ECOLOGY, PHYSIOLOGY AND BEHAVIOUR OF EQUIDS: DEVELOPING MARKERS FOR INDIVIDUAL STATUS AND POPULATION HEALTH

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ABSTRACT

Understanding the causes of poor population performance is vital for effective species conservation. This thesis aimed to develop physiological and behavioural markers for individual status and population health in two equid species, using faecal hormone monitoring and social network analysis. First, individual physiology and population structure were evaluated in Welsh mountain ponies (*Equus caballus*) in response to an annual management practice involving the temporary translocation of the whole population and the removal of several stallions. An increase in mare faecal corticosterone metabolite concentrations was observed between days 5-10 post-release, however a high degree of variability was observed between individuals. Further investigation is required to validate faecal glucocorticoid measurements in response to an acute stressor. Social network structure was disrupted after the removal of key individuals, signified by an increase in network connectivity and a decrease in the strength of individual ties. Stallion faecal androgen metabolite concentrations were elevated after a time lag of five days and remained elevated until the end of the sampling period, possibly as a result of intensified male competition. These results indicate that targeted removals may have negative consequences for the stability of the social structure of the Welsh mountain pony population. Next, the ecology and physiology of the Cape mountain zebra (*Equus zebra zebra*) was investigated to identify the factors leading to poor population growth. Habitat quality, quantified by the abundance of palatable grass and nutrient content within reserves, was positively correlated with three measures of population performance: population growth rate, zebra density, and the number of foals per mare (or female fecundity). Faecal glucocorticoid and androgen metabolite concentrations were assessed across seven populations; faecal glucocorticoids were elevated aseasonally in populations in low quality habitat, and faecal androgens were significantly higher in populations with heavily male-biased adult sex ratios. Populations in low quality habitat may be chronically stressed as a result of poor nutrition, and excess stallions may cause increased male competition. Both hormones were negatively correlated with population performance. These results indicate two separate causes of poor population growth: low habitat quality and sex ratio skew. The Cape mountain zebra is an example of a partial refugee species, where some populations perform poorly due to their preservation in suboptimal habitat. Physiological markers of individual status were successfully identified, and can be used to indicate population health, to evaluate on-going conservation management strategies, and potentially to inform species response models to future perturbations.

DECLARATION

No portion of the work referred to in thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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CHAPTER ONE – GENERAL INTRODUCTION

The global loss of biodiversity, across all taxa and within all ecosystems, is one of the most important issues faced by society to date. Whilst more than 12% of the world's land cover is protected and investment in conservation activities is increasing, populations continue to decline (Butchart *et al.*, 2010). Significant losses include some of the world's largest carnivores (Ripple *et al.*, 2014) and herbivores (Ripple *et al.*, 2015), with repercussions extending to ecosystem dynamics and functioning (Estes *et al.*, 2011). However, the combined efforts of conservation researchers, practitioners and policymakers have succeeded on many occasions in preventing extinctions, reversing population declines, and highlighting current and future threats (Dobson & Lyles, 2000; Butchart, Stattersfield & Collar, 2006; Rodrigues, 2006; Donald *et al.*, 2007; Hoffmann *et al.*, 2010, 2011; Simberloff *et al.*, 2011; Sodhi *et al.*, 2011; Balmford, 2012; Deinet *et al.*, 2013; Chapron *et al.*, 2014; Madliger *et al.*, 2016), particularly when compared to if no action had been taken at all (Hoffmann *et al.*, 2015). The passion for conservation remains pervasive in the face of increasingly pessimistic scenarios. Conservation scientists continue not only to identify broad-scale declines and losses, but pledge to save the species that remain (Ripple *et al.*, 2016). As such, the future of conservation science lies in identifying knowledge gaps and areas of weakness, and generating new methods to achieve practical and attainable goals. This thesis aims to contribute to that progress.

Protected area bias and the refugee species concept

The establishment of protected areas has been widely supported and is perceived as one of the most effective techniques for conservation (Leverington *et al.*, 2010), however they are known to have some weaknesses. There is a disparity between the level of risk and the amount of protection an area receives (Hoekstra *et al.*, 2005), and the location of protected areas is biased towards remote areas of high altitude ("rock and ice" landscapes) where the pressure of land conversion is already low (Joppa & Pfaff, 2009). These regions generally have low productivity, and often inadequately represent the variation in vegetation types and the optimal range of many species of fauna (Scott *et al.*, 2001). As such, the entire range of many extant species does not coincide with any protected area at all (Rodrigues *et al.*, 2004a). Termed ‘gap species’, they include many critically endangered fauna (Rodrigues *et al.*, 2004b). Moreover, there are many more species whose range only partially overlaps with any protected area (‘partial gap species’); while difficult to elucidate on a large scale this overlap may not be enough for effective conservation (Rodrigues *et al.*, 2004b). In fact, there is limited evidence in general about the effectiveness of protected areas at protecting habitats and fauna (Geldmann *et al.*, 2013).

An additional and potentially more insidious problem is described by the ‘refugee species’ concept (Kerley, Kowalczyk & Cromsigt, 2012), where a species undergoes severe range contraction and then persists in areas of suboptimal habitat as its core or preferred habitat has become unavailable. Fretwell's (1972) free distribution theory of habitat selection discusses how population fitness is greatest in high quality habitats and declines in marginal habitat; these can be termed ‘source’ and ‘sink’ populations (Pulliam, 1988). Sink populations usually have a lower density of individuals and are maintained by immigration from source populations (Pulliam & Danielson, 1991), however when habitat loss and range fragmentation lead to the isolation of such populations they may be at risk of extinction due to depressed growth rates and poor reproductive success (Kerley *et al.*, 2012). An important aspect of the refugee species concept is that when species isolation occurs over several generations, the perception of a species’ core habitat can change (Kerley *et al.*, 2012). Also referred to as ‘shifting baseline syndrome’, this has been discussed in detail with regard to fishing stocks (Pauly, 1995; Pinnegar & Engelhard, 2008), and has been identified as a real concern for conservation policy making (Papworth *et al.*, 2009). It can result in the active conservation of species in unsuitable ecological conditions due to a historical misconception of its optimal habitat or niche, with management strategies such as translocations and introductions targeted towards suboptimal habitat (Kerley *et al.*, 2012). Convincing evidence for refugee status has been compiled for the European bison, *Bison bonasus* (Kerley *et al.*, 2012) and the Mediterranean monk seal, *Monachus monachus* (Bocherens *et al.*, 2015; González, 2015), however refugee species may occur much more commonly than is currently recognised.

Instances of gap species (where the range is absent from any protected area) and refugee species (where the range is confined to suboptimal habitat within a protected area) pose clear threats to species conservation. As with partial gap species, it is possible that there are partial refugee species, where some populations are managed in marginal habitat. The prevalence of partial refugee species is not well known and alleviating the effects, which could include low reproductive or survival rates, could be challenging as there is often limited potential for expansion into better quality habitat (Kerley *et al.*, 2012). However, any improvement is impossible if there is no awareness of the problem. Therefore, the development of a method for identifying partial refugee species would not only improve our understanding of species ecology, but also provide valuable data for the effective expansion and establishment of protected areas.

The small population problem

In addition to the creation of refugee species, habitat loss and range fragmentation can often lead to small, isolated populations with limited dispersal opportunities. Small populations are more susceptible to the loss of genetic diversity through random genetic drift and subsequent inbreeding depression (Lande, 1988), which threaten to spiral into an ‘extinction vortex’ (Caughley, 1994). Moreover, they are more vulnerable to demographic stochasticity (Lande, 1993), which can result in large fluctuations in demography (e.g. sex ratio) that reduce effective population size (Nunney, 1993). Evolutionary theory suggests that sex ratios should remain balanced due to selection towards the rarer sex (Werren & Charnov, 1978), however empirical evidence has shown that severe fluctuations in sex ratios can lead to population collapse (Le Galliard *et al.*, 2005). Furthermore, small populations may experience Allee effects (Allee *et al.*, 1949), where individuals occurring in small numbers or at low densities have a reduced fitness, for example due to difficulty in finding mates and dispersing into new social units, or decreased survivorship as cooperative behaviours become less effective (Courchamp, Clutton-Brock & Grenfell, 1999). Multiple Allee effects could be present in a population simultaneously, however the strength of each component is difficult to quantify and empirical evidence is scarce (Berec, Angulo & Courchamp, 2007). In addition, Allee effects may confound our interpretation of species distribution and habitat preferences as conspecific attraction may drive increased densities of individuals into areas of marginal quality (Courchamp *et al.*, 1999). Whilst thoroughly discussed theoretically, the prevalence and impact of Allee effects or demographic skew is not well understood empirically. By monitoring population demography and aspects of social behaviour, we may be able to determine the occurrence of these issues and how they affect individual fitness and population performance, thereby informing future management strategies.

The issues faced by small and fragmented populations discussed here, namely population size, density, demography and environmental quality, could each contribute to poor population performance. To further complicate matters, demographic and environmental factors are difficult to assess in isolation, particularly in non-experimental situations. They undoubtedly interact with each other, for example population demography and density can affect how a species responds to environmental challenges (Coulson *et al.*, 2001). To develop a better understanding of how environmental and demographic factors impact on performance, we need to identify markers that provide insight into how a species or individual perceives its environment and how it responds to various stressors. These ‘biomarkers’ must be relatively easy and quick to collect on a broad scale, as well as minimally invasive and practical in an *in situ* setting, in order to be viable for use in conservation research. Moreover, the integration of different disciplines, such as physiology and behaviour, to provide multiple indicators will provide a holistic assessment of population health and the response to perturbations. The need for such empirical measures, also referred to as evidence-based conservation, has been recognised in the literature for over a decade, yet we still rely heavily on anecdotal and experience-based information to inform management decisions (Sutherland *et al.*, 2004; Legge, 2015). Even in countries that are relatively well resourced there is a struggle to sufficiently identify, monitor and – perhaps most importantly – adequately assess the outcome of conservation objectives; the situation in developing countries, where the majority of biodiversity hotspots are found, can only be worse (Cook, Hockings & Carter, 2010). Next, I outline the approach and the physiological and behavioural markers used to evaluate population health in relation to environmental and demographic factors during this PhD research.

Broad-scale physiological markers

The identification and mechanistic understanding of broad-scale physiological patterns, termed macrophysiology, is a hugely promising discipline for use in conservation research. By measuring patterns or variation in physiological traits across a species range we can directly inform (and predict) species abundance and distribution patterns (Chown, Gaston & Robinson, 2004). Macrophysiology incorporates aspects of physiological ecology, functional biogeography, and conservation physiology (Chown & Gaston, 2016). The latter is a relatively new field that employs various physiological disciplines to assess species responses to anthropogenic disturbances, environmental challenges and management interventions (Wikelski & Cooke, 2006). Conservation physiology has been successful in identifying both causes of decline and effective conservation strategies (Madliger *et al.*, 2016), however it is generally used on relatively small spatial and temporal scales. Macrophysiology by definition operates on a broad geographic or temporal scale, however it remains an underutilised approach in conservation research with the majority of studies focused on global temperature change and the marine environment (Osovitz & Hofmann, 2007; Richard *et al.*, 2012; Chown & Gaston, 2016). Moreover, macrophysiology has yet to be employed as a tool to evaluate on-going conservation management strategies.

A broad-scale physiological assessment of population health requires the use of reliable and relatively easy to collect biomarkers. A frequently measured physiological marker for health and stress are glucocorticoids, a group of steroid hormones that have a primary role in energy balance through glucose and salt regulation, and secondly regulate the stress response (Landys, Ramenofsky & Wingfield, 2006). Glucocorticoids fluctuate constantly in response to environmental conditions and challenges, a process termed ‘allostasis’ (McEwen & Wingfield, 2003). Such fluctuations promote physiological and behavioural changes that are an adaptive response to daily or seasonal change, however large perturbations result in ‘allostatic overload’, where energy demand exceeds availability

(McEwen & Wingfield, 2003). This triggers an elevation in glucocorticoids above normally modulated levels, prompting the major physiological changes associated with an acute stress response, such as energy diversion towards cardiovascular function and the suppression of reproductive physiology and behaviour (Sapolsky, Romero & Munck, 2000; Sheriff *et al.*, 2011). Potential stressors include exposure to predators or humans, severe weather events, increased physical activity, and nutritional stress or food deprivation, the latter of which directly influences allostasis due to a reduction in available energy (McEwen & Wingfield, 2003; Busch & Hayward, 2009). While an acute increase in glucocorticoids in response to a challenge can increase survivorship in the short-term, prolonged elevation caused by repeated exposure to a stressor(s) can lead to chronic stress (or “distress”; Linklater 2000). During chronic stress, acute glucocorticoid increases can become more pronounced and take longer to return to baseline levels (Busch & Hayward, 2009). This physiological state can result in many deleterious effects, including suppression of the immune system and cell growth, neuronal cell death, severe protein loss and the inhibition of reproductive behaviour, leading to a reduction in fitness (Busch & Hayward, 2009; Sheriff *et al.*, 2011; Dantzer *et al.*, 2014). Therefore, the identification of potential stressors in poorly performing populations should help target conservation efforts and improve species survival rates.

Another group of steroid hormones that are measured less frequently for conservation purposes are androgens, which are primarily associated with male reproduction and behaviour. Androgens have a variety of functions, including promoting changes to muscular physiology and testes size, regulating spermatogenesis and improving sperm quality, and the development of secondary sexual characteristics (Wingfield, Lynn & Soma, 2001; Miles *et al.*, 2007; Malo *et al.*, 2009; Ezenwa, Ekernas & Creel, 2012). In addition, they have a direct inductive effect on social behaviours related to breeding, dominance and aggression (Rose, Holaday & Bernstein, 1971; Lincoln, Guinness & Short, 1972; Albert *et al.*, 1986; Miller *et al.*, 1987; Muehlenbein & Watts, 2010). Androgens increase physical performance and competitive ability to allow males to achieve greater reproductive success, and they are usually elevated seasonally or during specific life history stages (Wingfield *et al.*, 2001).

However they are associated with high costs, such as suppression of immune function leading to increased parasite burden (Malo *et al.*, 2009; Muehlenbein & Watts, 2010), reduction in parental care (Silverin, 1980), aggression towards mating partners and offspring, and overall high energetic costs (see reviews: Wingfield *et al.*, 2001; Miles *et al.*, 2007). Therefore the fitness benefits gained by having high androgen levels are balanced by the potential drawbacks, which could act as a constraint to the upper limits an individual is exposed to (Reed *et al.*, 2006). Given their association with male social behaviour and competition, androgen measurements may provide insight into the effect of demographic skew on individual physiology.

Faecal hormone measurement

Measuring hormone levels and interpreting the results can prove challenging, as there are many factors that may affect their modulation. Hormone levels can vary for a variety of intrinsic reasons such as age, sex, reproductive status, as well as seasonal or temporal fluctuations; in addition the age, condition and storage protocol of the samples can create variation (Millspaugh & Washburn, 2004). In particular, the actions and causes of fluctuating glucocorticoids can be difficult to interpret, as they can both suppress and stimulate a variety of physiological responses (Sapolsky *et al.*, 2000), and the relationship between glucocorticoid levels and fitness is not always consistent (Bonier *et al.*, 2009). As such there is remarkable disparity in the literature about what constitutes chronic stress (Dickens & Romero, 2013). Noninvasive sampling techniques are preferable for use on species of conservation concern, as they are often more practical and there is less disturbance to the animal. Noninvasive methods for hormone measurement include the collection of faeces, urine, hair and feathers (Dantzer *et al.*, 2014). Faecal hormone measurements reflect the cumulative exposure of an individual to circulating hormone levels over a species-specific time period (Dantzer *et al.*, 2014), making them more reliable indicators of chronic conditions compared to instantaneous measures of circulating hormones from blood samples (Millspaugh & Washburn, 2004). However, as faecal measurements actually quantify the metabolites of circulating hormones that are broken down by bacteria they usually show more variation or ‘noise’, making patterns in the data more difficult to elucidate and potentially misleading (Goymann, 2012). Moreover, they can be strongly influenced by diet (Wasser *et al.*, 1993). This said, integrated hormone measures are considered more beneficial for conservation research as they reflect baseline hormone levels and incorporate all contributing intrinsic and extrinsic factors (Madliger & Love, 2013). Measuring both faecal glucocorticoid and androgen concentrations across several populations should provide a good estimate of population health, and allow variation in individual physiology to be quantified at a high enough resolution to identify patterns of association with the environment or population demography.

Social networks in conservation research

Another useful source of information about individual status and population dynamics is behavioural data, which is also relatively easy and non-invasive to collect. Behavioural studies have widespread potential for use in conservation biology (Sutherland, 1998), and there have been some successful applications (Buchholz, 2007). However, the two disciples are still not considered to be well integrated (Angeloni *et al.*, 2008), with poor communication between researchers and practitioners cited as a root cause (Greggor *et al.*, 2016). Behavioural traits can give an indication both of the status of the animal and of its environment (Berger-Tal *et al.*, 2011), and have been shown to vary between high quality and suboptimal habitat (Blondel *et al.*, 2006; Augé *et al.*, 2011). In addition, the aggregation or grouping of individuals is assumed to vary depending on ecological conditions (Linklater, 2000), therefore this be used as a measure of how they perceive their environment. Moreover, behavioural measures may be an ideal method to assess the impact of Allee effects (Stephens & Sutherland, 1999) and demographic imbalance (Le Galliard *et al.*, 2005).

Social network analysis has widespread potential for application within threatened ecosystems as a natural bridge between behaviour and conservation. Social networks characterise population structure using patterns of association or interaction (Croft, James & Krause, 2008; Krause, Lusseau & James, 2009); data is collected at the level of the individual making it a relatively easy and accessible method for population monitoring. Social network analysis allows the strength of relationships and changes in network position or structure to be quantified (Wey *et al.*, 2008), which can be compared across environmental gradients (Sundaresan *et al.*, 2007). Moreover, social network analysis could be used as a primary marker to monitor the health and welfare of domestic, captive and wild animal populations (Kleinhappel *et al.*, 2016). The integration of social network analysis with measures of individual physiology could increase its potential for application, as it provides a multidisciplinary assessment of an individual or population. Many hormonal markers are associated with social behaviours (e.g. Creel et al*.,* 2013) and social network position (Boogert, Farine & Spencer, 2014), making them ideal for use alongside network analysis. This thesis utilises both social network analysis and faecal hormone sampling to detect differences in population structure and individual physiological status, in relation to environmental pressures and management interventions.

Study species

This research was conducted on two equid species: the Welsh mountain pony (*Equus caballus*) in the United Kingdom and the Cape mountain zebra (*Equus zebra zebra*) in South Africa (Figs 1 and 2). The Carneddau Welsh mountain pony population occurs in the Snowdonia National Park in North Wales. The study population consists of more than 300 recorded individuals that inhabit an area of approximately 35-40 km^2 of commons land. The Welsh mountain pony population originates from domesticated stock and remains closely related to other Welsh pony breeds (Winton *et al.*, 2013). Both feral horses and mountain zebra share the same social structure: it is an example of female defence polygyny, where groups of unrelated females are defended by a single (or sometimes multiple) breeding stallion, with excess stallions forming bachelor groups (Klingel, 1971). Males in a bachelor group do not have access to females and many never breed at all, and whilst breeding stallions are known to herd mares and exhibit threatening behaviour there does not seem to be a physical determinant that distinguishes them (such as larger body size). The dominance of a stallion is dependent on the proximity and size of the mare group, and is not site-specific due to the free-roaming nature of the herds (Linklater, 2000). Interestingly, feral horse populations occur across a wide range of environmental conditions yet show fairly

Figure 1.1. Carneddau Welsh mountain ponies in Snowdonia National Park spend their lives with a lovely view of the surrounding landscape.

stable patterns of social organisation (Linklater, 2000). Long-term affiliations between unrelated individuals can reduce harassment and increase reproductive success (Cameron, Setsaas & Linklater, 2009), with stallions playing a key role in regulating and maintaining long-term relationships (Linklater *et al.*, 1999). The Welsh mountain pony population provides an ideal study system to investigate the effects of annual management practices – where the population is temporarily moved to adjacent farmland and some individuals are permanently removed – on social network structure and physiological status. Few studies have investigated the impact of removals on population structure and individual physiology, and it is prescient given that many endangered equids share the same social structuring and are subject to translocations as part of management strategies.

The second study sub-species, the Cape mountain zebra, underwent large-scale population declines due to excessive hunting and habitat loss, leaving three relict populations. Active conservation since the mid-20th century has resulted in their reintroduction across the majority of their historic range, with numbers now in excess of 4791 individuals in more than 75 sub-populations (Hrabar & Kerley, 2015). However, many difficulties are still faced in the

Figure 2. Cape mountain zebra in Gamkaberg Nature Reserve are one of three naturally surviving populations; here they occur in the highly diverse but grass-poor fynbos habitat.

management of this subspecies as it forms a complex metapopulation on both formally protected and privately owned land. Of the three relict populations, only one has provided founder individuals for the majority of new populations, and a prolonged bottleneck and subsequent isolation has resulted in the populations becoming genetically distinct from one another (Moodley & Harley, 2006). Consequently, a large proportion of the remaining genetic diversity remains stagnant and unrepresented, and is under threat of being lost altogether. The translocation of individuals from these populations has been limited by poor population growth rates. Most of the Cape mountain zebra's current (and historic) range is found within the Cape Floristic Region in South Africa, one of 25 global biodiversity hotspots that contains an estimated 44% of all the plant species in southern Africa (Myers *et al.*, 2000; Goldblatt & Manning, 2002). As selective grazers, Cape mountain zebra feed primarily on grass species with a high protein content (Grobler, 1983). However, over 80% of the Cape Floristic Region is covered by the fynbos biome, which consists of a wide array of fine-leaved shrubs (Cowling & Heijnis, 2001). The two poorly performing relict (and many newly founded) populations occur here, and less than 40% of each reserve is viable habitat for

Cape mountain zebra (Watson *et al.*, 2005; Watson & Chadwick, 2007). There is considerable evidence that Cape mountain zebra migrate seasonally between habitat types and predominantly select areas with high grass cover (Penzhorn, 1982; Grobler, 1983; Winkler & Owen-Smith, 1995; Boshoff & Kerley, 2001; Watson *et al.*, 2005; Watson & Chadwick, 2007; Smith *et al.*, 2008). Historically, Cape mountain zebra occupied grassland habitats and would have only persisted in low densities in the fynbos biome (Faith, 2012). It is possible that the confinement of Cape mountain zebra populations to areas of suboptimal montane habitat has caused a reduction in fitness that can be observed in poor population performance. This thesis examines the environmental and demographic correlates of Cape mountain zebra population performance and individual physiology through an assessment of habitat quality and faecal hormone monitoring. The results can be used to evaluate the efficacy of the on-going conservation management plan, and support a multi-faceted approach to population monitoring.

Aims and chapters

The main aims of this PhD were:

1) To develop physiological and behavioural markers of individual status and population health in two equid species using faecal hormone measurement and social network analysis

2) To use these measures to quantify individual responses to management interventions and aspects of population health over a large scale

3) To identify the environmental and demographic correlates of population performance in the Cape mountain zebra, and provide an assessment of the current management plan.

The chapter titles are as follows:

- Chapter 1: General Introduction
- Chapter 2: Validation of faecal glucocorticoid metabolites in response to an annual management event in free-living Welsh mountain ponies.
- Chapter 3: The removal of key stallions leads to a breakdown in social network structure and increase in androgens in feral Welsh mountain ponies (*Equus caballus*) – ***in preparation for submission to Animal Behaviour***.

- Chapter 4: Recognition and management of ecological refugees: A case study of the Cape mountain zebra – ***published in Biological Conservation: 203, 207-215***.
- Chapter 5: Macrophysiology uncovers relationships between demography, habitat and population performance in a vulnerable species, the Cape mountain zebra – ***currently under review at Functional Ecology***.
- Chapter 6: General Discussion

Following the general introduction, the second chapter assessed the physiological stress response of Welsh mountain pony mares to annual management practices as a potential biological validation for faecal glucocorticoid measurement. The third chapter uses both social network analysis and faecal hormone sampling to assess the impact of the removal of several key individuals from the Welsh mountain pony population. These chapters show the utility of a combined approach when assessing the outcome of management interventions, and the Welsh mountain ponies proved an ideal system to test and develop the methods used. In the fourth chapter, I identified two correlates of poor performance across the Cape mountain zebra metapopulation: habitat quality and sex ratio skew. The fifth chapter aimed to identify possible mechanisms for these correlations using faecal glucocorticoid and androgen measurements to assess the physiological status of individuals across seven populations. Together, these chapters present a detailed assessment of the health and status of the Cape mountain zebra metapopulation in relation to its environment and variable demography, as well as highlighting the need for a reassessment of its preferred ecological niche and potential changes to its management plan. When combined, this thesis presents two effective methods for evaluating species ecology and population health using a multidisciplinary approach. I show how physiological and behavioural characteristics can be measured in tandem to determine the different effects of environmental (habitat quality) and demographic (sex ratio skew) pressures, as well as the impact of management interventions. Both datasets are collected at the level of the individual yet can be expanded population-wide, making them highly relevant for conservation purposes and flexible in their focus. This research has many applications for conservation science, including population monitoring, the evaluation of management techniques, and the formation of new conservation strategies.

Approach and timeline

The Welsh mountain pony population was identified as a potentially useful study system for social network research almost a decade ago, and several years of demography and association data had been collected regularly since that time (Stanley *et al., in press*; Tierney, 2007; Stanley & Shultz, 2012). This meant that the methodology for social network data collection was already established and many individuals were already documented in a photographic identification database. This provided an ideal base to add more individuals to the existing database and expand the focus of the research to include faecal hormone sampling. When fieldwork began in the first year of the PhD, the database was updated and re-catalogued to ensure all entries were correct, and a new system for in-field identification was developed. There were several avenues and potential research questions to explore; firstly, could group dynamics and the strength of individual relationships be assessed over time by further investigating social networks; secondly, what are the distinguishable characteristics of stallions that maintain a breeding group compared to those that do not; and thirdly, what are the potential impacts of the annual management practices currently in use for this population?

To begin, a study was conceived to assess the impact of annual management practices on focal individuals by measuring faecal glucocorticoid levels, with the intention of using this as a biological validation of this measure. This study proceeded as planned, with sample collection taking place at the beginning of the second year of the PhD. In addition, regular survey trips were made during the first year of the PhD to collect social network and proximity data, and the area normally visited was expanded to incorporate more individuals and groups. As data collection progressed an unexpected opportunity presented itself as during the annual roundup several stallions were removed from the population, which had not been previously anticipated. The targeted removals acted as a perturbation to the existing population structure, meaning that we could investigate the resilience of the social network to disturbance. Ideally, data collection for this study would have continued on a regular basis for several months following the disturbance event to assess the long-term impacts; however this was not possible as fieldwork for another chapter was already scheduled. Therefore, this study focuses on the short-term impacts of individual removals, the results of which could be applicable to other species with similar social structures.

In addition to assessing the impact of annual management practices, we began preliminary investigations into a third study that would evaluate stallion profiles in relation to their breeding success or fitness, using faecal androgen measurements, an analysis of heterozygosity levels and parentage, and potentially faecal parasite burden. It was thought that stallion faecal samples could be obtained during the first study and alongside the collection of social network data during the first and second year of the PhD. During the first few months of sample collection we found it difficult to collect repeat samples from many of the stallions, as they were sighted infrequently (there is usually only one stallion per group, and the number of groups sighted per survey is restricted while waiting to collect samples). At this point, the large-scale sample collection for the Cape mountain zebra project was about to begin, requiring long periods of fieldwork out of the country. As such, there was not enough time to collect sufficient data to investigate the Welsh mountain pony stallions any further, though it would still be an excellent avenue of research in the future. Therefore, this thesis presents two chapters about the Welsh mountain pony population, where the majority of data collection took place during the first year of study and data analysis for each chapter taking place during the final year of the PhD.

Research on the Cape mountain zebra had also begun prior to this PhD; a number of reserves had been surveyed with the intention of being used for social network analysis, and an initial investigation had been made into quantifying habitat quality using Geographic Information Systems. The subspecies was of particular interest as it had been under a fairly intensive conservation reintroduction programme for several decades, resulting in a large number of isolated sub-populations of different sizes that are distributed across a landscape of varying ecological conditions. The history of the metapopulation, (e.g. when populations were founded and where the founders originated) had been well documented. This, combined with the relatively good records of current population sizes and translocation events, presented a unique opportunity to examine population dynamics and ecology at a broad scale in order to identify the causes of poor performance in important relict populations. Moreover, the method for field extraction of faecal hormone metabolites had recently been validated in captive black rhinoceros (*Diceros bicornis*; Edwards *et al.*, 2014), and the Cape mountain zebra presented an ideal subject to test this approach in the field. The abundance of palatable grass species within reserves was a focal point of the project from the beginning, and the vegetation mapping for the habitat quality scoring was completed in the first year of study. Two field trips were also undertaken in the first year, during which faecal samples were collected for diet analysis and suitable populations for further study were identified. These were selected as being not too large (re-sighting the same individual would be too difficult) or too small (sample sizes would be too small), and the absence of large dangerous mammals such as lions and elephants. Towards the end of the first year, the plan for faecal hormone sampling was developed, including the timeframe, resources and equipment required, and the target populations. At the beginning of the second year of the PhD the protocol for the in-field hormone extraction was tested on several Welsh mountain pony individuals, and enzyme immunoassays could detect faecal glucocorticoid and androgen metabolites in the samples. The collection of zebra faecal samples took place during the second and third year of study, taking approximately seven months in total.

The Cape mountain zebra project was hugely successful. There were several challenges encountered, for example finding the same individuals was not always easy, nor was locating faecal samples in the wide variety of habitat types. Time was often constrained, and we experienced the usual logistical issues associated with fieldwork. However, there were a number of triumphs, including one of the largest faecal hormone metabolite investigations of a large, wild mammalian species ever conducted, and the engagement and support of several important stakeholders who have reflected on our research outcomes for the future Cape mountain zebra management plan. There was only one issue that could not be resolved during the project, which was the measurement of faecal thyroid metabolites alongside glucocorticoids as an indicator of nutritional status. All hormone metabolites can be stored and extracted together using the in-field sampling protocol, however the thyroid assay protocol had not been previously described or validated for the in-field extraction technique and several problems arose during this process. First, there were issues with the enzyme immunoassay buffer causing interference during the assay; second, it took time to find the right sample dilution to use; and third, it was difficult to validate the assays by parallelism. Each of these issues were eventually resolved, however there was still a high level of variation in thyroid metabolites measurements among reserves and no distinctive patterns could be discerned. In correspondence with the laboratory responsible for running the assays it became apparent that it was possible that cross-contamination between samples had occurred during the process of solving the earlier issues. In lieu of this final irreversible problem, the thyroid hormone samples were excluded from the final presentation of the results. While faecal thyroid hormones remain a potentially important topic to investigate in this subspecies, the final results presented in chapter five using faecal glucocorticoid and androgen measurements are convincing and intuitive, and provide a novel contribution to our knowledge of the ecology and physiology of this species.

The chapters concerning the Cape mountain zebra contain more of the major themes and results put forward in the thesis, however they have been placed in order after the pony chapters for two reasons. Firstly, the pony chapters introduce the reader to the methodologies later applied at a larger scale to the Cape mountain zebra (particularly if including the large scale assessment of population structure described in Appendix 3). Secondly, the pony population was identified as a model species (due to its locality and existing identification database) on which to examine aspects of population management that could then be scaled up or transferred to other species. Therefore, the positioning of the pony chapters at the beginning of the thesis is logical, and builds towards the broader issues discussed in the zebra chapters at the end of the thesis.

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CHAPTER TWO

Validation of faecal glucocorticoid metabolites in response to an annual management event in free-living Welsh mountain ponies

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Abstract

Faecal glucocorticoid measurements are used frequently to monitor the response of captive and wild animals populations to potential stressors. The excretion of glucocorticoid metabolites can differ due to a variety of factors, therefore a validation may be required to be sure of measuring biologically meaningful fluctuations. Validations involving a pharmacological induction of the glucocorticoid response are often impractical for free-ranging animal populations, therefore monitoring the response to a disturbance event can help quantify the impact of management actions and serve as a biological validation. We measured faecal corticosterone metabolites in eight Welsh mountain pony mares (*Equus caballus*) in response to an annual management event, where individuals are temporarily relocated and physically handled, and four mares had their un-weaned foals removed. Faecal corticosterone metabolite concentrations were significantly elevated on days 5, 7 and 10 post-release, with two mares showing four- and six-fold increases compared to baseline levels. Faecal corticosterone metabolites showed significant diurnal fluctuations, however there was no effect of individual age or foal removal. Overall, there were high levels of variation between individuals in their glucocorticoid response. The time taken for an increase in faecal corticosterone metabolites was slightly later than observed in most other studies, which could be due to a restricted food intake during the holding period. The return of faecal corticosterone concentrations to baseline levels within two weeks of release suggests that the impact of annual management actions are not long lasting. This study is the first investigation into measuring the faecal glucocorticoid response in the Carneddau Welsh mountain pony population, and provides a range of faecal corticosterone metabolite concentrations that could be used as a guide for future work on this population. However, the results highlight the difficulty of using faecal measurements to assess fine-scale responses to events, given the variation in individual response and the time lag observed.

Introduction

Noninvasive measurement of faecal hormone metabolites has become a widespread technique for monitoring the health of domestic, captive and wild animal populations. Many studies aim to evaluate physiological stress through the measurement of glucocorticoid metabolites, often in response to unfavourable environmental conditions, disturbances, or specific management events. Faecal measurements are integrated: they represent the amount of hormone circulating over a species-specific (gut transit) time period and are therefore more representative of long-term exposure, which can be more useful for population monitoring than a pinpoint sample (Dantzer *et al.*, 2014). An acute increase in glucocorticoids in response to a stressor can increase survivorship and fitness in the short-term, however repeated exposure to one or more stressors can lead to an altered baseline state and an inability to respond to future threats (Busch & Hayward, 2009). A persistent change in baseline glucocorticoids in response to cumulative stressors, termed either chronic stress or 'distress', results in a diversion of energy from core functions and has a biological cost (Busch & Hayward, 2009; Linklater, 2010). Even the return of glucocorticoids to baseline levels can be potentially misleading, as it is not necessarily safe to assume that there are no continuing adverse effects (Linklater *et al.*, 2010).

There are many factors that can lead to variation in glucocorticoid measurements, such as sample age, condition, overall mass and storage prior to analysis, as well as differences in assay technique and antibody selection (Millspaugh & Washburn, 2004). In addition, there are many biological reasons that glucocorticoids may vary within an individual, for example age, sex, reproductive status, seasonal and temporal fluctuations, and diet (Millspaugh & Washburn, 2004; Goymann, 2012). In order to quantify deviations in faecal glucocorticoid metabolite levels that are representative of circulating glucocorticoid levels and that are biologically meaningful, it is often necessary to perform a physiological and/or biological validation (Touma & Palme, 2005). A physiological validation involves a pharmacological induction of the glucocorticoid response, usually through the injection of adrenocorticotrophic hormone (ACTH), with an associated monitoring period to follow the pattern of faecal metabolite excretion. ACTH challenges have been performed on a wide variety of mammalian species, including carnivores (Goymann *et al.*, 1999; Young *et al.*, 2004; Benhaiem *et al.*, 2012; Ludwig *et al.*, 2013), mice (Good, Khan & Lynch, 2003), hares (Sheriff, Krebs & Boonstra, 2010), and various domestic species (Möstl *et al.*, 1999; Kleinsasser *et al.*, 2010). However, extensive physiological validation may not be possible for wild or free-ranging populations as the costs of capture and release are high and post-challenge monitoring can be difficult. Moreover, capture itself could provoke an acute stress response. Therefore, a biological validation may be more appropriate, and less invasive. Such validations involve sequential sampling of faecal glucocorticoid metabolites before and after a known stressful event (Palme, 2005). Biological validations have been conducted successfully on several species of different taxa (Touma & Palme, 2005), including two endangered equid species (Franceschini *et al.*, 2008; Vick *et al.*, 2012).

Translocation events are often used for the biological validation of faecal glucocorticoid metabolites, as they are usually associated with some degree of stress (Dickens, Delehanty & Romero, 2010). Several large herbivore species have been successfully monitored during movement to a new enclosure or location, where a significant increase in faecal glucocorticoids was observed for at least several days post-capture, followed by a return to baseline levels (Millspaugh *et al.*, 2007; Viljoen *et al.*, 2008; Franceschini *et al.*, 2008; Vick *et al.*, 2012), or in some cases a long-term suppression (Linklater *et al.*, 2010). The success of translocation events is of high importance to wild and captive animal managers as there are can be negative consequences such as increased individual mortality rates (Pinter-Wollman, Isbell & Hart, 2009) or changes to the acute stress response (Dickens, Delehanty & Romero, 2009). The measurement of faecal glucocorticoids provides an insight into individual health in response to such a challenge, and is therefore a useful monitoring tool to assess the long lasting effects of translocations for wild and captive populations alike.

This aim of this study was to assess the physiological response of free-living Welsh mountain pony (*Equus caballus*) mares to a temporary translocation event as a potential biological validation of faecal glucocorticoid metabolite measurement. Despite not yet being recognised as a rare breed, the Carneddau Welsh mountain ponies are a genetically distinct population that may hold an important reserve of genetic diversity and adaptation to upland habitat (Winton *et al.*, 2013). They are subject to natural selection pressures, and the relative impact of environmental and social factors on their health and fitness is not well understood. A biological validation of faecal glucocorticoid metabolites should identify a biologically meaningful range for future work on the Welsh mountain pony population.

Methods

Study area and management practices

The Carneddau mountain range is found in Snowdonia National Park, North Wales (53.22°N, 3.95°W). Focal individuals in this study occurred across a 35 – 40 km^2 area of commons land, used primarily for sheep farming and recreational hiking. Annual management practices occur in the autumn, and involve the relocation of individuals onto adjacent farmland where they are retained for 1 – 2 days. Factors that contribute to this event being considered a stressor include: active chasing by people on foot and by vehicle, confinement to an enclosed space, separation from members of normal social group and mixing with unfamiliar individuals, manual handling through a livestock crush, and, if necessary, being administered an ear tag. In addition, individuals are sometimes removed from the population such as those in poor condition or juvenile males; on this occasion 20 adult or juvenile stallions were removed.

Sample collection

The location and membership of breeding groups in the study area has been recorded since 2007, with individuals continuously added to a database which details their age-sex classification and contains photographs showing distinctive markings (coat colour, face and leg markings, ear tags etc). Ponies were observed from a distance of 10 – 20m and identified using the photographic database. Faecal samples were collected within an hour of defecation: a large portion of the sample was collected, mixed thoroughly by hand, and a subsample was removed for storage. Samples were frozen at -18 °C for 7-10 months prior to extraction.

The sampling period ran from late August to early December 2014, with the annual roundup occurring on 15-16th November. Faecal samples were collected from eight adult mares from two focal groups; four mares had their male foals removed during the roundup but neither group lost its breeding stallion. Mares ranged in age from 3.5 to an estimated 10-12 years old (some were already adults when the population was first surveyed in 2007). Repeat samples were collected in the 12 weeks before the roundup, resulting in a minimum of three baseline samples per individual. The time sequence of the roundup event and post-release sampling was as follows: ponies were rounded up and driven onto farmland on day -2 and kept on a fenced paddock overnight, on day -1 ponies were put through a livestock crush and had their tails trimmed, then on day 0 individuals were moved back onto the commons land of Snowdonia National Park. The ponies were left for 24 hours to return to their social group and home range, after which faecal samples were collected on days 1, 2, 3, 5, 7, 10, 14 and 18 such that each mare was sampled at least six out of eight times over this period. This gave a total of 85 faecal samples for the eight mares.

Hormone extraction and analysis

Corticosterone metabolites were extracted from thawed faecal samples using a modified protocol described by Walker, Waddell & Goodrowe (2002). In brief, samples were thoroughly mixed by hand, weighed (0.5 g ± 0.003 g), combined with 5 ml of 90% methanol, vortexed and shaken overnight on an orbital shaker. The following day, each sample was vortexed and centrifuged for 20 min at 598g. The supernatant was decanted, dried under air, re-suspended in 1 ml of 100% methanol and stored at -20 °C until analysis.

Faecal corticosterone metabolites were analysed using enzyme immunoassays (EIA) as described by Edwards *et al.* (2014). Each EIA used an antiserum (polyclonal corticosterone CJM006); corresponding horseradish peroxidase-conjugated label (C. J. Munro, University of California, Davis) and standards (Sigma-Aldrich, UK) on a Nunc-Immuno Maxisorp (Thermo-Fisher Scientific, UK) microtitre plate. The procedure was as follows: 50 µl per well of antiserum (1:15 000 dilution in coating buffer) was loaded and incubated overnight at 4°C, plates were washed with wash solution (0.15 m NaCl, 0.05% Tween 20) five times, and standards (3.9–1000 pg per well) or samples diluted in EIA buffer were loaded at 50 µl per well followed by 50 μl per well of horseradish peroxidase (1:70 000 dilution in EIA buffer). Following incubation the dark for 2 h at room temperature, plates were washed with wash solution five times and incubated with 100 μl per well of room temperature substrate [0.4 mm 2,2-azino-di-(3-ethylbenzthiazoline sulfonic acid) diammonium salt, 1.6 mm H2O2 and 0.05 m citrate, pH 4.0] and left to develop at room temperature in the dark. Following incubation, developed plates were measured at 405 nm. Pony faecal extracts eluted from cartridges were diluted as necessary in EIA buffer and run in duplicate (50 µl) on respective EIAs.

EIAs were biochemically validated for measuring corticosterone in pony faecal extracts through parallelism ($R^2 = 0.93$, $F_{1,7} = 87.24$, $p < 0.001$) and matrix interference assessment ($R^2 = 1.00$, $F_{1,7} = 21160.99$, $p < 0.001$). The cross-reactivities of corticosterone antibodies have been reported elsewhere (Watson *et al.*, 2013), and only data with an intra-assay coefficient of variation (C_V) less than 10% and inter-assay C_V less than 15% were accepted and used for statistical analysis.

Data handling and statistics

A linear mixed effect model was used to examine differences in faecal corticosterone metabolite concentrations using the *lme4* package in R (Bates *et al.*, 2015; R Development Core Team, 2016). Sampling day was included as a fixed factor in the model, along with age of individual, time of day, and a categorical variable denoting whether each mare had their foal removed. A separate model also included an interaction term between sampling period and foal status to assess if mares responded differently if they had their foal removed. Pony ID was included as a random factor. To estimate the influence of outliers on the linear mixed effects model we calculated Cook's distance (Cook, 1979) using the *influence.ME* package in R (Niewenhuis, te Grotenhuis & Pelzer, 2012). Pairwise comparisons between sampling days were conducted using Wilcoxon's signed-rank tests. Faecal corticosterone metabolite concentrations were log-transformed to normalise model residuals.

Results

The individual faecal glucocorticoid response of each mare to the annual roundup can be viewed in Fig 2.1. The most notable increases included one mare that exhibited a four-fold increase in faecal corticosterone metabolite concentrations seven days after release (49.07 ng/g compared to baseline levels of 10.39 ± 2.44 ng/g; Fig 2.1a), and another with faecal corticosterone levels more than six times higher than that of baseline levels five days post-release (91.77 ng/g compared to baseline levels of 14.08 ± 2.00 ng/g; Fig 2.1b). When pooled together, significantly elevated faecal corticosterone levels were observed on days 5, 7 and 10 of the sampling period ($F_{8,68} = 2.18$, $p = 0.04$; Fig 2.2), with mean levels 1.5 to 2 times greater than baseline (day 5: 22.33 ± 10.16 ng/g, day 7: 17.79 ± 5.07 ng/g, day 10: 16.52 ± 5.12 ng/g). There were high levels of variation between mares in their glucocorticoid response (Table 2.1).

All Cook's distance values were below 0.2, far below the cut off value of 1 initially proposed by Cook & Weisberg (1982). However, one data point had a high value relative to the rest (Cook's distance of 0.19 compared to the second highest of 0.10). This data point corresponded to the highest corticosterone measurement

Table 2.1 Details of the eight focal mares, their baseline faecal corticosterone metabolite measurements and the levels observed between days 5-10 of the sampling period (mean ± standard deviation).

Mare	Age in years	Band	Foal removed?	Baseline faecal corticosterone	Day 5-10 faecal corticosterone
1	10+	Marsh	No	10.39 ± 4.23	28.60 ± 17.73
2	5	Marsh	No	10.86 ± 5.42	17.32 ± 8.77
3	10+	Marsh	No	9.62 ± 5.33	20.74 ± 3.40
4	10+	Aber	No	11.18 ± 3.22	10.61 ± 9.23
5	3	Marsh	Yes	14.08 ± 3.48	43.99 ± 41.44
6	7	Aber	Yes	7.14 ± 1.57	10.59 ± 1.95
7	5	Marsh	Yes	14.65 ± 13.12	8.86 ± 2.14
8	3	Aber	Yes	10.11 ± 4.21	10.30 ± 1.20

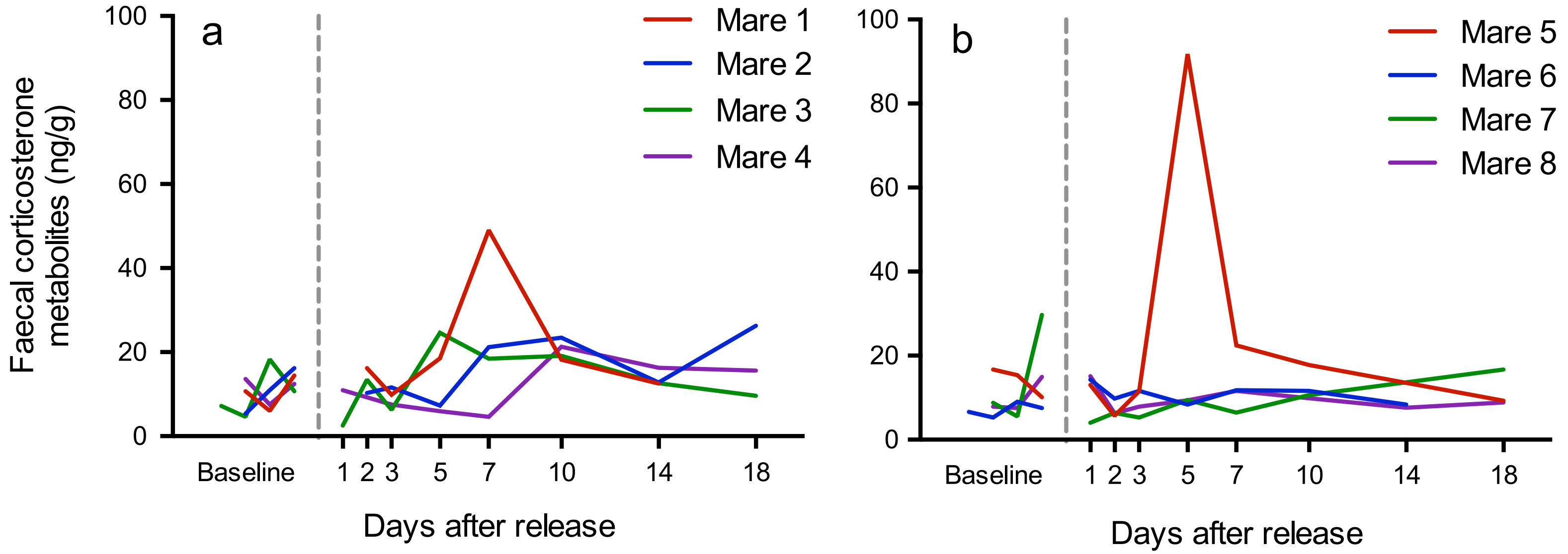

Figure 2.1. Individual faecal corticosterone metabolite measurements in free-living Welsh mountain pony mares are shown post-release after a temporary translocation event. All mares had foals prior to the event, during which four remained (a) and four were removed from the population (b). Dashed line (---) indicates the day of release after the two-day holding period.

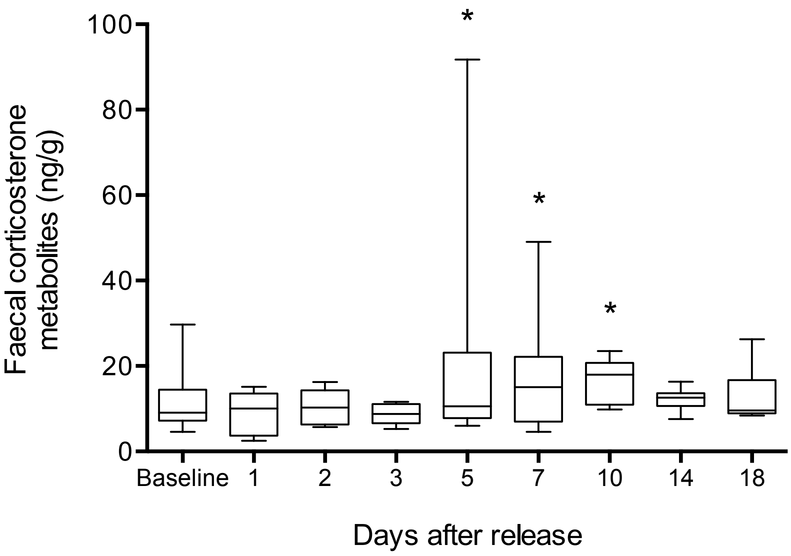

Figure 2.2. Welsh mountain pony mare faecal corticosterone metabolite levels in the days following release after a temporary translocation event. Box plots show the median, upper and lower quartiles while the whiskers show the range of the data. Significant elevations in faecal corticosterone levels were observed between days 5-10 (where * indicates $p < 0.05$).

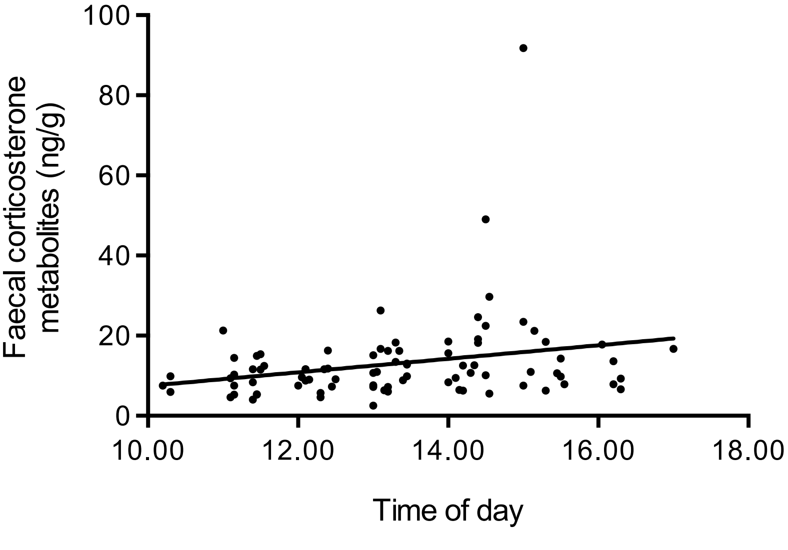

Figure 2.3. Faecal corticosterone metabolite concentrations in free-living Welsh mountain pony mares increase throughout the day during daylight hours, peaking between 14.00 and 16.00 in the afternoon.

(91.77 ng/g on day 5). Removing this data point resulted in the relationship between faecal corticosterone metabolites and sampling day becoming slightly less significant ($F_{8,67} = 2.01$, $p = 0.05$). The other notable high data point had a Cook's distance of 0.08, and removing both values resulted in the relationship between faecal corticosterone and sampling day becoming marginally significant ($F_{8,66} = 1.89$, $p = 0.08$). Wilcoxon's signed rank tests to compare sampling days found that faecal corticosterone levels on day 10 were significantly higher than baseline levels and days 1 – 3 (baseline: $W = 0.18$, $p < 0.01$; day 1: $W = 40$, $p = 0.04$; day 2: $W = 46$, $p = 0.04$; day 3: $W = 60$, $p < 0.01$).

The interaction term between sampling day and foal removal was not significant for faecal corticosterone ($F_{8,60} = 1.48$, $p = 0.18$), nor was there an overall effect of foal removal on mare faecal corticosterone metabolite concentrations ($F_{1,5} = 0.72$, $p = 0.44$), and no effect of individual age ($F_{1,5} = 1.28$, $p = 0.31$). Faecal corticosterone concentrations showed temporal variation during sampling hours, being lowest in the morning and highest between 14.00-16.00 in the afternoon ($F_{1,68} = 8.23$, $p < 0.01$; Fig 2.3).

Discussion

The aim of this study was to assess the acute stress response of focal Welsh mountain pony mares in response to an annual management event, and determine whether this could be used as a biological validation of faecal glucocorticoid metabolite measurement for this population. In general, we observed less of a response than expected, with an initial time delay of approximately five days before any acute stress response was measured at all. There was a high degree of variation between individuals, where some appeared to exhibit an acute stress response and others having a much more muted reaction. We discuss the potential reasons for these observations, and reflect on whether this study is adequate for the purpose of validating faecal glucocorticoid metabolites.

Faecal glucocorticoid metabolite concentrations represent the cumulative excretion of circulating hormone over the gut passage time, which is species-specific (Touma & Palme, 2005). Passage time through the equine gastrointestinal tract can vary depending on factors such as diet, body weight and pregnancy, and can vary from 20 – 60 hours depending on the material (Van Weyenberg, Sales & Janssens, 2006). In the current study corticosterone metabolites in mare faecal samples were not significantly elevated until five days after they were returned to their original habitat. Pharmacological validation studies in ponies have shown peaks in faecal glucocorticoid metabolite concentrations approximately 24 hours after ACTH administration (Palme *et al.*, 1996; Möstl *et al.*, 1999), although it was as long as 50 hours for Grevy's zebra (*Equus grevyi*; Franceschini *et al.*, 2008). However, it is more common for non-pharmacologically induced faecal glucocorticoid responses to take longer to appear, for example after castration horse stallions showed significant increases 48 hours later (Merl *et al.*, 2000), whereas for the Persian onager, *Equus hemionus onager*, peak elevations were observed between 1-6 days after capture depending on the individual (Vick *et al.*, 2012). Other species also show a lag response, for example faecal glucocorticoid increases took more than 10 days to appear after enclosure work began in captive African elephants (*Loxodonta africanus*; Watson *et al.*, 2013), and in a male spotted hyena (*Crocuta crocuta*) peak elevations were observed three days after translocation despite the response to an ACTH challenge taking less than 24 hours (Goymann *et al.*, 1999). Importantly, in this study individuals were housed in paddocks in high densities with limited food, therefore they ate very little until released back onto the commons land on Day 0. This could have caused an increased delay in the appearance of faecal hormone metabolites in addition to normal gut transit time. Whatever the reason for the time delay it leaves a window of uncertainty about the level of adrenal activity, particularly immediately following the first stages of the roundup. It is possible that the first peak in circulating glucocorticoids occurred on day -2 and the metabolites were excreted on day -1 or day 0, then following this was a period of decreased sensitivity of the hypothalamic–pituitary–adrenal (HPA) axis, as has been observed after translocation events in birds (Dickens *et al.*, 2009).

There was considerable variation between individuals in the level of glucocorticoid response, as two mares showed much more pronounced increases in faecal corticosterone metabolite concentrations than the others. These measurements do affect the overall trend of the data and therefore the pooled data must be interpreted with caution. This said, faecal corticosterone levels on day 10 were significantly higher than baseline and there were no outlier measurements on that day. Similar studies have found high individual variability in terms of the degree of response, the amount of fluctuation between samples, and the time taken to acclimate to new conditions (Franceschini *et al.*, 2008; Vick *et al.*, 2012). It is possible that the expected acute increase in faecal glucocorticoids for the other individuals in this study was missed due to incomplete sampling, as we only collected one sample per day from each individual. A study on greylag geese (*Anser anser*) found that it was necessary to collect a minimum of three faecal samples within three hours of exposure to an acute stressor to accurately estimate the glucocorticoid response (Scheiber, Kralj & Kotrschal, 2005). Therefore, we would advise future studies attempting to validate faecal glucocorticoid metabolites for this population to collect samples more frequently from focal individuals, or if possible to collect every sample. This would ensure that the acute stress response, if present, is not missed due to an inadequate sampling effort. Given the level of individual variation that can be observed it may be necessary to increase the number of focal individuals in the study. Many experimental validation studies have very small samples sizes (sometimes just two individuals; Touma & Palme, 2005), however these are usually conducted under controlled laboratory conditions. Given the difficulty of monitoring individuals in free-living conditions, increasing the sample size may be a way to absorb some of the variation observed between individuals. In addition, the current study only included adult females, and therefore further validation studies would be required for males and possibly for different age categories. Moreover, we observed diurnal variations in faecal corticosterone metabolites that may reflect fluctuations in metabolism or activity and rest (McEwen & Wingfield, 2003). This may be important to consider when attempting to measure time-delayed responses as well as temporally matching samples more generally when assessing correlative patterns of faecal glucocorticoids and a supposed stressor (Edwards *et al.*, 2013).

In many of the biological validation studies described above, the period of glucocorticoid elevation lasted for several days or even weeks, depending on the species and the nature of the stressor. In this study, pony faecal corticosterone metabolite concentrations had returned to baseline levels within two weeks of the disturbance event. This appears reasonable given that the stressor was no longer present and individuals were returned to their original environment. However, it is surprising that the removal of several of the mares' foals did not provoke an acute stress response. A study on mare cortisol levels found evidence for an acute stress response immediately after their foals were weaned, which then declined after approximately 24 hours (Malinowski *et al.*, 1990). Given the potential problems encountered with sampling adequacy in the current study, it is possible that the acute stress response to foal removal was missed, particularly if it is so short-lived. We would again recommend that a more thorough sampling protocol be used to further investigate the effect of foal removal on mare faecal glucocorticoid levels, where preferably all samples are collected in the days following foal removal.

Overall, these results suggest it may be difficult to use faecal glucocorticoid measurements to assess fine-scale responses to events, particularly in non-experimental systems where stressors may not be as severe, such as behavioural interactions (Edwards *et al.*, 2013). The disruption of stable social groups during the roundup period may have resulted in potentially stressful encounters in the days following, as individuals moved around to return to their home range (Nuñez *et al.*, 2014). Whilst all the focal mares in this study had returned to their home range by Day 1 of sampling, it is possible that interactions with unknown individuals from outside of their social groups were more likely during this time. However, this study shows that it is very difficult to interpret the causes of acute increases in faecal glucocorticoid levels without tracking each individual throughout the study period, as even a known stressful event did not result in consistent responses. Faecal measurements are an integrated average of the circulating glucocorticoids that an individual has secreted, metabolised and excreted, and may be better indicators of chronic conditions or stressors (Dantzer *et al.*, 2014). Faecal hormone measurements are becoming an increasingly common method to measure the impact of capture and translocation for a variety of species (Harper & Austad, 2001; Viljoen *et al.*, 2008; Franceschini *et al.*, 2008; Aguilar-Cucurachi *et al.*, 2010; Vick *et al.*, 2012), and to assess the welfare status of domestic animals (Beerda *et al.*, 1997; Palme, 2012). Our results suggest that this approach may be unsuitable for this application under certain circumstances, particularly in uncontrolled environments when focal animals cannot be monitored all the time.

In conclusion, faecal glucocorticoid metabolites were successfully quantified for free-living Welsh mountain pony mares in response to a disturbance event, however the level of variation and apparent lack of response for some individuals means this study does not represent a conclusive validation of the stress response. Further studies should focus on increasing the number of samples collected, and should investigate the response in males and juveniles. The baseline and peak metabolite levels observed here could be used to guide future studies investigating further stressors for this population. More generally, it will be important to define an accurate and repeatable methodology for faecal glucocorticoid validation in wild populations, as this technique is becoming increasingly popular and many species remain unsuitable for experimental validation procedures (such as pharmacological induction).

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CHAPTER THREE

The removal of key stallions leads to a breakdown in social structure and increase in androgens in Welsh mountain ponies

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ABSTRACT

Animal social networks may rely on the presence of key individuals that maintain social stability by exhibiting certain behaviours. Removing these individuals from the population can result in large changes to social network structure, the consequences of which are not well understood. Combining social network analysis with noninvasive sampling of physiological markers can give a holistic assessment of population structure and individual status. We use social network analysis and faecal hormone sampling to determine the impact of the removal of key stallions on social structure and individual physiology in free-living Welsh mountain ponies (*Equus ferus caballus*). The removal of herd stallions led to a breakdown in social network structure, signified by a decrease in clustering and tie strength, and an increase in individual connectivity as more individuals were connected by at least one tie. Importantly, an increase in closeness centrality was observed after the disturbance event, indicating that removals led to a breakdown of internal structure and discrete groups. In addition, we found an increase in stallion faecal androgen and glucocorticoid concentrations eight days after release, possibly as a result of increased male competition. This study concurs with previous evidence that population structure relies heavily on key individuals, as the removal of herd stallions resulted in marked changes to social networks. Therefore, care should be taken when considering removals and translocations. Social network analysis proved to be a useful tool for assessing the impact of a population management intervention.

INTRODUCTION

Social network analysis has widespread potential to assess population level responses to environmental fluctuations (Godfrey et al. 2013; Henzi et al. 2009; Rubenstein & Sundaresan 2007) and disturbance events (Ansmann et al. 2012; Dufour et al. 2011; Formica et al. 2017). Animal social networks have been shown to remain stable despite variation in individual behaviour and ecological conditions (Godfrey et al. 2013), and to display adaptive resilience to permanent environmental change (Ansmann et al. 2012). However, while networks may remain resilient to the random loss of individuals, they are vulnerable to the removal of those that maintain the integrity of social structuring, for example by having high betweenness scores (Williams & Lusseau 2006). The removal of key individuals that provide an important role in the maintenance of social stability can result in disruption to network structure (Flack et al. 2005). This could have consequences for population dynamics and social evolution (Kurvers et al. 2014), and could be particularly relevant for captive and wild populations that are often the focus of targeted removals or translocations.

There is now a wealth on information on the different methods of data collection and analysis of animal social networks (Croft et al. 2011; Farine & Whitehead 2015). Networks are constructed using associative or interactive behaviours, allowing the strength of relationships and changes in network position or structure to be quantified (see reviews Wey et al. 2008; Krause et al. 2009). A strength of social network analysis lies in the ability to assess social structure at the level of the individual, group, or population in a standardised format (Makagon et al. 2012). Moreover, the integration of social network analysis with other disciplines, such as physiology, to provide a holistic assessment of population health and performance could increase its potential for application to *in situ* conservation. Faecal hormone measurements could be particularly useful alongside social network analysis as hormonal markers are associated with many aspects of social behaviour (e.g. Creel *et al*. 2013), and social network position (Boogert et al. 2014). Androgens, the male reproductive hormones, can have direct inductive effects on behaviours related to breeding, dominance and aggression in males (Wingfield et al. 2001). Glucocorticoids (or ‘stress’ hormones) are also associated with dominance and aggression (Mehta & Josephs 2010), however they are more commonly used as an indicator of general health as they fluctuate with environmental conditions and in response to stressors (Busch & Hayward 2009). Measuring both endocrine markers simultaneously can give a more comprehensive overview of an individual's physiological status in response to a disturbance event, e.g. Linklater *et al*. (2010).

Feral horse populations occur in a wide variety of environmental conditions yet show broadly similar patterns in social organisation (Linklater 2000). Their social structure is an example of a fission-fusion society that employs female defence polygyny, where groups of unrelated females are defended by a single (or sometimes multiple) breeding stallion, with excess stallions forming bachelor groups (Linklater 2000). Several physiological correlates of social behaviour have been identified in horses, such as body condition and parasite load (Linklater et al. 1999) and glucocorticoid levels (Nuñez et al. 2014). Long-term affiliations between unrelated individuals can reduce harassment and increase reproductive success (Cameron et al. 2009). Stallions play a key role in regulating and maintaining long-term relationships between conspecifics (Linklater et al. 1999), therefore social network stability may rely on their presence. However, the impact of the removal of key herd stallions on social network structure has yet to be elucidated. In the Welsh mountain pony population, individual relationships remain stable during periods when individual turnover is low (Stanley et al. *in press*) (Stanley et al., *in press*). However, how targeted removals impact on social networks in terms of group stability or the strength of relationships is currently unknown.

The aim of this study was to assess the robustness of social network structure in a free-living Welsh mountain pony (*Equus ferus caballus*) population following the removal of several herd and bachelor stallions. In addition, we assessed individual physiological responses by monitoring faecal androgen and glucocorticoid hormone concentrations in adult stallions. Welsh mountain ponies are derived from domestic stock, yet are now genetically distinct and may hold an important reserve of genetic diversity and adaptation to upland habitat (Winton et al. 2013). They are subject to natural selection pressures and the relative impact of both environmental and social factors on their health and fitness is not well understood.

METHODS

Study Animals

Carneddau Welsh mountain ponies are found in the Carneddau mountain range, Snowdonia National Park, North Wales (53.22°N, 3.95°W). The population consists of more than 300 recorded individuals observed over an area of approximately 35-40 km^2 of commons land, where the altitude ranges from 287 – 610 m above sea level. The land is used primarily for sheep farming and recreational hiking; as such the ponies are well habituated to humans, though not to direct physical contact. Each individual can be uniquely identified using their age-sex classification and a photographic database that depicts coat colour, face and leg markings, and the presence of ear tags and notches. Demographic data for the population has been recorded on a regular basis since 2007, including the location and membership of breeding groups. In addition, social network structure has also been investigated in this population in relation to differential sex investment and the strength of individual bonds (Stanley & Shultz 2012; Stanley et al. *in press*) The population is essentially unmanaged aside from an annual roundup event, where individuals are driven down from the mountains onto adjacent farmland and kept on a paddock for one to two days. During this time, individuals are often separated from other members of their herd for an extended period, and all individuals are passed through a livestock crush where (if necessary) they are given ear tags for identification. Individuals considered surplus to the population, usually males, and those in poor condition are sometimes removed. On this occasion several stallions were removed from the population; known individuals included three herd stallions, three bachelor stallions and 9 juveniles less than two years old. It is thought that more individuals (belonging to other farmers) were also removed, as at least two other herd stallions did not return to their home range and were never sighted again.

Social Network Analysis

Demography and proximity data for the present study was collected between March 2014 and July 2015, with the roundup event occurring on 15-16th November 2014. The survey area is enclosed by walls or fences on the northern, eastern and western sides, whereas a hill with a peak altitude of 770 m above sea level determined the southern boundary (the valley and slopes leading to the summit were included). The ~35 km survey area was split into three sections, with one section surveyed per day. Each survey consisted of approximately 10 – 12 km of hiking, and the number of individuals seen per day ranged from 9 – 79 (mean = 34). Upon encountering a group, the name of each pony present and a GPS location was recorded. The approximate distance in metres between all individuals was documented, where individuals less than ~100 m apart and that moved as a cohesive unit were considered to be associated with each other. A histogram showing the frequency of distances between GPS coordinates between each group and the next group sighted for each survey day are shown in Figure 3.1. The mean distance between sequentially sighted groups was 446 m (range = 64 – 1795 m). Association matrices were constructed for each day of sampling (one matrix per day) where individuals that were seen within the same group but not close together (15 – 100 m apart) were given a score of 1, and individuals that were seen very close together (< 15 m apart) or interacted with one another (for example by grooming) were given a score of 2. Where GPS coordinates indicated groups that were previously recorded as separate were in fact within 100 m of each other they considered associated an given a score of 1 (8 out of 112 distances). There were 20 instances where

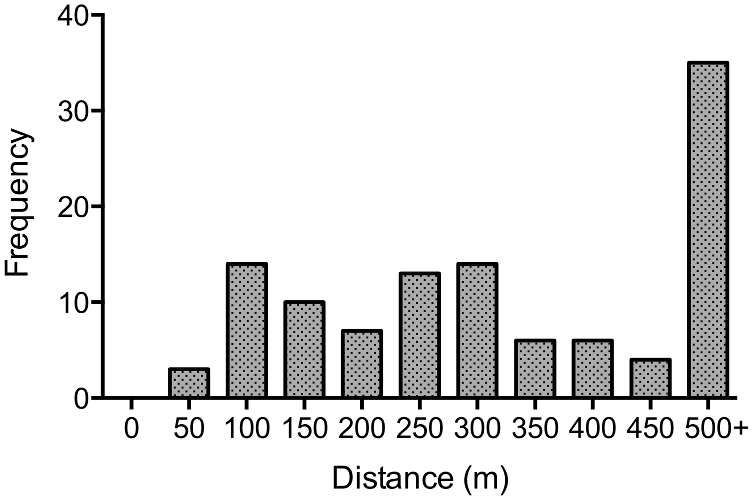

Figure 3.1. A histogram of recorded distances between Welsh mountain pony groups, measuring by GPS coordinates from each group to the next group sighted on each survey day.

sequentially sighted groups were between 100 – 200 m of one another; 15 of these were recordings of individuals from larger social groups that had split into smaller sub-groups, and the remaining five were between two breeding groups (shown in blue and green on Fig 3.2) that have partially overlapping home ranges. In all cases the distance was considered too large to class these groups as associated by proximity and they were recorded separately. This resulted in 23 matrices for before the roundup event (March to mid-November 2014) and 20 for the period after (16 in mid-November to December and a further four in June and July 2015). Individuals were retained in final networks if they were sighted at least three times both before and after the roundup, except for seven stallions (one herd stallion, one bachelor stallion, and five juvenile males) that were sighted three or more times prior to their removal from the population (this gave 69 individuals present in networks before the roundup and 62 present after). Ponies were sighted between 3-19 times (mean = 10.30 ± 0.15). Matrices from before and after the roundup event were summed to give weighted association indices, calculated using a modified version of the simple ratio index (Farine & Whitehead 2015), where edge weight was calculated as:

$$E_{AB} = \frac{x_{SUM}}{2x_{COUNT} + y_{AB} + y_A + y_B}$$

where x_{SUM} is the sum of associations between individuals *A* and *B*, x_{COUNT} is the number of times *A* and *B* have been sighted together (where x_{COUNT} multiplied by two is the maximum possible association score), y_{AB} is the number of times both *A* and *B* were observed but not together, y_A is the number of times only individual *A* was seen, and y_B is the number of times only *B* was seen. This gave two weighted, undirected association matrices (before and after the roundup) with associations between individuals ranging from 0 to 1, where 0 means they were never sighted in the same group and 1 that they were within 15 m of each other every time they were sighted.

Network metrics were calculated using the *igraph* (Csardi & Nepusz 2006) and *tnet* (Opsahl 2009) packages for R (R Development Core Team 2016), and can be viewed in Table 3.1. We focussed on measures that incorporate edge weight in their calculation, but also include

Table 3.1. A description of the social network measures used, whether they were calculated at the global or local level, if they use binary or weighted edges, and the R package used to calculate them.

Network metric	Description	Global or local	Binary or weighted	R package	Sources
Degree	The number of nodes to which the focal node is directly connected, or adjacent. Mean degree is used as a network level measure.	Both	Binary	*igraph*	(Freeman 1978)
Edge density	The number of edges or ties in a network divided by the total possible number of ties.	Global	Binary	*igraph*	(Wasserman & Faust 1994)
Strength	The sum of the weights of all edges connected to a node (the weighted equivalent of degree).	Local	Weighted	*tnet*	(Barrat et al. 2004) (Newman 2004)
Degree centrality	A weighted degree centrality measure that incorporates both degree and edge weight using a tuning parameter, α. Here, α was set at 0.5 so that the measure favours degree but also positively values edge weight.	Local	Weighted	*tnet*	(Opsahl et al. 2010)
Closeness centrality	The normalised sum of the length of the shortest paths between the focal node and all other nodes in the network. Here, we use a method that incorporates both the number of intermediary nodes and tie weights to find the shortest path, using a tuning parameter, α. We set α at 0.5, favouring fewer intermediary nodes with weaker ties for the shortest path length. To overcome the problem of many disconnected components where paths between nodes can be infinite, the measure is calculated using the sum of the inversed distances (rather than the inversed sum of distances).	Local	Weighted	*tnet*	(Freeman 1978) (Dijkstra 1959) (Opsahl et al. 2010)
Betweenness	The frequency with which the focal node is part of the shortest paths connecting pairs of other nodes. Here, we incorporate the number of intermediary nodes and tie weights into the calculation of shortest path length using the method described for closeness centrality.	Local	Weighted	*tnet*	(Wasserman & Faust 1994) (Opsahl et al. 2010)
Reachability	The proportion of other nodes the focal node can reach in a given number of steps (two in this study).	Local	Binary	*igraph*	(Wasserman & Faust 1994)
Transitivity	A standard measure used to assess the tendency of nodes to cluster together ('cliquishness') at both a global (network) and local (node) level. Global transitivity is the number of closed triplets divided by the number of closed and open triplets, where a triplet is three nodes connected by two or three edges. Local transitivity is calculated as the number of actual ties between neighbouring nodes divided by the total number of possible ties.	Both	Binary	*igraph*	(Wasserman & Faust 1994) (Watts & Strogatz 1998)
Clustering	A measure of clustering at the level of the network or node that takes into account edge weight. For global clustering, the value of closed triplets (calculated by the geometric mean of edge weights) is divided by the value of all triplets. Local clustering is calculated by summing the value of closed triplets centred on the node, divided by the value of all triplets centred on the node.	Both	Weighted	*tnet*	(Barrat et al. 2004) (Opsahl & Panzarasa 2009)

some that use a binary presence or absence of ties. The number of connected components and size of the largest component were calculated for each network, as well as three network-level (global) metrics. Eight additional metrics were calculated at the node, or local, level (see Table 3.1 for details). Several of the weighted measures calculated in the *tnet* package use a tuning parameter, α, which allows for both the number of ties and tie weight to be taken into account (Opsahl et al. 2010). The α value can be adjusted depending on the study system and the hypothesis to be tested. We used $\alpha = 0.5$, favouring the number of ties over tie weight (but positively valuing both). We chose this as *Equus caballus* populations normally show fairly high levels of group clustering (Linklater 1998) and so a single inter-group association between individuals (represented by a weak tie) could be very meaningful for population structuring.

Faecal Sample Collection

Faecal samples for hormone metabolite analysis were collected from late August to early December 2014, with baseline samples collected before the annual roundup on 15-16th November. The time sequence of the roundup event was as follows: ponies were rounded up and driven onto farmland on day -2 and kept on a fenced paddock overnight; on day -1 ponies were put through a livestock crush, had their tails trimmed and were given an ear tag if not already present; on day 0 they were moved back onto the commons land of Snowdonia National Park. Individuals were left for 24 hours to return to their social group and home range, after which faecal samples were collected opportunistically from day 1 onwards from various adult stallions. Due to data collection for another study, only four samples were collected during the first three days post-release. Subsequent to that, eight samples were collected between days 5-7, 13 were collected between days 8-12, and a further 13 during days 14-18. With 21 baseline samples this totalled 59 faecal samples collected from 10 herd stallions and four bachelor stallions, ranging from 1-13 samples per individual across the entire sampling period.

Ponies were observed and identified from a distance of 10-20m. Faecal samples were collected within an hour of defecation. The sample was mixed thoroughly by hand and a subsample was removed for storage. Samples were frozen at -18 °C for 7-10 months prior to extraction. The time of defecation and the day of sampling were recorded.

Hormone Extraction and Analysis

Corticosterone and testosterone metabolites were extracted from thawed faecal samples using a modified protocol described by Walker et al. (2002). In brief, samples were thoroughly mixed by hand, weighed (0.5 g ± 0.003 g), combined with 5 ml of 90% methanol, vortexed and shaken overnight on an orbital shaker. The following day, each sample was vortexed and centrifuged for 20 min at 598g. The supernatant was decanted, dried under air, re-suspended in 1 ml of 100% methanol and stored at -20 °C until analysis.

Faecal corticosterone and testosterone metabolites were analysed using enzyme immunoassays (EIA) as described by (Edwards et al. 2014). Each EIA used an antiserum (polyclonal corticosterone CJM006 or polyclonal testosterone R156/7); corresponding horseradish peroxidase- conjugated label (C. J. Munro, University of California, Davis) and standards (Sigma-Aldrich, UK) on a Nunc-Immuno Maxisorp (Thermo-Fisher Scientific, UK) microtitre plate.

For corticosterone, the procedure was as follows: 50 µl per well of antiserum (1:15 000 dilution in coating buffer) was loaded and incubated overnight at 4°C, plates were washed with wash solution (0.15 m NaCl, 0.05% Tween 20) five times, standards (3.9–1000 pg per well) or samples diluted in EIA buffer were loaded at 50 µl per well, followed by 50 µl per well of horseradish peroxidase (1:70 000 dilution in EIA buffer). For testosterone, non-specific goat anti-rabbit γ-globulin (IgG; R2004; Sigma) was diluted in coating buffer, then loaded, 1.0 µg in 250 µl per well, on microtitre plates and incubated overnight at room temperature. The non-specific IgG was then discarded, and 300 µl per well of Tris blocking buffer (0.02 m Trizma, 0.300 m NaCl and 1.0% bovine serum albumin, pH 7.5) was added and incubated for a minimum of 2 h at room temperature, plates were washed with wash solution five times, EIA buffer was loaded at 50 µl per well, standards (2.3–600 pg per well) or samples diluted in EIA buffer were loaded at 50 µl per well, followed by 50 µl per well of horseradish peroxidase (1:40 000 in EIA buffer) and 50 µl per well of antiserum diluted 1:25 000 in EIA buffer. Following incubation the dark for 2 h at room temperature, plates were washed with wash solution five times and incubated with 100 µl per well of room temperature substrate [0.4 mm 2,2′-azino-di-(3-ethylbenzthiazoline sulfonic acid) diammonium salt, 1.6 mm H2O2 and 0.05 m citrate, pH 4.0) and left to develop at room temperature in the dark. Following incubation, developed plates were measured at 405 nm. Pony faecal extracts eluted from cartridges were diluted as necessary in EIA buffer and run in duplicate (50 µl) on respective EIAs.

Enzyme immunoassays were biochemically validated for measuring corticosterone and testosterone in pony faecal extracts, through parallelism (corticosterone: $R^2 = 0.93$, $F_{1,7} = 87.24$, $p < 0.001$ and testosterone: $R^2 = 0.99$, $F_{1,7} = 617.27$, $p < 0.001$) and matrix interference assessment ($R^2 = 1.00$, $F_{1,7} = 21160.99$, $p < 0.001$ and testosterone: $R^2 = 0.99$, $F_{1,7} = 810.91$, $p < 0.001$). The cross-reactivities of testosterone and corticosterone antibodies have been reported elsewhere (deCatanzaro *et al.* 2003; Watson *et al.* 2013, respectively), and only data with an intra-assay coefficient of variation (C_V) less than 10% and inter-assay C_V less than 15% were accepted and used for statistical analysis.

Statistical Analysis

Linear mixed effect models were used to examine differences in degree, strength, degree centrality, closeness centrality, betweenness and reachability using the *lme4* package in R (Bates et al. 2015), whereas local transitivity and clustering were assessed using generalised linear mixed models using a penalised quasi-likelihood (*MASS* package; Venables & Ripley 2002). Each network metric was the dependent variable, with the roundup event (before or after), individual age class (adult or juvenile) and sex included as fixed factors, and pony ID included as a random factor. Absolute ones were removed from transitivity and clustering using $x' = (x(N-1) + 0.05)/N$, where N is the sample size. Betweenness scores were normalised between 0 and 1 by dividing by the highest value. We tested if dyadic weights were correlated before and after the roundup using Quadratic Assignment Procedure (QAP) regression analyses in the *sna* package in R (Butts 2016). A QAP regression analysis uses Monte Carlo simulations to randomly rearrange association weights between dyads and assesses the likelihood that the observed matrix occurred by chance; this is desirable to avoid statistical bias introduced when correlating dyadic data (Krackhardt 1988). As the matrices contain a high number of zero values (where individuals were never seen together), we used subsets of the before and/or after matrices that contained dyadic relationships that had a positive value both before and after the roundup. We also used the same method to compare the association weights between adult mares before and after the roundup.

Linear mixed effect models were also used to assess faecal glucocorticoid and androgen metabolite concentrations. Fixed factors were sampling period, time of day and herd/bachelor status; pony ID was included as a random factor. The sampling period was grouped into five categories: Baseline, 1-3, 5-7, 8-12 and 14-18 days. Pairwise comparisons were calculated using the *glht* function in the *multcomp* package in R (Hothorn et al. 2008). Faecal corticosterone and androgens were log-transformed to normalise model residuals. The C_V of faecal androgens and glucocorticoids was calculated for before and after the roundup for any individual with three or more samples using: C_V = standard deviation/mean. The difference in C_V before and after the roundup was assessed using an unpaired t test. To estimate the influence of outliers on the linear mixed effects model we calculated Cook's distance (Cook 1979) using the *influence.ME* package in R (Niewenhuis et al. 2012). To assess outliers, (Cook & Weisberg 1982) suggest a simple cut-off value of 1, however we use a more conservative cut-off value of 4/N, where N is the sample size (Bollen & Jackman 1985).

RESULTS

Social Networks

The roundup and removal of key stallions resulted in marked changes to the Welsh mountain pony population structure. There were fewer components in the post-roundup network, as a number of smaller groups formed one large inter-connected component (Table 3.2, Fig 3.2). Connectivity across the post-roundup network was higher as individual reachability and betweenness scores increased (reachability: $\beta = 0.11 \pm 0.02$ SE, $t_{61} = 5.13$,

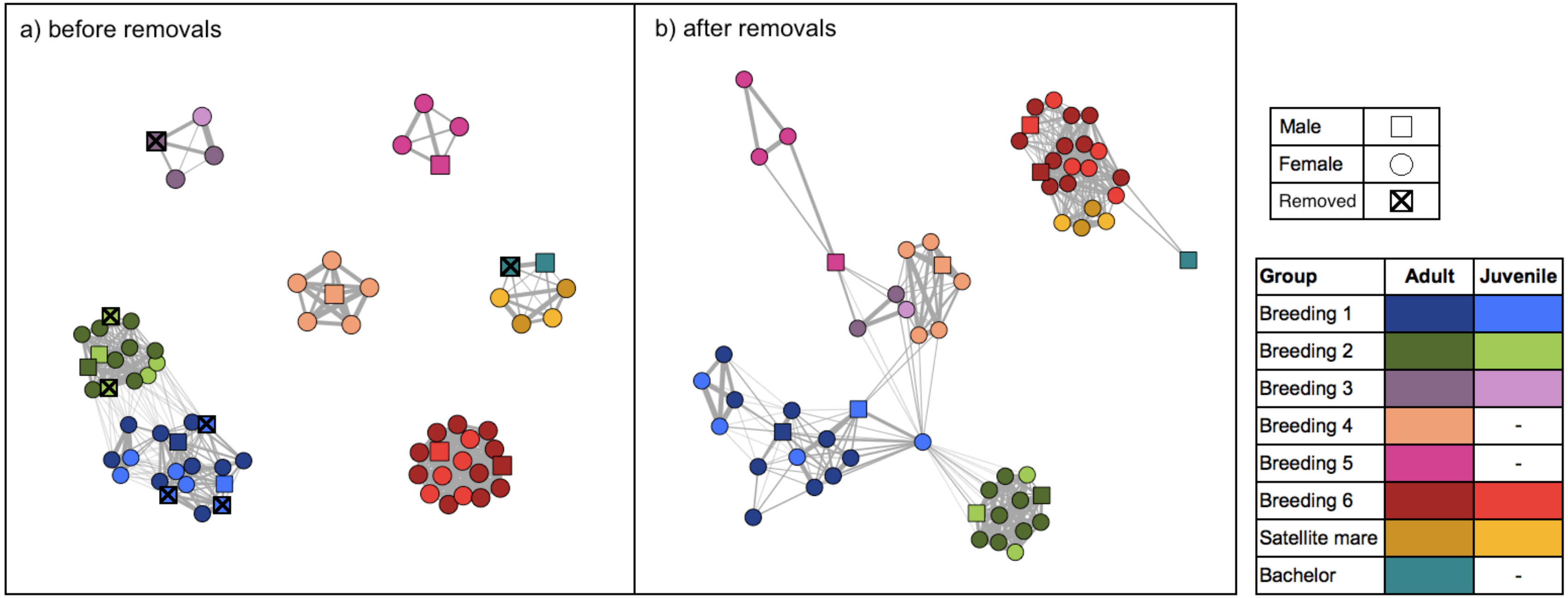

Figure 3.2. Network visualisations for weighted associations between Welsh mountain ponies before (a) and after (b) the annual roundup event and removal of key herd stallions. Different colours depict social groups, and edge weights are indicated by thickness of grey lines.

Table 3.2 Global social network measures calculated for weighted Welsh mountain pony association networks before and after a disturbance event

	Before roundup	**After roundup**
Number of individuals in network	70	62
Number of connected components	6	2
Largest component size	32	40
Edge density	0.18	0.19
Global transitivity	0.85	0.84
Global clustering	0.93	0.90

$p < 0.001$; betweenness: $\beta = 8.18$ e-05 ± 3.99 e-05 SE, $t_{61} = 2.05$, $p = 0.04$), and closeness centrality scores were significantly higher (Fig 3.3; $\beta = 0.09 \pm 0.01$ SE, $t_{61} = 8.76$, $p < 0.001$). However, there was a decrease in the strength of ties between individuals (Fig 3.3; $\beta = -0.39 \pm 0.18$ SE, $t_{61} = -2.25$, $p = 0.03$). The level of clustering within the network was significantly decreased post-roundup when calculated using weighted edges ($\beta = -0.62 \pm 0.22$ SE, $t_{61} = -2.87$, $p < 0.01$), however this was not significant when using binary presence or absence ties ($\beta = -0.35 \pm 0.23$ SE, $t_{61} = -1.52$, $p = 0.13$). There were no significant differences between the number of ties per individual or degree centrality after the roundup (degree: $\beta = -0.36 \pm 0.64$ SE, $t_{61} = -0.57$, $p = 0.57$; degree centrality: $\beta = -0.43 \pm 0.27$ SE, $t_{61} = -1.61$, $p = 0.11$). When examining demographic differences, juveniles had significantly more ties than adults ($\beta = 2.89 \pm 1.23$ SE, $t_{66} = 2.31$, $p = 0.02$) and marginally significantly higher degree centrality, closeness centrality and reachability scores (degree centrality:$\beta = 1.23 \pm 0.67$ SE, $t_{66} = 1.86$, $p = 0.07$; closeness centrality: $\beta = 0.03 \pm 0.02$ SE, $t_{66} = 1.79$, $p = 0.08$; reachability: $\beta = 0.06 \pm 0.03$ SE, $t_{66} = 1.93$, $p = 0.06$). There were no significant differences between sexes for any network metric.

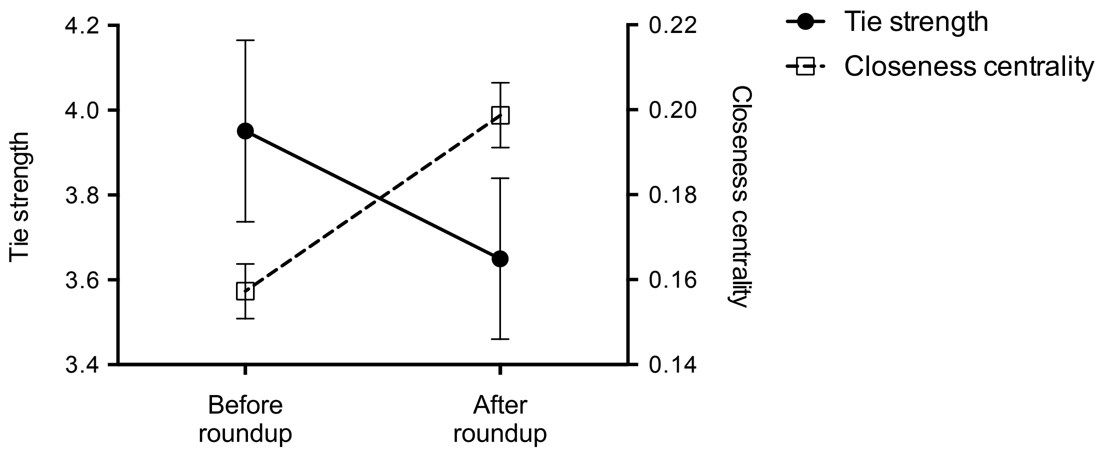

Figure 3.3. The strength of individual ties decreases and network connectivity increases in a Welsh mountain pony population after a disturbance event where several stallions were removed.

Using QAP regression analyses, we found that networks from before the roundup were highly correlated with those after the roundup, both for all individuals and for only adult females (all individuals: $r = 0.89$, $p < 0.001$; adult females $r = 0.85$, $p < 0.001$).

Stallion Androgen and Glucocorticoid Response

Stallion faecal androgen concentrations remained at levels similar to baseline for seven days post-release after the roundup. However, between days 8-12 of the sampling period a significant increase in faecal androgens was observed ($F_{4,40} = 5.48$, $p < 0.01$; Fig 4), followed by a further increase between days 14-18 to a maximum mean androgen concentration of 15.05 ± 2.59 ng/g compared to baseline levels of 7.44 ± 1.25 ng/g. Stallion faecal glucocorticoid metabolite concentrations followed a similar pattern and were also significantly elevated during days 8-12 of sampling, however they declined towards the end of the sampling period ($F_{4,40} = 3.53$, $p = 0.01$; Fig 4). Within-individual variation in faecal androgen concentrations was significantly higher after the roundup ($t = -3.10$, $df = 8.24$, $p = 0.01$), and within-individual variation in faecal glucocorticoid concentrations increased, but not significantly ($t = -2.66$, $df = 3.50$, $p = 0.07$). There were no differences between herd and bachelor stallions or with time of day for faecal androgen (herd/bachelor: $F_{1,12} = 0.18$, p =

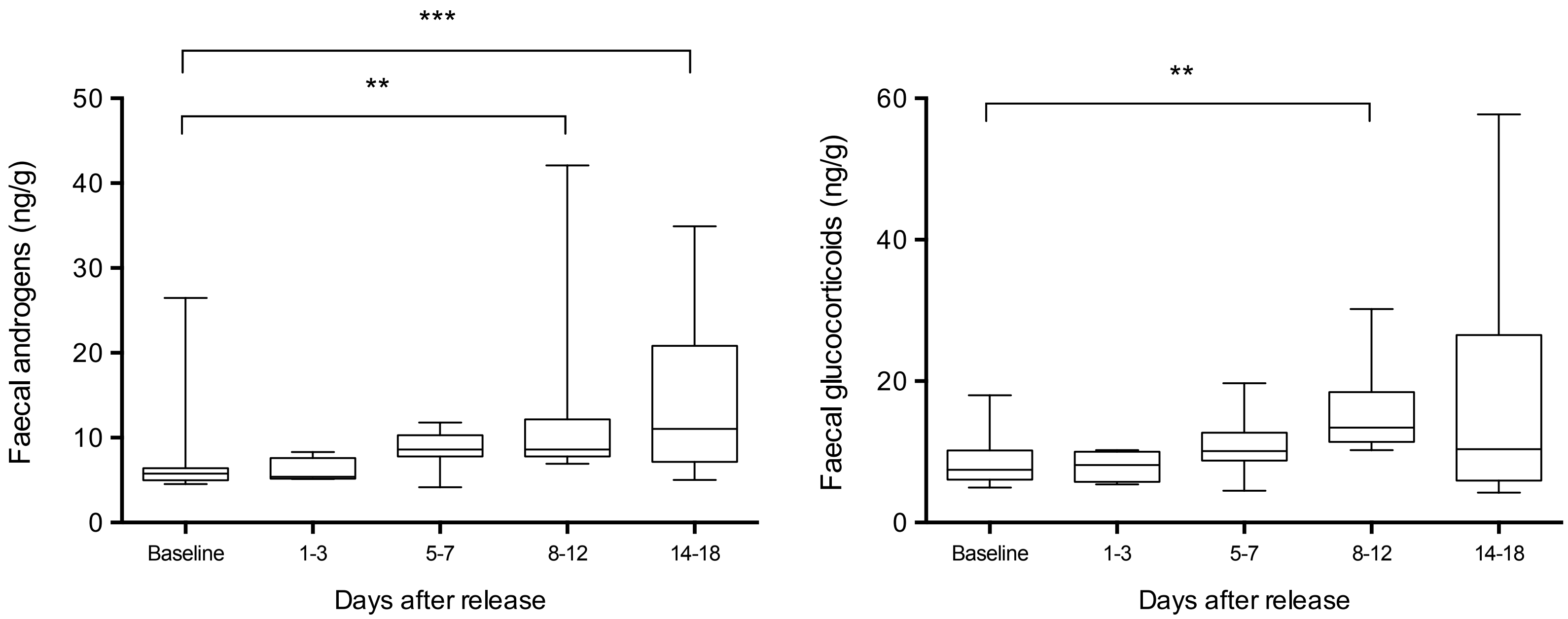

Figure 3.4. Faecal androgen (left) and glucocorticoid (right) metabolite levels in Welsh mountain pony stallions after release from a two-day roundup period and removal of several stallions. Faecal samples were collected opportunistically throughout the sampling period. Pairwise comparisons reveal significant differences, where ** indicates $p < 0.01$ and *** indicates $p<0.001$. Box plots show the median, upper and lower quartiles while the whiskers show the range of the data.

0.68, time of day: $F_{1,40} = 0.40$, $p = 0.53$) or glucocorticoid (herd/bachelor: $F_{1,12} = 0.02$, $p = 0.90$, time of day: $F_{1,40} = 0.56$, $p = 0.46$) metabolite concentrations.

Cook's distance values for the faecal androgen model were all below 0.15. Three data points exceeded the 4/N cut off value; to assess the contribution of these data points to the trend observed we removed all three and re-ran the model. The relationship between sampling day and androgen levels was the same as previously observed, with a higher level of significance ($F_{4,38} = 9.26$, $p < 0.001$). For faecal glucocorticoid models all Cook's distance values were also below 0.15. There were four data points with Cook's distance values higher than the cut off value, however when these were removed the relationship between sampling day and glucocorticoids also remained significant ($F_{4,36} = 4.47$, $p < 0.01$).

DISCUSSION

Disruption to Social Network Structure

The roundup and removal of stallions from the Welsh mountain pony population resulted in large-scale disruption to social network structure. Initially there was a high level of clustering across the population, consisting of breeding groups, mare groups (often a sub-group from a large breeding group) and small bachelor groups. After the removal of key stallions, individuals were less clustered and had higher closeness centrality scores, indicating greater connectivity across the network. In addition, individuals had on average lower tie strengths, which is concerning as individuals in this population tend to form stable strong social bonds when there are no major perturbations to the population (Stanley et al. *in press*). Furthermore, the strength of social relationships has been correlated with reproductive success in feral horses (Cameron et al. 2009). The QAP regression analysis showed that the strength of dyadic relationships were correlated before and after the roundup event, this is unsurprising as one would not expect large numbers of individuals to abandon their existing social groups and form completely new bonds, even despite the disruption that the removals caused. Similarly, mares also showed high fidelity to their existing relationships as dyadic relationships between females were highly correlated. Social networks have been shown to remain stable in the face of random removals and disturbances to the environment (Formica et al. 2017). However, networks can be more susceptible to disruption if individuals with key social roles, for example with high betweenness scores, are targeted (Williams & Lusseau 2006). The marked changes to Welsh mountain pony network structure indicate that stallions have an important role in maintaining social stability in *Equus caballus* populations, likely because they form long-term bonds with mares that reduce aggression between individuals (Linklater et al. 1999) performing a function analogous to 'policers' found in primate social systems (Flack et al. 2006). Social network position is associated with individual survival (Lehmann et al. 2015) and fitness (Solomon-Lane et al. 2015), therefore the removal of individuals from any population should be considered carefully. The consequences may be even more prescient for endangered equid species that share a similar social structure, such as the African wild ass (*Equus africanus*) or the Przewalski's horse (*Equus ferus przewalksii*).

Closeness centrality: benefits and costs

A high level of centrality is generally assumed to be beneficial, with central individuals experiencing increased fitness (Formica et al. 2012; Lehmann et al. 2015; Stanton & Mann 2012). However, whether an individual incurs a cost or a benefit from an increase in closeness centrality, or from greater connectivity across a network overall, has yet to be conclusively decided. Theoretically, a decrease in the shortest path length between individuals would create greater efficiency in the transmission of information: a shorter path means less time leading to lower costs (Freeman 1978). Empirical evidence has shown that greater social connectivity at a young age can increase the likelihood of achieving a high social rank in the future (McDonald 2007). However, increasing the efficiency of information transfer could also lead to higher rates of disease transmission; studies have shown that overall structure and individual network position can influence disease dynamics in wild animal populations (Webber et al. 2016; Weber et al. 2013). Moreover, being highly connected to conspecifics can increase the occurrence of agnostic interactions (Leu et al. 2016). In the Welsh mountain pony population, the symptoms of a disrupted network were shorter average path lengths but weak associative ties. This was due to a greater level of movement between groups by some individuals, thereby connecting otherwise discrete components. In free-living horse populations long-term associations create strong bonds between individuals, which reduce aggression between conspecifics and boost reproductive success (Cameron et al. 2009). Moving between groups can provoke a physiological stress response (Nuñez et al. 2014), and social dispersers often have a poor body condition and decreased fitness relative to individuals that change groups less frequently (Linklater et al. 1999). Therefore, an increase in closeness centrality as a result of the removal of key stallions could have negative consequences for individual health and fitness across the population. Importantly, the disturbed network may be vulnerable to disease outbreak, where individuals with the highest closeness centrality scores are more susceptible and the most likely to spread infection (Corner et al. 2003). The likelihood of disease or parasite transmission may depend on characteristics of the pathogen (e.g. length of infection period, Cross et al. 2004), or the strength of individual ties (Godfrey et al. 2010). Our results show that it is important to consider the costs and benefits to increased levels of connectivity both at the individual level and for the population as a whole, particularly when investigating the role of social networks in disease transmission and population dynamics.

Stallion Endocrine Response

While stallions did not show an immediate endocrine response to the roundup event, stallion androgen levels were elevated eight days after release and remained so until the end of the study period. As androgen levels are directly linked to behaviours associated with dominance and male competition (Lincoln et al. 1972; Muehlenbein & Watts 2010; Rose et al. 1971), the removal of breeding stallions from the population could have resulted in heightened competition between stallions for available mares, leading to an increase in faecal androgen concentrations. In addition, stallion faecal glucocorticoids also increased eight days post-release, then subsequently declined but remained highly variable. This trend is comparable to faecal glucocorticoid concentrations observed in Welsh mountain pony mares, also measured in response to the roundup event (Lea et al. unpublished data). Despite the relatively small sample size the physiological response observed in stallions appears reliable, and is intuitive given the level of disruption to the underlying social structure of the population. Elevated faecal glucocorticoids could be a result of stressful social interactions between conspecifics, as both stallion harassment and mare-mare aggression are more likely to occur during movement events (Linklater, 2000; Nuñez et al. 2014). While the hormone profiles are by no means conclusive, they do give an additional insight into the status of the remaining stallions in the pony population and suggest that there may be a physiological disruption as well as a behavioural one as a result of targeted removals. Importantly, we cannot conclude from this study how long the effects of the disturbance persisted, which is a highly importance factor when trying to determine overall impact. Further investigation should attempt to address this issue and aim to collect a more comprehensive profile of faecal hormone samples to provide a conclusive result.

Management Recommendations

The annual roundup event is a necessary management practice for the purpose of monitoring the otherwise undisturbed Welsh mountain pony population. However, the results of this study suggest that the removal of stallions should be approached with caution, as there may be significant behavioural and physiological consequences. It is likely that any physiological response to the roundup event itself would return to normal once the ponies are released and the stressor is gone However, it is possible that the disruption to normally stable social groups caused by stallion removal may create additional stressors after the event, thereby generating a further endocrine response. Moreover, if the removal of stallions were to be consistent and frequent this would likely create a female-biased population, the impact of which is at the moment unknown. Surveys of the current population have found very few bachelor stallions present, which are an important aspect of introducing competition and stallion turnover in equid sociality (Linklater, 2000). If individual removals are considered necessary to limit population growth, then we suggest that they are conducted randomly (e.g. removing every tenth individual through the gate) to avoid biasing the sex ratio of the population and to minimise disruption.

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CHAPTER FOUR

Recognition and management of ecological refugees: a case study of the Cape mountain zebra

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Abstract

Anthropogenic activities have led to long-term range contraction in many species, creating isolated populations in ecologically marginal and suboptimal habitats. ‘Refugee’ species have a current distribution completely restricted to suboptimal habitat. However, it is likely that many species are partial refugees, where one or more populations are managed in ecologically unsuitable habitat. Here, we develop a framework to assess potential refugee populations in marginal habitats using a model species: the Cape mountain zebra. We assessed habitat quality by the abundance and palatability of grass and diet quality using proximate nutrient and element analysis. High grass abundance was associated with higher population growth rates and zebra density and less skewed adult sex ratios. Furthermore, faecal nutrient and dietary element quality was also positively associated with grass abundance. Our results show that poorly performing populations were characterised by suboptimal habitat, supporting the hypothesis that the Cape mountain zebra has refugee populations. In addition, we found more variance in sex ratio and population growth rates in smaller populations suggesting they may be more at risk for random stochastic effects, such as a biased sex ratio, compounding poor performance. We show how the ‘refugee’ concept can be applied more generally when managing species with fragmented populations occurring across marginal habitats. More broadly, the results presented herein highlight the importance of recognizing the range of habitats historically occupied by a species when assessing ecological suitability. Identifying and mitigating against refugee, relict and gap populations is especially critical in the face of on-going environmental change.

Introduction

Current protected area coverage

Globally, protected areas are biased towards areas that have low value for human conversion for agriculture or development (“rock and ice” landscapes, Joppa & Pfaff, 2009). Given these biases, it is unsurprising that many species have distribution ranges that do not coincide with formally protected areas (Rodrigues *et al.*, 2004a). In fact, over 92% of critically endangered fauna can be considered as ‘gap species’, as their ranges do not occur within any protected area (Rodrigues *et al.*, 2004b). Many more species can be considered as ‘partial gap species’, where only a small proportion of their range is protected (Rodrigues *et al.*, 2004b; Maiorano, Falcucci & Boitani, 2006).

Whilst inadequate range overlap with protected areas is a clear impediment to successful conservation, confinement of a species in poor quality or unsuitable habitat is an equal but often overlooked problem. Thus, a simple focus on protected area overlap with species’ ranges may not be an appropriate measure of adequate protection. In addition to overlap, habitat suitability of protected populations needs to be considered. Species that are restricted or managed in marginal habitats may have poorer long-term prognosis than is apparent by evaluating protected area coverage. An extreme case is the ‘refugee species’ (Kerley, Kowalczyk & Cromsigt, 2012), where anthropogenic pressures across a species’ historical distribution leaves little available optimal habitat, and management interventions now restrict species to lower quality areas of their range where fitness is reduced. Whilst a gap species has a range that is absent from any protected area, a refugee species’ range is confined to a protected area consisting of suboptimal or inappropriate habitat. The refugee concept builds on the ideal free distribution theory of habitat selection (Fretwell, 1972): population density will be highest in optimal habitats, but individuals will disperse into low quality habitat creating a gradient from high performing ‘source’ to low density ‘sink’ populations that are maintained by immigration from source populations (Pulliam, 1988) where reproduction and mortality rates vary across sites (Pulliam & Danielson, 1991). Habitat loss and fragmentation can lead to populations that are restricted to poor quality habitat. These populations will have a higher extinction risk than large, high-density populations in optimal habitats (Pulliam & Danielson, 1991), due to slow population growth rates and/or low population densities (Kerley *et al.*, 2012). Convincing evidence for refugee status has been compiled for the European bison (*Bison bonasus*) and the Mediterranean monk seal (*Monachus monachus*; Kerley, Kowalczyk & Cromsigt, 2012; Bocherens *et al.*, 2015; González, 2015).

Importantly, when range contraction occurs over several decades or longer, relict populations can be perceived as occurring in 'natural' or core habitat, due to the acceptance of an altered state as a baseline. 'Shifting baseline syndrome' has been discussed in detail with regard to fishing stocks (Pauly, 1995; Pinnegar & Engelhard, 2008), and has been identified as a real concern for conservation policy-making (Papworth *et al.*, 2009). Counter-productive management strategies can be implemented under shifted baselines, for example translocations and introductions may be targeted towards areas of suboptimal habitat. As with partial gap species (those with a portion of their range outside a protected area) it is likely there are many cases of partial refugees, with at least some populations actively managed in protected, but marginal, habitat. In fact, many species may be both partial gap and partial refugees, where their current distribution is limited to poorly protected, suboptimal habitats. Thus, long-term conservation of species that have undergone extensive range contraction demands the recognition that relict populations may not occur in optimal habitats across their historical distribution. Successful management and conservation of such species relies on the implementation of novel interventions to overcome such constraints (Kerley, Kowalczyk & Cromsigt, 2012).

A second consequence of long-term range contraction is habitat fragmentation leading to small, isolated populations. Such populations are more vulnerable to extinction as a result of multiple processes including environmental and demographic stochasticity and inbreeding (Lande, 1998). Thus, refugee populations are likely to be small, stochastic and isolated (Lesica & Allendorf, 1995), which can also result in Allee effects, where population performance is reduced in small or low-density populations. However, the causes of Allee effects are notoriously hard to document in vertebrate populations (Courchamp, Berec &

Gascoigne, 2008). Thus, historical fragmentation can impose both ecological and demographic challenges for populations.

Cape mountain zebra as a partial refugee

The Cape mountain zebra (*Equus zebra zebra*) is a candidate for partial refugee status [see Kerley, Kowalczyk & Cromsigt (2012) for assessment criteria]. During the 20th and 21st centuries they underwent a large-scale population decline due to excessive hunting, persecution and habitat loss, leaving three relict populations with fewer than 80 total individuals. Active conservation has resulted in reintroduction across their historic range, with numbers now in excess of 4791 individuals (Hrabar & Kerley, 2015). Although this represents a great improvement and a rare conservation success story, many difficulties are still faced in their management. Cape mountain zebra occur as a complex of more than 75 small, fragmented and isolated populations, on both formally protected and privately-owned land. Individuals cannot freely disperse between these populations, rendering long-term natural metapopulation dynamics impossible without human intervention. Apart from one historical translocation, slow and stochastic growth in two of the relict populations has precluded the removal of individuals, such that 95% of the global population derives from the single relict population in the Mountain Zebra National Park. The prolonged bottleneck and isolation has resulted in the relict populations (and their daughter populations) becoming genetically distinct from one another (Moodley & Harley, 2006). Consequently, a large proportion of the remaining genetic diversity remains unrepresented by the majority of the subspecies, and is under threat of being lost altogether if the two relict populations (Gamkaberg Nature Reserve and Kammanassie Nature Reserve) are not secured.

Cape mountain zebra are an ideal model species for understanding how confinement to marginal habitat impacts on population performance because the current populations, both relict and reintroduced, occur across a range of habitat types. Most of the Cape mountain zebra's current (and historic) range is found within the Cape Floristic Region in South Africa (Hrabar & Kerley, 2015; Boshoff *et al.*, 2016). The northeastern areas are characterised by summer rainfall and escarpment grasslands, whereas the southwest is dominated by winter-rainfall, with fynbos and succulent Karoo vegetation communities and low grass cover, much of which is not suitable for grazing animals (Boshoff *et al.*, 2002; Kerley *et al.*, 2003). The distribution of protected areas within the Cape Floristic Region is heavily biased towards marginal upland habitat (Rouget, Richardson & Cowling, 2003a), with few lowland areas large enough to support even small populations of large mammal herbivores (Kerley *et al.*, 2003). Paleontological evidence suggests that Cape mountain zebra occupied open grassland, and persisted in low densities in fynbos habitat (Faith, 2012). There is evidence that, where possible, Cape mountain zebra seasonally move between habitat types and predominantly select areas with high grass cover (Penzhorn, 1982; Grobler, 1983; Winkler & Owen-Smith, 1995; Smith *et al.*, 2008).

Two of the relict populations, Gamkaberg Nature Reserve and Kammanassie Nature Reserve, are dominated by fynbos vegetation and it has been suggested that less than 40% of each reserve is appropriate habitat for Cape mountain zebra (Watson *et al.*, 2005; Watson & Chadwick, 2007). Most importantly, these populations have been actively managed in upland areas with restricted access to year-round grass-rich habitats and drinking water, which is likely a key factor leading to limited population growth. As relict populations were restricted to upland fynbos habitat in recent memory, these areas have been perceived by managers as core habitats for Cape mountain zebra, despite poor population performance. Thus, introductions and translocations of individuals into similar, and more arid, habitats have been supported as a key part of the species management plan (Novellie *et al.*, 2002).

Here, we assess partial refugee status in the Cape mountain zebra by evaluating variation in habitat quality and population performance across reserves. We predict that: 1) populations in grass-poor habitats will have slower population growth rates and lower population densities, and 2) habitat quality (grass availibility) and population performance will be associated with nutrient profiles derived from faecal diet analysis. We use three measures of population performance, which reflect different aspects of populaiton health: population growth rate, zebra density and foal:mare ratio. We then provide a framework for identifying refugee populations both in Cape mountain zebra and other potential refugee species, and discuss the importance and application of this concept within conservation biology.

Methods

Vegetation index

Habitat assessments were made for a subset of 21 Cape mountain zebra populations (both public and private reserves, representing 28% of extant populations) where long-term population records were available, and which varied in terms of grassiness. We developed a *perceived grass vegetation index* (VI) that incorporates fine-scale differences in vegetation communities by qualitatively assessing the abundance of palatable grass species within each reserve. Although this technique does not quantify the biomass of palatable grass, it provides a systematic and repeatable assessment of grass dominance and richness that can be readily estimated across populations. The majority of the resources used (vegetation map and reserve boundaries) are freely available online (SANBI, 2006). Where geo-referenced maps were not available for private reserves, boundary information was obtained from reserve managers and shapefiles were created using Google Maps, with imagery provided by 2015 AfriGIS (Pty) Ltd, Google Imagery 2015, CNES/Astrium, CNES/Spot Image, DigitalGlobe, Landsat and TerraMetrics TruEarth Satellite Imagery. The boundary of each reserve was overlain on the National Vegetation Map of South Africa, Lesotho and Swaziland (Mucina & Rutherford, 2006) using ArcGIS Desktop v.10, and the area of all vegetation types occurring in the reserve was estimated. Each defined vegetation type has an associated list of 'Important Taxa' that have a high abundance or frequent occurrence within the landscape, and also highlights dominant taxa (Mucina & Rutherford, 2006). The number of graminoid species in this list (excluding Cyperaceae, Juncaceae and Restionaceae) was used as a proxy for grass abundance; dominant species were weighted by a factor of two (see Table A1.1 in Appendix 1 for list of vegetation types and number of grass species present). Each grass species was ranked in terms of palatability (1 = low, 2 = medium, 3 = high) using information from published resources and online databases (see Table A1.2 for full list of species; (Quattrocchi; van Breda, Nel & Bayer, 1990; van Oudtshoorn, 2012; Andersson *et al.*, 2015). Graminoid species listed in the 'Endemic Taxa' section (Mucina & Rutherford, 2006) were included. Graminoids, mainly endemic to a particular vegetation type, were excluded if there was no information available for palatability, which constituted 42 out of 154 described species or 65 out of 558 (~12%) of total occurrences. This resulted in a vegetation index for each vegetation type (VI_V) present within a reserve that was calculated by:

$$\mathrm{VI_v} = \Sigma\mathrm{PT} + \Sigma 2\mathrm{PD}$$

where T is the total number of non-dominant grass species listed in the vegetation type description, D is the number of dominant grass species listed and P is the palatability of each grass species found within that vegetation type.

Finally, a standardised VI for each reserve was calculated as:

$$\mathrm{VI} = \frac{\sum(\mathrm{CVI_V})}{\mathrm{VI_{MAX}}}$$

where C indicates the percentage area of each vegetation type within a reserve and VI_{MAX} is the highest VI attained by any reserve in this study. The grass cover of three reserves was adjusted to reflect anthropogenic removal of fynbos habitat and replacement with disturbed, cultivated grasslands containing highly palatable grass species (De Hoop Nature Reserve: 1.70%, Swartberg Private Game Reserve: 21.52% and Welgevonden Game Farm: 41.72%). The boundaries of these disturbance grasslands were demarcated using Google Maps® and given the same VI_V as the high quality Karoo Escarpment Grassland as transformation of land in these reserves was managed for grazing stock and therefore constitutes a high abundance of palatable grass species.

Diet quality

Faecal analysis has been used to estimate diet quality across several wild herbivore taxa (Erasmus, Penzhorn & Fairall, 1978; Putman, 1984; Hodgman, Davitt & Nelson, 1996; Mésochina *et al.*, 1998; Landman *et al.*, 2013). We used this approach to evaluate Cape mountain zebra diet quality variation across reserves. During April and May 2014, a total of 106 faecal samples were collected from Mountain Zebra National Park, Gamkaberg Nature Reserve, De Hoop Nature Reserve, Camdeboo National Park, Mount Camdeboo Private Reserve and two sites in Karoo National Park (the Potlekkertjie Loop and the Mountain View area in the east). Fresh samples (< 1 hour) were collected from as many individuals as possible, although if necessary older samples were also collected and age estimated (< 24 hours or > 24 hours). Samples were oven dried at 60°C and milled using a 1 mm sieve, then subject to proximate diet analysis to estimate the content of crude protein, ash, fat, acid detergent fibre (ADF) and neutral detergent fibre (NDF) in the Cedara Feed Laboratory, South Africa (for full protocols see Goering & van Soest (1970) and AOAC (1980). The minerals Ca, Mg, K, Na, P, Zn, Cu, Mn, and Fe were determined by dry-ashing samples and atomic absorption spectroscopy. Faecal crude protein content from the proximate diet analysis was converted to faecal N by dividing by 6.25 (Rivera & Parish, 2010). Crude protein represents all sources of nitrogen in the faeces and is directly positively related to the palatability and digestibility of the diet (Erasmus *et al.*, 1978), while ash represents the inorganic minerals. Conversely, the fibre content (shown here as NDF and ADF) coincides with low digestibility, where NDF represents the total amount of fibre in the diet and ADF the component of that fibre that is indigestible (Rivera & Parish, 2010). N and P are the most frequently measured elements as indicators of forage quality and both are limiting nutrients in South Africa (Wrench, Meissner & Grant, 1997; Grant, Peel & van Ryssen, 2000).

Study sites and population performance measures

Collection of demographic data took place at the following reserves (Fig 1): Bakkrans Nature Reserve, Camdeboo National Park, De Hoop Nature Reserve, Gamkaberg Nature Reserve, Karoo National Park, Mount Camdeboo Private Reserve, Mountain Zebra National Park, Samara Private Game Reserve, Swartberg Private Game Reserve and Welgevonden Game Farm. Data were collected during seven sampling periods: Aug – Sep 2010, Feb – June 2011, Dec – Mar 2012, Nov – Dec 2013, Apr – May 2014, Jan – May 2015 and Sep – Dec 2015; eight reserves were visited a minimum of four times across these sampling periods whilst the remaining two were visited twice. Each visit entailed a minimum of three reserve-wide surveys. Surveys were conducted both by vehicle and on foot, using public access and/or management roads. Whilst surveys are likely biased towards the areas covered by roads, the use of binoculars from key vantage points enabled most reserves to be surveyed extensively. The most notable exception to this is Karoo National Park, which is large, mountainous, and has a large section in the west not covered by a road network. Upon encounter, overall group size and each individual's sex and age class (foal ≤12 months, sub-adult 13-36 months, adult >36 months) were recorded. Where possible, and for the majority of zebra sighted, each individual was photographed and repeated sampling of individuals

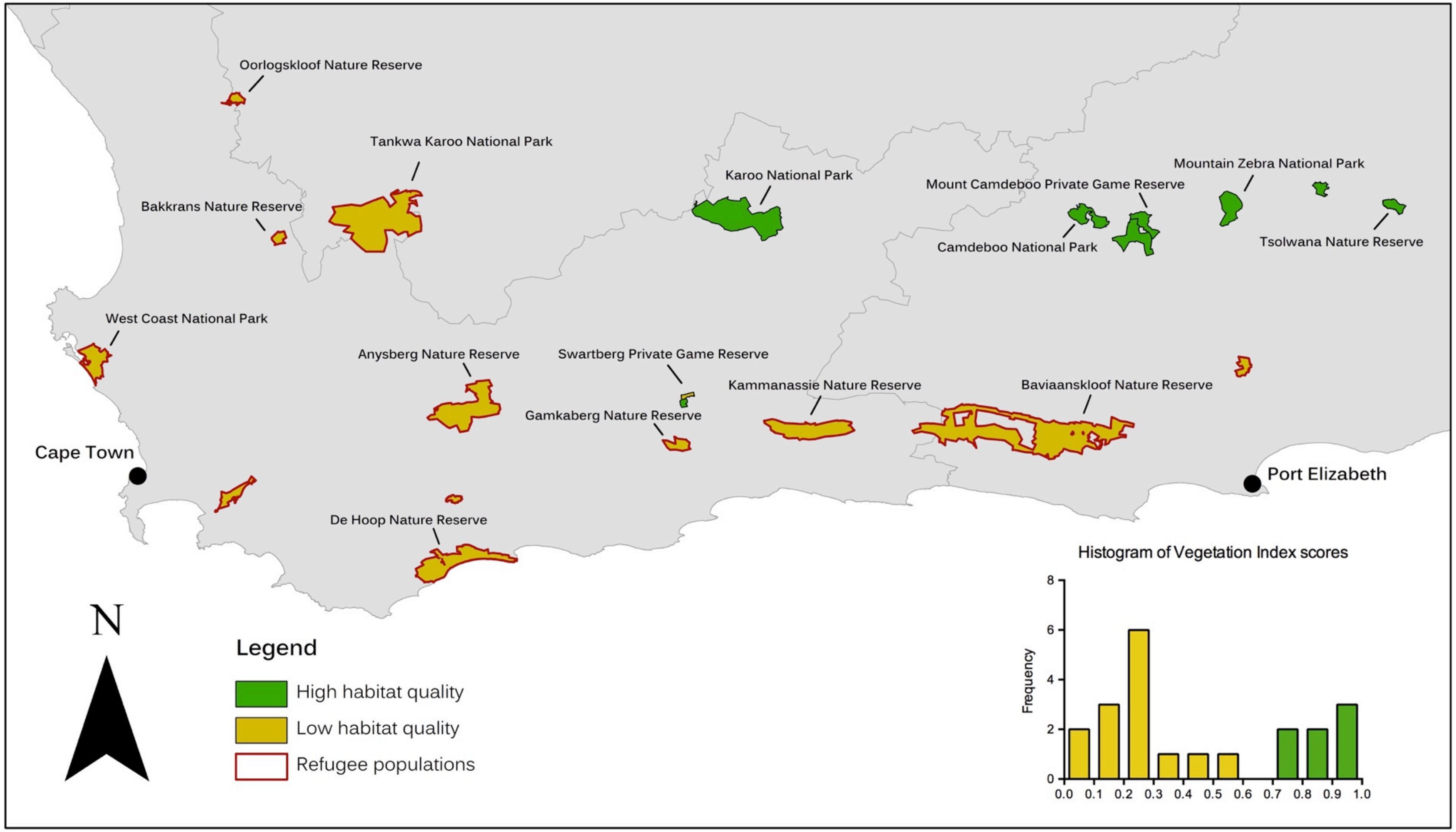

Figure 4.1 Variation in Vegetation Index scores across populations where population performance and demographic data were available. Some focal populations are labelled.

was accounted for using HotSpotter identification software (Crall *et al.*, 2013). The number of foals per mare observed at each reserve was averaged over all sampling periods to give the mean number of foals per mare (foal:mare). In addition, the following information for a further 16 populations was made available by the management team at each reserve from their records and 2015 ground and aerial counts: founding year, founding number, current population size, number of introductions and removals and the reserve area. Population growth rate was calculated using:

$$\mathrm{N_t = N_o e^{rt}}$$

solved for r:

$$\mathrm{r = \frac{\ln N_t - \ln N_o}{t}}$$

where r is the rate of increase, N_t is the current population size, N_o is the starting population size (or founding number), t is the time in years, e is the mathematical constant and ln the natural log. Current population size was adjusted for any recorded introduction or removal events since the population was founded: introductions were added to the founder population number and removals were added to the current population size. Populations range from 5500 - 146000 ha, with the youngest founded nine years ago and the oldest relict population, Mountain Zebra National Park, established 79 years ago. Population density was calculated as current population size/reserve area (number of individuals/ha). Poorly performing populations were defined as scoring at least 50% worse than the best performing population on each measure. Mountain Zebra National Park was excluded from this calculation as it has an extremely high density of individuals that likely reflects its substantially longer time since establishment than any other reserve in similar habitat, and if included would unfairly make all other populations poorly performing on this measure. The three measures (growth rate, density and foal:mare) reflect different aspects of performance. Newly founded populations may still be growing quickly as they have not yet have reached carrying capacity, whereas older established populations, such as in Mountain Zebra National Park, support a high density but have relatively moderate population growth rates. The observed number of foals per mare gives an indication of female fecundity at each site. In addition, the mean number of adult males and adult females observed from surveyed populations were used to calculate adult sex ratio (male:female).

Statistical analyses

Statistical analyses were performed using RStudio v.0.98.976 and IBM SPSS Statistics v.22. To reduce the proximal nutritional components (crude protein, ash, fat, NDF and ADF) to fewer explanatory variables, we performed a principal component analysis (PCA). Fat was removed as it formed its own principal component (PC), leaving the remaining four variables loading on to one PC (crude protein and ash loading positively and ADF and NDF loading negatively) that explained 46.6% of the variation with a Keiser-Meyer-Olkin (KMO) score of 0.610. A second PCA was performed on the mineral components of the diet analysis; Ca and K were removed as they did not load with the other minerals; the remaining eight minerals loaded onto two PCs (PC1: N, P, Na, Zn, Mg and PC2: Cu, Fe, Mn). The two components explained 65.6% of the variation in the analysis with a KMO score of 0.714. VI scores had a bimodal distribution (see histogram in Fig 1), and are presented as both a categorical factor with 'high' and 'low' grass categories and a continuous variable in the performance measure analyses. Diet quality was compared across reserves and with categorical VI using Generalized Linear Models (GLM), with age of sample included as a factor and subsequently removed if not significant ($p > 0.05$). GLMs were used to evaluate relationships between continuous and categorical VI and population growth rate, foal:mare ratio, zebra density and with continuous VI and adult sex ratio. Reserve size and years since establishment were included as fixed factors in the population performance analyses, and removed if not significant. Zebra density was cube root transformed before statistical analysis to normalise the data. F tests were used to compare variance in sex ratio and population growth rate between small (< 50 individuals) and large populations (*stats* library, R Development Core Team, 2016).

Results

Habitat and diet quality

The distribution of VI scores was bimodal; reserves with less than half the abundance of palatable grass than that of the highest of this study were characterised as having low habitat quality for Cape mountain zebra (Fig 1). High VI scores are associated with summer rainfall areas on the Karoo escarpment where there is high abundance of palatable C4

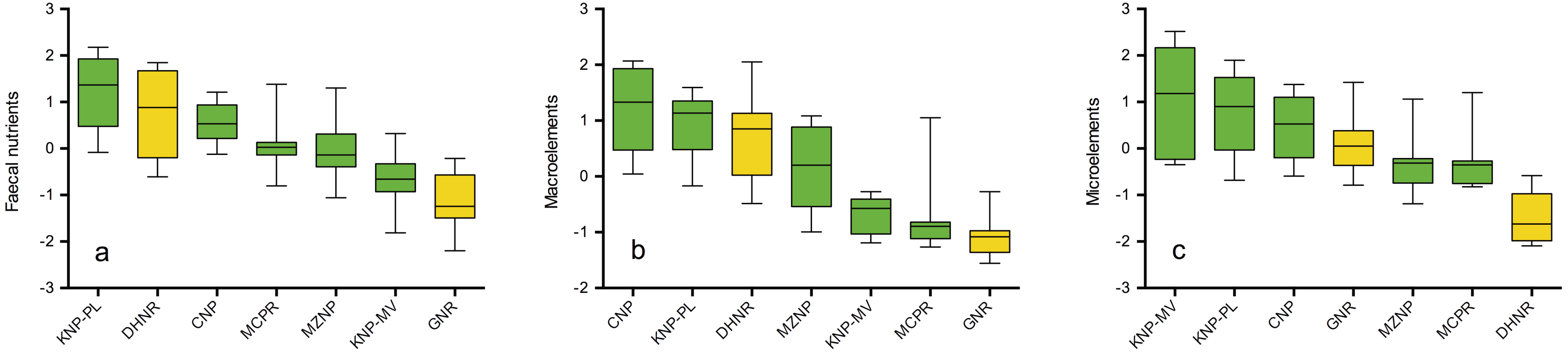

Figure 4.2 Mean values of a) proximate diet component (crude protein, ash, acid detergent fibre (ADF) and neutral detergent fibre (NDF) content, where crude protein and ash load positively on the principal component and ADF and NDF load negatively), b) macroelement and c) microelement content of Cape mountain zebra fecal samples across reserves. Reserve abbreviations are: CNP: Camdeboo National Park, DHNR: De Hoop Nature Reserve, GNR: Gamkaberg Nature Reserve, KNP-MV: Karoo National Park, Mountain View, KNP-PL: Karoo National Park, Potlekkertjie Loop, MCPR: Mount Camdeboo Private Reserve; MZNP: Mountain Zebra National Park. Colours represent the Vegetation Index score as in Fig 1. Boxes show the median, upper and lower quartiles while the whiskers show the range of the data.

grasses, whereas low VI scores are found in arid and/or winter rainfall regions, primarily, but not exclusively, in the fynbos biome. The levels of faecal nutrients ($F_{6,98} = 26.29$, $p < 0.001$) and macro ($F_{6,99} = 34.46$, $p < 0.001$) and microelements ($F_{6,99} = 18.52$, $p < 0.001$) varied significantly between reserves (Fig 2). Faecal nutrient ($F_{1,104} = 9.74$, $p < 0.01$), macroelement ($F_{1,104} = 10.90$, $p < 0.01$) and microelement ($F_{1,104} = 14.87$, $p < 0.001$) content were all positively associated with VI. However, between-reserve differences in faecal diet profiles accounted for more variation than did grassiness. Overall, faecal samples from Camdeboo National Park and Karoo National Park Potlekkertjie Loop contained the highest levels of crude protein, macro and microelements, while samples from Gamkaberg Nature Reserve contained the lowest (Fig 2; Table A1.3).

Population performance indicators

Performance measures and VI scores for each reserve can be viewed in Table 4.1. Population growth rate was significantly correlated with zebra density and foal:mare ratio (R = 0.60, 95% C.I. 0.24 to 0.82, $p < 0.01$ and R = 0.72, 95% C.I. 0.16 to 0.93, $p = 0.02$, respectively). There was a positive trend between zebra density and foal:mare ratio, though not significant (R = 0.61, 95% C.I. -0.03 to 0.90, $p = 0.06$). All three performance measures were significantly higher in reserves with a high VI (Fig 3), when treated as a categorical variable (population growth rate: $\beta = -0.05 \pm 0.02$ s.e., $t_{1,19} = -3.01$, $r^2 = 0.29$, $p < 0.01$; zebra density: $\beta = -0.09 \pm 0.03$ s.e., $t_{2,18} = -3.33$, $r^2 = 0.44$, $p < 0.01$; foal:mare ratio: $\beta = -0.16 \pm 0.05$ s.e., $t_{1,8} = -3.45$, $r^2 = 0.55$, $p < 0.01$). Population growth rate and zebra density increased significantly with VI (Fig 3) as a continuous variable (population growth rate: $\beta = 0.07 \pm 0.03$ s.e., $t_{1,19} = 2.22$, $r^2 = 0.16$, $p = 0.04$; zebra density: $\beta = 0.14 \pm 0.04$ s.e., $t_{2,18} = 3.62$, $r^2 = 0.48$, $p < 0.01$), and there was a positive trend between continuous VI and foal:mare ratio ($\beta = 0.20 \pm 0.09$ s.e., $t_{1,8} = 2.27$, $r^2 = 0.32$, $p = 0.05$). Zebra density decreased with increasing reserve size in both models ($\beta = -7.61e-07 \pm 3.52e-07$ s.e., $t_{2,18} = -2.16$, $r^2 = 0.48$, $p = 0.04$ and $\beta = -8.12e-07 \pm 3.63e-07$ s.e., $t_{2,18} = -2.24$, $r^2 = 0.44$, $p = 0.04$).

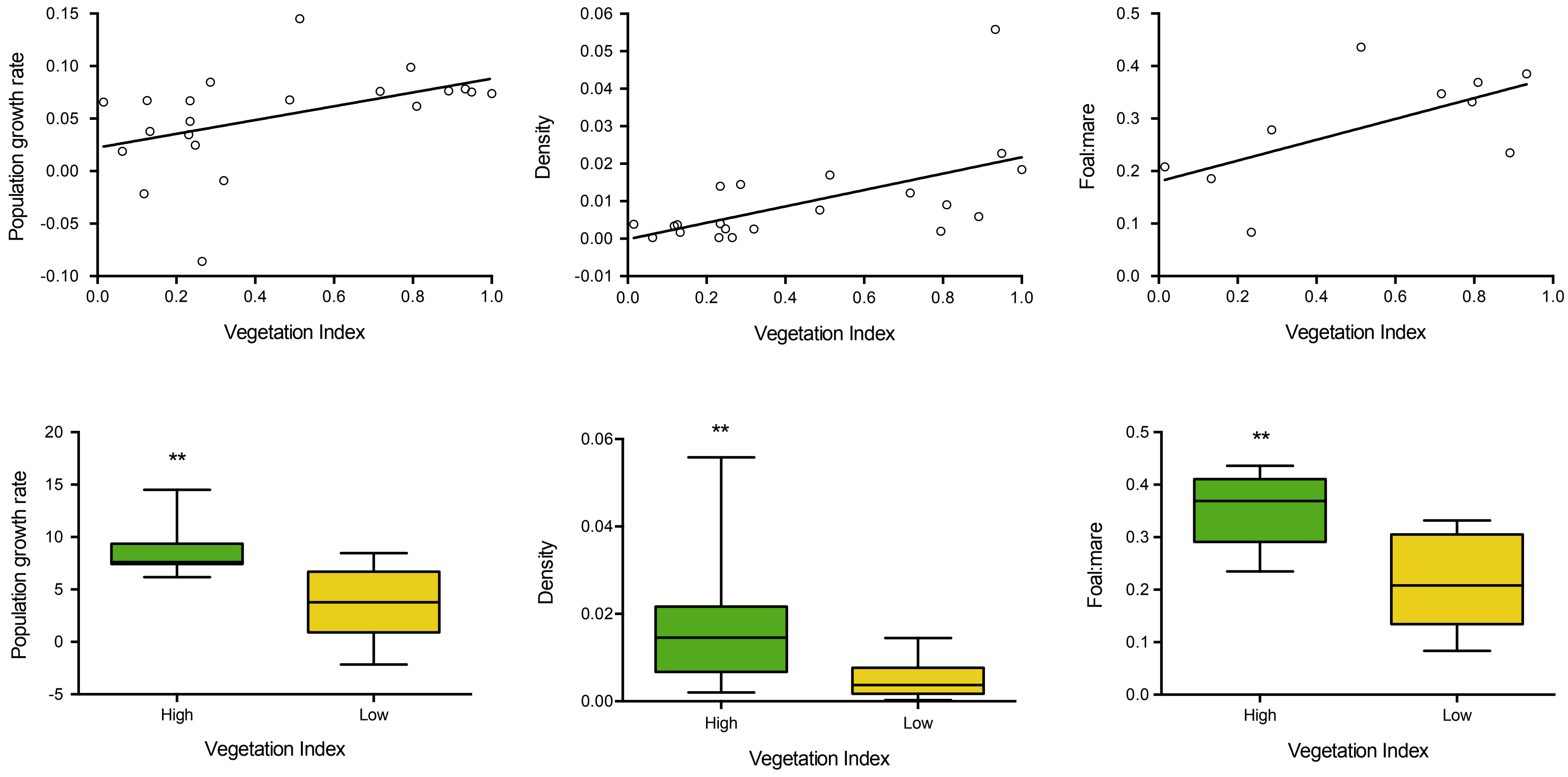

Figure 4.3 Population growth rate, zebra density (individuals ha^{-1}), and female fecundity (foal:mare ratio) of Cape mountain zebra are all positively associated with habitat quality (Vegetation Index), shown as both a continuous and a categorical variable. Boxes show the median, upper and lower quartiles while the whiskers show the range of the data.

Table 4.1 Population performance measures, relative assessment of palatable grass abundance (Standardised Vegetation Index), and refugee status for 21 Cape mountain zebra populations

Reserve	Standardised Vegetation Index	Current population size	Population growth rate	Density (individuals/ha)	Mean foal:mare (± s.d.)	Refugee population?
Anysberg Nature Reserve	0.06	23	0.019	0.0003		Yes
Bakkrans Nature Reserve	0.02	43	0.066	0.0038	0.208 ± 0.06	Yes
Baviaanskloof Wilderness Area	0.32	51	-0.009	0.0026		Yes
Bontebok National Park	0.25	9	0.025	0.0027		Yes
Camdeboo National Park	0.72	236	0.076	0.0122	0.347 ± 0.13	No
Commandodrift Nature Reserve	0.95	136	0.075	0.0227		No
Coppermoon Private Reserve	0.49	46	0.068	0.0077		Yes
De Hoop Nature Reserve	0.13	100	0.038	0.0017	0.186 ± 0.06	Yes
Gamkaberg Nature Reserve	0.24	42	0.047	0.004	0.083 ± 0.12	Yes
Hottentots-Holland Nature Reserve	0.27	6	-0.086	0.0003		Yes
Kammanassie Nature Reserve	0.13	80	0.067	0.0037		Yes
Karoo National Park	0.81	843	0.062	0.0091	0.369 ± 0.06	No
Mount Camdeboo Private Reserve	0.89	71	0.076	0.0059	0.235 ± 0.11	No
Mountain Zebra National Park	0.93	1191	0.078	0.0558	0.385 ± 0.12	No
Oorlogskloof Nature Reserve	0.12	19	-0.022	0.0034		Yes
Samara Private Game Reserve	0.8	26	0.099	0.002	0.332 ± 0.04	No
Swartberg Private Game Reserve	0.29	29	0.085	0.0145	0.278 ± 0.16	No
Tankwa Karoo National Park	0.23	41	0.035	0.0003		Yes
Tsolwana Nature Reserve	1	144	0.074	0.0185		No
Welgevonden Game Farm	0.51	34	0.145	0.017	0.436 ± 0.02	No
West Coast National Park	0.23	42	0.067	0.014		Yes

Demography and population performance

For the subset of populations with demographic data, sex ratio and population growth rate variance was higher across small populations (<50 individuals) than large populations ($F_{4,4}$ = 21.81, p = 0.01 and $F_{8,11}$ = 4.26, $p < 0.05$, respectively). The ratio of adult males to adult females was negatively associated with VI (β = -0.96 ± 0.41 s.e., $t_{1,8}$ = -2.33, , r^2 = 0.33, p = 0.05), such that populations in grass poor habitats were more likely to have a male biased sex ratio. Adult sex ratio was marginally, but not significantly, associated with foal:mare (β = -0.14 ± 0.06 s.e., $t_{1,8}$ = -2.04, p = 0.08, r^2 = 0.34), but not with population growth rate (β = -2.45 ± 1.82 s.e., $t_{1,8}$ = -1.35, p = 0.22, r^2 = 0.18) or zebra density (β = -0.004 ± 0.004 s.e., $t_{1,8}$ = -1.11, r^2 = 0.15, p = 0.30,), suggesting that VI had a stronger impact on population performance than demography.

Identification of refugee populations

The distribution of scores in the standardised Vegetation Index was bimodal (see histogram in Fig 1); therefore populations in the lower half were highlighted as potential refugees. Populations were subsequently confirmed as refugees if they performed poorly in one or more of the population performance measures (scoring at least 50% worse than the highest performing population on each measure). Using this criterion, we identified 12 refugee populations out of a total of 21 (i.e. 57%) in this study (Fig 4).

Discussion

A model partial refugee species: the Cape mountain zebra

Our results support the hypothesis that the Cape mountain zebra has refugee populations, which are confined to protected areas containing suboptimal habitat within its historic range and are characterised by poor population performance. The habitats currently occupied by Cape mountain zebra differ substantially in terms of their grass availability and nutrient and elemental dietary composition. We identified 12 refugee populations of Cape mountain zebra in this study, which have low habitat quality and score poorly in at least one performance measure. Populations in habitats with less palatable grasses performed worse across all performance indicators (population growth rate, foal:mare and density) and had more male-

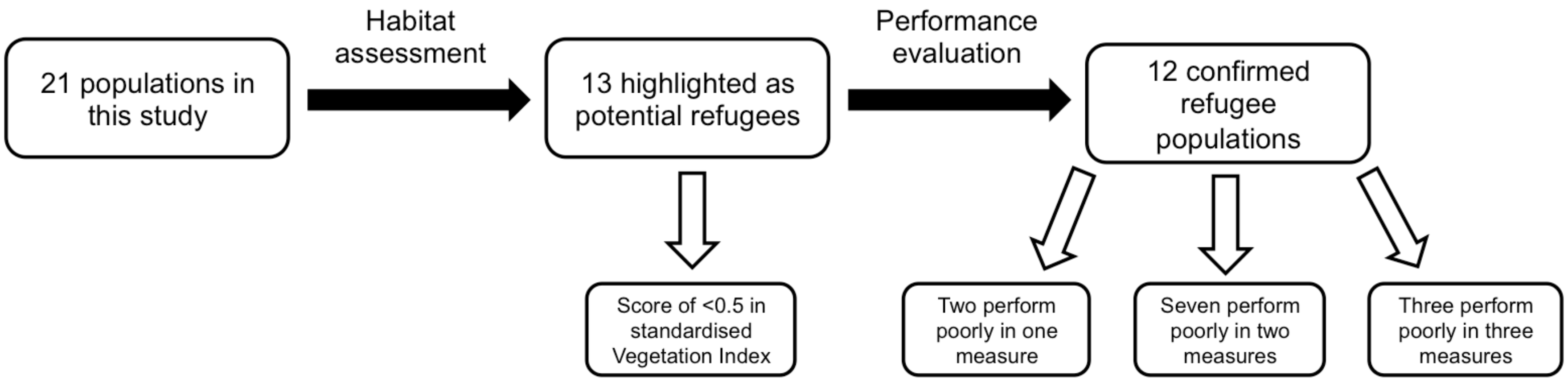

Figure 4.4 Potential refugee populations were identified as those with relatively low habitat quality. Of these, 12 were confirmed as refugee populations as they performed at least 50% worse than the highest performing population in at least one of the following performance measures: population growth rate, zebra density (individuals ha^{-1}) and foal:mare ratio.

biased adult sex ratios. Low density populations were also associated with lower growth rates and male biased sex ratios were weakly associated with female fecundity (foal:mare)., This which raises two questions: 1) how does demography impact on population growth rates?, and 2) what is the cause of skewed sex ratios? That smaller populations had higher sex ratio and population growth rate variance could either be the result of random demographic stochasticity or could suggest differential fecundity or survivorship in small populations. This also highlights potential feedback between habitat quality, population demography and long-term performance.

The three populations that contain the majority of the genetic diversity of the sub-species (Gamkaberg, Kammanassie and De Hoop Nature Reserves; Moodley & Harley, 2006) were all identified as refugee populations, suggesting that habitat, and specifically palatable grass availability, is limiting performance for these key populations. Moreover, other refugee populations recently re-introduced into reserves with a lower abundance of palatable grasses (e.g. Anysberg Nature Reserve and Bakkrans Nature Reserve) have performed worse than the two relict populations. These results corroborate studies that have argued that only small areas of some reserves have sufficient grazing for Cape mountain zebra (Watson *et al.*, 2005; Watson & Chadwick, 2007; Smith *et al.*, 2011; Weel *et al.*, 2015). It is important to note that there are seasonal rainfall differences between reserves that could affect diet quality at different times throughout the year. It is likely this is a contributing factor leading to between-reserve differences in diet quality accounting for more variation than VI in this study. This said, faecal samples collected from individuals utilising different vegetation communities in the same reserve (Karoo National Park) reflect marked differences in nutrient and element quality (Fig 2, Table A.3). A large proportion of the Cape mountain zebra's historic range covers the Cape Floristic Region, where soil nutrients are often low (Goldblatt & Manning, 2002). The widespread transformation of lowland areas, where rainfall and fertility are sufficient for agriculture, has pushed wildlife out of these areas (Rouget *et al.*, 2003b). Whilst the overall lower abundance of grasses in this region would have likely meant that Cape mountain zebra would have occurred at lower densities here than in the east, the inability of individuals to migrate to lowland habitat on a seasonal basis (Kerley *et al.*, 2003), and on a larger scale between populations, is inhibiting the performance of individual populations and the metapopulation as a whole. This results in small populations that are vulnerable to the impacts of demographic stochasticity.

Whilst some Cape Floristic Region species have been completely extirpated, including the global extinction of the blue antelope *Hippotragus leucophaus* (Kerley *et al.*, 2009), others, such as Cape mountain zebra, now have disjunct populations often restricted to lower quality, ecologically unsuitable habitat within their historical distribution. To improve the performance of refugee populations, Cape mountain zebra require access to suitable habitat, either through land acquisition or by active management of the currently available habitat (e.g. burning regimes or ecological restoration in heavily grazed areas; Watson *et al.*, 2005; Watson & Chadwick, 2007; Smith *et al.*, 2011). Ideally, this should be focused on securing the poor performing key relict populations, with the intention of increasing numbers to such an extent that individuals can be translocated to new areas of good quality habitat to form mixed stocks with individuals from other populations. Most importantly, our results highlight the on-going management of Cape mountain zebra populations in inappropriate habitat, often due to a misconception of what constitutes optimal, or core, habitat by conservation managers.

Implications and uses of the partial refugee species concept

Of the 21 Cape mountain zebra populations in this study, over half were identified as refugees. We propose a framework to assess species and populations for refugee status (outlined in Table 4.2). A potential partial refugee is likely to have undergone extensive range reduction, resulting in disjointed populations that occur across a range of environmental conditions. Refugee populations occur where there is low ecological suitability, which could be due to a number of factors including resource limitation, anthropogenic disturbance, or predation; such populations have little or no opportunity to disperse in order to find more suitable conditions. In confirming refugee status, it is important to develop objective measures of one or more ecological correlates and population performance, such as those used here, and a thorough analysis of the species' historical distribution and ecology. In some cases, where there is limited information available from historical records, population performance data from either closely related or ecologically similar species may provide a better baseline than do the potential refugee populations.

The prevalence of partial refugee species globally is potentially quite high, given the aforementioned bias of protected areas towards marginal upland habitat (Joppa & Pfaff, 2009). Partial gap species, or those species where protected areas cover fragments of their range, are particularly likely to also be partial refugee species. Using the framework provided here, such species could be readily identified. For example, the Mediterranean monk seal has already been identified as a refugee species, due to a reduction in its prehistoric range resulting in confinement to suboptimal breeding habitat (González, 2015). However, the Hawaiian monk seal (*Monachus schauinslandi*) is a likely candidate for partial refugee status as population growth rates vary widely, and physiological evidence suggests that poorly performing populations are resource limited (Gobush, Booth & Wasser, 2014).

A key factor in the refugee species concept is the negative impact of active management, where the confinement process has been reinforced due an inaccurate perception of what is ecologically suitable for the species (Kerley, Kowalczyk & Cromsigt, 2012), the latter aspect being termed ‘shifting baseline syndrome’ (Pauly, 1995). Identifying and understanding such misconceptions, and then managing accordingly, is vital for improvement in conservation planning. As is often the case, the conservation needs of one species may clash with another (such as that of Cape mountain zebra with the endemic fynbos flora); this said the needs of all species cannot be properly considered if they are not understood. Once refugee populations have been identified, the principal mitigation strategies are to expand and manage protected areas to promote more optimal ecological conditions and/or to translocate individuals to more suitable areas. This can only be successful if the historical distribution and ecology of the species is properly understood, and has broader implications for the areas targeted as conservation priorities. The indicators developed when evaluating refugee status could be used to assess new areas for species range expansion; indeed the framework presented here has the potential to be expanded and used to assess the

Table 4.2 Criteria for the assessment of candidate partial refugee species and potential mitigation strategies for refugee populations (following Kerley et al. 2012), with the Cape mountain zebra used as a model example.

	Initial indicators	Identification of refugee populations	Mitigation of partial refugee status
A candidate (partial) refugee species	• Range reduction or population declines due to anthropogenic disturbance • Disparity between current and historical distribution • Exists as complex of fragmented populations with restricted movement and/or dispersal opportunities • Occurs across heterogeneous landscape that can be expected to vary in habitat quality	• Evaluate variation in population performance (e.g. reproductive rates, mortality) • Identify and measure important ecological factors across range (such as habitat/diet quality) • Identify correlates of ‘marginal’ poorly performing populations	• Change in perception of optimal or core habitat by managers • Expansion of poor quality reserves to include more optimal habitat • Translocation of individuals from refugee populations to suitable habitat • Management and restoration of limiting ecological factor
A model partial refugee species: the Cape mountain zebra	• Reduced to three populations totalling ~80 individuals in mid-20th century due to hunting and habitat loss • Historical preference for grassy habitats (Faith, 2012; Boshoff *et al*., 2015) • Currently >75 populations found across diverse habitat types in southern mountainous South Africa with restricted dispersal (Hrabar & Kerley, 2015)	• Population growth rate, zebra density and foal:mare ratio vary between populations • These measures are positively associated with habitat and diet quality • Potential refugee populations have a low standardised Vegetation Index score • Confirmed refugee populations score poorly in at least one performance measure (12 out of 21 in this study)	• Immediate focus on poorest performing and key relict populations: De Hoop, Gamkaberg and Kammanassie Nature Reserves • Where possible expand reserves into lowland areas with increased grass cover (priority for Gamkaberg and Kammanassie Nature Reserves) • Form mixed stocks via translocation of individuals to areas of high grass abundance (priority for De Hoop Nature Reserve) • Active management of existing land to promote an increase in grass abundance (e.g. burning, ecological restoration)

suitability of protected areas for ecological communities rather than just single species. In any case, future conservation planning needs to avoid preconceived ideas about habitat suitability and incorporate a way to assess marginality and whether or not species (or communities) are adequately protected. More broadly, our results highlight the issue of inferring ecological preferences from current distribution patterns. Species distribution and climate envelope models are often based on current distribution patterns (Kerley, Kowalczyk & Cromsigt, 2012). If ranges have undergone historical contraction, especially into marginal areas, then inferred suitability and adequacy of protected areas derived from such models would be strongly biased (Cromsigt, Kerley & Kowalczyk, 2012). Using poorly informed assessments of habitat suitability or solely anecdotal evidence may grossly over-estimate the conservation value of existing protected areas. The importance of understanding ecological suitability within a historical context becomes even more critical in the face of on-going anthropogenic environmental change. As habitats shift with land use and climate change, the current distribution of protected areas may be inappropriate to meet our future conservation goals.

Conclusions

The conservation of species or populations in ecologically unsuitable conditions is extremely problematic and ineffective, and has far-reaching consequences for broad-scale conservation planning. A core issue lies in the active management of a species in suboptimal habitat due to the inaccurate or poorly informed perceptions of its historical distribution and ecology. Here, we provide a framework for identifying such species and apply it to a model species, the Cape mountain zebra. We identify 12 out of 21 populations as ecological refugees, due to low habitat and diet quality and poor performance. The framework provided here can be used to assess other species for refugee status, and will be most important in highlighting misconceptions by conservations managers in what habitats a species occupied historically, and where it should be conserved now.

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CHAPTER FIVE

Macrophysiology uncovers relationships between demography, habitat and population performance in a vulnerable species, the Cape mountain zebra

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Abstract

Effective intervention strategies to mitigate species declines require an understanding of the causes of poor population growth. Macrophysiology uses biomarkers to understand variation in individual health or status over large temporal or spatial scales. Here, we use a macrophysiological approach to identify the ecological and demographic correlates of poor performance in the Cape mountain zebra metapopulation. We use two noninvasive biomarkers: faecal glucocorticoids as a measure of chronic stress, and faecal androgens as an indicator of male physiological status. We found that faecal glucocorticoid concentrations were elevated aseasonally in low quality habitat, relative to individuals in high quality habitat. In addition, faecal androgen concentrations were higher in populations with male-biased adult sex ratios and a high proportion of non-breeding stallions, suggesting sex ratio imbalance may lead to an intensification of male competition. Finally, both faecal glucocorticoids and androgen concentrations were negatively associated with female fecundity and population growth rates. Thus, we identified two separate causes of poor performance in Cape mountain zebra populations: habitat marginality and demographic imbalance. More broadly, we advocate physiological biomarkers as indicators of population health, and as a way to evaluate the impact of variable habitat quality and demography on population performance. In addition, macrophysiology can be used to assess the efficacy of management interventions and to inform models of species responses to future environmental change.

Introduction

Understanding and mitigating against the factors leading to global biodiversity loss is a major societal challenge. In an ever-changing environment new problems arise quickly, therefore on-going evidence-based evaluation of population health and the efficacy of interventions and management is essential. The emerging field of conservation physiology evaluates the physiological status or response of an individual to short- and long-term environmental challenges (Wikelski & Cooke 2006; Madliger *et al.* 2016). In addition to documenting causes of decline, conservation physiology can also provide tools to assess the impact of management interventions (Cooke *et al.* 2013). Broad-scale spatial and temporal physiological patterns, termed macrophysiology, can inform and predict species abundance and distribution patterns (Chown, Gaston & Robinson 2004), and can identify the impact of environmental change on individual health, and possibly fitness (Chown & Gaston 2008, 2016). However, macrophysiology is underutilised in conservation research, appearing infrequently in leading ecology, and particularly conservation biology, journals (Chown & Gaston 2016). Moreover, macrophysiology has yet to be employed as a tool to evaluate on-going conservation strategies and practices. Here we use a macrophysiological approach to assess ecological and demographic correlates of poor performance in Cape mountain zebra (*Equus zebra zebra*), a partial refugee species (Lea *et al.* 2016).

Individuals often respond differently to the same stressor both physiologically and behaviourally (Cockrem 2012). However, if enough individuals are sampled it should be possible to scale up individual physiological profiles to provide insights into ecological challenges at the scale of the population or ecosystem, for example to indicate habitat quality (Homyack 2010). Resource availability is a key determinant of population dynamics and individual fitness (Krebs *et al.* 1995). Variation in environmental conditions across a species' range typically results in relatively high and low quality habitats that can sustain different densities of individuals (Pulliam & Danielson 1991). As a whole, populations in low quality habitat will have reduced individual survival and reproductive rates, leading to lower densities and growth rates (Pulliam & Danielson 1991), and as such may require immigration of individuals from high quality habitat to be sustainable (Pulliam 1988). A metapopulation assessment of physiological markers should provide a window into variation in individual condition, and subsequently population performance, across environmental gradients. This requires measuring physiological parameters across populations occurring in habitats of varying quality as indexed by population performance.

In addition to the challenges imposed by poor quality habitats, population demography and density can affect population dynamics and performance (Boonstra *et al.* 1998; Clutton-Brock *et al.* 2002), and ultimately how populations respond to environmental challenges (Coulson *et al.* 2001). Individuals in low density or small populations may have reduced fitness due to Allee effects, caused by difficulty in finding mates, dispersing into new social units, or depressed fitness in small groups and aggregations (Courchamp, Clutton-brock & Grenfell 1999). Furthermore, small populations are more vulnerable to demographic stochasticity (Lande 1993), resulting in large fluctuations in demography, such as biased sex ratios, that reduce effective population size (Nunney 1993). The relationships between ecology, demography and fitness are complex and difficult to elucidate. Measuring physiological markers across populations with varying ecological and demographic conditions may be an ideal way to determine the relative impacts of these factors on individual health, and consequentially population performance.

One of the challenges in wildlife physiology is identifying a set of non-invasive markers that are informative about individual condition and responses to perturbations. A frequently used marker for health and ‘stress’ are glucocorticoid hormones, a group of steroid hormones that have a primary role in energy balance and, at a secondary level, regulate the stress response (Landys, Ramenofsky & Wingfield 2006). Glucocorticoids fluctuate in response to environmental conditions and stressors, such as severe weather events, exposure to predators or humans, and nutritional stress or food deprivation (McEwen & Wingfield 2003; Busch & Hayward 2009). Changes to the social environment can also cause a stress response, and glucocorticoids can promote social behaviours related to dominance and reproduction (Reeder & Kramer 2005; Mehta & Josephs 2010). In fact, a clear relationship between social dominance and glucocorticoids has not been established, with both dominant and subordinate individuals experiencing relatively high glucocorticoid levels (Creel 2001; Abbott *et al.* 2003; Sands & Creel 2004; Creel *et al.* 2013). An acute elevation in glucocorticoids can increase survivorship and fitness in the short-term, however repeated exposure to one or more stressors can lead to an altered baseline state and changes in the acute stress response, creating an inability to respond to future threats (Busch and Hayward 2009). This state of chronic stress (or "distress"; Linklater 2010) is thought to have many deleterious effects, including suppression of the immune system and growth, neuronal cell death, and the inhibition of reproductive behaviour, ultimately reducing fitness (Busch & Hayward 2009). Interestingly, chronic activation of the stress axis could be perceived as an adaptive mechanism that occurs in some (but not all) species, where the stress response is prolonged yet does not have any pathological consequences and in fact promotes phenotypic plasticity (Boonstra 2013). Nevertheless, it is essential that we identify the causes and consequences of chronic stress responses for species of conservation concern to increase our understanding of their ecology and to maximise reproduction and survival.

Other potentially useful endocrine markers that are used less frequently in the context of conservation physiology are reproductive hormones. Androgens are a group of steroid hormones that are associated with the development of secondary sexual characteristics, including an increase in muscular development and testes size, and the regulation of spermatogenesis and sperm quality (Wingfield, Lynn & Soma 2001; Miles *et al.* 2007; Malo *et al.* 2009; Ezenwa, Ekernas & Creel 2012). In addition, androgens have a direct inductive effect on social behaviours related to breeding, dominance and aggression (Rose, Holaday & Bernstein 1971; Lincoln, Guinness & Short 1972; Albert *et al.* 1986; Miller *et al.* 1987; Muehlenbein & Watts 2010). In some cases higher androgen levels may be associated with male reproductive success (Edwards *et al.* 2015), however elevated androgen levels can impose high costs such as immune suppression (Malo *et al.* 2009), reduced parental care (Silverin 1980), aggression towards mating partners and offspring, and overall high energetic costs (see reviews Wingfield et al. 2001, Miles et al. 2007). Male androgen levels have been shown to fluctuate depending on the sex ratio of the population (Sipari *et al.* 2016), and severe male bias can promote aggressive behaviour towards females resulting in lower survival and fecundity (Le Galliard *et al.* 2005). Given the association between androgen levels and intrasexual competition, population level variation in androgens could provide insight into the impact of population demography (i.e. sex ratio skew) on individual physiology. Moreover, a combination of both biomarkers (glucocorticoids and androgens) should provide a good estimation of overall population health.

The Cape mountain zebra (*Equus zebra zebra*) is an ideal model species to assess the impacts of both environment and demography on population performance using macrophysiological approach. Having undergone massive population declines, the Cape mountain zebra has been actively conserved for several decades and has recovered well, now numbering over 4700 individuals from a bottleneck of 80 (Hrabar & Kerley 2015). Though the status of this subspecies is currently being revised, they are listed as “Vulnerable” on the IUCN Red List (Novellie 2008), and face several conservation management issues. They are managed as a metapopulation of at least 75 isolated populations, which range in size from less than 10 to over 1,000 individuals, with movement between populations requiring active translocation (Hrabar & Kerley 2015). Cape mountain zebra social structure is an example of female defence polygyny: populations consist of breeding groups with a single stallion, usually no more than five unrelated mares and their offspring, with excess stallions forming ‘bachelor’ groups (Penzhorn 1979; Linklater 2000). The populations vary considerably in terms of performance and the majority of individuals are sourced from one relict population with few mixed stocks, and the three naturally surviving populations are now genetically distinct (Moodley & Harley 2006). Across the metapopulation, variation in habitat quality is associated with performance, and populations vary in their demography with some exhibiting a male-biased adult sex ratio (Lea *et al.* 2016). As some populations are actively conserved in low quality habitat, we have identified Cape mountain zebra as a partial ‘refugee’ species, due to a historic misconception of its habitat preferences (Lea *et al.* 2016).

The aim of this study was to use broad-scale physiological measurements to identify the mechanisms by which resource limitation and demographic imbalance influence population performance in Cape mountain zebra. We noninvasively sampled glucocorticoid and androgen hormone metabolites in adult Cape mountain zebra across seven populations, which vary in habitat quality (grass abundance), demography (adult sex ratio) and performance (growth rate, zebra density and female fecundity). We hypothesised that a) faecal glucocorticoid concentrations would be elevated in populations in low quality habitat (du Dot *et al.* 2009; Balestri *et al.* 2014; Gobush, Booth & Wasser 2014), b) faecal glucocorticoids would reflect seasonal variation in temperature and rainfall such that they would be elevated during cold or dry conditions (Landys *et al.* 2006; Vick *et al.* 2012; Balestri *et al.* 2014), and c) faecal androgen concentrations would be elevated in populations with a male-biased adult sex ratio and high proportion of non-breeding bachelor stallions (Le Galliard *et al.* 2005). In addition, using model selection criteria we aimed to elucidate which factors (environmental or demographic) were the best predictors of faecal glucocorticoid and androgen measurements.

Methods

Study populations

Cape mountain zebra are found in the Eastern and Western Cape provinces of South Africa. The majority of their historic and current range is in the Cape Floristic Region, but also extends northeast into Nama-Karoo, thicket and grassland habitats and northwest into the Succulent Karoo biome (Boshoff *et al.* 2016). The Cape Floristic Region has a Mediterranean climate and is known for its unusually high biodiversity and proportion of endemic species, particularly flora (Cowling & Holmes 1992). The dominant vegetation type is fynbos, forming a shrubland habitat that varies in type and diversity depending on altitude and burning patterns (Campbell & Van der Meulen 1980). The abundance of C_3 and C_4 grass species [the Cape mountain zebra's primary food source; Penzhorn 1988)] in this region varies with rainfall, soil temperature, composition and nitrogen content, as well as altitude, grazing intensity and age of the vegetation (Cowling 1983).

Seven populations were sampled, these being in the Bakkrans Nature Reserve, Camdeboo National Park, De Hoop Nature Reserve, Gamkaberg Nature Reserve, Mount Camdeboo

Private Game Reserve, Swartberg Private Game Reserve and Welgevonden Game Farm. Four of these reserves experience a peak in rainfall seasonally (two in summer, two in winter) and the other three have no definitive rainy period. Sampling occurred in Jan-May and Sep-Dec 2015 (late summer/autumn and in the spring), such that each reserve was sampled soon after the rainy period or at the end of the dry season (seasonal reserves), or at two time points during the year (aseasonal rainfall). The exception to this was De Hoop Nature Reserve where samples were collected once in the spring after the winter rainfall period.

Zebra identification and demography

At each site, the zebra population was surveyed by vehicle and on foot, and an individual identity database was constructed. For each zebra group encountered, the age-sex classification of each individual was recorded and photographs were taken, which were used to identify individual zebra using HotSpotter^{TM} pattern species instance recognition software (Crall *et al.* 2013). Data collected during this study were complemented with data from previous years (a further 2 – 6 surveys of each population conducted between 2010 – 2014) to calculate the overall mean observed adult sex ratio (male:female) and mean observed proportion of bachelor (non-breeding) stallions, using the identity database to ensure no replications of the same individual were included. We estimated that the proportion of each population sampled during each survey visit was 0.58 ± 0.24 SD, based on the most recent census data for the individual populations (Hrabar & Kerley 2015). In addition, the estimated mean number of foals per mare (foal:mare ratio) was used as a measure of female fecundity and population performance. Additional performance measures include population growth rate since population establishment and zebra density (number of individuals/ha), which were calculated from current population estimates and records (Lea *et al.* 2016).

Sample collection and extraction

We used noninvasive faecal hormone measurements to estimate glucocorticoid and androgen metabolites levels in the Cape mountain zebra. Faecal hormone measurements are deemed far less risky to the animal and are considered more useful than blood samples for measuring chronic stress, as they represent circulating hormone levels experienced during the gut passage time rather than a pinpoint measure and are therefore more indicative of chronic exposure (Dantzer *et al.* 2014). In addition, they are not biased by the restraint or handling procedures used to collect blood samples (Sheriff *et al.* 2011).

Using the zebra ID database, repeat samples were collected from known individuals such that 3-6 individuals were sampled at least three times per population, for both sampling trips (except for Bakkrans Nature Reserve, where repeat samples could not be collected during the second sampling trip). We collected repeat samples from five individuals across both seasons (with 3+ samples for each season, excluding outliers). From an additional six individuals we collected three repeat samples from one season and two for the other season. A further 10 individuals were sampled across both seasons where a single sample was collected during one visit and 1-3 during the other. Finally, within the *same* season four samples were collected for 12 individuals, three samples for 29 individuals, two samples for 27 individuals, and single samples for 74 individuals (totalling 365 samples from 163 individuals). We observed individuals from approximately 30-100 m, either from a vehicle or on-foot, using binoculars to identify zebra and detect defecation events. Reserve staff regularly monitor all populations and the majority of reserves are open to members of the public, therefore the zebra are partially habituated to humans. Faecal samples were collected within an hour of defecation, thoroughly mixed and a sub-sample (~100 g) kept for hormone extraction. When it was not possible to extract faecal samples immediately they were stored in freezers at approximately -18°C for up to a couple of weeks (23 - 54% of the number of samples collected, depending on reserve).

We followed a modified version of an in-field extraction technique (Edwards *et al.* 2014) that utilises HyperSep™ octyl bonded silica (C8) cartridges (Thermo-Fisher Scientific, UK; see Appendix 2.1).

Hormone measures and validation

To retrieve the sample for analysis, 5 ml of 100% methanol was pushed through the cartridge, air dried, resuspended in 1 ml 100% methanol and stored at −20°C. Faecal corticosterone and testosterone metabolites were analysed using enzyme immunoassays (EIA) following Edwards et al. (2014). Each EIA used an antiserum (polyclonal corticosterone CJM006 or polyclonal testosterone R156/7); corresponding horseradish peroxidase- conjugated label (C. J. Munro, University of California, Davis) and standards (Sigma-Aldrich, UK) on a Nunc-Immuno Maxisorp (Thermo-Fisher Scientific, UK) microtitre plate. Details of the full procedure can be found in the Supplementary material Appendix 2.2.

Habitat and climate

An estimation of the abundance of palatable grass within reserves was taken from Lea et al. (2016). In brief, the boundaries of each reserve were overlaid on the National Vegetation Map of South Africa, Lesotho and Swaziland (Mucina & Rutherford 2006). The abundance of palatable grass found in each vegetation type was estimated using the list of dominant and important taxa in the vegetation description, and each grass species identified was given a palatability score of 1 (low), 2 (medium) or 3 (high). The number of species listed (with dominant species weighted by a factor of two) were summed and multiplied by the mean palatability score, to give an overall score for each vegetation type. Reserve scores were then calculated based on the proportion of each vegetation type within each reserve, and standardised between 0 and 1. Grass abundance for the seven reserves in this study was ranked as ‘high’ or ‘low’ based on the bimodal distribution of the grass index scores.

Weather stations are up to 50 km away from some reserves. Therefore, we used the estimated historical average monthly temperatures between 1990 – 2012 (The World Bank Group 2016) for each reserve’s GPS location and the sampling month, which reflect seasonal fluctuations for that area. We were not aware of any extreme weather conditions for the year of sampling (2015) and data from the nearest weather stations reflected normal seasonal variation in temperature and rainfall, therefore we believe the historical data to reflect consistent seasonal variation in climate. Rainfall season was categorised as wet, dry, or year-round depending on the location of the reserve and the month of sampling.

Statistical Analysis

We performed an analysis of faecal sample dry material content that could potentially be used to account for variation in faecal moisture content due to differences in diet (Wasser et al. 1993; see Appendix 2.3).

Linear mixed effect models were used to evaluate the relationships between faecal glucocorticoid and faecal androgen concentrations and all other parameters, using the *lme4* (Bates *et al.* 2015) package in R. The first two models assessed whether either hormone varied between reserve, with reserve included as a fixed factor and zebra ID and sampling trip as random factors. Next, we evaluated the relationships between faecal glucocorticoids or androgens and habitat, seasonality, population demography and performance variables using univariate models (due to small number of populations sampled). Fixed factors included were: grass abundance (high or low), rainfall season (wet, dry or year-round), average temperature for the month of sampling, adult sex ratio (male:female), the proportion of bachelors, population growth rate, log-transformed zebra density, and female fecundity (foal:mare ratio). Where faecal androgen concentration was the dependent variable, faecal glucocorticoid concentration was included as a fixed factor in each model to account for any potential correlations between the two steroids (see Appendix 2.4 for details). Random factors included in each model were: zebra ID, sex (for glucocorticoids only), reserve and sampling trip. We also used models with interactions terms when assessing habitat and seasonality (incorporating both the interaction term and main effects). For the interaction between grass abundance and rainfall season, data were grouped by each combination of the two categorical variables (e.g. high grass/dry season, low grass/year-round and so on) so that pairwise comparisons could be conducted using Tukey's post hoc analyses (*glht* function in *multcomp;* Hothorn et al. 2008). In addition, we assessed trends between each hormone and the number of days since sampling began in each reserve (as a precautionary measure of disturbance), time of day, and the effect of freezing samples prior to extraction, and no significant effects were found ($p > 0.05$ for all models). Due to right skewed distributions faecal glucocorticoids were square root transformed and faecal androgens were log-transformed; model fit was checked by confirming that the residuals were normally distributed. Faecal glucocorticoid and faecal androgen concentrations more than three standard deviations from the mean were excluded prior to all analyses (glucocorticoids: $n = 5$, androgens: $n = 3$).

Models are shown with the following selection criteria: Akaike information criterion (AIC; Akaike 1973), Schwarz criterion, otherwise known as Bayesian information ciriterion (BIC; Schwarz 1978), and marginal R^2, calculated using the sem.model.fits function in the R package *piecewiseSEM* (Lefcheck 2015). Model selection criteria are the same for AIC and BIC, where the model comparison value ΔAIC or ΔBIC is calculated as $\mathrm{AIC/BIC}_i$ – $\mathrm{AIC/BIC}_{min}$, where $\mathrm{AIC/BIC}_i$ is the current model and $\mathrm{AIC/BIC}_{min}$ is the model with the lowest value (Burnham & Anderson 2002). When comparing models, ΔAIC values between 0-2 have similar support to the best fit model, ΔAIC between 4-7 have considerably less support, and ΔAIC > 10 have essentially no support (Burnham & Anderson 2002). Similarly, Raftery (1995) describes the evidence corresponding to ΔBIC values of 0-2 as weak, 2-6 as positive, 6-10 as strong, and > 10 as very strong. To estimate the influence of outliers on linear mixed effects models we calculated Cook's distance (Cook 1979) for all best fit models using the *influence.ME* package in R (Niewenhuis, te Grotenhuis & Pelzer 2012). To assess outliers, Cook & Weisberg (1982) suggest a simple cut-off value of 1, however we use a more conservative cut-off value of 4/N, where N is the sample size (Bollen & Jackman 1985).

Results

In total, 365 faecal samples were collected from 163 adult Cape mountain zebra across the seven study populations. Of these, 162 samples were collected from 89 stallions for faecal androgen analysis. Cape mountain zebra faecal glucocorticoid and faecal androgen metabolite concentrations varied between reserves ($F_{6,159} = 10.57$, $p < 0.001$ and $F_{6,81} = 19.96$, $p < 0.001$, respectively).

Table 5.1. Demographic and environmental factors associated with faecal glucocorticoid metabolite concentrations in Cape mountain zebra, ranked by Bayesian information criterion (BIC). For all models with two-way interactions (*), both main effects and the interaction term were incorporated into the model.

Model	Predictors	AIC	BIC	ΔAIC	ΔBIC	Marginal R^2		Estimate ± SE	df	t	p
Environment and demography											
1	Habitat quality*Temperature	567.2	602.27	0	0	0.27		-0.43 ± 0.06	1,20	-7.02	**<0.001**
	Habitat quality							6.92 ± 1.07	1,161	6.46	**<0.001**
	Temperature							0.18 ± 0.04	1,20	4.77	**<0.001**
2	Habitat quality*Rainfall season	570.26	612.98	3.06	10.71	0.25	*year-round ~ wet*	1.02 ± 0.49	2,18	2.11	**<0.05**
							year-round ~ dry	2.15 ± 0.49		4.44	**<0.001**
	Habitat quality							-2.04 ± 0.45	1,161	-4.56	**<0.001**
	Rainfall season						*year-round ~ wet*	-0.58 ± 0.20	2,18	-2.86	**0.01**
							year-round ~ dry	-0.81 ± 0.20		-4.00	**<0.001**
3	Temperature*Rainfall season	593.92	636.64	26.72	34.37	0.22	*year-round ~ wet*	-0.24 ± 0.05	2,17	-5.19	**<0.001**
							year-round ~ dry	-0.06 ± 0.08		-0.76	0.46
	Temperature							0.09 ± 0.04	1,17	2.53	**0.02**
	Rainfall season						*year-round ~ wet*	4.06 ± 0.86	2,17	4.74	**<0.001**
							year-round ~ dry	1.11 ± 1.40		0.79	0.44
4	Habitat quality	600.99	628.25	33.79	25.98	0.10		-0.69 ± 0.13	1,161	-5.09	**<0.001**
5	Rainfall season	604.73	635.86	37.53	33.59	0.11	*year-round ~ wet*	-0.47 ± 0.09	2,20	-5.06	0.55
							year-round ~ dry	-0.06 ± 0.10		-0.61	**<0.001**
6	Proportion of bachelors	614.3	641.56	47.10	39.29	0.03		1.61 ± 0.57	1,161	2.81	**<0.01**
7	Temperature	611.82	639.08	44.62	36.81	0.07		0.08 ± 0.02	1,21	-4.25	**<0.001**
8	Adult sex ratio	625.13	652.39	57.93	50.12	<0.01		0.08 ± 0.08	1,160	0.96	0.34
Population performance											
1	Foal:mare ratio	604.01	631.27	0	0	0.08		-1.68 ± 0.38	1,161	-4.46	**<0.001**
2	Population growth rate	616.17	643.42	12.16	12.15	0.06		-0.05 ± 0.01	1,161	-3.70	**<0.001**
3	log Zebra density	617.33	644.59	13.32	13.32	0.04		-0.19 ± 0.06	1,161	-3.07	**<0.01**

Table 5.2. Demographic and environmental factors associated with faecal androgen metabolite concentrations in Cape mountain zebra stallions, ranked by Bayesian information criterion (BIC). For all models with two-way interactions (*), both main effects and the interaction term were incorporated into the model

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Model	Predictors	AIC	BIC	ΔAIC	ΔBIC	Marginal R^2		Estimate ± SE	df	t	p
Environment and demography											
1	Proportion of bachelors	225.63	247.11	-0.83	0	0.30		3.31 ± 0.55	1,86	6.06	**<0.001**
	Glucocorticoids							*0.19 ± 0.07*	*1,65*	*2.85*	*0.01*
2	Adult sex ratio	226.78	248.26	-1.98	1.15	0.30		0.44 ± 0.07	1,86	6.22	**<0.001**
	Glucocorticoids							*0.24 ± 0.06*	*1,65*	*3.73*	*<0.001*
3	Habitat quality	235.65	257.14	-10.85	10.03	0.27		-0.79 ± 0.15	1,86	-5.16	**<0.001**
	Glucocorticoids							*0.15 ± 0.07*	*1,65*	*2.12*	*0.04*
4	Habitat quality*Temperature	230.62	258.12	-5.82	11.01	0.35		-0.03 ± 0.07	1,6	-0.41	0.69
	Habitat quality							-0.71 ± 1.16	1,86	-0.61	0.54
	Temperature							0.11 ± 0.04	1,6	2.81	**0.03**
	Glucocorticoids							*0.14 ± 0.07*	*1,65*	*1.99*	*0.05*
5	Habitat quality*Rainfall season	224.8	258.28	0	11.17	0.36	*year-round ~ wet*	2.76 ± 0.49	2,4	5.64	**<0.01**
							year-round ~ dry	2.19 ± 0.49		4.44	**0.01**
	Habitat quality							-3.06 ± 0.47	1,86	-6.52	**<0.001**
	Rainfall season						*year-round ~ wet*	-1.02 ± 0.20	2,4	-4.97	**<0.01**
							year-round ~ dry	-0.77 ± 0.19		-3.97	**0.02**
	Glucocorticoids							*0.08 ± 0.07*	*1,65*	*1.12*	*0.27*
6	Temperature	261.21	282.70	-36.41	35.59	0.09		-0.02 ± 0.02	1,7	0.90	0.4
	Glucocorticoids							*0.29 ± 0.07*	*1,65*	*4.05*	***<0.001***
7	Temperature*Rainfall season	249.73	283.21	-24.93	36.10	0.29	*year-round ~ wet*	-0.20 ± 0.05	2,3	-4.08	**0.03**
							year-round ~ dry	-0.49 ± 0.09		-5.66	**0.01**
	Temperature							0.17 ± 0.04	1,3	4.59	**0.02**
	Rainfall season					0.11	*year-round ~ wet*	3.59 ± 0.93	2,3	3.86	**0.03**
							year-round ~ dry	8.06 ± 1.42		5.68	**0.01**
	Glucocorticoids							*0.21 ± 0.07*	*1,65*	*3.01*	*<0.01*
8	Rainfall season	262.96	287.46	-38.16	40.35	0.10	*year-round ~ wet*	-0.06 ± 0.07	2,6	-0.48	0.65
							year-round ~ dry	-0.05 ± 0.12		-0.43	0.68
	Glucocorticoids							*0.27 ± 0.07*	*1,65*	*3.65*	***0.001***
Population performance											
1	Foal:mare ratio	220.36	241.84	0	0	0.32		-2.49 ± 0.37	1,86	6.71	**<0.001**
	Glucocorticoids							*0.12 ± 0.07*	*1,65*	*1.80*	*0.08*
2	log Zebra density	241.03	262.51	20.67	20.67	0.24		-0.31 ± 0.07	1,86	-4.48	**<0.001**
	Glucocorticoids							*0.20 ± 0.07*	*1,65*	*2.93*	*<0.01*
3	Population growth rate	241.96	263.44	21.60	21.60	0.24		-0.07 ± 0.01	1,86	-4.78	**<0.001**
	Glucocorticoids							*0.12 ± 0.07*	*1,65*	*2.93*	*<0.01*

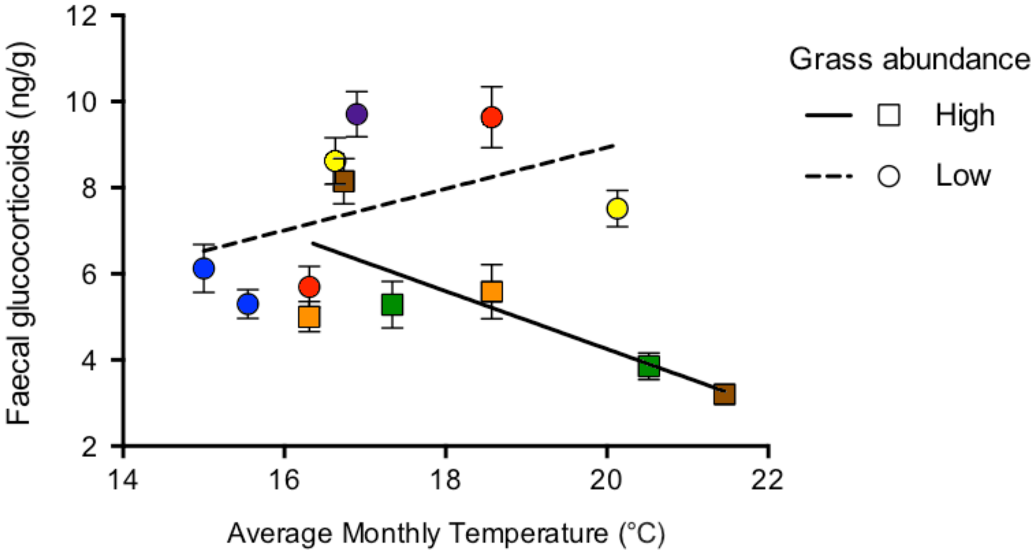

Figure 5.1. The relationship between faecal glucocorticoid metabolite concentrations and the average monthly temperature differs depending on whether individuals are in reserves with high or low grass abundance. Reserves were sampled at two time points indicated by matching colours.

Glucocorticoids

Faecal glucocorticoid metabolite concentrations varied between populations with different habitat quality. The best-fit model incorporated grass abundance, average temperature and their interaction term (Table 5.1). During colder months faecal glucocorticoid levels were similar in all habitat types, however in warmer months they were elevated in low quality habitat, and lower in high quality habitat (Fig 5.1). The model including a habitat by rainfall interaction held less support when considering AIC (ΔAIC = 3.06; Table 5.1), and even weaker support when using BIC (ΔBIC = 10.71). Though this interaction is a worse predictor of faecal glucocorticoids, we present this relationship in Fig 5.2A as it is of high ecological relevance to the Cape mountain zebra. During the dry season individuals in both high and low grass habitats had similar faecal glucocorticoid concentrations, however in the wet season populations with high grass abundance experienced a decrease in faecal glucocorticoid levels whilst populations with low grass abundance did not (Fig 5.2A). In populations where rainfall remains fairly consistent throughout the year, individuals in less grassy habitats had elevated faecal glucocorticoids (Fig 5.2A). Thus, in populations with less

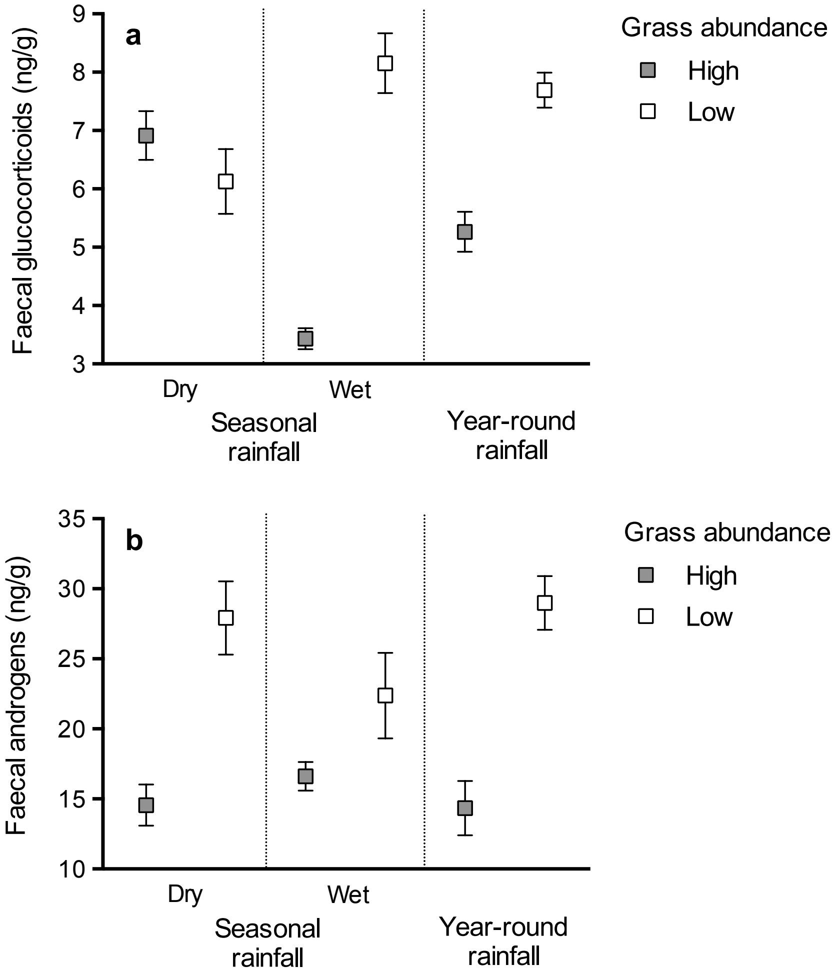

Figure 5.2. Cape mountain zebra individuals show variation in faecal a) glucocorticoid and b) androgen metabolite concentrations in response to different rainfall patterns and grass abundance.

grassy habitat, faecal glucocorticoids are consistently elevated and individuals show a different physiological response to seasonal fluctuations in climate. Models incorporating demographic factors were the poorest predictors of faecal glucocorticoid concentrations (Table 5.1). Finally, female fecundity a better predictor of faecal glucocorticoid concentrations that population growth rate or zebra density, where individuals in populations with high numbers of foals per mare had lower faecal glucocorticoid concentrations (Table 5.1).

Androgens

Populations with a male-biased adult sex ratio, as measured by either male/female ratio or the proportion of bachelor stallions in the population, had elevated faecal androgen metabolite concentrations (Table 5.2, Figs 5.3A, 5.3B). This relationship was driven primarily by two populations (Bakkrans Nature Reserve and Gamkaberg Nature Reserve) with strongly male-biased sex ratios. There was no difference in model fit between sex ratio and the proportion of bachelors when assessed using ΔAIC or ΔBIC (Table 5.2). However, there was disparity between the two selection methods as AIC also identified the interaction term between habitat quality and rainfall season as an equivalently good predictor as the demographic measures. In comparison, ΔBIC for this model was 11.01, indicating that the demographic models were far stronger. Other environmental variables had considerably less

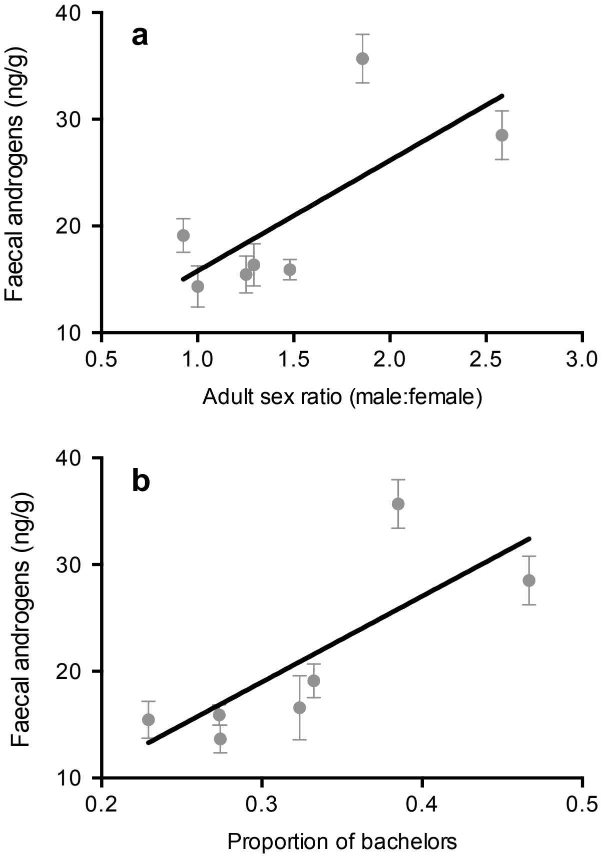

Figure 5.3. Cape mountain zebra faecal androgen metabolite concentrations vary with population demography. Faecal androgen concentrations are elevated in populations with a strongly male-biased sex ratio (a) and a high proportion of bachelors (b).

support from both AIC and BIC (Table 5.2). The relationship between habitat quality, rainfall season and faecal androgens is shown in Fig 5.2B, where stallions in reserves with low grass abundance had higher faecal androgen concentrations in all but the wet season, when they declined to similar levels as those observed in high quality habitat populations. In addition, female fecundity was an excellent predictor of faecal androgen concentrations, where higher numbers of foals per mare were associated with increased androgens, Finally, faecal glucocorticoids were a significant predictor of faecal androgens (where high glucocorticoids correlated with high androgens) for most, but not all, models (Table 5.2).

Cook’s distance values were calculated for all best fit models; all were below 0.15. There were several outliers for each model that exceeded the 4/N cut off value, except for one (where faecal glucocorticoids were the dependent variable and female fecundity was the predictor) that had no outliers. For each model, we excluded the outliers and reran the analysis; all models retained the same level of significance as was previously observed.

Discussion

We have conducted a broad-scale evaluation of individual physiology across seven Cape mountain zebra populations that exhibit variation in habitat quality, demography and population performance. Faecal glucocorticoid concentrations were better predicted by environmental factors by both model selection criteria, such that they were elevated aseasonally in individuals in low quality habitat. AIC and BIC differed slightly in their selection of the best model for faecal androgen concentrations, where both criteria favoured demographic variables but AIC also selected the habitat quality and rainfall season interaction term as an equivalently good predictor. AIC and BIC are similarly structured, however BIC penalises additional parameters more strongly and tends to select the more parsimonious model (Zucchini 2000). Both are used in the fields of ecology and evolution (Johnson & Omland 2004; Posada & Buckley 2004), and which is the more appropriate choice is still up for debate (Aho, Derryberry & Peterson 2014). Both AIC and BIC favour models with predictors related to sex ratios, which is intuitive given the close association between androgens and aspects of male competition (Wingfield *et al*. 2001). Therefore, it is clear that demography is a key factor when considering stallion hormone profiles, where faecal androgen concentrations were elevated in populations with a male-biased sex ratio and an excess of non-breeding stallions. Finally, both hormones were negatively correlated with female fecundity. We discuss how these results provide insight into the causes of poor population performance in the Cape mountain zebra, and make suggestions for the future management of this subspecies.

Elevated faecal glucocorticoids in poor quality habitats

The best model for describing variation in Cape Mountain zebra faecal glucocorticoid concentrations incorporated an interaction between grass abundance and the average monthly temperature, as faecal glucocorticoids were generally higher in less grassy habitat and declined as temperature increased. The significant interaction between grass abundance and rainfall season indicates that Cape mountain zebra individuals in poor quality habitat did not experience seasonal fluctuations in faecal glucocorticoid concentrations, whereas those in high quality habitats did. This indicates that these individuals could be suffering from chronic stress as a result of poor nutrition (Busch & Hayward 2009). Habitat quality was associated with the timing of peak rainfall, as summer rainfall areas promote the growth of palatable C_4 grasses and provide extra sources of drinking water. Conversely, in the winter rainfall regions where habitat quality is low, grass productivity is lower and is more likely to consist of less palatable C_3 grass species (Cowling 1983). It is possible that low winter temperatures induced a stress response to modulate metabolic rate (Crespi *et al.* 2013). Demographic variables, namely adult sex ratio and the proportion of bachelor stallions, were less useful for predicting variation in faecal glucocorticoid concentrations. Interestingly, the significant positive trend between faecal glucocorticoids and the proportion of bachelor stallions, in contrast to the lack of a relationship between the former and adult sex ratio, suggests that a surplus of non-breeding stallions leads to an increase in social harassment and aggression by these bachelors.

Stallion androgen response

Adult sex ratio and the proportion of bachelor stallions were the best predictors of faecal androgen concentrations in Cape mountain zebra stallions. Stallions in populations with a heavily male-biased sex ratio had substantially higher faecal androgens (at least 1.5 times greater) than those with more even sex ratios. Elevated androgen levels are problematic for two main reasons: 1) the costs of androgen production may result in reduced fitness, and 2) elevated testosterone may be associated with an increased frequency of aggressive or disruptive social interactions. An increase in aggression rate is concerning as it can lead to a reduction in mare condition and reproductive success (Linklater *et al.* 1999), creating a scenario analogous to Caughley's (1994) ‘extinction vortex’ where demographic imbalance is exacerbated and threatens to spiral towards extinction (Le Galliard *et al.* 2005). A key driver of the formation of stable social bonds in equids is thought to be harassment from non-breeding stallions (Rubenstein 1994; Linklater 2000).

Unbalanced adult sex ratios can result from either differential survival rates or a bias in birth sex ratios, with the latter potentially influenced by maternal condition and the perceived levels of inter- and intrasexual competition (Clutton-Brock & Iason 1986). The Trivers-Willard hypothesis (Trivers & Willard 1973) suggests that mothers in poor condition should produce more daughters, however we do not find support for this. Previous evidence suggests that socially dominant Cape mountain zebra mares (who tend to be in better condition) will have more female offspring that are subsequently more likely to survive (Lloyd & Rasa 1989). However, our surveys suggested roughly even birth sex ratios across populations (19 male/19 female foals in high quality habitat and 7 male/9 female foals in low quality habitat). This leaves differential survival rates as a more likely scenario, where females are more strongly impacted by resource limitation. If so, this is in contrast to other findings for wild equid populations where male mortality rates are higher, typically leading to female-biased sex ratios (Berger 1983; Garrott 1991; Monard *et al.* 1997). Our models suggest that variation in faecal androgen concentrations is better explained by demography, however it is worth noting that the two populations with heavily male-biased sex ratios were also both in low quality habitat. Elevated faecal androgens were also associated with less grassy habitats and with the dry and perennial rainfall seasons. As such, the relative impact and relationship between environmental and demographic factors is complex and difficult to determine.

What are the causes of poor population performance?

As environment and demography are closely linked it is difficult to elucidate which factors are directly influencing individual physiology, most likely it is a combination of several. However, our results indicate some order of influence of the predictors used on both physiological measures. Faecal androgens were more strongly associated with demography than directly with the environment, which is intuitive as they have a primary role in moderating competitive social behaviour (Wingfield *et al.* 2001). However, elevated faecal glucocorticoids appeared to occur principally in response to environmental stressors. Thus, we have identified two possible causes of poor performance in Cape mountain zebra populations. Firstly, nutritional issues cause a reduction in female fecundity and population growth rates, which can occur when a chronic increase in glucocorticoids leads to a diversion of energy away from reproduction (McEwen & Wingfield 2003). Given that individuals in resource-limited populations exhibit chronically high faecal glucocorticoid concentrations in the absence of seasonal variation, it is possible that they would be less resilient to future threats or perturbations (Busch & Hayward 2009). A chronic elevation of faecal glucocorticoids could be considered an adaptation to a persistent stressor (Boonstra 2013), allowing the individual to survive long enough for the stressor to disappear so they can reproduce, or to reproduce in spite of it. Interestingly, maternal chronic stress can also affect offspring phenotype, resulting in an increase in fitness (Dantzer *et al.* 2013). It is possible that chronic stress acts as an adaptive trait in Cape mountain zebra individuals occurring in low quality habitat, as populations are managing to persist over time (albeit at low densities). However, given that there is a clear negative correlation between female fecundity and faecal glucocorticoid levels, and that population performance is significantly lower in areas of low quality habitat (Lea *et al.* 2016), we would suggest that chronically elevated glucocorticoid levels as a result of poor nutrition are detrimental to Cape mountain zebra fitness.

Secondly, demographic imbalance has led to elevated faecal androgen concentrations in stallions, which may have fitness consequences (Reed *et al.* 2006). In this study the elevation in faecal androgens was only observed in populations with extremely male biased sex ratios – where male:female exceeded 1.5:1 – suggesting that individuals and populations may be resilient to small demographic fluctuations. Both of these populations are small (<50 individuals) and occur in low quality habitat, therefore either habitat quality or demographic stochasticity (or a combination) could have caused sex ratio skew. Along with faecal glucocorticoids, high faecal androgen concentrations are associated with lower female fecundity and population growth rates.

These findings have wide ranging implications for our understanding of ecology and conservation management. As shown by Lea et al. (2016), some Cape mountain zebra populations are maintained in marginal habitat due to a misconception of what is ecologically suitable for this subspecies. To improve the performance of important relict subpopulations it will be necessary to expand reserves to include grassier habitats, or translocate individuals to other areas. For conservation managers, sex ratio skew may also be an early warning sign of problems within a population. Both factors should be examined in future Cape mountain zebra conservation management.

Macrophysiology in conservation

Macrophysiology has been identified as a valuable research area to help identify threats to biodiversity and elucidate the impacts of environmental change (Chown & Gaston 2008). However, the breadth of potential applications is not yet reflected in the literature, with the majority of studies that employ a macrophysiological approach restricted to marine organisms and global temperature change (Osovitz & Hofmann 2007; Richard *et al.* 2012; Chown & Gaston 2016). Macrophysiology unifies aspects of functional biogeography, physiological ecology and conservation physiology, yet also distinguishes itself from all three, most notably the latter two which are considered comparatively small-scale (Chown & Gaston 2016). Despite its clear applications for the field of conservation biology, a search for the term “macrophysiology” receives zero hits in two leading conservation journals, Conservation Biology and Biological Conservation. Whilst distinctive themes can offer different perspectives, a combination of terms and approaches is best served to effectively inform conservation work, as has been suggested by Ellis et al. (2012).

To our knowledge this is the first time a macrophysiological approach has been used to evaluate the efficacy of an on-going conservation management plan, by identifying physiological correlates to ecological factors that limit performance in a large terrestrial mammal. Few studies have assessed physiological measures across more than one subpopulation of a wild animal species. Faecal glucocorticoid concentrations have been linked to food availability across barren-ground caribou (*Rangifer tarandus*) populations (Joly, Wasser & Booth 2015), and to habitat degradation in collared brown lemur (*Eulemur collaris*; Balestri et al. 2014). However, no study has assessed the relative impact of both ecological and demographic factors on individual physiology across several subpopulations of a single species. In the Cape mountain zebra study system it was possible to compare the physiology of individuals in marginal habitat with those in more optimal conditions. For some species this may not be possible, as they may only now exist in suboptimal habitat (Kerley, Kowalczyk & Cromsigt 2012), and interpreting physiological markers without a comparison may be difficult. Given the current bias of protected area location towards unproductive land (Joppa & Pfaff 2009), this will be increasingly prevalent (Kerley *et al.* 2012). Physiological measures across an environmental gradient can also improve the accuracy of envelope or habitat models of species distributions (Chown & Gaston 2008). These models typically take a top-down approach by relating occurrence data to underlying environmental conditions. However, incorporating bottom-up niche models of individual functional responses to varying environmental conditions has the potential to drastically improve their predictive power. A combined bottom up and top down approach is especially powerful for informing models of species resilience and responses to future environmental change.

Conclusions

We used a macrophysiological approach to identify possible causes of poor population performance in the Cape mountain zebra metapopulation. We find that elevated faecal glucocorticoid concentrations occur aseasonally in populations with low habitat quality, and that stallion faecal androgen concentrations are higher in response to heavily male-biased adult sex ratios. Both hormones are negatively associated with female fecundity and population growth rates, suggesting there are possible fitness consequences for chronic increases in either hormone. Effective conservation management strategies for small populations with increasingly restricted ranges require a thorough understanding of species ecology and physiology in order to maintain viable populations that can survive future challenges. We advocate the use of macrophysiology, alongside other physiological and ecological disciplines, to assess conservation management strategies and identify current and future threats to biodiversity decline.

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CHAPTER SIX – GENERAL DISCUSSION

Conservation science has evolved rapidly in recent decades, and has an essential role in species conservation and management. This includes evaluating on-going management strategies to ensure that the best approach and methods are being employed. This thesis investigates two aspects of conservation management and strategy, firstly by assessing the impact of a specific management intervention, and secondly by looking more broadly at the ecological and demographic factors that can affect population performance. I evaluate the use of faecal hormone monitoring and social network analysis as markers for individual status and population health, using two equid species as models. In the Welsh mountain pony population I showed that targeted removals resulted in major disruption to population structure, potentially as a result of losing individuals that play a vital role in the maintenance of social structure. In addition, I highlighted the difficulty of using faecal hormone measurements to assess the impact of acute stressors in wild populations. For the Cape mountain zebra, I performed a broad-scale assessment of habitat quality and individual physiology, by successfully sampling faecal hormone metabolites across seven populations. From this, I describe two potential causes of poor performance for this subspecies, and identify several refugee populations that are likely being conserved in suboptimal habitat. The approach used here of measuring physiology across several populations to assess variation in population performance is novel, and these techniques have the potential to be applied to other species to investigate whether they are being conserved in their optimal habitat.

The Welsh mountain pony population: key findings and conclusions

The Welsh mountain pony population present an ideal study system to assess the impact of a commonly used management intervention, namely the removal or translocation of individuals. The ponies are relatively easy to study due to their proximate location and unique individual markings. Importantly, their social structure is similar to that of several endangered equid species where the outcome of translocation events is of high concern.

Here, I used faecal hormone monitoring of several focal individuals to measure the acute stress response to the initial capture of a large proportion of the population, followed by an assessment of perturbation to social network structure after the removal of several herd stallions. The findings of this work can be used to inform the managers of the Welsh mountain pony population, and could also be scaled up to highlight aspects of translocation events that require further investigation in other species.

Using faecal glucocorticoids to measure an acute stress response

The study presented in the second thesis chapter assessed the acute physiological response of focal individuals to the annual roundup event, whereby faecal glucocorticoid metabolites were measured in eight focal mares. It was hypothesised that the relocation, two-day confinement, and physical handling of individuals would illicit a stress response that could be tracked through faecal sampling and provide a biological validation of faecal glucocorticoid metabolites for this population. All of the focal mares had un-weaned foals prior to the event and belonged to two breeding groups. Neither group lost the resident stallion during the roundup event, however four of the mares had their male foals removed from the population. The characteristic pattern of an acute stress response was only observed in two individuals, with a delay of five days before detection in faecal samples. There was a high degree of variation between individual responses, with the remaining six mares showing a more muted response. Moreover, there was no evidence of foal removal acting as a stressor to any of the focal mares. Overall, the results were inconsistent and could not be used as a conclusive validation of faecal glucocorticoid measurement for this population.

This study highlighted some of the potential pitfalls that can occur when using faecal hormone measurements to assess an acute stress response. Faecal samples are thought to be better at capturing the physiological status of an individual over a longer (species specific) time period, avoiding the pulsatile fluctuations in glucocorticoids that are observed from blood samples (Sheriff *et al.*, 2011; Dantzer *et al.*, 2014). However, here we observed such a high degree of within- and between-individual variation that it is difficult to conclusively determine the acute response to a known stressor. Based on these findings, future studies aiming to measure an acute stress response in free-living animal populations using faecal hormone measurements should focus on collecting as many samples as possible from focal individuals, ensure that sample collection begins as soon as possible after the stressful event to ensure that the response is captured, and extend sampling longer than may be anticipated to account for any time delay in the appearance of faecal metabolites. This supports evidence from greylag geese (*Anser anser*), where a minimum of three samples were required to reliably estimate faecal glucocorticoids within a window of just a few hours following an acute stressor (Scheiber, Kralj & Kotrschal, 2005). This approach has been used successfully for a variety of captive and domestic species (Möstl *et al.*, 1999; Schwarzenberger, 2007; Palme, 2012), and to assess the impact of translocation events when individuals remain captive for a sufficient period to reliably collect repeat samples (e.g. Goymann *et al.*, 1999; Vick *et al.*, 2012). However, this would be a difficult method to apply both to the Welsh mountain ponies and to other wild or free-living animal populations, as it is often not possible to keep track of individuals for extended periods. Studies that have measured the faecal glucocorticoid response to translocation events in free-living populations have done so by increasing the sample size (Viljoen *et al.*, 2008; Aguilar-Cucurachi *et al.*, 2010) or by collecting repeat samples from individuals for several months following relocation (Franceschini *et al.*, 2008). However, this may be impractical for many study populations or species, and it may be necessary to only measure the response of individuals that are confined to smaller areas and can be easily monitored (such as throughout holding periods during translocations).

More generally, this study indicates that the interpretation of faecal glucocorticoid measurements is difficult and that one or a few samples may give an unreliable representation of individual physiological status. Faecal glucocorticoids are increasingly being used to identify different types of stressors for the purpose of conservation (Dantzer *et al.*, 2014; Madliger *et al.*, 2016), therefore it is vital that we fine-tune methods of sample collection to ensure accuracy and the correct interpretation of factors that influence the stress response. Moreover, faecal hormone measurements are also used to investigate the physiological basis of basis social behaviours and the impact of social stressors (Foley, Papageorge & Wasser, 2001; Sands & Creel, 2004; Nuñez *et al.*, 2014), however the relationships between faecal glucocorticoids and various social factors can be highly variable (Creel *et al.*, 2013). The current study on Welsh mountain ponies showed that the time delay between the stressor and the measured response is a disadvantage of faecal hormone measurements, and concurs with previous evidence that faecal samples may need to be time-matched to accurately determine the physiological response to social behaviours (Edwards *et al.*, 2013). Overall, this study indicates that there are several possible limitations to the use of faecal hormone measurements for evaluating species responses to acute stressors in wild populations. Given the difficulty in addressing some of the issues with sampling frequency and individual variation, faecal hormone monitoring may be a more useful tool to assess baseline glucocorticoid levels and the impact of long-term or 'chronic' stressors in free-living populations.

Impact of removals on network structure

During the roundup event several Welsh mountain pony stallions were removed from the population. The third thesis chapter evaluated population structure and individual social network position in response to these removals, and in addition faecal glucocorticoid and androgen metabolites were quantified for a subset of the remaining adult stallions. First, individual association data from over 40 surveys were combined to create two weighted networks for before and after the removal event. The networks characterised the strength of individual ties by the proportion of times they were sighted together and the proximity of individuals. The disruption to social network structure after the removal of herd stallions was considerable: clustering was significantly lower as several discrete components had dissolved into one large interconnected group, connectivity across the network was higher as more individuals were connected by at least one tie, while individuals on average weaker tie strength than before. In addition, an increase in faecal androgen concentrations was observed eight days after release that remained elevated until the end of the study period, which could indicate increased levels of male competition in the population.

The impact of removals on social network structure has been discussed theoretically, where networks are thought to remain stable to the random removal of individuals yet are highly vulnerable to the loss of key individuals (Makagon, McCowan & Mench, 2012; Kurvers *et al.*, 2014). When modelling the effect of random deaths or removals from a population of bottlenose dolphins (*Tursiops* spp.), Lusseau (2003) found that the overall social network structure remained resilient even when losing up to 20% of the population. Similarly, Formica *et al.* (2016) found that tie strength and betweenness scores were repeatable across networks after experimentally removing individuals. However, other studies have found that the removal of key individuals, i.e. those that are highly connected or display a high frequency of behaviours necessary for group cohesion, can result in a disruption to network structure (Flack, Krakauer & de Waal, 2005; Williams & Lusseau, 2006). Our results align with these findings, as similar disruption was observed in the Welsh mountain pony population following the removal of herd stallions. The significance of the perturbation becomes apparent when considering the level of disruption to social structuring caused by removing just 20 individuals from a population of over 300, the majority of which were younger than two years old and had not left their natal groups, and only five of which were herd stallions. The stability of mare-mare and mare-stallion social bonds is thought to be an adaptation in equid social structuring that reduces the level of harassment received by mares, thereby increasing their reproductive success (Rubenstein, 1994; Sundaresan, Fischhoff & Rubenstein, 2007; Cameron, Setsaas & Linklater, 2009). Moreover, *Equus caballus* stallions are thought to perform a vital role in maintaining group cohesion (Linklater, 2000). The longevity of the changes to population structure observed in this study were not evaluated; it would be expected that social networks would stabilise over time following a disturbance (Formica *et al.*, 2017), however they can never return to exactly how they were in the Welsh mountain ponies as several individuals are no longer present. A novel aspect of this study is the addition of faecal hormone monitoring to include both a physiological and behavioural overview of individual response. A small number of studies have used hormone measurements alongside social network analysis, for example to assess the impact of developmental stress on social network position (Boogert, Farine & Spencer, 2014) and the evaluate the physiological correlates of network structure (Solomon-Lane *et al.*, 2015).

However, to the author's knowledge the current study is the first to use social network analysis and faecal hormone measurement to assess the impact of individual removals.

In addition to the disruption to social structure, the removal of stallions could affect the sex ratio of the population. Though not a focus of this thesis, there is some evidence to this effect from the surveys conducted during data collection, as by the final year of study the ID database contained 204 confirmed females, 108 confirmed males, and a further 17 individuals of unknown sex (essentially comprising an almost 2:1 female to male sex ratio). Bachelor stallions may be more difficult to spot during surveys if they occur in smaller groups and therefore it would be useful to confirm population demographics during the roundup when most individuals are present. If confirmed, the Welsh mountain pony population could be an interesting study population to investigate the consequences of a female biased sex ratio on population dynamics and other related fields. Overall, this study shows that targeted removals from the Welsh mountain pony population need more careful consideration in order to minimise impact on social structuring. It may be beneficial to identify central or key individuals prior to removals to avoid selecting individuals that are or high social importance; this has been achieved using social network analysis for captive populations of African lion (*Panthera leo*) as part of a reintroduction programme (Dunston *et al.*, 2017).

Implications for using translocations as a conservation tool

Together, chapters two and three provide interesting insight into the effect of management interventions on population stability and individual physiology in the Welsh mountain ponies, and present both the usefulness and the challenges of using these markers to assess individual and population-level responses to perturbations. The use of both physiological and behavioural measures highlighted different aspects of the impact of the disturbance event, giving a more comprehensive overview than a single measure. In particular, the resilience of social networks and the time taken to recover following perturbation requires further investigation; while the Welsh mountain pony population is of low conservation concern, other more endangered species may exhibit similar disruption in response to removals, translocations or hunting practices. Translocations have been a key part of the conservationist’s toolbox for a long time (Griffith *et al.*, 1989) and are conducted for a variety of purposes and to achieve a diverse range of goals (Seddon, 2010). In particular, they are crucial for the management of the Cape mountain zebra as the only means of individual movement between subpopulations. That the loss of a small number of specific individuals can effect such large changes to social structuring in the Welsh mountain ponies is concerning. In addition, mortality events could produce the same effects as was observed here, therefore changing environmental conditions could be important as individuals of a certain demographic (e.g. lactating females, older individuals) may be more at risk. Whether changes to structuring and individual network position result in decreases in fitness or population growth rates, both for Welsh mountain ponies and other species, is also unknown, though it has been shown previously that social network position can influence individual survival (Lehmann, Majolo & McFarland, 2015) and fitness (Solomon-Lane *et al.*, 2015). Future studies on the impact of targeted removals on population structure could investigate whether disturbed networks recover after a period of time or if they settle at a new baseline; in addition it would be beneficial to assess the reproductive success of individuals and/or the growth rate of the overall population. While there are many avenues for future research this work also provides a valuable contribution to our knowledge of the social dynamics of the Carneddau Welsh mountain pony, which has been relatively poorly studied. An executive summary of the key findings and management recommendations for the Welsh mountain ponies can be found at the end of this chapter.

The Cape mountain zebra: identifying causes of poor population performance

An aspect of species conservation that has received little attention is that of the potential contrast between a species’ current range and its historical distribution, in light of habitat loss and range contraction. ‘Refugee’ species or populations may no longer have access to their preferred habitat and are restricted to areas of low ecological suitability. In some cases, the species management plan may even be targeted towards areas of suboptimal habitat, therefore it is essential that we develop methods to identify refugee species or populations so that their ecological requirements are properly understood and they can be conserved effectively. The Cape mountain zebra metapopulation provided a model system to develop an approach to identify populations that may be preserved in suboptimal habitat, and to investigate the consequences of this for individual physiology. This was achieved using a broad-scale assessment of habitat suitability that was related to measures of population performance, and by measuring faecal hormone metabolites across an ecological gradient, and with varying population demography. Using these methods, we identified the underlying causes of poor population growth in the Cape mountain zebra and in doing so provided an evidence base for conservation managers.

A comprehensive assessment of ecology and physiology

Habitat quality, defined by the abundance of palatable grass, was identified as a key factor from the outset and was assessed using existing vegetation maps for 21 populations. This measure was positively correlated to population growth rate, individual density, and female fecundity, leading to the identification of 12 refugee populations that occur in suboptimal habitat and perform poorly on at least one performance measure. This included three populations that hold the majority of the remaining genetic diversity for the subspecies. In addition, adult sex ratios (male:female) were negatively associated with habitat quality, suggesting that populations in less grassy habitat have lower reproduction or survival rates, and that females may be more severely impacted. The large-scale assessment of individual physiology confirmed that individuals in low quality habitat exhibit elevated baseline levels of faecal glucocorticoids indicative of chronic nutritional stress. Moreover, stallion physiological status was also associated with sex ratio skew, where faecal androgen levels were significantly higher in two populations with heavily male-biased sex ratios and the highest proportions of non-breeding bachelor stallions. Both hormones were negatively associated with female fecundity, suggesting a link between habitat quality, demography, and individual fitness. In addition to these findings, a large scale assessment of population structure in the Cape mountain zebra (detailed in Appendix 3) found that network clustering and mean group size formed a nonlinear relationship with habitat quality, and networks were least clustering in populations with male-biased sex ratios (Jackson *et al*., *in review*). These results align well with the data presented in this thesis, and suggest that populations with male-biased sex ratios may have both distinct social structures and characteristic individual physiological profiles due to the disruption of breeding groups. Moreover, these studies represent the collection, handling and analysis of data from markedly different disciplines, yet present parallel results and discuss complementary themes and conclusions.

The results presented in chapters four and five suggest two potential causes of poor population performance in the Cape mountain zebra: low quality habitat and male-biased sex ratios. Nutritional stress is known to result in an elevation in glucocorticoids in other mammalian species (du Dot *et al.*, 2009; Balestri *et al.*, 2014; Joly, Wasser & Booth, 2015) and in birds (Astheimer, Buttemer & Wingfield, 1992; Kitaysky *et al.*, 1999), however to the author's knowledge no study has assessed this over such a large number of populations of the same species before. What is also particularly concerning is that Cape mountain zebra individuals in low quality habitat did not show the same seasonal fluctuations in faecal glucocorticoid levels as those in high quality habitat. Chronic stress can result in changes to the sensitivity of the hypothalamic-pituitary-adrenal (HPA) axis, meaning individuals may be less capable of responding to future perturbations and may have an altered acute stress response (Rich & Romero, 2005; Dickens, Delehanty & Romero, 2009; Busch & Hayward, 2009; Sheriff *et al.*, 2011). Baseline glucocorticoids and fitness are not always negatively correlated, and the relationship can vary depending on the stressor, the species, the population, or even the life history stage of the individual (Bonier *et al.*, 2009). Moreover, under natural environmental circumstances chronic activation of the stress axis should be considered an adaptive trait designed to promote fitness in the presence of a long-term stressor (Boonstra, 2013). In the Cape mountain zebra faecal glucocorticoids were negatively correlated with female fecundity, suggesting that there is a fitness consequence as a result of chronic nutritional stress. However, populations of Cape mountain zebra that are confined to suboptimal habitat do not necessarily represent this species in its 'natural' environment, as it may be subjected to a more persistent stressor than would be normally expected as a result of habitat loss and range contraction.

The elevation of stallion faecal androgens in heavily male-biased populations may be a result of intensified male competition, and could lead to increased rates of aggression towards conspecifics (Sapolsky, 1983; Albert *et al.*, 1986; Wingfield, Lynn & Soma, 2001). Aggression in equids can result in a decrease in reproductive rates (Rubenstein, 1994; Linklater *et al.*, 1999; Sundaresan *et al.*, 2007), and male-biased sex ratios can threaten to spiral towards extinction (Le Galliard *et al.*, 2005). In addition, androgens are physiologically costly and when not required could divert energy away from more beneficial functions (Wingfield *et al.*, 2001). The cause of sex ratio skew in male-biased populations is not clear. If the source is resource limitation, then it could be due to biased birth sex ratios or differential survival rates. During our surveys we observed fairly even foal sex ratios, leaving the latter option more likely. However, as both heavily male-biased populations were also small (less than 50 individuals), it is possible that demographic stochasticity is the source of sex ratio skew. Population demography, density and the environment are intrinsically linked, and different combinations of characteristics can determine population dynamics (Coulson *et al.*, 2001). Therefore, it is difficult to establish a direct cause and effect relationship between the environment or demography and Cape mountain zebra physiology, as either factor (or likely both) will have an effect. For the vast majority of species it will not be possible to conduct experimental studies to assess the relative impact of each factor, as it would be too time-consuming and costly. Therefore, we must rely on correlative studies such as this to inform management decisions, and it will be important to use markers from multiple disciplines (e.g. physiological and behavioural) to give a broad overview of the ecology of the target species.

Implications for conservation science and practice

Using the assessment of habitat quality and population performance, 12 out of 21 Cape mountain zebra populations were identified as ‘refugees’, where confinement to suboptimal habitat appears to be impacting on fitness. Importantly, the identification of a (partial) refugee species requires a change in perception of what constitutes optimal habitat for that species, which may have been misinterpreted due to a shifting baseline (Pauly, 1995). The Cape mountain zebra’s current distribution is fairly indicative of its historic range; however in some cases it has been restricted to areas where it would historically have occurred at low densities, and as such performs comparatively poorly in these habitats. Cape mountain zebra have been shown to migrate seasonally between habitat types at different altitudes, yet in many places has been extirpated from lowland areas as a result of anthropogenic land conversion (Kerley *et al.*, 2003). As range contraction has occurred over several generations there has been a change in perception of the species' preferred habitat type. This has even been reaffirmed by its common name – the Cape 'mountain' zebra. Given the bias of protected areas towards less productive high altitude habitats (Joppa & Pfaff, 2009), this subspecies is unlikely to be the only example of this problem. For example, the mountain lion (*Puma concolor*) is an ecologically diverse species that can persist in a range of environmental conditions (Dickson & Beier, 2002). The species used to be prevalent across North America, but is now restricted almost entirely to remote locations in the west (Sweanor, Logan & Hornocker, 2000). Not coincidentally, protected areas in the US are also biased towards remote, high altitude regions (Scott *et al.*, 2001), and conflict between humans and mountain lions is not uncommon as they often enter areas of human habitation (Braun, 1991). The 'mountain' lion may be so called because it now persists in mountainous areas as the only habitat remaining to it after anthropogenic land conversion, and may be another example of a species where there is a misperception of its preferred habitat. The bias introduced by a species' common name in inferring habitat preferences has received little attention by conservation biologists so far, however it may play an important role in the development of awareness about refugee species in the future.

When Kerley *et al*. (2012) first outlined the refugee species concept using the example of the European bison (*Bison bonasus*) there were no other populations to provide comparative performance data, as the entire species is now confined to marginal habitat. The Cape mountain zebra metapopulation has therefore provided a model system not only to exemplify partial refugee status, but as evidence to support the original concept. Mitigating against refugee status is challenging, as the solution (regaining access to optimal habitat) is confounded by the cause of the problem (the land has already been lost). Moreover, the conservation requirements of different species can sometimes clash, such as for the Cape mountain zebra and the endemic flora of the Cape Floristic Region. However, a major barrier to success is an unawareness of the problem, making identifying refugee species vital both for improving species management plans and for the future planning of protected areas. In the case of the Cape mountain zebra, the overall metapopulation currently holds almost 5,000 individuals and in many ways the conservation efforts for this subspecies represent a huge success. This said there are still some concerns regarding the stability of some populations, most importantly the two naturally surviving ones that hold two-thirds of the remaining genetic diversity. The genetic status of the metapopulation was assessed over a decade ago (Moodley & Harley, 2006) yet little progress has been made towards integration, perhaps in part due to fear of losing individuals through risky translocation events. However, this will need to be a priority in order to create populations with the best chance of survival in the long-term.

Interestingly, the issue of sex ratio skew had been already been raised for at least one formally managed Cape mountain zebra population (Smith *et al.*, 2011), and anecdotally described by private landowners who are faced with very low resale values for males. However, the prevalence and potential negative impacts of male-biased sex ratios has been outlined most effectively here, as to the author's knowledge this work constitutes the most detailed and broad assessment of population demography to date. Demographic imbalance is potentially the most important factor when determining minimum viable population sizes (Lande, 1988), and could quickly lead to extinction in smaller populations (Caughley, 1994). If conservation managers can identify demographic imbalance in its early stages, preventative measures could be taken to avoid an accelerated decline. The ability to sustainably hunt Cape mountain zebra stallions, if appropriately monitored, may act to increase growth rates in afflicted populations by freeing up resources and lessening male harassment of females (Linklater *et al.*, 1999). This is currently prohibited as Cape mountain zebra are listed on the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) Appendix I, however a proposal to transfer the species to Appendix II is in progress (Graham Kerley, *pers. comm.*). Finally, introductions and translocations need to take into consideration the existing demography of the original and new population to avoid worsening sex ratio skew. In light of the findings for the Welsh mountain ponies and those outlined by Jackson *et al*., (*in review*) in Appendix 3, it may also be useful to assess the impact of translocation events on social network structure in the Cape mountain zebra in order to maximise potential growth rates. This is unlikely to be undertaken by conservation managers as it is time and labour intensive, however it would be a highly valuable avenue to explore for conservation research as it may have applications for a variety of other species.

Critical appraisal of the methodology and approach

The use of some of the methods employed during this project require critical appraisal to evaluate their success and their accessibility for conservation managers. First, when assessing habitat quality we used vegetation maps made freely available online by the South African National Biodiversity Institute (SANBI), compiled by Mucina & Rutherford (2006). This was perhaps an unusual approach as many other large-scale assessments of grass abundance have used the normalised difference vegetation index (NDVI), a measure of primary productivity (essentially greenness) derived from satellite imagery. The NDVI has wide ranging applications for remote sensing in conservation research (Turner *et al*., 2003) and for assessing species responses to environmental change (Pettorelli *et al*., 2005). However, we decided it was unsuitable for use in this project for a variety of reasons. First, NDVI measurements can be error prone in arid to semi-arid habitats and mountainous areas (Huete, 1988; Kerr & Ostrovsky, 2003), both of which constitute parts of the Cape mountain zebra's range. In addition, an assessment of green cover provides no information about the grass species present and their palatability, and could completely miss areas of land where grass is very sparse, but present. For example, Bakkrans Nature Reserve is very arid and has no grassland habitat, yet six grass species occur there. In this case, our assessment of grass availability picked up on fine-scale differences that an NDVI would not, and the latter also does not include any measure of palatability. Finally, during a preliminary assessment of the available NDVI data at the beginning of this project we found that the spatial resolution was poor in relation to the size of the protected areas in this study (however this may no longer be the case). Thus, after exploration we decided that it was not appropriate for our analyses. We tried to make the formula for the vegetation index relatively simple to increase its accessibility for managers and other researchers, as well as being replicable and useable for broad scale analyses. The vegetation maps used are likely more diverse for the purpose of habitat evaluation in South Africa because they describe in detail lists of important taxa not restricted solely to grass species; this is particularly relevant given that the Cape region of South Africa is a known for its high levels of plant diversity and species of conservation concern (Cowling *et al.*, 2003). In addition, the use of the SANBI vegetation map supports the production of a freely available high quality dataset by an in-country organization, and we hope our successful application here will encourage other researchers and conservation managers to do the same.

It is also worth evaluating the validity and efficacy of the measures used to indicate population performance for the Cape mountain zebra, namely population growth rate, individual density and the number of foals per mare. The first two were calculated using census and historical data, which is relatively good for this subspecies as it has been closely monitored for several decades. One challenge for population growth rate was how to account for introductions and removals, as their incorporation may bias the resulting value. We chose to add introduction events to the number of founding individuals, and the number of removals to the estimation of current population size. The addition of further introductions to the founding number would potentially negatively impact growth rate, however for the majority of populations this effect is likely to be small. Individuals are often introduced soon after the population has been established and later introductions are rare. Thus, it is more reasonable to account for introductions in the starting abundance rather than the final abundance. In contrast, removals are more common recently rather than early in the population’s history. There may be an effect of density dependence on growth rates, though even for Mountain Zebra National Park where density is extremely high and there has been a large number of removals, the population growth rate was not the highest observed (or even the second highest). Overall, it is unlikely that we have disproportionately inflated (or deflated) the growth rates, however it is certainly an important factor to consider.

The density of individuals in a given area is a frequently used metric for conservation purposes, and is a fundamental part of conservation theory and population dynamics (Caughley, 1994; Lande, 1998; Stephens & Sutherland, 1999; Courchamp, Berec & Gascoigne, 2008). The number of individuals sighted in a given area (or the number of dung piles as a proxy) has been used to determine habitat preferences for a number of Cape mountain zebra populations (Watson *et al.*, 2005; Watson, Kraaij & Novellie, 2011; Watson & Chadwick, 2007; Smith *et al.*, 2011). However, inferring habitat preferences from individual densities can be problematic as individuals may occur in an area for reasons other than the habitat, for example because of the presence of conspecifics (Skagen & Yackel Adams, 2011). An additional issue is the interpretation of density measurements for populations that are fenced and have no dispersal opportunities outside active translocation events, such as the Cape mountain zebra. This could bias measurements of habitat preferences as individuals may occur in certain habitats because they have nowhere else to go. Here, we use density measures as an indication of how many individuals each unit area of habitat can support; that reserves are fenced should actually make this a more accurate estimate. We do not investigate habitat preferences, focusing on the quality of the overall habitat available to each population. Moreover, zebra density is correlated with population growth rates indicating that it is capturing some aspect of population performance. Finally, estimates of female fecundity were based on surveys counting the number of foals per mare, which reflect a snapshot of an individual's reproductive status at any given time. While tracking individual reproductive rates for longer periods may be a more accurate reflection of fitness, this is an impractical method to use to assess the status of an entire species. We found that foal:mare ratio was a reliable measure of performance as it was correlated to population growth rate, habitat quality, and individual physiology. In an ideal world there would be data reflecting a year-by-year assessment of population size and demography for each population, however the reality is that this is not the case. Few species will have high quality demographic data for each population, however measuring foal:mare ratio could be a suitable approach to estimate reproductive rates across fragmented populations.

Applying this approach to other species

While the primary aim of this project was to identify the correlates of population performance in the Cape mountain zebra, it was also envisioned that the approach to study design and data collection would be relevant and applicable to other species of conservation concern. For the evaluation of refugee species status a framework was outlined in chapter three, using the Cape mountain zebra as a model species. This requires a relevant measure of ecological suitability that will be species-specific, though could be interchangeable between some species with similar ecological preferences (for example the measure of habitat quality used for the Cape mountain zebra could be applied to other grazing ungulates in South Africa). For terrestrial species the assessment of ecological suitability may refer to habitat quality (Kerley, Kowalczyk & Cromsigt, 2012), while for marine species it could be focused more specifically on food abundance (González, 2015). Both refer to general resource availability, a key determinant of the density of individuals a particular area of range can support (Pulliam, 1988; Dunning, Danielson & Pulliam, 1992). In addition, some measure of fitness or reproductive success is required in order to assess variation across the species' range. This data may not exist for many species, and this may represent the most important barrier to implementing this framework. While gathering enough empirical evidence to conclusively determine refugee status may be difficult, the identification of potential refugee species, through observations of varying population performance or a disparity between current and historic range, is still highly valuable.

In addition to identifying refugee species, this thesis research has shown that broad-scale physiological measurements is an effective way of evaluating conservation management plans by identifying key correlates of individual health and performance across populations. This approach used a noninvasive, field-based sampling technique that was found to be highly practical and could be applied to a diverse range of species. Both the Cape mountain zebra and the Welsh mountain pony study systems were comprised of individuals with highly distinctive markings that could be used for individual identification. This meant we could target known individuals to collect repeat samples and introduce control somewhat for individual variation in our analyses. This approach would not be suitable for species that are generally similar in appearance such as many ungulates, not least because collecting repeat samples would be close to impossible (depending on the size of the population). However, even using a distinctly patterned species as a model study system, we struggled to collect repeat samples from individuals across seasons, as individuals were simply not re-sighted. Even so, we found strong relationships between individual physiology and environmental and demographic measures due to a large overall sample size, indicating that sampling at a large enough scale may be enough to overcome the lack of repeat measurements for non patterned species. It is my belief that a macrophysiological approach could be of huge utility for conservation research as a way of informing conservation management strategies through empirical, intuitive and understandable measures of individual physiology and ultimately population ecology and fitness.

Areas for future research

There are several ways in which this research can be expanded upon both to further develop the methodology and to investigate other factors affecting performance in the two focal species, as well as others. Firstly, the relationships between environment, demography and individual physiology could be further clarified by measuring faecal thyroid and female reproductive hormone metabolites. The former are regulated partly in response to food availability (Flier, Harris & Hollenberg, 2000) and are suppressed when resources are limited (Bahnak *et al.*, 1981; Schew, McNabb & Scanes, 1996; du Dot *et al.*, 2009; Gobush, Booth & Wasser, 2014; Cristóbal-Azkarate *et al.*, 2016); an assay suitable for faecal metabolite measurement has been used successfully for several species (Wasser *et al.*, 2010). Secondly, measuring reproductive hormones would give an indication of whether females are cycling properly, becoming pregnant, or aborting the foetus due to inadequate resource availability or social harassment (Rutberg & Greenberg, 1990; Wade & Schneider, 1992; Pluháček & Bartoš, 2000). Such a study would require intensive sample collection from targeted populations, as repeat samples would be needed over the course of the entire female ovulatory cycle. To assess changes in population structure over time, ‘dynamic’ social network analysis could highlight more fine-scale differences in network position based on season (Rubenstein *et al.*, 2015). In addition, the use of Global Positioning System

(GPS) collars would open up several avenues for future research, such as fine-scale habitat use and activity budgets (Cagnacci *et al.*, 2010; Tomkiewicz *et al.*, 2010), and a record of individual associations judged by their proximity (Prange *et al.*, 2000). However, GPS collars are currently very expensive and would require that the animal be anaesthetised at least once, which is not always possible. Moreover, they are not without problems as collars sometimes fail or collect data inaccurately (Johnson, Heard & Parker, 2002; Frair *et al.*, 2010), and their high cost can result in poorly designed experiments and small sample sizes (Hebblewhite & Haydon, 2010).

In addition to method development, future work could focus on investigating other factors linked to performance and fitness. The assessment of resource availability for the Cape mountain zebra focused on grass abundance, whereas water is also a frequently limiting resource. Combining measures of food and water availability would give a more detailed assessment of habitat quality, and may highlight which resource is more important providing useful data for conservation managers. In addition, long-term data on individual survival and reproductive success would give certainty to the performance measures used and the relationships identified with the environment and demography. It may be possible to collect this long-term data for a species like the Welsh mountain pony, which is more accessible, easier to find and more habituated to the presence of humans. However, it is unlikely that such detailed information on individual fitness could be collected for most wild animal populations, and particularly for long-lived mammals like the Cape mountain zebra. As such, conservation science must proceed in a far from ideal world, making best use of the data we do have and attempting to collect more where possible. Finally, to truly test whether macrophysiology and macroecology have a place in the future of conservation research, the techniques outlined here should be applied to other species, such as other endangered equid species, (e.g. Grevy's zebra or the onager), species with good historical data like primates, and 'charismatic' species including other African megafauna.

Carneddau Welsh mountain ponies: impacts of management on individual physiology and population structure

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Project Summary

This research project aimed to assess the impacts of annual management practices on individual physiology and population structure in Welsh mountain ponies. The findings of this project have increased our knowledge of the ecology of a relatively poorly studied population, and could provide valuable information about a frequently used management technique (translocation) in the field of conservation.

Background

Carneddau Welsh mountain ponies are a free-living population of several hundred individuals that have been isolated long enough to become genetically distinct from other pony breeds. They are essentially unmanaged aside from a single management event that occurs annually in the autumn, where they are rounded up, temporarily relocated to adjacent farmland for two days, and new individuals are tagged for identification. Occasionally, male individuals of varying ages are removed as a method of controlling population size. We expected that this management intervention would provoke a stress response in the ponies due to the physical exercise and handling by humans, therefore we set out to measure the physiological response in eight focal mares to the roundup in the autumn of 2014. In addition, we measured changes in social network structure across the population after stallion removal and tracked a subset of the remaining stallions' testosterone levels through faecal hormone sampling.

Methods

The acute stress response was measured using faecal hormone monitoring, a method that had not previously been validated for this population. We collected 3-6 faecal samples from eight mares before the roundup to show baseline hormone levels, then collected one sample per day from each individual on days 1, 2, 3, 5, 7, 10, 14 and 18 after release from the roundup. We measured faecal glucocorticoid (stress hormone) metabolites using enzyme immunoassays and looked for significant elevations on each sampling day compared to baseline levels. Social networks were based on association data (individuals that grouped together, and their proximity within that group) collected during demographic surveys of the population that took place during 2014 and 2015. We used a subset of 62 individuals that were sighted three or more times both before and after the roundup, and constructed weighted networks (one for each condition) based on the strength of association between each individual. We looked for significant differences between social network measures such as the level of clustering, connectivity, and the number and strength of ties per individual.

Findings and Conclusions

We found that there was a time delay of five days before any indication of a stress response was observed in the eight focal mares, and then only two individuals exhibited the peak in faecal glucocorticoids that we expected to measure in response to the roundup event. There was a lot of variation between individuals, and while the study did provide some guidelines measurements of faecal glucocorticoid measurements it was not conclusive enough to be used as a biological validation. Despite losing only one herd stallion, one bachelor stallion, and five juveniles in our subset of the population, we found significant changes to social network structure. There was a disruption to group clustering, as individuals were more likely to move between groups resulting in the original six disconnected breeding groups becoming two large, interconnected components. We also found that the strength of ties between individuals was weaker following the roundup. Finally, stallion faecal testosterone levels were elevated from eight days after release until the end of the sampling period, suggesting that there may be increased male competition as a result of removing other adult stallions.

The results of these studies have some interesting implications. First, it became clear that our sampling protocol for faecal sample collection was not sufficient to give conclusive results about the physiological response of mares to the roundup event. We collected a single sample per individual per day, whereas we would now recommend that several or all samples be collected in the days following the roundup event to ensure that the response is not missed. However, using this approach in wild populations may be difficult as it is often difficult or impossible to keep track of individuals all the time. In addition, our evaluation of social networks indicated that stallions play an important role in maintaining a stable social structure, and their removal can cause large-scale disruption. We cannot be sure how long the effects of this lasted, or whether it had repercussions for reproductive rates in mares. Each of these areas would be an ideal avenue for future study, and the Welsh mountain ponies are a model population to investigate the impacts of management practices given their location, their moderate habituation to the presence of humans, and the ease by which they can be individually identified.

Management Recommendations

- If removals are necessary, select individuals at random rather than targeting stallions. For example, selection criteria could be every tenth individual to pass through the gate, or the last ten individuals remaining in the livestock crush at the end of the day. This would help keep numbers low while maintaining an even sex ratio, and would leave some bachelor stallions present that can provide natural levels of competition for herd stallions.
- Record the number of ponies capturing during each roundup event, so that population size and growth rate can be monitored over time. This would mean any changes in reproductive rates following individual removals could also be assessed.
- Consider choosing 20-25 individuals (of varying age and sex) to be held in a separate paddock for up to a couple of weeks following the roundup so that faecal samples can be collected to measure the acute physiological response. This would allow for a comprehensive biological validation of faecal glucocorticoid measurements and provide a scale that the impact of other stressors (e.g. environmental or social) can be compared to.

The Cape mountain zebra: an investigation into the environmental and demographic causes of poor population performance

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Project Summary

The aim of this project was to determine the causes of variable population performance in the Cape mountain zebra. Our approach and methods can be applied to other species to highlight cases where management strategies may be targeted towards suboptimal habitat. In addition, our findings have contributed to a review of the conservation management plan for this subspecies.

Background

The Cape mountain zebra is a subspecies endemic to the Cape region of South Africa. It has recovered from large-scale historical declines due to conservation efforts, and now exists as a metapopulation of over 5,000 individuals across more than 75 subpopulations. Despite this success, two out of the three naturally surviving populations perform poorly, and have become genetically distinct from each other and the rest of the metapopulation. It is vital that the growth rate of these populations is maximised so that individuals can be translocated to form mixed populations. Cape mountain zebra populations are diverse in size, demography and density and occur across a range of environmental conditions. This provided an ideal study system to investigate how habitat quality and demography can impact population performance.

Methods

We evaluated variation in population performance for 21 populations using both historical and current census data, recording when the population was founded and by how many individuals, the number of translocations and removals, and the current population size. We also surveyed 10 populations between 2010-15, visiting each population at least twice and sampling a minimum of three times during each visit. When sighted, each zebra was photographed and given an age-sex classification. From these data, we estimated the mean number of foals per mare and the mean adult sex ratio for each population. In addition, we developed a measure for habitat quality that estimated the abundance and palatability of grass species for 21 Cape mountain zebra populations. We also performed proximate diet analyses on 106 faecal samples collected from six reserves during Apr – May 2014. This involved analysing faecal samples for protein, fibre and nutritional element content. Finally, we used noninvasive faecal hormone measurements to measure glucocorticoid and androgen metabolites. We collected repeat samples were collected from known individuals; a total of 365 faecal samples were collected from 163 adult Cape mountain zebra across seven populations. Of these, 162 were collected from 89 stallions for faecal androgen analysis. Faecal samples were collected within an hour of defecation, thoroughly mixed and a sub-sample kept for hormone extraction. We followed a modified version of an in-field extraction technique that allows extracted hormone metabolites to be stored at room temperature for several months (Edwards *et al.*, 2014). Faecal corticosterone and testosterone metabolites were analysed using enzyme immunoassays following Edwards *et al*. (2014).

Findings and Conclusions

Grass abundance separated into two categories across reserves, showing there are areas of high and low habitat quality. Reserves in the north and east of the range had much more grass than those in the south and west. In addition, protein and nutritional element content was higher in faeces from grass-rich reserves. Importantly, we found that population growth rates, zebra density, and female fecundity (the number of foals per mare) were positively correlated with grass abundance. Moreover, Cape mountain zebra populations in low quality habitat were more likely to have male-biased adult sex ratios. These results indicate that low habitat quality is a factor in poor population performance.

We then related faecal glucocorticoid and androgen hormone levels across populations to habitat quality and adult sex ratios. Glucocorticoids were generally higher in less grassy habitat and declined as temperature increased. Moreover, Cape mountain zebra individuals in poor quality habitat had elevated faecal glucocorticoids across seasons, whereas those in high quality habitats had low glucocorticoid levels in the warm, wet summer months. This suggests that individuals in populations with low grass abundance may be chronically stressed as a result of low resource availability. In addition, we found that stallions in populations with heavily male-biased sex ratios had elevated faecal androgen levels, possibly as a result of increased male competition. Both hormones were negatively associated with female fecundity, indicating that compromised individual condition leads to poor population performance.

Overall, we have highlighted two possible causes of poor performance in the Cape mountain zebra: low habitat quality and sex ratio skew. In addition, we identified 12 out of 21 Cape mountain zebra populations as ‘refugees’, in that they are restricted to low quality habitat and have relatively poor growth rates. Populations that become isolated in suboptimal habitat are at risk as over time the perception of their core habitat may change; this means that identifying potential refugee species is essential for conservation strategies to be effective. The approach used here of measuring individual physiology across several populations to assess important factors for population performance is novel, and we believe that the techniques can be applied to other species (both captive and *in situ*) relatively easily. Finally, we make recommendations based on our results for all relevant stakeholders to contribute to the continual improvement of the management plan for this subspecies.

Management Recommendations

The immediate priorities for Cape mountain zebra management should be to maximise growth in relict populations (Gamkaberg and Kammanassie Nature Reserves) and the mixing of genetic stocks. We have several suggestions for how this could be achieved, which are outlined as follows:

- Translocate up to half of the current group of bachelor stallions at Gamkaberg Nature Reserve to another population in high quality habitat. This would relieve pressure on the Gamkaberg population, both in terms of resource availability and stallion harassment towards females, and create a mixed population. Individuals could be moved to an existing population of small- to medium-size, or form a new population elsewhere. Importantly, the habitat should be highly suitable for Cape mountain zebra to promote quick growth, therefore a privately-owned population may be a more suitable target for translocations as they can manage the land for this purpose. This could also be considered for Kammanassie Nature Reserve depending on the demography of the population, which is unknown to the author at this time.

- While land acquisition (through purchasing or the formation of a conservancy) and active land management are often costly and time-consuming and could contradict with the conservation strategies for other species, they should be considered a priority for Gamkaberg and Kammanassie Nature Reserve to increase growth rates. At Gamkaberg, recent improvements include the opening of 1000km of grassy fynbos habitat and the on-going instalment of additional water points, and in addition a large fire has burnt approximately 90% of the reserve. These events should be highly beneficial for the Cape mountain zebra population in the long term, and the impact on reproductive rates should be closely monitored.
- The population at De Hoop Nature Reserve should be reduced, and due to their high genetic diversity individuals should be relocated to form mixed populations in suitable habitat as was outlined above for Gamkaberg Nature Reserve. ALL translocations should take into account the demography of the current and target population to avoid worsening sex ratio skew.
- More generally, reliable population monitoring could be introduced throughout the Cape mountain zebra metapopulation, using pattern identification software such as Hotspotter or identification books for small populations. This will allow managers to keep track of foaling rates and demography, and to observe potential warning signs such as sex ratio skew more quickly.

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APPENDIX 1

Table A1.1. The vegetation types found across all reserves, with the number and mean palatability score of the grass species present

Biomes and Vegetation Types	**Total number of grass species**	**Number of dominant grass species**	**Mean palatability score**
Afrotemperate, Subtropical and Azonal Forests			
Southern Afrotemperate Forest	1	1	2.00
Albany Thicket			
Camdeboo Escarpment Thicket	20	8	2.05
Eastern Cape Escarpment Thicket	20	9	1.95
Gamka Thicket	13	4	2.33
Gamtoos Thicket	24	4	2.43
Groot Thicket	13	6	2.00
Kowie Thicket	12	9	2.45
Azonal Vegetation			
Albany Alluvial Vegetation	1	1	3.00
Cape Estuarine Salt Marshes	8	6	2.50
Cape Inland Salt Pans	4	1	2.00
Cape Lowland Alluvial Vegetation	6	0	2.43
Cape Lowland Freshwater Wetlands	6	1	2.50
Cape Seashore Vegetation	6	4	1.50
Muscadel Riviere	2	0	3.00
Southern Karoo Riviere	2	1	3.00
Tanqua Wash Riviere	2	0	2.00
Fynbos			
Albertinia Sand Fynbos	1	0	3.00
Baviaanskloof Shale Renosterveld	4	0	2.50
Bokkeveld Sandstone Fynbos	1	0	2.00
Boland Granite Fynbos	12	6	2.00
Central Inland Shale Band Vegetation	1	0	1.00
De Hoop Limestone Fynbos	2	1	3.00
Eastern Inland Shale Band Vegetation	1	0	3.00
Eastern Ruens Shale Renosterveld	9	4	2.00
Elgin Shale Fynbos	6	0	2.00
Elim Ferricrete Fynbos	5	0	1.33
Hawequas Sandstone Fynbos	6	4	1.33
Hopefield Sand Fynbos	2	2	2.50
Kango Conglomerate Fynbos	11	0	2.00
Kango Limestone Renosterveld	3	1	2.67
Kogelberg Sandstone Fynbos	6	4	1.75
Kouga Grassy Sandstone Fynbos	18	0	1.57
Kouga Sandstone Fynbos	5	0	1.75
Langebaan Dune Strandveld	5	1	2.50
Matjiesfontein Quartzite Fynbos	0	0	0
Matjiesfontein Shale Fynbos	1	0	1.00

Matjiesfontein Shale Renosterveld	6	0	2.33
Montagu Shale Renosterveld	2	1	2.00
Nieuwoudtville-Roggeveld Dolerite Renosterveld	2	2	2.00
North Kammanassie Sandstone Fynbos	3	2	3.00
North Rooiberg Sandstone Fynbos	5	5	1.75
North Swartberg Sandstone Fynbos	3	0	2.33
Overburg Dune Strandveld	3	0	2.33
Potberg Sandstone Fynbos	0	0	0
Roggeveld Shale Renosterveld	6	0	2.50
Saldanha Flats Strandveld	4	1	2.50
Saldanha Granite Strandveld	6	0	2.80
Saldanha Limestone Strandveld	3	0	2.33
South Kammanassie Sandstone Fynbos	0	0	0
South Rooiberg Sandstone Fynbos	4	1	1.50
South Swartberg Sandstone Fynbos	4	2	1.75
Suurberg Quartzite Fynbos	10	9	1.67
Suurberg Shale Fynbos	5	1	2.00
Swartberg Shale Fynbos	3	0	2.00
Swartruggens Quartzite Fynbos	1	0	1.00
Swellendam Silcrete Fynbos	8	0	2.00
Uniondale Shale Renosterveld	3	0	1.67
Vanrhynsdorp Shale Renosterveld	8	2	2.00
Western Coastal Shale Band Vegetation	4	2	2.00
Grassland			
Karoo Escarpment Grassland	20	9	2.00
Queenstown Thornveld	24	18	1.96
Tarkastad Montane Shrubland	20	11	1.85
Nama-Karoo			
Albany Broken Veld	10	4	2.20
Eastern Lower Karoo	15	5	1.93
Eastern Upper Karoo	23	10	2.00
Gamka Karoo	15	3	1.93
Upper Karoo Hardeveld	25	10	2.00
Western Upper Karoo	11	4	1.73
Succulent Karoo			
Agter-Sederberg Shrubland	5	1	2.00
Eastern Little Karoo	4	0	2.50
Kobee Succulent Shrubland	1	0	2.00
Little Karoo Quartz Vygieveld	0	0	0
Roggeveld Karoo	11	4	2.56
Tanqua Escarpment Shrubland	4	0	2.00
Tanqua Karoo	8	2	2.13
Western Little Karoo	1	0	0
Willowmore Gwarrieveld	10	0	2.00

Table A1.2. A list of the grass species found across all vegetation types and their palatability scores (1 = low, 2 = medium, 3 = high).

Grass species	Notes	Palatability score	Sources
Alloteropsis semialata* subsp. *eckloniana	Black seed grass. Grazed in the young stages	2	Andersson et al. 2015, Quattrocchi 2006, van Oudtshoorn 2012
Aristida adscensionis	Sixweeks three-awn, bunchgrass, perennial	1	Andersson et al. 2015, van Oudtshoorn 2012
Aristida barbicollis	Closely related to *A. congesta*	1	van Oudtshoorn 2012
Aristida canescens	Resembles *A. junciformis*	1	van Oudtshoorn 2012
Aristida congesta	Tassel three-awn, densely tufted short-lived perennial, low forage yield, hardy	1	Andersson et al. 2015, van Breda et al. 1990, van Oudtshoorn 2012
Aristida diffusa	Copper wire grass, used for erosion control	1	van Oudtshoorn 2012
Aristida junciformis	Perennial, hard and unpalatable, used for erosion control	1	Andersson et al. 2015, van Oudtshoorn 2012
Brachiaria serrata	Red top grass, perennial, slow-growing, low leaf production	2	Andersson et al. 2015, van Oudtshoorn 2012
Bromus pectinatus	Japanese brome, introduced European grass, annual, grows in disturbed soil	2	van Oudtshoorn 2012
Cenchrus ciliaris	Blue buffalo grass, perennial. Palatability decreases as plant matures. Hardy	3	Andersson et al. 2015, van Breda et al. 1990, van Oudtshoorn 2012
Chaetobromus involucratus	Hartebeest grass, leafy evergreen, widespread on sandy and loamy soils	3	van Breda et al. 1990, van Oudtshoorn 2012
Chloris virgata	Feather finger grass, hardy, grows in disturbed areas	2	Andersson et al. 2015, van Oudtshoorn 2012
Cladoraphis cyperoides	Looks more like a reed than a grass. Seldom grazed	1	van Oudtshoorn 2012
Cladoraphis spinosa	Spiny love grass, perennial, lives in desert regions	1	van Oudtshoorn 2012
Cymbopogon marginatus	Closely related to *C. popischilii*	1	van Oudtshoorn 2012
Cymbopogon popischilii	Narrow-leaved turpentine grass or lemongrass, bitter taste, repels insects	1	van Oudtshoorn 2012
Cymbopogon proxilus	Closely related to *C. popischilii*	1	Quattrocchi 2006
Cynodon dactylon	Bermuda grass, fast-growing and tough	3	Andersson et al. 2015, van Breda et al. 1990
Cynodon incompletus	Annual creeping grass, variable, hardy, has rhizomes	3	Quattrocchi 2006
Digitaria argyrograpta	Silver finger grass, native pasture species	3	Andersson et al. 2015, van Breda et al. 1990
Digitaria eriantha	Digit grass, variable, drought tolerant	3	Andersson et al. 2015, van Breda et al. 1990
Digitaria natalensis	Coast finger grass	3	Quattrocchi 2006
Diheteropogon filifolius	Wire bluestem, hard and unpalatable	1	van Oudtshoorn 2012
Ehrharta calycina	*Ehrharta* spp. – natural pasture grass. Veldt grass, variable and drought-tolerant perennial.	3	Andersson et al. 2015, Quattrocchi 2006, van Breda et al. 1990
Ehrharta capensis	Perennial, good quality, has small tubers just above root system	3	van Breda et al. 1990, van Oudtshoorn 2012
Ehrharta delicatula	Annual, leafy, grows among shrubs and rocky outcrops	3	van Breda et al. 1990
Ehrharta erecta	Veldt grass, perennial, loosely tufted	2	van Breda et al. 1990, van Oudtshoorn 2012
Ehrharta longiflora	Oat-seed grass, annual veldtgrass, rapid growth. Seldom common in veld	2	van Oudtshoorn 2012

Ehrharta melicoides	Hare grass, perennial, tufted, compact, dense	3	van Breda et al. 1990
Ehrharta villosa* var. *maxima	Closely related to *E. villosa* var. *villosa*	2	van Breda et al. 1990
Ehrharta villosa* var. *villosa	Pipe grass, used for erosion control on sandy dunes	2	van Breda et al. 1990
Elionurus muticus	Wire grass, bitter taste and wiry leaves. Indicator of overgrazing	1	van Oudtshoorn 2012
Enneapogon desvauxii	Eight day grass, short and densely tufted. Indicator of overgrazing	2	van Oudtshoorn 2012, van Oudtshoorn 2012
Enneapogon scaber	Rock nine-awned grass, perennial, grows on rocky outcrops. Uncommon	2	van Breda et al. 1990, van Oudtshoorn 2012
Enneapogon scoparius	Bottlebrush grass, perennial, grows in shade. Tough, unpalatable leaves	1	Andersson et al. 2015, van Oudtshoorn 2012
Eragrostis bergiana	Perennial, mat-forming. Mostly palatable, natural pasture species, cultivated fodder	2	Quattrocchi 2006
Eragrostis bicolor	Speckled vlei grass, perennial, leaves mostly basal. Average palatability but well utilised	2	van Oudtshoorn 2012
Eragrostis capensis	Heart-seed grass, leaves concentrated at base. Well utilised, low leaf yield	2	van Oudtshoorn 2012
Eragrostis chloromelas	Curly leaf, perennial, drought tolerant. Becomes unpalatable due to rolled leaves	2	Andersson et al. 2015, van Oudtshoorn 2012
Eragrostis curvula	Weeping love grass, long-lived perennial, dense root system. Cultivated pasture	2	Andersson et al. 2015, van Breda et al. 1990, van Oudtshoorn 2012
Eragrostis homomalla	Laxly tufted perennial	2	Quattrocchi 2006
Eragrostis lehmanniana	Lehmann's love grass. Valuable pasture, but low leaf production	2	Andersson et al. 2015, van Oudtshoorn 2012
Eragrostis nindensis	Wether lovegrass, polymorphic. Called "resurrection grass" as can quickly re-hydrate	2	Quattrocchi 2006, van Oudtshoorn 2012
Eragrostis obtusa	Dew grass, perennial, occurs in disturbed places. Low leaf yield	2	Andersson et al. 2015, van Breda et al. 1990, van Oudtshoorn 2012
Eragrostis procumbens	Usually in sandy soils, disturbed areas. *Eragrostis* spp. are native pasture species	2	Quattrocchi 2006
Eragrostis sabulosa	Perennial, creeping rhizomes	2	Quattrocchi 2006
Eragrostis sarmentosa	Perennial, mat-forming	2	Quattrocchi 2006
Eragrostis trichophora	Hairyflower lovegrass, tufted perennial	2	van Oudtshoorn 2012
Eustachys paspaloides	Fan grass, leafy, forms colonies	3	Andersson et al. 2015, van Breda et al. 1990, van Oudtshoorn 2012
Festuca caprina	Goat beard grass, perennial, variable, closely related to *F. scabra*	1	Quattrocchi 2006, van Oudtshoorn 2012
Festuca costata	Fibrous leaves, perennial, closely related to *F. scabra*	1	van Oudtshoorn 2012
Festuca scabra	Rough fescue, morphologically variable, sparsely distributed, green in winter	2	Andersson et al. 2015, van Oudtshoorn 2012
Fingerhuthia africana	Thimble grass, wide, hard leaves	2	van Breda et al. 1990, van Oudtshoorn 2012
Harpochloa falx	High leaf yield, average palatability. Tough later in season	2	Andersson et al. 2015, van Oudtshoorn 2012
Hemarthria altissima	Limpograss, perennial, short rhizomes, grows in many soil types	3	Andersson et al. 2015, van Oudtshoorn 2012
Heteropogon contortus	Speargrass, perennial, very variable. Common, relatively palatable, well grazed	2	Andersson et al. 2015, van Oudtshoorn 2012
Hyparrhenia hirta	Common thatching grass, perennial, drought and soil tolerant, stands heavy grazing	2	Andersson et al. 2015, van Oudtshoorn 2012

Karroochloa purpurea	Short-tufted, replaces *T. triandra* in overgrazed areas	2	Andersson et al. 2015, van Breda et al. 1990
Koeleria capensis	Crested koeleria, densely tufted, wiry but palatable	1	van Oudtshoorn 2012
Leersia hexandra	Swamp cut grass, aquatic, can withstand swamps drying out	3	Andersson et al. 2015, van Oudtshoorn 2012
Leptochloa fusca	Swamp grass, widespread. Can grow in brackish soil. Palatable and well utilised	3	van Oudtshoorn 2012
Leptochloa fusca (or Diplachne fusca)	Brown beetle grass/Kallar grass, salt tolerant, easily propagated	3	Andersson et al. 2015, van Oudtshoorn 2012
Melica decumbens	Staggers grass, evergreen, coarse leaves. Poisonous in large quantities	1	Andersson et al. 2015, van Breda et al. 1990, van Oudtshoorn 2012
Melica racemosa	Closely related and similar to *M. decumbens*	1	van Oudtshoorn 2012
Melinis repens* subsp. *repens	Natal red-top, annual, invasive, purple/pink flowers. Typical sight along roads	1	van Oudtshoorn 2012
Merxmuellera disticha	Mountain wire grass, densely-tufted, hard leaves, endemic	1	Andersson et al. 2015, van Oudtshoorn 2012
Merxmuellera dura	Closely related to *M. stricta*	1	van Breda et al. 1990, van Oudtshoorn 2012
Merxmuellera stricta	Cape wire grass, hard wiry leaves, endemic. Used as ornamental grass	1	van Breda et al. 1990, van Oudtshoorn 2012
Microchloa caffra	Pincushion grass, short tufted, indicates stressed conditions	1	van Oudtshoorn 2012
Microchloa kunthii	Kunth's smallgrass, perennial. Can hybridise with *M. caffra*	1	van Oudtshoorn 2012
Oplismenus hirtellus	Basket grass, grows in dense shade, low leaf production	1	van Oudtshoorn 2012
Oropetium capense	Dwarf grass, grows in shallow soil and rock crevices. Low leaf production	1	van Oudtshoorn 2012
Panicum colorata	Small buffalo grass, grows in fertile soil. Good grazing grass, cultivated as pasture	3	Andersson et al. 2015, van Oudtshoorn 2012
Panicum deustum	Broad-leaved Panicum, perennial, nutritious. Well utilised in winter	2	van Oudtshoorn 2012
Panicum maximum	Guinea grass, large tufted, fast-growing perennial	3	Andersson et al. 2015, van Oudtshoorn 2012
Panicum stapfianum	Highly variable, forms a complex	3	van Oudtshoorn 2012
Paspalum distichum	Knotgrass, summer-growing perrenial, tolerant to salinity	3	Andersson et al. 2015, van Oudtshoorn 2012
Paspalum vaginatum	Seashore paspalum, salt and submersion tolerant, perennial	3	van Oudtshoorn 2012
Pennisetum macrourum	Riverbed grass, hardy perennial, spear-like stem. Protects river banks from flooding	2	van Oudtshoorn 2012
Pennisetum thunbergii	Thinberg’s Pennisetum, hardy, grows at high altitude and in disturbed areas	2	van Oudtshoorn 2012
Pentameris macrocalycina	Perennial, variable. *Pentameris* spp. morphologically similar to *Pentaschistis* spp.	3	van Breda et al. 1990
Pentaschistis airoides	Common Annual Pentaschistis, forms small, delicate tufts, low leaf yield.	1	Andersson et al. 2015, van Oudtshoorn 2012
Pentaschistis aristifolia	Annual tufted grass. *Pentaschistic* spp. are native pasture	3	van Breda et al. 1990
Pentaschistis curvifolia	Tassel grass, densely tufted perennial. Average grazing value	2	van Oudtshoorn 2012
Pentaschistis eriostoma	Densely tufted	1	van Breda et al. 1990
Pentaschistis pallida	Pussytail grass, likes disturbed areas	1	van Oudtshoorn 2012
Pentaschistis patula	Annual, forms small, delicate tufts, low leaf production	1	Andersson et al. 2015
Pentaschistis rigidissima	Perennial, leaves pungent and rigid, found in arid habitats	1	Quattrocchi 2006
Polypogon	Annual beard grass, salt tolerant.	2	van Oudtshoorn 2012

monspeliensis	Introduced, could be good pasture for saline soil		
Schismus barbatus	Common Mediterranean grass, annual	3	van Breda et al. 1990
Schismus inermis	Mediterranean grass, *Schismus* spp. generally palatable	3	van Breda et al. 1990
Schismus scaberrimus	Mediterranean grass	3	van Breda et al. 1990
Secale strictum* subsp. *africanum	Wild rye, critically endangered, needs undisturbed soil on riverbanks	2	Andersson et al. 2015
Setaria lindenbergiana	Moutain bristle grass, dense tussocks, blue green colour	3	Andersson et al. 2015, van Oudtshoorn 2012
Setaria sphacelata	Golden bristle grass, tufted perennial, well cultivated	3	Andersson et al. 2015, van Oudtshoorn 2012
Setaria verticillata	Bur bristle grass, annual, grows under trees. Palatable but produces limited forage	2	van Oudtshoorn 2012
Sporobolus africanus	Parramatta grass, perennial, likes disturbed areas	2	van Oudtshoorn 2012
Sporobolus fimbriatus	Dropseed grass, tufted perennial. Palatable with high leaf yield	3	van Oudtshoorn 2012
Sporobolus ludwigii	Mat-forming dropseed grass	2	van Oudtshoorn 2012
Sporobolus nitens	Curly-leaved dropseed, perennial, grows in disturbed veld. Low leaf production	1	van Oudtshoorn 2012
Sporobolus virginicus	Good palatability, low grazing value	2	Andersson et al. 2015, Quattrocchi 2006
Stenotaphrum secundatum	St. Augustine grass, hardy perennial, variable in size, loses palatability with age	3	Andersson et al. 2015, van Oudtshoorn 2012
Stipagrostis ciliata	Tall bushman grass, annual and perennial, feather-like inflorescence	3	van Breda et al. 1990, van Oudtshoorn 2012
Stipagrostis obtusa	Small bushman grass, fine-leaved, grows in arid regions. Very palatable	3	van Breda et al. 1990, van Oudtshoorn 2012
Stipagrostis zeyheri* subs. *barbata	Cape bushman grass, natural sand stabiliser	1	Andersson et al. 2015
Tetrachne dregei	South African Cocksfoot, grows on slopes. Very palatable with high leaf production	3	Andersson et al. 2015, van Oudtshoorn 2012
Themeda triandra	Red grass, forms dense stands. Dominant in veld, very important grazing grass	3	Andersson et al. 2015, van Breda et al. 1990, van Oudtshoorn 2012
Trachypogon spicatus	Giant spear grass, forms dense coverings, protects soil from erosion	1	Andersson et al. 2015, van Oudtshoorn 2012
Tragus berteronianus	Carrot seed grass, annual, colonises hard compacted soil. Low grazing value	1	van Oudtshoorn 2012
Tragus koeleriodes	Perennial creeping grass. Low leaf production	1	Andersson et al. 2015
Tragus racemosus	Stalked burgrass, likes disturbed sites. Closely related to *T. berteronianus*	1	van Oudtshoorn 2012
Tristachya leucothrix	Hairy trident grass, perennial, moderate leaf production.	2	Andersson et al. 2015, van Oudtshoorn 2012

Table A1.3. Mean proximate component and elemental nutrient content of Cape mountain zebra faecal samples. Standard error of the mean is shown beneath in italics.

Reserve	Ash (% DM)	Fat (% DM)	Acid Detergent Fibre (% DM)	Neutral Detergent Fibre (% DM)	Crude Protein (% DM)	Faecal N (g/kg)	Ca (g/kg)	Mg (g/kg)	K (g/kg)	Na (g/kg)	P (g/kg)	Zn (ppm)	Cu (ppm)	Mn (ppm)	Fe (ppm)
Camdeboo National Park	19.70	2.71	56.35	78.11	7.36	11.78	6.31	3.15	5.82	2.56	3.85	41.80	6.19	127.65	1546.63
	± 0.90	*± 0.18*	*± 0.47*	*± 0.67*	*± 0.21*	*± 0.34*	*± 0.80*	*± 0.27*	*± 0.52*	*± 0.27*	*± 0.24*	*± 1.63*	*± 0.65*	*± 5.53*	*± 206.36*
De Hoop Nature Reserve	19.90	2.54	50.21	78.61	7.08	11.32	20.34	1.34	4.92	4.22	3.04	36.47	3.65	59.89	711.61
	± 1.45	*± 0.10*	*± 1.03*	*± 1.30*	*± 0.22*	*± 0.35*	*± 4.55*	*± 0.10*	*± 0.27*	*± 0.29*	*± 0.41*	*± 3.25*	*± 0.19*	*± 5.03*	*± 100.27*
Gamkaberg Nature Reserve	16.56	3.19	63.74	81.54	5.61	8.98	1.91	0.60	5.55	1.62	0.92	30.53	3.85	136.05	1449.43
	± 0.81	*± 0.14*	*± 0.80*	*± 0.63*	*± 0.17*	*± 0.27*	*± 0.39*	*± 0.03*	*± 0.10*	*± 0.09*	*± 0.04*	*± 1.26*	*± 0.17*	*± 5.38*	*± 172.43*
Karoo National Park: Mountain View area	20.54	3.89	56.76	83.17	5.44	8.71	2.17	1.93	5.74	0.19	2.10	32.95	5.75	121.60	2712.18
	± 0.93	*± 0.16*	*± 1.86*	*± 0.52*	*± 0.16*	*± 0.26*	*± 0.14*	*± 0.12*	*± 0.33*	*± 0.02*	*± 0.07*	*± 1.96*	*± 0.39*	*± 8.54*	*± 365.11*
Karoo National Park: Potlekkertjie Loop	22.04	3.55	56.01	71.96	6.98	11.17	7.04	2.22	8.17	2.13	3.57	49.96	6.58	121.83	1919.25
	± 0.87	*± 0.17*	*± 1.50*	*± 1.03*	*± 0.20*	*± 0.32*	*± 0.62*	*± 0.14*	*± 0.74*	*± 0.24*	*± 0.14*	*± 1.90*	*± 0.48*	*± 8.12*	*± 198.17*
Mount Camdeboo Private Reserve	19.92	3.28	56.56	78.59	6.27	10.03	2.10	1.20	7.97	0.31	1.94	29.59	3.64	119.13	778.98
	± 0.68	*± 0.22*	*± 0.40*	*± 1.24*	*± 0.27*	*± 0.43*	*± 0.42*	*± 0.33*	*± 0.58*	*± 0.04*	*± 0.26*	*± 3.33*	*± 0.25*	*± 9.32*	*± 217.80*
Mountain Zebra National Park	18.73	3.41	59.41	76.86	6.21	9.93	4.08	2.68	7.11	1.33	2.93	35.67	4.02	84.68	1340.31
	± 0.56	*± 0.12*	*± 0.96*	*± 0.51*	*± 0.19*	*± 0.31*	*± 0.35*	*± 0.36*	*± 0.32*	*± 0.22*	*± 0.17*	*± 1.44*	*± 0.13*	*± 2.67*	*± 119.10*

APPENDIX 2

Appendix 2.1: Field Hormone Extraction

Faecal samples were extracted in the field using a modified version of Edwards et al. (2014). Each faecal sample was thoroughly mixed, 0.5 g (±0.05 g) was weighed on a portable scale and transferred to an 8ml glass vial using clean tweezers, then 4 ml of 90% methanol was added to the vial which was capped and hand-shaken for 5 min. Faecal extracts were filtered by attaching a syringe filter (30mm diameter 0.2 μm PTFE; Thermo-Fisher Scientific, UK) to a 5 ml syringe, pouring as much of the liquid extract into the syringe as possible, and slowing pushing it through into a 15ml glass test tube.

To enable the filtered extract to be retained onto C8 cartridges (6ml HyperSep™ octyl bonded silica C8 cartridges; Thermo-Fisher Scientific, UK), 5 ml of distilled water was added to filtered extract to reach a concentration of ~40% methanol. Each C8 cartridge was primed just prior to loading the ~40% filtered extract by first adding 4 ml of 100% methanol followed by 4 ml of distilled water. Both liquids were pushed through the gel slowly (~2.5 ml/min) added using a 60 ml syringe with an adapter (60104-259; Thermo-Fisher Scientific, UK) attached to the C8 cartridge. The 100% methanol was pushed through the gel completely prior to adding the 4 ml of distilled water as per the manufacturers instructions (Thermo Scientific 2011). The ~40% methanol faecal extract was then loaded onto the primed C8 cartridge, again by pushing through with attached 60 ml syringe and adaptor (at ~2.5 ml/min), ensuring it is was not pushed all the way the gel until the entire sample had been added. This was followed by 2 ml of distilled water, this time leaving the distilled water to sit just above the gel. Each end of the cartridge was then sealed with Parafilm® and stored upright at room temperature until analysis.

Appendix 2.2: Hormone Enzyme Immunoassay (EIA)

Each EIA utilized an antibody (polyclonal corticosterone antiserum CJM006 or polyclonal testosterone R156/7 supplied by CJ Munro, University of California, Davis, CA), horseradish peroxidase conjugated label [corticosterone or testosterone; prepared according to Munro and Stabenfeldt (1984)] and standards (corticosterone or testosterone; Sigma-Aldrich, UK).

For corticosterone EIA, the loading procedure was as follows: (i) 50 µl per well of antiserum 1:15,000 dilution in coating buffer (0.05M NaHCO3, pH 9.6) was loaded onto a 96-well mircotitre plates (NUNC Immuno Maxisorp; Thermo-Fisher Scientific) and incubated overnight at 4°C; (ii) plates were washed with wash solution (0.15 m NaCl, 0.05% Tween 20) five times; and (iii) standards (3.9 – 1000 pg per well) or samples diluted (1:10) in EIA buffer (0.1M NaPO4, 0.149M NaCl, 0.1% bovine serum albumin, pH 7.0) were loaded at 50 µl per well in duplicate; followed by (iv) 50 µl per well of horseradish peroxidase (1:70,000 dilution in EIA buffer).

For testosterone, the loading procedure was as follows: (i) non- specific goat anti-rabbit γ-globulin (IgG; R2004; Sigma) was diluted in coating buffer, then loaded, 1.0 µg in 250 µl per well, on plates and incubated overnight at room temperature. The non-specific IgG was discarded, and 300 µl per well of Tris blocking buffer (0.02 m Trizma, 0.300 m NaCl and 1.0% bovine serum albumin, pH 7.5) was added and incubated for a minimum of 2 h at room temperature; (ii) plates were washed with wash solution five times; (iii) EIA buffer was loaded at 50 µl per well; (iv) standards (2.3 – 600 pg per well) or samples diluted in EIA buffer (1:30) were loaded at 50 µl per well in duplicate; followed by (v) 50 µl per well of horseradish peroxidase (1:40,000 in EIA buffer); and (vi) 50 µl per well of antiserum 1:25,000 in EIA buffer.

Following loading, for both the corticosterone and testosterone EIA, plates were incubated in the dark for 2 h at room temperature, then washed with wash solution five times and incubated with 100 µl per well of room temperature substrate [0.4 mm 2,2′-azino-di-(3-ethylbenzthiazoline sulfonic acid) diammonium salt, 1.6 mm H2O2 and 0.05 m citrate, pH 4.0) and left to develop at room temperature in the dark and measured 405 nm at an optical density 0.8 to 1.0.

EIAs were validated for measuring corticosterone and testosterone for field extracted zebra faeces, through parallelism (corticosterone: $R^2 = 0.966$, $F_{1,7} = 197.09$, $p < 0.001$; testosterone: $R^2 = 0.995$, $F_{1,7} = 1392.70$, $p < 0.001$). Additionally, there was no evidence of matrix interference, as addition of diluted faecal extract to standards did not alter the amount expected (corticosterone: $R^2 = 0.995$, $F_{1,6} = 1110.20$, $p < 0.001$; testosterone: $R^2 = 0.982$, $F_{1,6} = 332.33$, $p < 0.001$).

The cross-reactivities of testosterone and corticosterone antibodies have been reported elsewhere (deCatanzaro *et al.* 2003; Watson *et al.* 2013). Intra- and inter-assay coefficients of variation were maintained at < 10% for corticosterone EIAs and < 15% for testosterone EIAs.

Appendix 2.3: Analysis of faecal sample dry weight

Approximately 5 g wet weight from each faecal sample was air dried completely and re-weighed. From this a percentage of dry matter was calculated for each sample that could be used to correct for variation across reserves due to variation in faecal moisture content caused by dietary differences (Wasser *et al.* 1993). First, we used a linear mixed effect model to determine if faecal sample dry weight varied between reserves, with reserve included as a fixed factor and zebra ID and sampling trip as random factors, and found that there was significant variation between populations ($F_{6,156} = 13.15$, $p < 0.001$). We then took several steps to determine if faecal sample dry weight was affecting the outcome of our results: first, we adjusted hormone metabolite concentrations by multiplying by sample dry weight (%), and assessed how much they differed using a Pearson's product moment correlation. Next, we built models using both adjusted and non-adjusted hormone concentrations as the dependent variable, and compared model outputs for the environmental and demographic variables. Finally, we compared within-individual variation by calculating the coefficient of variation (CV) in adjusted and non-adjusted glucocorticoid and androgen concentrations for individuals with three or more samples ($n = 62$ individuals), then using Wilcoxon signed rank tests to see if accounting for dry weight decreased variation in faecal hormone metabolites.

The adjusted and non-adjusted values were highly correlated for both hormones (glucocorticoids: $R = 0.99$, 95% C.I. 0.98 – 0.99, $p < 0.001$; androgens: $R = 0.99$, 95% C.I. 0.99 – 1.00, $p < 0.001$). We observed no differences in trends between adjusted or non-adjusted hormone concentrations and any predictive variable (see Tables A2.1 and A2.2 for model outputs for adjusted hormone concentrations); almost all significance levels remained the same and the order of model selection was highly comparable. In addition, when comparing within-individual variation (CV) in hormone metabolite concentrations calculated from either adjusted or non-adjusted values, we found no significant differences (glucocorticoids: $W = 2215$, $p = 0.67$; androgens: $W = 275$, $p = 0.80$). As such, whilst accounting for faecal sample dry weight may in some cases reduce variation in hormone metabolite concentrations, we find no evidence that it is required for our analyses and prefer to present faecal glucocorticoid and faecal androgen results in non-adjusted concentrations in the main text. This is also preferential for comparison of raw measurements between studies.

Table A2.1. Demographic and environmental factors associated with faecal glucocorticoid metabolite concentrations in Cape mountain zebra **adjusted for faecal sample dry weight (%)**, ranked by Bayesian information criterion (BIC). For all models with two-way interactions (*), both main effects and the interaction term were incorporated into the model.

Predictors	BIC	ΔBIC	R^2		Estimate ± SE	df	t	p
Environment and demography								
Grass*Temperature	403.62	-	0.28		0.18 ± 0.03	1,20	6.36	**<0.001**
Grass					-2.81 ± 0.49	1,161	-5.70	**<0.001**
Temperature					-0.10 ± 0.03	1,20	-5.94	**0.02**
Grass*Rainfall season	417.33	13.71	0.27	*wet*	0.23 ± 0.13	2,18	1.74	**<0.001**
				dry	-0.45 ± 0.14		-3.24	
Grass					0.39 ± 0.08	1,161	4.64	**<0.001**
Rainfall season				*wet*	-0.24 ± 0.09	2,18	-2.62	**<0.001**
				dry	0.30 ± 0.09		3.22	
Grass	421.45	17.83	0.14		0.36 ± 0.06	1,161	6.23	**<0.001**
Rainfall season	440.58	36.96	0.10	*wet*	-0.33 ± 0.07	2,20	-4.65	**<0.001**
				dry	0.01 ± 0.08		0.13	
Temperature	441.25	37.63	0.07		-0.06 ± 0.01	1,21	-4.32	**<0.001**
Proportion of bachelors	441.43	37.81	0.04		1.41 ± 0.43	1,161	3.29	**0.001**
Temperature*Rainfall season	442.61	38.99	0.21	*wet*	-0.19 ± 0.03	2,17	-5.40	**<0.001**
				dry	-0.11 ± 0.06		-1.72	
Temperature					0.08 ± 0.03	1,17	2.90	**<0.001**
Rainfall season				*wet*	3.22 ± 0.65	2,17	4.98	**<0.01**
				dry	1.90 ± 1.05		1.80	
Adult sex ratio	452.49	48.87	0.01		0.11 ± 0.06	1,161	1.87	0.06
Sex	455.61	51.99	0.00		-0.03 ± 0.06	1,161	-0.44	0.66
Population performance								
Foal:mare ratio	429.98	-	0.09		-1.38 ± 0.28	1,161	-4.94	**<0.001**
Population growth rate	440.81	10.83	0.08		-0.05 ± 0.01	1,161	-4.42	**<0.001**
log Zebra density	442.96	12.98	0.06		-0.18 ± 0.05	1,161	-3.75	**<0.001**

Table A2.2. Demographic and environmental factors associated with faecal androgen metabolite concentrations in Cape mountain zebra stallions **adjusted for faecal sample dry weight (%)**, ranked by Bayesian information criterion (BIC). For all models with two-way interactions (*), both main effects and the interaction term were incorporated into the model.

Predictors	**BIC**	**ΔBIC**	**R^2**		**Estimate ± SE**	**df**	**t**	**p**
Environment and demography								
Adult sex ratio	245.10	-	0.35		0.50 ± 0.07	1,86	7.27	**<0.001**
Glucocorticoids					*0.26 ± 0.07*	*1,71*	*3.89*	*<0.001*
Proportion of bachelors	249.44	4.34	0.31		3.55 ± 0.55	1,86	6.50	**<0.001**
Glucocorticoids					*0.21 ± 0.07*	*1,71*	*2.89*	*<0.01*
Grass	257.27	7.83	0.29		0.55 ± 0.09	1,86	6.01	**<0.001**
Glucocorticoids					*0.12 ± 0.08*	*1,71*	*1.47*	*0.15*
Grass*Temperature	265.47	20.37	0.35		0.03 ± 0.04	1,69	0.70	0.49
Grass					0.17 ± 0.72	1,86	0.23	**<0.001**
Temperature					0.06 ± 0.03	1,69	2.08	**0.001**
Glucocorticoids					*0.12 ± 0.08*	*1,69*	*1.46*	*0.08*
Grass*Rainfall season	273.13	28.03	0.35	*wet*	-0.66 ± 0.20	2,67	-3.36	**<0.01**
				dry	0.04 ± 0.19		0.19	
Grass					0.72 ± 0.13	1,86	5.59	**<0.001**
Rainfall season				*wet*	0.36 ± 0.13	2,67	2.71	0.73
				dry	0.10 ± 0.14		0.74	
Glucocorticoids					*0.17 ± 0.08*	*1,67*	*2.20*	*0.18*
Temperature	289.03	43.93	0.11		0.02 ± 0.02	1,70	0.87	0.86
Glucocorticoids					*0.35 ± 0.08*	*1,70*	*4.32*	*<0.001*
Rainfall season	293.99	48.89	0.11	*wet*	-0.02 ± 0.12	2,69	-0.18	0.42
				dry	0.01 ± 0.12		0.06	
Glucocorticoids					*0.33 ± 0.08*	*1,69*	*4.01*	*<0.001*
Population performance								
Foal:mare ratio	245.14	-	0.34		-2.69 ± 0.38	1,86	-7.03	**<0.001**
Glucocorticoids					*0.13 ± 0.07*	*1,71*	*1.93*	*0.06*
log Zebra density	266.37	21.23	0.26		-0.34 ± 0.07	1,86	-4.74	**<0.001**
Glucocorticoids					*0.23 ± 0.08*	*1,71*	*2.98*	*<0.01*
Population growth rate	267.35	22.21	0.26		-0.08 ± 0.02	1,86	-5.05	**<0.001**
Glucocorticoids					*0.23 ± 0.07*	*1,71*	*3.11*	*<0.01*

Appendix 2.4: correlation between glucocorticoids and androgens

We used a Pearson's product-moment correlation and a linear regression model to assess the relationship between faecal glucocorticoid concentrations (square root transformed) and faecal androgen concentrations (log transformed), sampled from Cape mountain zebra stallions. The two hormones were significantly positively correlated (r = 0.33, 95% C.I. 0.17 to 0.45, $p < 0.001$), though the goodness of fit of the linear regression was poor ($\beta = 0.34 \pm 0.08$, t = 4.39, $R^2 = 0.10$; Fig. A2.1). To determine the degree of the covariance between the two hormone metabolites, we compared the output of linear mixed effect models for faecal androgens and the *subset* of stallion faecal glucocorticoids, to see if trends differed for environmental and demographic predictors. In addition, we used generalized linear models to determine trends between the mean faecal glucocorticoid and androgen concentration for each population (n = 7 for each) and environmental/demographic predictors, and compared these models using Akaike Information Criterion (AIC).

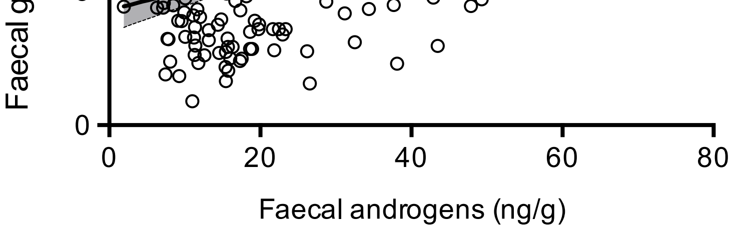

Figure A2.1. Faecal glucocorticoid and androgen metabolite concentrations are positively correlated (r = 0.33, 95% C.I. 0.17 to 0.45, $p < 0.001$), though the goodness of fit of the linear regression is poor ($\beta = 0.34 \pm 0.08$, t = 4.39, $R^2 = 0.10$). The linear regression line is shown with the 95% confidence interval.

Firstly, several environmental and demographic variables differed substantially in significance level and goodness of fit (R^2) between stallion faecal androgens and glucocorticoids (Table A2.3). Second, mean faecal glucocorticoid and androgen concentrations were better predicted by different variables: glucocorticoids were best predicted by habitat quality and least well by population demography, whereas the converse was true for androgens (Table A2.4). This evidence indicates that whilst showing a degree of correlation, the faecal glucocorticoid and androgen measurements are fluctuating in relation to different ecological variables. Nonetheless, as a precautionary measure we included faecal glucocorticoid metabolite concentrations as a fixed factor in every model where faecal androgen concentration was the response variable (outputs in Table 2 in the main text of Chapter 5).

Table A2.3. Linear mixed effect models showing the effect size and goodness of fit (R^2) of environmental and demographic predictors of stallion faecal glucocorticoid and androgen metabolite concentrations, where factors highlighted in bold show different significant levels and/or goodness of fit between the two hormones.

Predictor	Stallion glucocorticoids (n = 163)						Stallion androgens (n = 162)					
	R^2		Estimate ± SE	df	t	p	R^2		Estimate ± SE	df	t	p
Adult sex ratio	**0.03**		0.15 ± 0.09	1,88	1.77	**0.08**	**0.24**		0.48 ± 0.07	1,87	6.49	**<0.001**
Proportion of bachelors	**0.08**		2.11 ± 0.66	1,88	3.22	**<0.01**	**0.26**		3.68 ± 0.53	1,87	6.90	**<0.001**
Grass*Temperature	**0.29**		0.16 ± 0.04	1,6	3.85	**<0.01**	**0.35**		0.06 ± 0.04	1,6	1.56	**0.17**
Grass			-2.42 ± 0.76	1,88	-3.17	<0.001			-0.30 ± 0.66	1,87	-0.46	<0.001
Temperature			-0.08 ± 0.09	1,6	-2.85	0.74			0.04 ± 0.02	1,6	1.70	0.01
Rainfall season	**0.10**	*wet*	-0.42 ± 0.12	2,6	-3.65	**0.03**	**0.02**	*wet*	-0.17 ± 0.11	2,6	-1.50	**0.37**
		dry	-0.20 ± 0.12		-1.59			*dry*	-0.11 ± 0.12		-0.93	
Temperature	**0.04**		-0.05 ± 0.02	1,7	-2.32	**0.05**	**0.00**		-0.00 ± 0.02	1,7	-0.20	**0.85**
Grass	0.21		0.51 ± 0.09	1,88	5.64	<0.001	0.29		0.59 ± 0.08	1,87	7.73	<0.001
Grass*Rainfall season	0.26	*wet*	0.23 ± 0.23	2,4	1.03	0.19	0.32	*wet*	-0.54 ± 0.19	2,4	-2.87	0.10
		dry	-0.32 ± 0.22		-1.46			*dry*	-0.08 ± 0.18		-0.47	
Grass			0.47 ± 0.14	1,88	3.36	<0.001			0.76 ± 0.12	1,87	6.39	<0.001
Rainfall season		*wet*	-0.29 ± 0.15	2,4	-1.92	0.21		*wet*	-0.26 ± 0.13	2,4	2.06	0.94
		dry	0.06 ± 0.17		0.37			*dry*	0.09 ± 0.14		0.65	
Temperature*Rainfall season	0.19	*wet*	-0.18 ± 0.06	2,3	-3.25	0.10	-		-	-	-	-
		dry	-0.18 ± 0.10		-1.72							
Temperature			0.07 ± 0.04	1,3	1.59	0.08						
Rainfall season		*wet*	3.07 ± 1.06	2,3	2.90	0.10						
		dry	2.73 ± 1.67		1.64							

Table A2.4. Environmental, demographic and population performance factors associated with mean faecal glucocorticoid and androgen metabolite concentrations sampled from cape mountain zebra individuals from seven populations, ranked by Akaike Information Criterion (AIC).

Predictor	Mean ± SE	AIC	Estimate ± SE	df	t	p
Glucocorticoids	**6.60 ± 0.69**					
Grass abundance		26.74	2.68 ± 0.96	1,5	2.79	**0.04**
Proportion of bachelors		33.22	2.51 ± 10.25	1,5	0.25	0.82
Adult sex ratio		33.30	-0.04 ± 1.43	1,5	-0.03	0.98
Androgens	**20.89 ± 2.98**					
Proportion of bachelors		47.15	77.01 ± 27.72	1,5	2.78	**0.04**
Adult sex ratio		48.98	9.59 ± 4.38	1,5	2.19	**0.08**
Grass abundance		49.51	9.89 ± 4.89	1,5	2.02	0.10

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APPENDIX 3

Socioecological flexibility in the Cape mountain zebra meta-population: both ecology and demography impact on social network structure

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Abstract

Understanding how species, and populations, respond to environmental change is essential for predicting resilience and future viability. Although there are abundant examples for how range and phenology changes across ecological gradients, there is very little evidence for how social structure and demography does. Here we investigate how group size, social network structure and demography change across an environmental gradient in ten independent sub-populations of the Cape mountain zebra, *Equus zebra zebra*. Using network metrics from association matrices, we derived a size-independent and repeatable characterisation of social network structure at the population level based on two components: *network clustering* and *group variation*. Both network clustering and group size were highest in populations in intermediate habitats with balanced adult sex ratios. This suggests that population structuring is sensitive to both ecological and demographic constraints. Thus, network dynamics provide novel insight into resilience across environmental gradients as well as the relationship between ecological pressures and flexibility in social structuring.

Introduction

Habitat loss and fragmentation strongly predict extinction risk in birds and mammals and disproportionately affect species with small ranges (Brooks *et al.*, 2002). Range contraction often results in a matrix of fragmented populations (Hanski & Gilpin, 1991), some of which are confined to spatially peripheral or ecologically marginal habitats relative to the historical range (Kerley, Kowalczyk & Cromsigt, 2012). Populations in marginal habitat can provide insight into how species cope with environmental change. Behavioural flexibility has been well documented in many species (e.g. Clutton-Brock 1977, Lott 1984, Vaudo et al. 2014), and can help buffer populations to environmental challenges (Salido *et al.*, 2012). The socio-ecological model provides a framework for evaluating behavioural responses to the environment as it assumes that animal grouping patterns are a direct response to ecological challenges, such as predation and resource availability (Jarman, 1974; Emlen & Oring, 1977; Linklater, 2000; Dammhahn & Kappeler, 2009). Another factor that can link habitat quality to social behaviour and potentially performance is the demographic composition of the population. The ratio of adult males to adult females has been shown to impact the number and size of social units in primates (Ohsawa & Dunbar, 1984). Male-biased populations may also suffer from social instability due to increased male-male compeition and harassment of females (Greives, Casto & Ketterson, 2007). Thus change in population structure may reflect underlying biases in secondary (birth) sex ratios or subsequent mortality that ultimately are the consequence of ecological stress.

Relating differences in population structure to underlying ecological pressures is not straightforward. Group typology often varies little between closely related species, as well as between populations of the same species (Linklater, 2000; Shultz, Opie & Atkinson, 2011). In contrast, group size can be highly variable both within and between populations such that average group size may not accurately reflect within population variation, association patterns or group composition. There is a need, therefore, to develop methods that can describe consistent population level differences in behaviour and structure.

Quantifying the *structure* rather than the size or density of populations, by evaluating demography and how animals associate, group, and use space, can reveal how individuals respond to pressures such as resource limitation, demographic imbalance or predation. Social network analysis allows population structure to be characterised using individual association patterns (Lusseau & Newman, 2004; Croft, James & Krause, 2008; Whitehead, 2008; Krause, Lusseau & James, 2009). In a social network, nodes represent individuals and ties are the associations between individuals (e.g. based on proximity or behaviours such as grooming or aggression). As such, a social network can quantify the underlying structure of a population (Hinde, 1976; Croft *et al.*, 2008) and highlight individual and population level responses to the environment. For example, differences between Grevy's zebra (*Equus grevyi*) and onagers (*Equus hemionus*) in association preferences, the fidelity of tie choices, and the degree of sub-structuring in association networks has been attributed to differences in food, water and predation pressure between the two species (Rubenstein, 1994; Sundaresan *et al.*, 2007a). Typically, social network analyses are performed on association data gathered over long periods of time for a single population, with multiple repeated encounters of individuals (Croft *et al.*, 2008). Intensive sampling will highlight the nature of individual ties, but may not be necessary for characterising structure at the population level. Although there are compelling theoretical and applied reasons for understanding social responses at a macro-ecological scale, there is currently no framework in place to characterise these features.

Comparing network structure across independent populations is challenging because surface characteristics, such as size, can have a considerable influence on network metrics (Anderson *et al.*, 1999; but see Faust & Skvoretz 2002; Faust, 2006; Croft *et al*., 2008). One solution for comparing networks is the NetSimile approach, which uses multiple moments (e.g. mean, standard deviation, skew and kurtosis) from network metrics derived from nodes or egos (focal nodes with specific characteristics; Berlingerio *et al.*, 2012). Whereas mean metrics may, or may not, vary across network typologies, additional moments provide information about variation in association patterns between nodes. Moreover, by capturing the variation as well as the central tendency for each of the metrics, the resulting features describing the network are not as influenced by network size. For example, Berlingerio *et al.* (2012) used a NetSimile approach to derive network features that were not correlated with network size, and were able to compare networks that varied in size by a factor of 100. Furthermore, capturing information about the variation in the features of nodes or egos can account for nuanced differences between individual association patterns across two networks.

The Cape mountain zebra: a model

The Cape mountain zebra, *Equus zebra zebra*, occurs in the Cape Floristic Region of South Africa (Novellie, 2008). Following global population declines, Cape mountain zebra were critically endangered in the 1970s, with fewer than 80 individuals. However, subsequent conservation management has resulted in a large population increase (Millar, 1970; Novellie *et al.*, 2002; Novellie, 2008; Hrabar & Kerley, 2015). The meta-population is now spread over more than 75 sub-populations, many of which are on privately owned land with very few individuals. These sub-populations range in size from fewer than 10 individuals at Bontebok National Park to over 1000 individuals at Mountain Zebra National Park (Hrabar & Kerley, 2015). Furthermore, two (Gamkaberg Nature Reserve and Kammanassie Nature Reserve) of the three relict populations represent less than 3% of the total population (Hrabar & Kerley, 2015).

Cape mountain zebra show a preference for habitats with a high number of palatable grass species, including protein rich grasses such as *Themeda triandra* (Grobler, 1983; Watson & Chadwick, 2007; Weel *et al.*, 2015; Lea *et al.*, 2016). The Cape Floristic Region is characterised by fynbos shrubland vegetation, which has a high diversity of plant species but a low abundance of grass species (Campbell & Van der Meulen, 1980). In addition to diet preferences, palaeontological evidence from ungulate assemblages in the Pleistocene-Holocene transition indicate that reductions in open grassland habitat severely reduced the number *Equus zebra* (Faith, 2012). More recently, the conversion of land to agriculture and intensive grazing have also had a large impact on the availability of grassland habitat (Faith, 2012). Many studies have emphasised the importance of grass availability in the maintenance of stable Cape mountain zebra populations (Watson *et al.*, 2005; Watson & Chadwick, 2007; Smith *et al.*, 2011; Weel *et al.*, 2015; Lea *et al.*, 2016). Cape mountain zebra have been introduced into several arid habitats with limited grass, and these populations have shown limited growth (Hrabar & Kerley, 2015; Lea *et al.*, 2016).

Across the *Equus* genus, social structure varies with ecological conditions (Jarman, 1974; Rubenstein, 1994; Janson & Goldsmith, 1995) from tight family groups to large fission-fusion aggregations. Cape mountain zebra most commonly occur in discrete, independent family groups, where one male monopolises breeding, or in less stable bachelor groups that are mainly comprised of adult and sub-adult males (Fig. A1; Penzhorn, 1984; Novellie, 2008). Given that male harassment impacts on group structure and female reproduction in equids (Rubenstein, 1994; Sundaresan, Fischhoff & Rubenstein, 2007b), the prevalence of bachelor groups (and thus the demographic composition of the population) may play a pivotal role in the overall social structure of populations. Variation in habitat quality across the current Cape mountain zebra range is also reflected in population performance: population growth, density and female fecundity (the foal to mare ratio, foal:mare) are significantly lower in reserves with a lower availability grassland habitat (Lea *et al.*, 2016). Furthermore, some Cape mountain zebra populations show characteristics associated with Allee effects. Population density is positively associated with population growth rates and female fecundity, and adult sex ratio varies more in small populations (Lea *et al.*, 2016).

The Cape mountain zebra meta-population is distributed across a diverse landscape that differs in habitat quality, providing a novel opportunity to investigate how habitat quality influences population structure. Here, we assess whether population-level association patterns are influenced by habitat quality and demography across 10 independent Cape mountain zebra populations. We predict that structuring may collapse at ecological extremes and this can reflect the limits of behavioural resilience for a species. We also investigate whether the demographic composition (adult sex ratio) of the population is related to social network structure; male biased sex ratios could lead to more, smaller social units (Ohsawa &

Dunbar, 1984) or to increased instability. While resource availability has been shown to affect sociality in animals (Henzi *et al.*, 2009; Beck *et al.*, 2012; Foster *et al.*, 2012), to our knowledge this is the first study to take a macroecological approach to measuring social network structure in a large number of independent populations. Importantly, understanding how variation in habitat quality is associated with social network structure and demography may provide insight in to the mechanisms underpinning decline in marginal populations.

Methods

Data collection and group recognition

We collected data between 2010 and 2015 throughout daylight hours and carried out a total of 120 days of sampling. Ten focal populations were used for this study; Bakkrans Nature Reserve, Camdeboo National Park, De Hoop Nature Reserve, Gamkaberg Nature Reserve, Karoo National Park, Mount Camdeboo Private Game Reserve, Mountain Zebra National Park, Samara Private Game Reserve, Swartberg Private Game Lodge, and Welgevonden Game Farm (Fig. 1). In comparison to typical social network studies, the sampling effort per population was lower in the current study, with an average of 12 sampling days for each population. However, we argue that the data gathered for assessing general features of population-level association patterns is adequate for three reasons. First, previous work has shown that Cape mountain zebra individuals show a high tie fidelity within social groups with individual associations remaining stable for several years (Penzhorn, 1984). Therefore, fewer repeated encounters were required to elucidate an individual's position within a social network. Second, across study populations, generally a high proportion of the population was sampled within a sampling period (see Table A3.3 for details). Third, we evaluate the repeatability of the network metrics to determine their reliability.

For each zebra encountered, we recorded both their GPS location and age-sex classification, and took photos. Photographs were then used to identify individual animals using HotSpotter^{TM} pattern species instance recognition software (Crall *et al.*, 2013). We used the individual identification and age-sex data to compile a 'snapshot' of the population

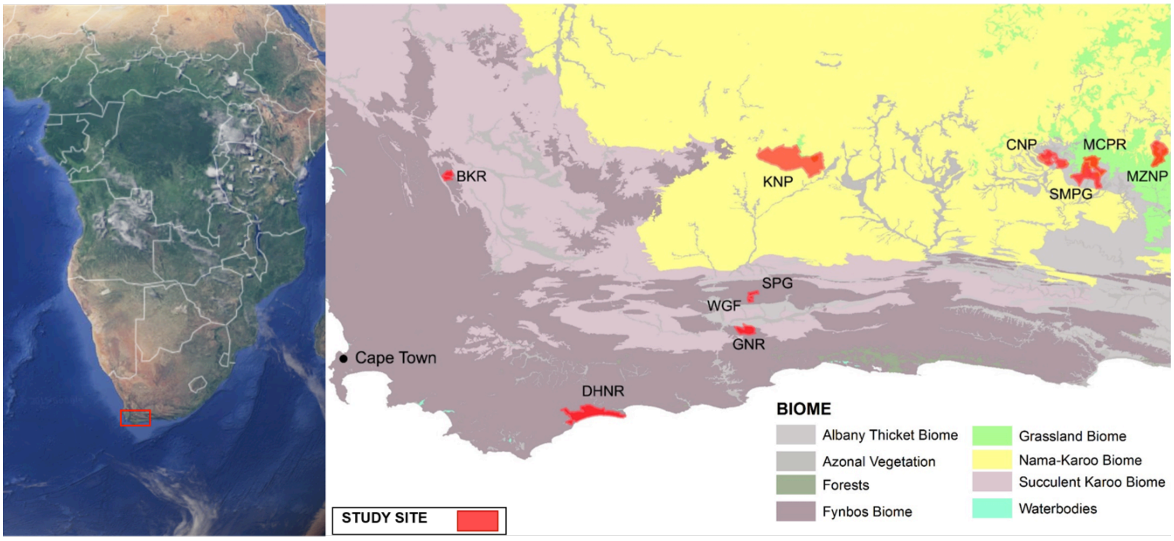

Figure A3.1. Focal Cape mountain zebra sub-populations in the Eastern and Western Cape provinces of South Africa and the underlying biome in each focal population. Vegetation data gathered from Mucina and Rutherford (2006). Reserves abbreviated as follows: Bakkrans Nature Reserve (BKR), Camdeboo National Park (CNP), De Hoop Nature Reserve (DHNR), Gamkaberg Nature Reserve (GNR), Karoo National Park (KNP), Mount Camdeboo Private Game Reserve (MCPR), Mountain Zebra National Park (MZNP), Samara Private Game Reserve (SMPG), Swartberg Private Game Lodge (SPG) and Welgevonden Game Farm (WGF).

demography for the sampling period. Following the assumptions of the ‘gambit of the group’, individuals were considered to be associated if they were observed in a group during an encounter (Croft *et al.*, 2008; Franks, Ruxton & James, 2010). Individuals were assumed to be members of a group when they were within ~50 m of other zebra and moved together as a cohesive unit. Individuals, especially stallions, from separate groups occasionally interacted (including both aggressive and passive encounters); where individuals separated from their social group and interacted we recorded an additional association between the interacting dyad/s only. In addition to association patterns, we extracted all group sizes as well as the population mean group size in our analyses. Surveys conducted at Samara Private Game Reserve during 2010 and 2011 did not incorporate a photographic ID database. Without photographic IDs there is an increased probability that there was pseudo-replication in individual encounters. However, we think this is unlikely for the following reasons: i) individuals normally display high within-group fidelity (Penzhorn, 1984) and so are unlikely to leave and join other groups readily, and ii) groups would have to travel several kilometres unseen across a mostly open plateau area in order to be recounted in a single sampling day.

Ecological variable extraction

Data on the availability of palatable grass species within reserves was taken from Lea *et al*. (2016), where the percentage cover of distinct vegetation types within each reserve was calculated by overlaying reserve boundaries on the National Vegetation Map of South Africa, Lesotho and Swaziland, compiled by Mucina & Rutherford, (2006). Each vegetation type/community has an associated list of common and dominant species. A standardised palatable 'grassiness' index for each vegetation type found in a reserve was estimated by totalling the number of Graminoid species, with dominant species weighted by a factor of two, and ranking each grass species for its palatability (high, medium or low). For each reserve, the grass abundance scores were multiplied by the percentage area cover of that vegetation type and summed. These scores were then standardised between zero and one by dividing by the maximum value across reserves, such that zero represents very little palatable grass and one represents a high coverage of grass rich vegetation communities.

Social network construction

The association data from individuals within and between groups were used to construct matrices, from which binary social networks were derived. Nodes within each of these networks are individual zebra, and ties are associations between those individuals. Data from multiple sampling days were grouped by visit and were used to construct an association network. Networks were only retained for subsequent analyses if they met the following criteria: i) there were at least three sampling days (range 3-9) within each sampling period for a particular population, and ii) there were at least 15 individuals identified in each sampling period. A total of 22 association networks were used in subsequent analyses. Three networks were constructed for Camdeboo National Park (Feb 2013, Feb 2015, Oct

2015), Gamkaberg Nature Reserve (Nov 2013, Mar 2015, Oct 2015), Mount Camdeboo Private Game Reserve (Feb 2013, Feb 2015, Nov 2015) and Mountain Zebra National Park (Dec 2012, Jan 2013, May 2014), one for Karoo National Park (Feb 2013) and Welgevonden Game Farm (Apr 2015), and two for each of the remaining populations.

Social network analysis

We used the *igraph* package in R (Csardi and Nepusz 2006, R Core Team 2014) to calculate network metrics (shown in Table A3.4). Network comparisons were initially made using seven standard whole-network metrics (density, reach 2, mean degree, transitivity, eigenvector centrality, community size and modularity), which were reduced to two components using a principle component analysis (PCA, for details see Appendix 3.2). However, the score of principal component one was significantly associated with network size, highlighting the need for a size-independent approach. Size-independent comparisons between association networks were made using a simplified version of the NetSimile approach (Berlingerio *et al.*, 2012). Three undirected network metrics (degree, node–wise clustering coefficient, and the mean clustering coefficient of each nodes' ties) used in the NetSimile approach were calculated for each node and summary statistics were calculated for each metric across the network (Table A3.1). Metrics were dropped if they showed a lack of variation across populations. The means and moments of each metric were collated as a 'signature' vector for each network, which could be compared to the other networks (Berlingerio *et al.*, 2012).

Table A3.1. Network metrics and summary statistics used to compare 10 independent sub-populations of Cape mountain zebra. Metrics were calculated for each network at the level of each node, i.e all individuals.

Network Metric	Description	Summary Statistics
Degree	The number of ties of node *i*	mean, median, standard deviation, skewness, kurtosis
Clustering coefficient	The local, undirected transitivity for node *i*	mean, median, standard deviation
Average clustering coefficient of each nodes ties	For the local network of node *i,* this metric describes the average local, undirected transitivity for all nodes in the local network (excluding node *i*).	mean, median, standard deviation

The metrics within each signature vector were not independent, and so the dimensions of the signature vectors were again reduced using a PCA with a varimax rotation using the *psych* package (Croft *et al*., 2008; Revelle, 2011; R Core Team 2014). The components retained for further analysis were those with eigenvalues greater than one (Jackson, 1993). The Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy (MSA) was used to test whether signature vector features were suitable for a PCA (Kaiser, 1970; Revelle, 2011).

To verify whether the signature vectors used in this analysis were independent of network size, the principal component scores were regressed against network size using univariate linear mixed effects models in the *nlme* package, with a single random effect of reserve (Pinheiro *et al.*, 2014).

The availability of palatable grass species is affected by annual climatic variation and there was reduced sampling over the study period with respect to other social network analyses, highlighting the need to assess the sampling effort and the influence of season on measures of population structure. Therefore, to investigate whether the sampling effort was sufficient to extract robust features of population structure and to test the effect of seasonality, we performed repeatability tests on principal component scores and mean group size for all reserves with multiple networks (eight reserves) using the *rptR* package of R (Schielzeth, Stoffel & Nakagawa, 2017). The grouping variable used for repeatability tests was reserve, and tests were carried out without the addition of fixed effects. We assessed the uncertainty of repeatability measures using 1000 parametric bootstrapping iterations and assessed the significance of repeatability using 1000 re-sampling permutations (Schielzeth *et al.*, 2017).

To examine the effect of habitat quality and demography on social network structure, we analysed the principal component scores against grassiness and adult sex ratio (male:female) using linear mixed effects models with a single random effect of reserve, implemented using the *nlme* package (Pinheiro *et al.*, 2014). With 22 network features, we examined both univariate and multi-variate models to evaluate evidence for independent effects of demography and environment on social structure. We used AIC model selection to determine the best-fit model from our predictors (Burnham & Anderson, 2002; Johnson & Omland, 2004). For each possible model, we report the AIC value, the change in AIC from the best fit model and the model weight. The model weight is calculated as model likelihood, exp(-0.5*ΔAIC), divided by the sum of likelihoods over all models (Burnham & Anderson, 2004). We report model summary statistics to demonstrate the relative predictive power of each predictor. Parameter weights are calculated by summing the weights over all models containing each parameter. Finally, we estimate marginal r^2 for the fixed effects of all linear mixed effects models using the *r.squaredGLMM* function in package *MuMIn* (Barton, 2013).

Finally, we tested whether the availability of palatable grass affected the demographic structure of populations; namely overall network size, the adult sex ratio, mean group size within networks and the size of all observed groups within networks (both overall and family groups). The relationship between all group sizes within networks and other structural and grass availability was tested using generalised linear mixed effects models (GLMMs) with Poisson distributions and a random effect of reserve in the *lme4* package (Bates, Maechler & Bolker, 2012). Mean group sizes were analysed with respect to habitat availability using linear mixed effects models with a single random effect of reserve (Pinheiro *et al.*, 2014), and all other demographic parameters were regressed against grassiness using univariate linear models.

Results

Two metrics of social network structure

A total of 1368 zebra were present in all networks between 2010 and 2015 and the size of networks ranged from 17 to 269 individuals. The final PCA with eight network metric summary statistics yielded a two-component solution that explained 85% of the variation in the subset (Table A3.7; Fig. A3.5), with a KMO MSA score of 0.63 (Table A3.8), indicating that the signature features obtained were suitable for a PCA. These two components were used in all subsequent analyses of network structure, demography and ecology (summary of population measures given in Table A3.3).

For principal component one, the mean clustering coefficient of each node and the mean and median of the average clustering coefficient of each node's ties loaded positively and the standard deviation of both clustering coefficient measures loaded negatively (Table A3.7). In subsequent analyses, we term principal component one '*network clustering*', such that populations with a high network clustering score have more discrete family harem and bachelor groups, with less variable structure across groups than populations with low clustering (Fig. A3.3). For principal component two, the mean node-wise degree, the standard deviation of node-wise degree and the skewness of node-wise degree all loaded positively (Table A3.7), and we term this component *group variation.* Populations with high *group variation* scores have both larger and more variable group sizes; mean group size across sampling periods was correlated strongly with group variation ($\beta = 1.00 \pm 0.24$, $t_{11,11} = 4.09$, $p = 0.002$). Moreover, the population *group variation* score is associated with all observed group sizes ($n = 324$, $\beta = 0.10 \pm 0.03$, $z = 3.13$, $p = 0.002$, marginal $r^2 = 0.04$). In contrast, *network clustering* was less strongly associated with both mean ($\beta = 0.69 \pm 0.32$, $t_{11,11} = 2.16$, $p = 0.05$) and observed group size ($n = 324$, $\beta = 0.08 \pm 0.05$, $z = 1.72$, $p = 0.09$, marginal $r^2 = 0.032$). The weaker relationship between *network clustering* and both group size measures suggests that *network clustering* captures additional variation in population structure.

The comparison of social network structure using a NetSimile approach was size-independent, and there was no significant correlation between network size and either *network clustering* ($\beta = 0.002 \pm 0.006$, $t_{8,12} = 0.33$, $p = 0.75$; Fig. A3.6A) or *group variation* ($\beta = 0.006 \pm 0.004$, $t_{8,12} = 1.50$, $p = 0.17$; Fig. A3.6B). Importantly, however, groups were significantly larger in larger networks ($n = 324$, $\beta = 0.001 \pm 4 \times 10^{-4}$, $z = 3.45$, $p < 0.001$, marginal $r^2 = 0.04$), highlighting the utility of assessing population structure independently of group or network size.

Table A3.2. The relationship between adult sex ratio, habitat quality and two measures of population structure across 10 Cape mountain zebra populations.

	Model	Network score	Coefficient	r^2	AIC	ΔAIC	Model weight	t	p
Network clustering	*1*	*Quadratic*	0.51 ± 0.89	0.60	51.66	-	0.64	0.57	0.58
		grassiness +	-2.05 ± 0.77					-2.66	0.02
		Adult sex ratio	-0.92 ± 0.43					-2.11	0.07
	2	*Grassiness*	1.81 ± 0.83	0.51	53.59	1.97	0.24	2.18	0.05
		quadratic	-2.91 ± 0.81					-3.57	0.005
	3	*Adult sex ratio*	-1.49 ± 0.38	0.49	56.15	4.49	0.07	-3.93	0.004
	4	*Grassiness +*	-0.08 ± 0.75	0.47	56.94	5.28	0.05	-0.11	0.91
		Adult sex ratio	-1.53 ± 0.50					-3.04	0.02
	5	*Grassiness*	1.36 ± 0.85	0.18	62.32	10.66	0.003	1.61	0.15
Mean group size	*1*	*Grassiness*	1.61 ± 0.71	0.49	33.37	-	0.52	2.24	0.05
		quadratic	-1.65 ± 0.68					-2.44	0.03
	2	*Quadratic*	1.19 ± 0.97	0.47	34.59	1.29	0.27	1.22	0.24
		grassiness +	-1.32 ± 0.87					-1.53	0.16
		Adult sex ratio	-0.32 ± 0.48					-0.32	0.53
	3	*Adult sex ratio*	-0.93 ± 0.33	0.40	36.69	3.32	0.10	-2.83	0.02
	4	*Grassiness +*	0.45± 0.64	0.41	37.27	3.90	0.07	0.71	0.50
		Adult sex ratio	-0.75 ± 0.87					-1.77	0.12
	5	*Grassiness*	1.13 ± 0.57	0.27	38.32	4.95	0.04	1.98	0.08

We calculated the repeatability of population structure measures to assess whether sampling was adequate for each reserve and whether there was an effect of seasonality. *Network clustering* was highly repeatable across the eight reserves with multiple networks, suggesting that patterns of individual connectivity were consistent at the population level (*R* = 0.69, 2.5% = 0.15, 97.5% = 0.89, permutation p <0.01). Although *group variation* displayed lower repeatability across reserves (*R* = 0.31, 2.5% = 0, 97.5% = 0.70, permutation p = 0.13), mean group sizes across reserves were also highly repeatable (*R* = 0.81, 2.5% = 0.43, 97.5% = 0.94, permutation p = 0.001), suggesting that *network clustering* and group size reliably characterise population structure independently of sampling season. Thus, we take these two measures forward to evaluate structural variation between populations.

Habitat quality, demography and social network structure

In order to better understand how habitat quality and demography impact on population structure, we compared our two structural measures (group size and network clustering) with grassiness and adult sex ratio (male:female). Adult sex ratio exhibited a strong negative association with *network clustering* (Table A3.2; Fig. A3.2A), but was also related to mean group size (Table A3.2), meaning that male biased sex ratios were associated with poorly clustered networks and small groups. Furthermore, adult sex ratio was negatively associated with grassiness ($F_{1,8}$ = 4.47, p = 0.07; Fig. A3.2B). There is a strong suggestion of a negative quadratic relationship between grassiness and both group size and group variation (Table A3.2; Figs A3.2C, A3.2D). The best performing model for mean group size only included the quadratic grassiness term, whereas the (marginally) best performing model for network clustering included both the quadratic term for grass availability and adult sex ratio. This is

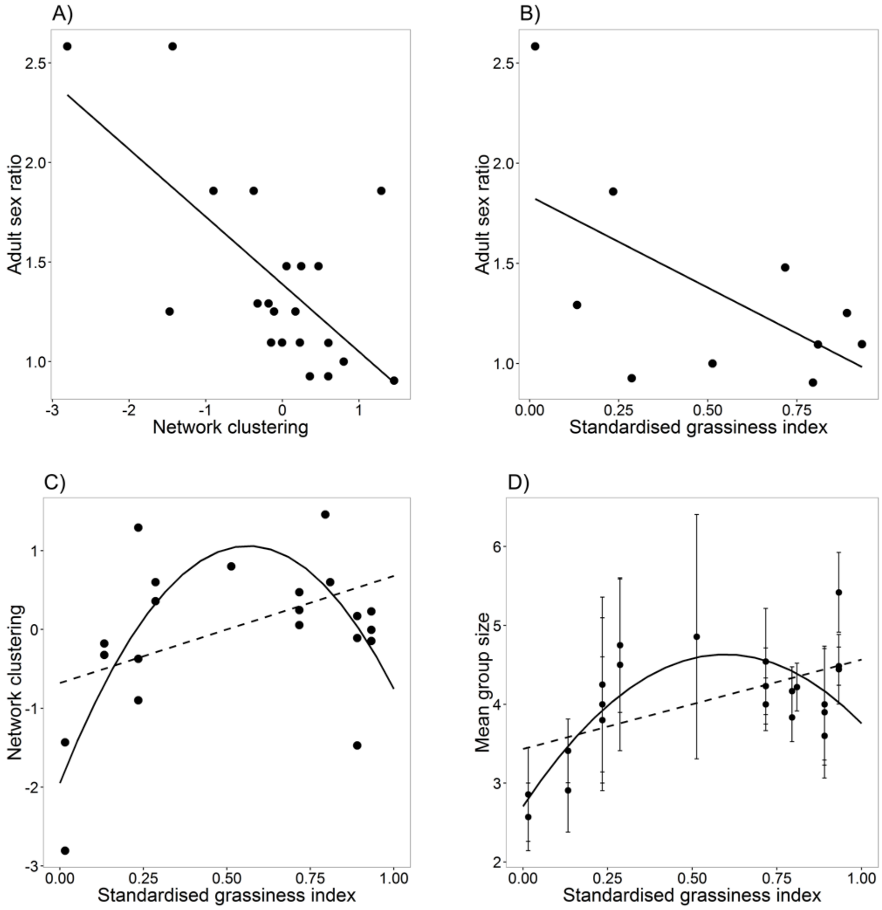

Figure A3.2. Demography and social network structure are driven by habitat quality. A) The correlation between *network clustering* and the adult sex ratio (ratio of males to females) of each reserve. B) Grassiness was associated with the adult sex ratio of each reserve. C) The relationship between grassiness and *network clustering*. D) The mean group size in each network was also associated with grassiness.

supported by high parameter weights for both adult sex ratio (w= 0.71) and quadratic grassiness (w= 0.88) for *network clustering*, but less support for adult sex ratio (w = 0.37) than quadratic grassiness (w= 0.79) for mean group size. Linear grassiness had little support from either measure of population structure (*network clustering* w = 0.05; mean group size w = 0.12). Together, these results suggest that in low quality habitats with less palatable grass, there is a greater ratio of males to females, and this is associated with smaller, more variable groups and lower network connectivity.

Discussion

Here, we develop a framework to document responses in social structure to the environment across a species' range using size-independent, repeatable features of social network structure at the population level. Both population-level social structure and demography were responsive to ecological gradients in the Cape mountain zebra, as low quality habitats were associated with smaller group sizes, a male-biased sex ratio, and lower *network clustering.* Moreover, our results suggest that *network clustering* and mean group size interact quadratically with habitat availability, which may also indicate that population structure is the most stable in intermediate habitats. The specific causes of demographic imbalance and loss of structuring in marginal habitats is unknown; male biased secondary sex ratios or higher female mortality could equally result in demographic imbalance (Lea et al. *in press*, Smith et al. 2008). Because clustering is strongly associated with adult sex ratio, we argue that a high proportion of males in the population can disrupt social structure through repeated, sometimes aggressive, harassment of family groups. Increased aggression and harassment in male-biased populations has previously been linked with social instability (Greives *et al.*, 2007). The impact of bachelor harassment on group and population stability has also been suggested in other equids, and bachelor disturbance and aggression can lead to reproductive failure (Rubenstein, 1994; Sundaresan *et al.*, 2007b). Furthermore, male faecal androgens, which may be associated with increased competition and aggression, are elevated in male-biased Cape mountain zebra populations (Lea et al. *in press*). Thus, a high proportion of males and bachelors in the population may destabilise network structure through aggression, by harassing and breaking down cohesive family groups.

The current results suggest that both sex ratio imbalance and habitat quality (and their interaction) impact on population structure, and this may provide additional insight into Allee effects in wild populations. Small or low-density populations typically exhibit poor population growth and heightened extinction risk (Dennis, 1989, 2002; Courchamp, Clutton-Brock & Grenfell, 1999; Boukal & Berec, 2002; Allen *et al.*, 2005). The potential causes of inverse density dependence in population growth are well documented, particularly in experimental systems, and include demographic stochasticity and genetic inbreeding or loss of heterozygosity (Courchamp *et al.*, 1999). For example, demographic imbalance such as an increase in the ratio of males to females was shown to result in an Allee effect in lizards, most likely through the increase in intraspecific aggression directed towards females by males (Le Galliard *et al.*, 2005). Concurrently, we propose that habitat marginality and subsequent demographic imbalance in poorly performing populations of Cape mountain zebra may cause Allee effects, and this impacts upon social structure, namely population connectivity. Demographic imbalance can be detrimental for poorly performing populations (Le Galliard *et al.*, 2005), and the breakdown of population connectivity may be a crucial indicator of population resilience (Foster *et al.*, 2012).

Habitat quality is associated independently with both social network structure and population performance in Cape mountain zebra (Lea *et al.*, 2016), but identifying the key driver of poor population performance was not possible in this relatively small subset of populations. However, reductions in connectivity may intensify the ecological and demographic pressures faced by declining populations. To fully understand the relationship between demography, population structure and viability, we would need evidence of network turnover and temporal variation from a wider range of populations with population performance data.

Few studies have used social networks to characterise population-level responses to ecological variation. Long term association data from Pacific orca, *Orcinus orca,* revealed that the abundance of a primary food source, Chinook salmon, *Oncorhynchus tshawytscha,* was significantly correlated with overall network connectivity (Foster *et al.*, 2012). Increased clustering coefficients, degree centrality and mean half weight indices were associated with higher salmon abundance (Foster *et al.*, 2012). Studies of cetaceans and primates have also related variation in the environment to social network structure (Henzi *et al.*, 2009; Beck *et al.*, 2012), but to our knowledge no other study has compared social network structure in a large number of independent populations of the same species. We show here an apparent gradient of social structure across habitats: at intermediate habitat quality networks are highly clustered, whereas at very low and high quality there is relatively lower clustering. The inflection point in *network clustering* suggests that there may be an optimum or maximum structuring for Cape mountain zebra populations. Our interpretation of this is that at low density or habitat quality, groups are smaller and less stable, with more fission-fusion structure. Conversely in high quality habitats, there is higher density and more between-group interactions, which results in lower *network clustering* scores.

Our results have several implications for conservation and population management. First, although there has long been a call for conservation action to be informed by animal behaviour (Geist & Walther, 1974; Harcourt, 1999; Lindell, 2008), the two disciplines are often still not integrated (Angeloni *et al.*, 2008). These results demonstrate that social network structure can be used as a tool in conjunction with demographics to help estimate population viability and guide conservation. For the case of the Cape mountain zebra, there is consensus between paleontological evidence (Faith, 2012), population performance measures (Lea *et al.*, 2016), physiological responses (Lea et al. *in review*) and now population structure, and successful strategies should utilise this evidence together to guide management. Second, the potential impact of differential harvesting, off-takes and translocation strategies must carefully consider the potential impact on source population structure to ensure that population viability is maintained. For example, leaving or creating biased sex-ratios may undermine population health and slow recovery rates.

At a global scale, natural habitats are increasingly fragmented and disturbed. Remnant populations are often small, isolated and potentially restricted to marginal habitats. Behavioural indicators are important for maintaining populations in these ‘islands of habitat’ (Buchholz, 2007). Whilst species with greater flexibility in behaviour and social structure may be more resilient to anthropogenic pressures in the short-term, altered behaviours could result in long-term reductions in population viability (Manor & Saltz, 2003; Berger-Tal *et al.*, 2011), which could affect the wider ecosystem (Wright *et al.*, 2010). Therefore, understanding how anthropogenic pressures and habitat marginality influence behaviour and sociality can be crucial for conservation management (Lindell, 2008). However, human impacts are often so great that the normal behaviour of animals is completely disrupted (Rubenstein, 2010). In addition to managing species in order to conserve the normal range of behaviours, we may also be able to use behavioural ‘norms’ as indicators of vulnerability or as indicators of population responses to management interventions.

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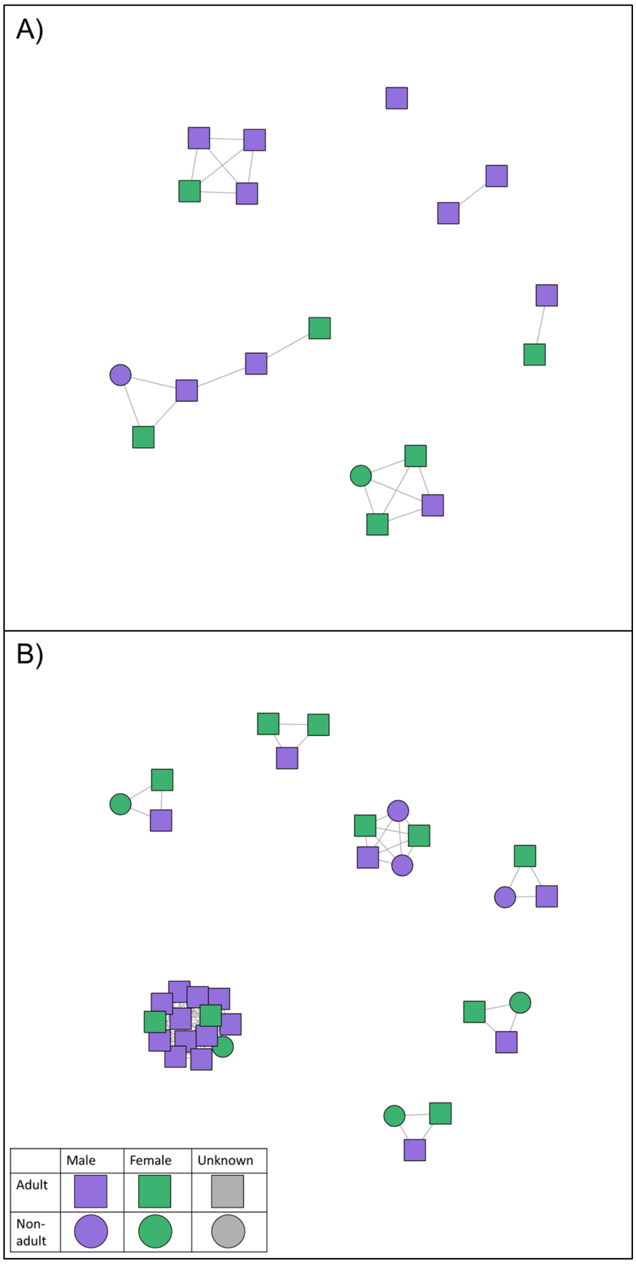

Figure A3.3. Two examples of the social network structure of Cape mountain zebra from A) Bakkrans Nature Reserve, September 2015, and B) Welgevonden Game Farm, April 2015. The colour and shape of each node denotes the age-sex classification of each individual within the network. Unknown individuals were primarily foals and young sub-adults. A) represents the population with the lowest *network clustering* score, exhibiting inter-group interactions. B) represents the population with the highest *network clustering* score, such that the population is comprised of discrete groups.

Table A3.3. Metrics describing 22 social networks of Cape mountain zebra across 10 reserves. Data on the size of each population in 2014/15 taken from Lea et al. (2016).

Reserve	Network	Rainfall Season	Standardised Grassiness Index	Adult Sex Ratio (Male: Female)	Network clustering	Group variation	Mean Group Size	Average number of repeated observations per individual	Mean network size	2014/15 Population Size	Proportion of the population sighted
Bakkrans Nature Reserve	May 2015	Dry	0.02	2.58	-1.57	-1.14	2.86	2.57	19	40	0.48
	September 2015	Wet			-2.97	-1.55	2.57	1.43			
Camdeboo National Park	February 2013	Wet	0.72	1.48	0.45	-0.03	4.23	1.38	63.5	236	0.27
	January 2015	Wet			0.43	2.23	4.54	1.88			
	October 2015	Dry			0.12	-0.71	4.00	1.61			
De Hoop Nature Reserve	February 2013	Dry	0.13	1.29	-0.45	-0.80	2.91	1.91	53.5	100	0.54
	September 2015	Wet			-0.28	-0.62	3.41	1.73			
Gamkaberg Nature Reserve	November 2013	Year-round	0.23	1.86	-0.41	-0.30	4.25	1.25	20	42	0.48
	March 2015	Year-round			1.21	-0.24	3.80	2.00			
	October 2015	Year-round			-0.78	0.51	4.00	2.50			
Karoo National Park	February 2013	Wet	0.81	1.09	0.64	0.33	4.22	1.03	135	843	0.16
Mount Camdeboo Private Reserve	February 2013	Wet	0.89	1.25	-0.18	-0.47	4.00	1.50	35.7	71	0.50
	February 2015	Wet			0.07	-0.57	3.60	3.30			
	October 2015	Dry			-1.25	1.02	3.90	2.90			
Mountain Zebra National Park	December 2012	Wet	0.93	1.10	0.24	1.42	5.42	1.03	185.7	1191	0.16
	February 2013	Wet			0.24	0.12	4.48	1.17			
	May 2014	Dry			-0.04	0.62	4.44	1.00			
Samara Private Game Reserve	September 2010	Dry	0.79	0.90	1.22	-1.09	3.83		24	26	0.92
	April 2011	Dry			1.20	-1.22	4.17				
Swartberg Private Game Reserve	April 2015	Year-round	0.29	0.93	0.40	0.34	4.50	3.83	23	29	0.79
	November 2015	Year-round			0.63	0.29	4.75	3.50			
Welgevonden Game Farm	April 2015	Year-round	0.51	1.00	1.09	1.86	4.86	2.57	34	34	1.00

Appendix 3.1. Principal component analysis on seven standard social network metrics characterising ten Cape mountain zebra populations.

The association networks were characterised using seven un-weighted network metrics (Csardi & Nepusz, 2006; Croft, James & Krause, 2008; Table A3.4). As these un-weighted network metrics were not independent, a principal component analysis (PCA) with an oblimin rotation, allowing factors not to be orthogonal, was carried out using the *psych* package of R, retaining only components with eigenvalues greater than one (Table A3.2; Fig A3.1; (Jackson, 1993; Croft, James & Krause 2008; Revelle, 2011; R Development Core Team, 2016). Principal component scores were assessed for size-independence against network size using the Pearson's product moment correlation coefficient and principal component one was significantly correlated with network size ($R = -0.72$, $p = 0.019$). This limited the ecological inferences that could be made using a standard network approach and highlighted the need for a size-independent approach.

Table A3.4. Seven un-weighted network metrics used to compare 10 independent sub-populations of Cape mountain zebra. Metrics adapted from Croft *et al.,* 2008 and Csardi & Nepusz, 2006.

Network metric	Description
Density	The ratio between the number of completed edges within a graph and the number of potential edges within the graph.
Reach 2	The mean proportion of a graph that is within a neighborhood of two for node *i*.
Mean Degree	The mean number of edges across all nodes within a graph
Transitivity	The ratio of node triangles and connected node triples within a graph i.e. the graph level probability of adjacent nodes that are connected to node *i* being connected.
Eigenvector centralisation	The graph level eigenvector centrality index.
Mean community size	The Mean size of communities within a network detected using a short random walk framework.
Modularity	The graph level modularity of the communities established using a short random walk framework i.e. the cohesiveness of modules within the graph.

Table A3.5. Standardised loadings from a principal component analysis of a subset of seven network metrics from ten Cape mountain zebra populations.

	Standardised loading	
Factor	**PC1**	**PC2**
Density	1.00	-0.09
Reach_2	1.00	-0.17
Mean Degree	0.22	0.91
Eigenvector Centralization	-0.94	-0.20
Mean community size	-0.16	0.98
Modularity	-0.96	-0.13
Eigen value	**3.90**	**1.89**
Proportion of variation	**0.65**	**0.31**

Appendix 3.2. Principal component analysis for Cape mountain zebra signature vectors

After the extraction of metrics calculated for all nodes within each network, the median node-wise clustering coefficient was excluded from the principal component analysis as there was no variation across parks (all values of 1). The initial principal component analysis yielded a three-component solution, which explained 84% of the variation in the 10 factors of the 22 signature vectors. However, the proportion of variation explained by the third component (16% variation explained) was far less than components one and two (68% cumulative variation explained; Table A3.6; Figure A3.4). Furthermore, the variables loading on to the third component, median node-wise degree and kurtosis of node-wise degree, had sampling adequacy scores below 0.5 (0.33 and 0.20, respectively), and so these variables were removed from subsequent analyses. The second and final principal component analysis on eight factors from 22 signature vectors yielded a two-component solution, which explained 85% of the variation of in the subset (Table A3.7, Fig A3.5).

Signature feature abbreviations

Network metric	
D	node-wise degree
T	node-wise clustering coefficient
aT	average clustering coefficient of each nodes ties

Summary statistic	
mn	mean
md	median
sd	standard deviation
sk	skewness
k	kurtosis

Table A3.6. Standardised loadings from a principal component analysis of 10 signature vector features from 22 Cape mountain zebra social networks. Factors and loadings highlighted in red are those excluded from subsequent analyses.

	Standardised loadings		
Factor	PC1	PC2	PC3
Dmn	0.23	0.92	0.19
Dmd	0.07	0.32	0.73
Dsd	0.00	0.98	-0.03
Dsk	0.31	0.68	0.23
Dk	-0.05	-0.01	0.82
Tmn	0.96	0.18	0.17
Tsd	-0.94	-0.11	0.21
aTmn	0.96	0.19	0.15
aTmd	0.74	0.18	0.39
aTsd	-0.92	-0.10	0.24
Eigen value	**4.28**	**2.50**	**1.60**
Proportion of variation	**0.43**	**0.25**	**0.16**

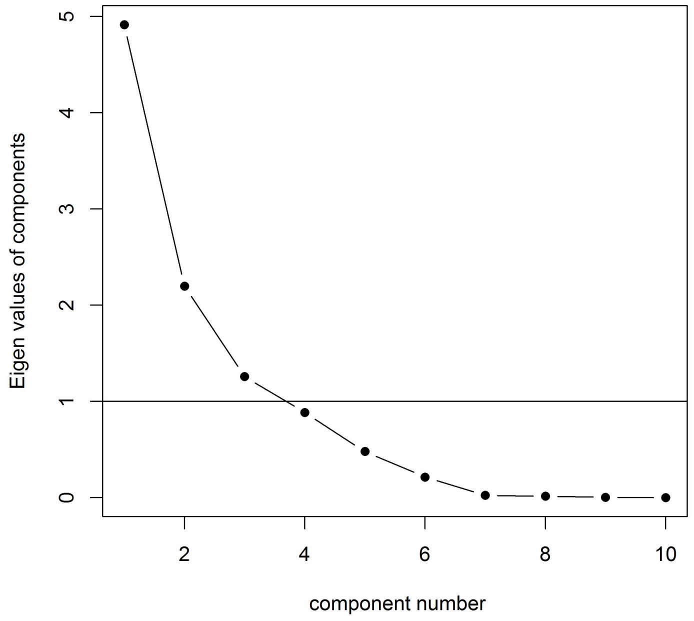

Figure A3.4. Scree plot of the change in Eigen values against the increase in the number of components for a principal component analysis of 10 network feature statistics characterising Cape mountain zebra populations.

Table A3.7. Standardised loadings from a principal component analysis of a subset of eight signature vector features from 22 Cape mountain zebra social networks.

Factor	**Standardised loading**	
	PC1	**PC2**
Dmn	0.22	0.92
Dsd	-0.02	0.96
Dsk	0.29	0.76
Tmn	0.96	0.22
Tsd	-0.93	-0.07
aTmn	0.96	0.23
aTmd	0.74	0.28
aTsd	-0.92	-0.06
Eigen value	**4.32**	**2.53**
Proportion of variation	**0.53**	**0.32**

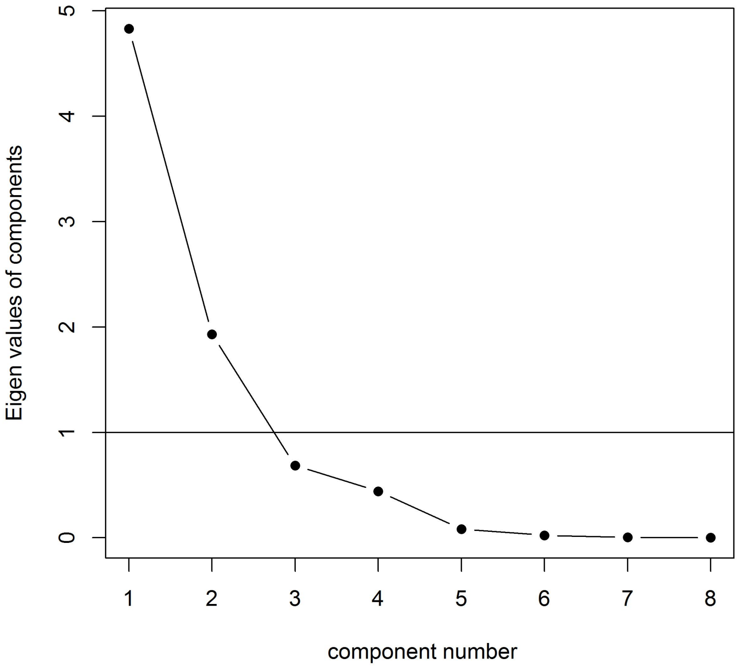

Figure A3.5. Scree plot of the change in Eigen values against the increase in the number of components for a principal component analysis of eight network feature statistics characterising Cape mountain zebra populations.

Table A3.8. Kaiser-Meyer-Olkin (KMO) measures of sampling adequacy (MSA) for a subset of eight signature vector features from 22 Cape mountain zebra social networks. Overall KMO highlighted in bold.

Overall KMO **0.63**							
MSA for each factor							
Dmn	Dsd	Dsk	Tmn	Tsd	aTmn	aTmd	aTsd
0.60	0.50	0.83	0.63	0.63	0.69	0.69	0.58

Appendix 3.3. Supplementary information: Social structure, network size and habitat availability

Overall network size was not associated with grass availability ($F_{1,8} = 3.88$, $p = 0.08$), but groups within networks were significantly larger where there was more palatable grass available ($n = 324$, $\beta = 0.36 \pm 0.09$, $z = 3.82$, $p < 0.001$, marginal $r^2 = 0.05$). However, family groups were not significantly larger where more palatable grass was available, despite a positive trend ($n = 247$, $\beta = 0.27 \pm 0.15$, $z = 1.79$, $p = 0.07$, marginal $r^2 = 0.02$).

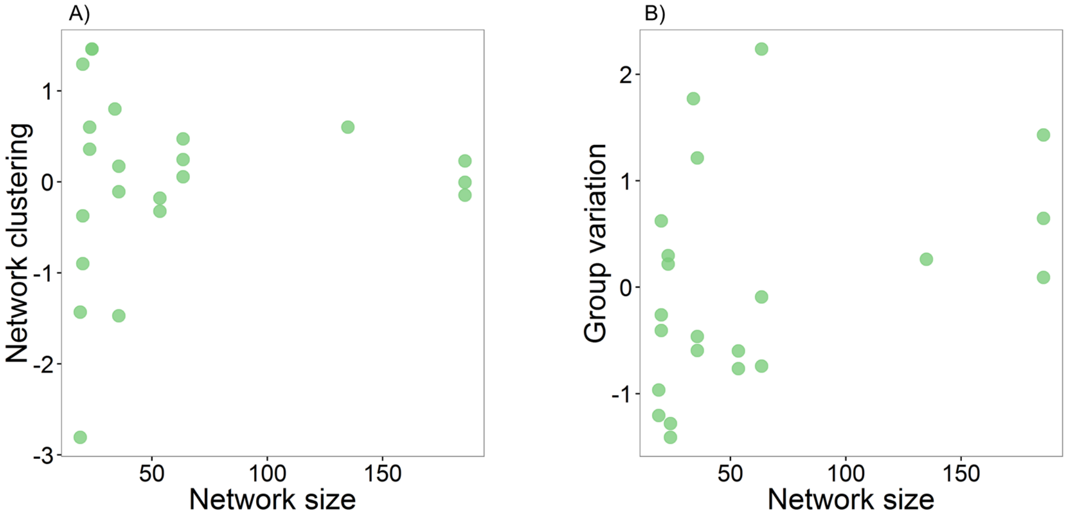

Figure A3.6. The effect of network size on two principal component scores characterising 22 Cape mountain zebra social networks. A) The correlation between network size and *network clustering*. B) The correlation between network size and *group variation*.

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