male, opposite results were obtained for the female. Similar differences between the sexes, in terms of the structure and quantity of FGMs excreted were also revealed in other mammals e.g. mice (Touma et al. 2003). To reveal further sex-dependent information about the presence and relative abundance of immunoreactive FGMs

in plains zebra faeces, analysis via high-pressure liquid chromatography (HPLC) would be useful.

Changes in FGM concentrations post defecation are expected (Möstl & Palme 2002), which makes it mandatory to keep the time between defecation and collection constant. However, the two assay systems (11, 17 DOA and 5β-3α-ol-11-one) demonstrated different reactions to the experimental setup. The 11, 17 DOA EIA demonstrated an initial decrease in FGM concentrations where after it continually increased. The 5β-3α-ol-11-one EIA demonstrated a continual decrease in FGM concentrations over the 24 hour period. These results correspond to those obtained in similar studies in domestic livestock (Möstl et al. 1999; 2002) and horses (Flauger et al. 2010) and underline the importance of the detailed validation of EIAs.

To summarise, the ACTH challenge test demonstrated that both the 11, 17 DOA and the 5β-3α-ol-11-one EIA are reliable to detect changes in FGM output in plains zebra. In contrast, the cortisol EIA seems not ideal to detect stress-related changes in FGM levels in this species. The separation (biological validation) procedure also identified both group-specific EIAs as equally suitable to detect biologically meaningful stress effects on FGM levels in this species. The degradation experiment further validated the use of the 5β-3α-ol-11-one EIA as it demonstrated a gradual decrease in FGM concentrations over the 24 hour period, compared to the 11, 17 DOA EIA that demonstrated a high variability in changes in FGM levels over the same period.

In this study we have not obtained sufficient information on the presence and relative abundance of immunoreactive glucocorticoid metabolites present in plains zebra faeces and therefore, we cannot further distinguish between the 11, 17 DOA and the 5β-3α-ol-11-one EIA

in terms of their ability to co-measure hormones of non-cortisol origin. Therefore, we recommend both EIAs as equally suitable to detect stress-related changes in FGM output in plains zebra at this stage. However, when conducting studies in the wild where fresh faecal samples are not that easy to obtain, the 5β-3α-ol-11-one EIA may be the assay of choice as it demonstrates a lower variability in changes in FGM levels over time. To reveal further information about the presence and relative abundance of immunoreactive FGMs in plains zebra faeces, analysis via high-pressure liquid chromatography (HPLC) would be necessary.

3.5.2 FGM levels, band size and sex in plains zebra

Significant differences in FGM levels are evident between the 6 different plains zebra bands as determined by both the 11, 17 DOA and 5β-3α-ol-11-one EIA (see section 3.4.4). Bands in DGR often differ in size (number of females and offspring), and the differing FGM levels between bands may be a result of this. Even though results appear inconsistent between the two EIAs when looking at band size as the only factor contributing to this, FGM levels were lower in a band of 6 individuals than those consisting of 4, 7 or 9 individuals. This may mean that in plains zebra, predation risk, vigilance levels and resource competition may affect FGM output on group level, and that a group size of 6 may be best to minimise these effects.

Due to the potential different effect band size has on FGM levels for males and females; it might be advisable to determine potential band size effects on FGM levels separately for the sexes. According to the 5β-3α-ol-11-one EIA, males in bands of greater size exhibit higher FGM values. A similar visual (but not significant) trend can be observed with the 11, 17 DOA EIA. In bands of a larger size, more females are present which increases their need to be protected by the resident male from predation and harassment (Rubenstein 1994). This may

increase allostatic load on the male in a band, through increased vigilance levels and reduced foraging. In bands with more members, the competition over available resources (scramble competition) may be greater which will increase nutritional stress (Beauchamp 2003) and the distance that members must move to access resources also increases because larger groups deplete food patches more rapidly (Silk 2007). Because reproduction in this species is not limited to a certain time of year (Rubenstein 1994), the presence of a high number of females may increase reproduction pressure as perceived by the male which may increase its allostatic load.

Individual vigilance levels in this population differ according to the group-size effect (see Chapter 2), with higher rates of vigilance recorded in smaller groups and vice versa. Therefore, it might be conceivable that a relationship exist between group size and changes in FGM output. However, no differences were found in FGM levels of females in bands of different sizes using both the 11, 17 DOA and 5β-3α-ol-11-one EIAs. A possible explanation may be that even if females exhibit a higher rate of vigilance, this will not alter FGM output per se, because this study was conducted in a predator-free environment. The sample size for samples collected from females was also extremely low (Section 3.3.3, Table 1), which might also explain why no relationship between group size and FGM levels was found in this study. Therefore, this experiment should be repeated not only with a larger sample size of focal bands, but also with an extended period of faecal sample collection.

Our study confirms that sex and group size should be taken into account when analysing differences in FGM levels in plains zebra. The limited information provided in this study indicates that there is a difference in FGM levels between the sexes, at least in the winter. This

contrasts with a study by Franceschini et al. (2008) on a closer-related species, the Grevy's zebra, that found that FGM levels between territorial males, non-lactating females and bachelor males are not significantly different. Interestingly, Franceschini et al (2008) did not detect any significant difference between seasons, which is in contrast to e.g. Chinnadurai et al. (2009) who described seasonal differences in FGM levels in plains zebra. Although it is limited, information is available from other mammals in terms of the link between group size and glucocorticoid output (Colobus monkey (*Procolobus rufomitratus*), Chapman et al. 2007; Ring-tailed lemur (*Lemur catta*), Pride 2005; degu (*Octodon degus)*, Ebensperger et al. 2011; great gerbil (*Rhombomys opimus*), Rogovin et al. 2003). These studies support our assumption that group size has to be taken into account when studying FGM output in social species.

To conclude, we successfully identified a non-invasive assay system suitable for measuring glucocorticoid metabolites in plains zebra, and to monitor the effect of gender-based and social factors on FGM levels in this species. This methodology can now be applied to future studies on the effect of ecological and other social factors, as well as conservation and management practices on the physiological welfare of plains zebra.

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Chapter 4

General Discussion

4.1 Group size, vigilance and its endocrine correlates in plains zebra

In Dinokeng Game Reserve, where adult predation risk is low, the group size effect on vigilance is still present, with a negative correlation between group size and individual vigilance. Therefore, even with low adult predation risk, benefits of grouping, like the collective ability to detect predators, reduced levels of individual scanning and increased foraging effort are still realised, and probably contribute to the maintenance of vigilance behaviour (Hunter and Skinner 1998; Childress and Lung 2003). Levels of collective vigilance remain constant with group size. Therefore, collective vigilance does not decrease in this low predation risk area, ensuring that while individuals reduce their own vigilance, each individual's ability of detecting a predator is not diminished. A similar maintenance of vigilance behaviour in a low predation risk area has previously been demonstrated in impala and wildebeest (Hunter and Skinner 1998).

Until now, the group-size effect on vigilance in plains zebra were only found in habitats with high predation risk (Scheel 1993), but because lions, the main predator of this species, were not resident in DGR during this study, other factors like resource availability, reproduction status, inter-group competition and social structures may have contributed to this effect and the level of risk as perceived by the population (Beauchamp 2007). In Laikipia, where mammalian predators are not abundant, zebras are mostly limited by food resources (Grange and Duncan 2006). The group size effect on individual vigilance levels may be due to an increase in inter-group competition for limited resources, as proposed by the 'scramble competition' hypothesis

(Beauchamp 2003; Cameron and du Toit 2005; Li et al. 2009). Also, when conditions are favourable for the birth and rearing of young, females will be lactating and will need to increase their feeding rate to meet their energy requirements. As a result, they will be spending less time being vigilant. In the stable social structure of plains zebra, females form strong bonds which may increase foraging time and decrease vigilance (Rubenstein 1994), strengthening the group-size effect on vigilance.

In such a low predation-risk environment vigilance may serve some other purpose which is sufficient to maintain it. Vigilance may be used to monitor the behaviour of conspecifics to increase knowledge of resources, follow mates and reduce intra-group aggression (Cameron and du Toit 2005; Lung and Childress 2007). When individuals are surrounded by band members that display high levels of vigilance, they may perceive a high risk of predation, and will increase their own vigilance even in the absence of a predator (Pays et al. 2009).

The vigilance behaviour of the DGR plains zebra population reflect significant seasonal changes, recognised by the higher level of individual vigilance levels displayed in the summer (wet) season. With the higher rainfall, food density increases, scramble competition decreases and with it feeding activity. Vegetation will be denser; therefore conditions are favourable for stalking predators, but the ability of plains zebra to see danger approaching will be lessened considerably. Therefore vigilance needs to be increased during this period.

In the winter (dry) season, competition for resources will be the predominant factor influencing vigilance levels. Good quality grazing is scarce which increases competition between bands and band members. Plains zebra mostly feed on low-quality grazing and thus they need to increase

their daily food intake even more in winter (Thaker et al. 2010), leaving less time for being vigilant. However, because food is limited during winter, group-members will need to obtain information from other group members on the location of possible resources, which will maintain vigilance levels, although not on the same level as recorded in summer. Some degree of anti-predator vigilance will still be required during winter, but because grass height is lower and visibility is improved, vigilance levels will be lower than in summer months.

Adult plains zebra in DGR is exposed to low predation risk, due to the absence of lions in the Reserve, but foals in bands are still vulnerable and may be preyed on by leopards and black-backed jackal (Estes 1991; Walton and Joly 2003). Interestingly enough, vigilance levels of bands without foals were higher than of bands with foals, which do not correlate with previous results found in this and other prey species (Burger and Gochfeld 1994; Hunter and Skinner 1998; Childress and Lung 2003). However, mountain goat females with young also do not show higher investment in vigilance (Hamel and Côté 2008). The presence of foals, as additional members of the group, may add to the group-size effect (many-eyes hypothesis), because foals were mainly found in large bands of sizes 4 to 9. Females with foals also find themselves in the predicament of trading-off their energetic requirements against the risk of offspring predation. These females will be lactating and rearing their young whereby nutritional stress and energetic costs will increase, leading to females increasing their feeding activity to compensate for this (White and Berger 2001). This results in lowered individual vigilance levels in females with foals, as time spent scanning will be reduced.

Due to the overall low predation risk in the game reserve, the necessity of increasing vigilance in response to the presence of foals in a band is greatly diminished. Band structure is also relatively stable and individuals will be able to actively defend themselves against attacking predators (Kruuk 1972). Collective vigilance remains constant between bands with and without foals, meaning that risk effects do not increase due to the presence of foals in a band.

Individual vigilance levels in this population differ according to the group-size effect, with higher rates of vigilance recorded in smaller groups and vice versa. Therefore, it might be possible that changes in FGM levels in individuals are associated with differences in group size. Bands of plains zebra in DGR differ in their size i.e. the number of females and offspring in a particular band, which seems to be associated with differences in FGM output. Even though results appear inconsistent between the two EIAs (11, 17 DOA and 5β-3α-ol-11-one), at least the 5β-3α-ol-11-one EIA revealed significant differences in FGM output between bands, when using band size as the only co-variable in the analysis. FGM levels determined by the 5β-3α-ol-11-one EIA were lower in a band of 6 individuals than those consisting of 4, 7 or 9 individuals. This may mean that in plains zebra, predation risk, vigilance levels and resource competition may affect FGM output on group level, and that from the four different band sizes monitored, a group size of 6 may be best to minimise these effects. However, further research would be necessary to explain the above mentioned assay-specific differences found in this study.

Males in bands of greater size, exhibit higher FGM levels, as determined by the 5β-3α-ol-11-one EIA, probably due to females' need for protection (Rubenstein 1994), reduced foraging, competition over resources (Beauchamp 2003) and reproduction pressure (Rubenstein 1994). In contrast, no differences were found in FGM levels of females in bands of different sizes using

both the 11, 17 DOA and 5β-3α-ol-11-one EIAs. A possible explanation may be that even if females exhibit a higher rate of vigilance, this will not alter FGM output per se, because this study was conducted in a predator-free environment. The sample size for samples collected from females was also extremely low (Section 3.3.3, Table 1), which might also explain why no relationship between group size and FGM levels was found in this study. Therefore, this experiment should be repeated not only with a larger sample size of focal bands, but also with an extended period of faecal sample collection.

Our study confirms that sex and group size should be taken into account when analysing differences in FGM levels in plains zebra. The limited information provided in this study indicates that there is a difference in FGM levels between the sexes, at least in the winter. This contrasts with a study by Franceschini et al. (2008) on a closer-related species, the Grevy's zebra that found that FGM levels between territorial males, non-lactating females and bachelor males are not significantly different. Interestingly, Franceschini et al (2008) did not detect any significant difference between seasons, which is in contrast to e.g. Chinnadurai et al. (2009), who described seasonal differences in FGM levels in plains zebra. Although it is limited, information is available from other mammals in terms of the link between group size and glucocorticoid output (Colobus monkey (*Procolobus rufomitratus*), Chapman et al. 2007; Ring-tailed lemur (*Lemur catta*), Pride 2005; degu (*Octodon degus)*, Ebensperger et al. 2011; great gerbil (*Rhombomys opimus*), Rogovin et al. 2003). These studies support our assumption that group size has to be taken into account when studying FGM output in social species.

Out of the three assays tested, the two group-specific EIAs (11, 17 DOA and 5β-3α-ol-11-one) seems to be most suitable to detect stress-related physiological responses in plains zebra. These

EIAs have also been successfully used in other Equidae (Möstl et al. 1999; Flauger et al. 2010) and mammal species (Möstl et al. 2002; Ganswindt et al. 2010; Hulsman et al. 2011) to determine species-specific responses to environmental and social stressors. This underlines the advantage of this type of EIAs in being applicable to a wider range of species (Hodges et al. 2010). In this study we have not obtained sufficient information on the presence and relative abundance of immunoreactive glucocorticoid metabolites present in plains zebra faeces and therefore, we cannot further distinguish between the 11, 17 DOA and the 5β-3α-ol-11-one EIA in terms of their ability to co-measure hormones of non-cortisol origin. Therefore, we recommend both EIAs as equally suitable to detect stress-related changes in FGM output in plains zebra at this stage. To reveal further information about the presence and relative abundance of immunoreactive FGMs in plains zebra faeces, analysis via high-pressure liquid chromatography (HPLC) would be necessary.

4.2 General conclusions

In plains zebra living in a low predation-risk environment the classic group-size effect on vigilance is still evident. Thus, our results suggest that in this species, this anti-predatory behavioural trait is maintained throughout different habitats and populations regardless of the level of predation risk they are exposed to. Vigilance is affected by seasons and the presence of foals, although not as expected, but other factors, including sex, breeding status, inter-group competition and within-group surveillance may have added to these effects. Prey species show differences between the sexes in terms of vigilance behaviour. In plains zebra, higher vigilance rates in males versus females have been detected (Simpson et al. 2011). In African ungulates, vigilance levels in males and females may also change with breeding status, with adult males

more at risk of being preyed upon in the breeding season and adult females during the gestation period (Thaker et al. 2010). Mothers may also spend more time rearing their young which increases nutritional stress and lead to females increasing their feeding activity and decreasing their vigilance levels, to compensate for this (White and Berger 2001). Intra-specific competition between males and inter-group competition between group members forces them to scan their surroundings to assess the activities and behaviours of rivals or conspecifics (Cameron and du Toit 2005). An increase in group size may shift the balance from anti-predator vigilance to social monitoring vigilance (Cameron and du Toit 2005; Lung and Childress 2007).

This data can be utilised in future studies to investigate the impact of the introduction of the main predator of plains zebra, the lion, into DGR or other habitats on the vigilance behaviour of the species. The differences between males and females but also the effect of habitat type, edge effects, location in band and social influences should be incorporated into studies on vigilance levels in this species. Hereby we will be able to fully understand how much the prevailing predation risk adds to vigilance levels and how much can be attributed to other factors. Comparisons between different prey species in similar habitats will further our knowledge of the "group-size effect" and the way in which predation risk influence this effect.

In this study we identified a reliable, non-invasive assay system as suitable for measuring glucocorticoid metabolites in plains zebra, which could be a valuable tool for monitoring the effect of gender-based and social factors on FGM levels in this species. This methodology can now be applied to future studies on the effect of ecological and other social pressures, as well as conservation and management practices on the physiological welfare of plains zebra, one of Africa's most iconic species.

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