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Responses to potential captivity-induced stressors in captive-born cheetah (*Acinonyx jubatus*): implications for behavioural and physiological stress and gastrointestinal health

by

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Submitted in partial fulfilment of the requirements for the degree of

PhD (Veterinary Science)

Department of Paraclinical Sciences

Faculty of Veterinary Science

University of Pretoria

South Africa

January 2023

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ABSTRACT

Stress is an intrinsic part of nature. As such, animals have evolved a repertoire of behavioural and physiological strategies to adapt to environmental variation. However, a growing concern in wildlife conservation is the apparent tendency for threatened species to have a reduced ability to adapt to variable environmental conditions, predisposing them to the deleterious outcomes of chronic stress. This thesis assessed different methods for measuring behavioural and physiological responses to psychological stressors in threatened species using the cheetah model. I simulated scenarios depicting conditions considered to be potential psychological stressors for captive cheetahs, namely (i) a more naturalistic reduced feeding days schedule, (ii) the provision of environmental enrichment (EE), (iii) participation in animal-visitor interactions (AVI), and (iv) the close proximity to other large predators. Using emerging biologging technology and more traditional measures of stress-related behavioural and physiological responses, variations in body temperature (T_b), heart rate (HR), locomotor activity (LA), behaviour, faecal glucocorticoid metabolite concentration, and faecal consistency score were documented. The circadian rhythm of T_b, HR, and LA demonstrated an increase in all parameters at around 0700 hours (shortly after sunrise) and again at about 1600 hours (shortly before sunset) for T_b. In Chapter 2, the findings encourage a more naturalistic reduced feeding days schedule to mediate the unnatural composition of horsemeat-based diets routinely fed to cheetahs in captivity and as an effective EE strategy. Chapter 3's findings encourage the provision of EE to enhance the welfare of captive cheetahs, particularly cognitive, sensory, and nutritional enrichment, while cautioning against social enrichment as an effective EE strategy. Chapter 4 reiterates the ambiguous impact of AVIs on the animals involved, including whether they are positive, neutral, or negative. The findings in Chapter 5 highlight the importance of trepidation when imposing minimally harmful negative events not to be contrary to the welfare of cheetahs in captivity. The thesis provides a holistic assessment

of cheetahs' resilience to the captive setting using a multi-method approach and, potentially, a basis on which appropriate management and husbandry protocols may be developed for the species that better accommodate its requirements.

ACKNOWLEDGMENTS

I would like to gratefully acknowledge the following groups of people, without whom this thesis project would not have been possible:

First and foremost, I am thankful to my supervisor, Prof. Adrian S. W. Tordiffe, for his encouragement, words of wisdom, and perseverance in guiding me through. In addition, I express my sincere gratitude to Prof. Gerhard Steenkamp for his surgical expertise during the implantation of the data loggers and Prof. André Ganswindt for his knowledgeable guidance on faecal glucocorticoid metabolite analysis.

Narinda Beukes and the staff at the Cango Wildlife Ranch and Conservation Centre, Oudtshoorn, South Africa, for their cooperation during the experimental trials. Special thanks to Craig Gouws, Tamblyn Williams, and the rest of the cheetah caretakers for their constant support of the research and assistance during the sample and data collection.

Dr Glen A. P. Carlisle for his tenured experience in the management and veterinary treatment of the study cheetahs.

Leo Ragazzo (PhD candidate, Cornell University College of Veterinary Medicine, Ithaca, NY, US) for his assistance in behavioural observations and faecal sample collection.

Dr Nicole Hagenah and the technicians at the Endocrine Research Laboratory, University of Pretoria, South Africa, for analysing steroid concentrations.

Finally, thanks to my family and partner, who have helped me reach this point in my academic career.

Financial support for this research was kindly given by the University of Pretoria Doctoral Research Bursary; the National Research Foundation, South Africa (reference number: SFH170622243855 and MND200714543782); and the Kevin Richardson Foundation.

DECLARATION

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Signature of student: 

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Date: 2023/01/31

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

GLOSSARY OF ACRONYMS/ABBREVIATIONS

α	Alpha
ACTH	Adrenocorticotropic hormone
ANS	Autonomic nervous system
AVI	Animal visitor interaction
AVP	Arginine vasopressin
bpm	Beats per minute
CNS	Central nervous system
CRH	Corticotropin-releasing hormone
CV	Coefficients of variation
DW	Dry weight
E	Epinephrine
ECG	Electrocardiogram
EE	Environmental enrichment
EIA	Enzyme immunoassay
fGCM	Faecal glucocorticoid metabolite
FCS	Faecal consistency score
GC	Glucocorticoid
GH	Growth hormone
GI	Gastrointestinal
GR	Glucocorticoid receptor
HPA	Hypothalamic-pituitary-adrenal
HRV	Heart rate variability
HR	Heart rate
HSD	Honestly significant difference

ID	Identification
IF	Intermittent fasting
IL	Interleukin
IM	Intramuscular
LA	Locomotor activity
LC	Locus coeruleus
MAD	Median absolute deviation
MMRM	Mixed model repeated measures
NE	Norepinephrine
ODBA	Overall dynamic body acceleration
PA	Protected areas
QI	Quality Index
RIA	Radioimmunoassay
SD	Standard deviation
SIH	Stress-induced hyperthermia
SNS	Sympathetic nervous system
T_a	Air temperature
T_b	Body temperature
T_c	Core body temperature
WAZA	World Association of Zoos and Aquariums

Chapter 1: Introduction

In the broad scope of wildlife conservation to protect already declining populations of various species, the question of what renders a species extinction prone is fundamental. Resilience, or the ability to adapt to adverse or stressful stimuli, is an intrinsic part of this. Those individuals who manifest fewer deleterious effects of stress are resilient (Lui *et al.*, 2018). Millenia of selective evolutionary pressures has been a feature of every organism's existence. As such, animals have evolved a repertoire of behavioural and physiological strategies to adapt to environmental variation. This adaptive capability is highly differentiated between species and even individual animals. A possible underlining factor for these interspecific differences is an ecological specialisation of species' biological traits (Colles *et al.*, 2009; Foden *et al.*, 2008). There is an apparent trend whereby generalist species show plasticity in their phenotypes under variable environmental conditions and are, as a result, able to thrive in the face of adversity (Richmond *et al.*, 2005). While specialist species exploiting narrow ecological niches demonstrate sensitivity to environmental change and increased extinction risk (Berry *et al.*, 2013; Colles *et al.*, 2009). Most environments on Earth experience spatial and temporal variation, still the staggering rate at which the natural world is currently being altered is driving the need for a greater understanding of the intrinsic biological mechanisms of resilience.

This thesis assessed different methods for measuring behavioural and physiological responses to psychological stressors in the cheetah, an International Union for the Conservation of Nature listed Vulnerable species (Durant *et al.*, 2015). Mounting anthropogenic threats, conservation concerns, and low genetic diversity make the cheetah an attractive model for exploring resilience to environmental variation in threatened species.

In this chapter, I will briefly review published literature describing the welfare of captive cheetahs, the adaptive stress response, and the advantages and limitations of traditional and novel methods to measure stress. I then set forth what I believe the significance of this thesis to be, how each chapter may contribute insight into the cheetah's response to specific aspects of captivity, and finally, the methodology used to conduct the research.

1.1 BACKGROUND AND SCOPE

1.1.1 Captive Welfare of the Cheetah

With its morphological and physiological specialisations for prey-killing and locomotion (Meachen *et al.*, 2018), the cheetah is an iconic feline species. However, the cheetah is threatened throughout its remaining geographical range. Population declines are primarily attributable to human-wildlife conflict, loss of habitat and prey, and illegal wildlife trade (Durant *et al.*, 2017; Marker *et al.*, 2018; Nowell & Rosen, 2018; Tricorache *et al.*, 2018). Genetic homogeneity also reduces the species' resilience to ecological and environmental changes to which the world is increasingly subjected (Schmidt-Küntzel *et al.*, 2018). Another threat to the cheetah's long-term survival is its lack of genetic variation relative to other felids (O'Brien *et al.*, 1985; 1983). Protected areas (PA) such as wildlife reserves and national parks can safeguard many species. Living in low densities, cheetahs' home ranges cover over 1,500 km^2 (Marker *et al.*, 2018). The conservation potential of PAs depends on their size, connectivity with other PAs, setting within the broader landscape, management policy, and law enforcement (Cristescu *et al.*, 2018). Moreover, for cheetahs, living in PAs leads to greater intraguild competition and predation with larger or more dominant predators. Today, nearly 80% of wild cheetahs are, as a result, found outside of PAs and living alongside human communities (Durant *et al.*, 2017).

As the free-ranging population of cheetahs continues to decline, the species' survival depends on a holistic *in situ* and *ex situ* conservation approach (Schwartz *et al.*, 2018). Modern zoological institutions collectively constitute a powerful conservation resource. Cheetahs cared for in accredited zoos play various conservation roles, including raising awareness as display animals, connecting with the visitors as educational ambassadors, and supporting i*n situ* efforts through funding and involvement in conservation action planning (Schwartz *et al.*, 2018). Targeted zoo-based research, advances in husbandry management, animal welfare and health care, and increased knowledge of cheetah biology have facilitated a greater understanding of individuals living freely. Furthermore, zoological associations contribute to conservation through cooperatively managed *ex situ* breeding programmes (Schwartz *et al.*, 2018). If cheetah translocation programmes using captive-bred animals become feasible and a necessary conservation option, reserve populations in captivity could be indispensable for reintroducing numbers and genetic diversity. However, to offer such conservational benefits requires the maintenance of healthy, sustainable *ex situ* populations, eliminating the reliance on a further infusion from wild-caught cheetahs.

Animals in captivity generally receive veterinary care and are free from predation, starvation, and dehydration. As a result, they are often healthier, live longer, and breed more successfully than their conspecifics in the wild (Mason, 2010). However, there are significant differences (even among close taxonomic relatives) in species' ability to adapt to captivity (Clubb & Mason, 2007; Mellor *et al.*, 2021; Müller *et al.*, 2011). As some species have arguably acceptable or even good welfare, for others, poor conception rates, high infant mortality rates, and poor adult survivorship are significant limitations to establishing sustainable *ex situ* populations (Barnes *et al.*, 2002; Bashaw *et al.*, 2001; Clubb *et al.*, 2009). As evidenced by compromised welfare, it has been suggested that the latter species has psychological and

physiological requirements that are difficult to accommodate in captivity or not yet understood (Mason, 2010).

Worldwide, the sustainability of *ex situ* cheetah populations has been limited by the affliction of multiple unusual diseases (e.g., *Helicobacter*-associated gastritis, amyloidosis, glomerulosclerosis, and veno-occlusive disease) (Terio *et al.*, 2018), in addition to poor reproductive health and performance (Crosier *et al.*, 2018). Captive cheetahs, in particular, appear to be more susceptible to infectious diseases, the pathophysiology of which research in the 1980s attributed to the species' genetic uniformity (Heeney *et al.*, 1990; Munson *et al.*, 2005; O'Brien *et al.*, 1985; Schmidt-Küntzel *et al.*, 2018; Terio *et al.*, 2018). Polymorphism loss is a shared trait among threatened species (Munson *et al.*, 2005), demonstrating a propensity for poor welfare in captivity (Mason, 2010). The high disease incidence in geographically distinct populations of captive cheetahs further suggests a genetic predisposition (Munson *et al.*, 2005). However, in the 1990s, pathology studies indicated that morbidity and mortality in captive cheetahs were caused by degenerative rather than infectious diseases (Munson, 1993; Munson *et al.*, 1999). Moreover, that these diseases are rare, if at all present, in free-ranging cheetahs (Munson *et al.*, 2004a; 2004b; 2005; Thalwitzer *et al.*, 2010), cautions against attributing fitness loss to genetics alone and attests to the equal or greater importance of environmental factors, such as captivity-induced stress.

Several potential stressors could adversely affect animals living in captivity. Most conspicuous are confinement-specific, including restricted movement, forced proximity to humans, reduced feeding opportunities, and maintenance in abnormal social groups, as well as other, more subtle abiotic sources of stress (for review, see Morgan & Tromborg, 2007). It is thought that the cheetahs' genetic homogeneity has limited their adaptability to the captive setting, resulting in a chronic stress response that exacerbates and accelerates disease

pathogenesis (Munson *et al.*, 2005). In addition to their dietary and habitat specialism, the species' timidity and naturally wide-ranging lifestyle may be similarly unfavourable for adaptation to captivity (Driscoll *et al.*, 2009; Mason, 2010).

Multiple studies have now documented behavioural (e.g., stereotypies, such as pacing) and physiological chronic stress responses in captive cheetahs (Jurke *et al.*, 1997; Terio *et al.*, 2004; Wells *et al.*, 2004; Wielebnowski, 1999). Cheetahs in captivity have larger adrenal glands and higher baseline levels of corticoids than their free-living counterparts (Meachen *et al.*, 2018; Terio *et al.*, 2004). Corticoid levels differ between institutions and individuals residing at the same institution, suggesting that captive management and cheetah temperament could affect the stress response (Terio *et al.*, 2004; Wells *et al.*, 2004). Considering the species' susceptibility to stress-related diseases, we must carefully manage cheetahs in captivity.

Ex situ breeding is sometimes accompanied by hand-rearing of animal offspring because parental rearing fails or is not an option. Alternatively, raising ambassador animals intended to educate people on the plight of their wild conspecifics. Its "nonaggressive temperament and ease of trainability and handling when raised by experienced and qualified handlers" (Rapp *et al.*, 2018, p. 404) make the cheetah a model candidate for ambassador programmes. Positive early-life experiences with humans may down-regulate stress reactivity and reduce adult fearfulness (Bonato *et al.*, 2013; Feenders & Bateson, 2011; Hemsworth & Barnett, 1992), facilitating an adaptation to the captive environment. Whether or not zoos should hand-rear animals is a controversial topic. Cheetahs which are hand-reared cannot be reintroduced into the wild. Additionally, proper socialisation is essential for large felids to develop the correct behavioural repertoire for mating and copulation (Hampson & Schwitzer, 2016). For this reason, cheetahs raised by humans alone without conspecific contact are often no longer

included in *ex situ* breeding programmes. Despite this, ambassador cheetahs may fulfil a different educational role in conservation and are valuable additional sources of revenue.

Feeding and nutrition are fundamental to managing and caring for *ex situ* cheetah populations. Gastrointestinal (GI) disease, known to affect the majority of cheetahs in captivity (Fox *et al.*, 2021), is likely to confer significant welfare implications for affected animals and potentially contribute to the poor reproductive output of the species. Chronic stress is considered an aetiological agent in various diseases of captive cheetahs (Munson *et al.*, 2005) and is often cited in GI pathology (Munson *et al.*, 2005; Terio *et al.*, 2004). More recently, the role of nutrition in preventing, treating, and causing GI disease in cheetahs has been suggested (Lane *et al.*, 2012; Whitehouse-Tedd *et al.*, 2015). Of particular interest is the beneficial reduction in intestinal bacteria-derived putrefactants when cheetahs are fed a diet containing animal and plant fibres, i.e., non-digestible tissues (Depauw *et al.*, 2012a; 2012b; 2013; Kerr *et al.*, 2013). While findings in other species could prove applicable, the high incidence of GI diseases in *ex situ* cheetah populations compared to other large carnivores warrants a species-specific research focus. Nutritional and diet-related aspects that need further scientific attention include identifying the role of diet, specific nutrients (and their interactions), non-digestible tissues, dietary format, meal frequency, and feeding schedules in GI health, behaviour, and overall welfare (Whitehouse-Tedd *et al.*, 2018).

Determining how to care for cheetahs in captivity presents an ethical obligation for animals' well-being and practical consideration for *ex situ* breeding programmes. As wild cheetah populations continue to decline globally, successful captive propagation and the maintenance of as much genetic diversity grows in importance. Cheetah reproduction has improved over the years. However, significant disparities in reproductive success remain between geographic regions, with only the captive population of southern Africa being considered self-sustaining

(Marker *et al.*, 2018). Furthermore, our currently limited understanding of how cheetahs adapt to environmental variation is a significant barrier hindering *ex situ* conservation efforts and the management of free-ranging cheetahs. Wild-caught cheetahs temporarily held in captivity develop diseases like those afflicting captive-born cheetahs (Munson *et al.*, 2005), potentially impacting their survival when reintroduced into the wild. Capture, holding, and translocation can mitigate human-wildlife conflict and habitat fragmentation; however, reducing stress under these conditions is essential to realising their full conservation potential.

1.1.2 The Adaptive Stress Response

All living organisms must maintain a dynamic equilibrium of their vital bodily systems, or homeostasis, constantly challenged by intrinsic and extrinsic adverse forces termed stressors (Chrousos, 2009; Chrousos & Gold, 1992). Stress is, thus, defined as a state of threatened or perceived as threatened homeostasis (Charmandari *et al.*, 2005) and is counteracted by a repertoire of behavioural and physiological responses aiming to maintain or re-establish an optimal equilibrium, i.e., the adaptive stress response. The stress system is subserved by a complex neuroendocrine, cellular, and molecular infrastructure, extending into the central nervous system (CNS) and the periphery (Charmandari *et al.*, 2005; Nicolaides *et al.*, 2015; Kyrou & Tsigos, 2009; Tsigos *et al.*, 2020). The central components, located in the hypothalamus and brain stem, primarily include the parvocellular corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP) neurons of the paraventricular nuclei of the hypothalamus and the locus coeruleus(LC)/norepinephrine[NE] system (Charmandari *et al.*, 2005; Nicolaides *et al.*, 2015; Kyrou & Tsigos, 2009; Tsigos *et al.*, 2020). The peripheral components include the neuroendocrine hypothalamic-pituitary-adrenal (HPA) axis and the

efferent systemic sympathetic-adrenomedullary system and components functioning under the control of the parasympathetic system.

Activation of the HPA axis begins with the secretion of CRH and AVP by a group of neurons located in the PVN of the hypothalamus (Nicolaides *et al.*, 2015; Romero & Wingfield, 2015). Corticotropin-releasing hormone and AVP reach the anterior lobe of the pituitary gland through the hypophyseal portal system. The CRH is the principal hypothalamic regulator of the anterior pituitary adrenocorticotropic hormone (ACTH) secretion into the systemic circulation, while AVP contributes synergistically (Charmandari *et al.*, 2005; Kyrou & Tsigos, 2009; Tsigos *et al.*, 2020). Receipt of circulating ACTH in the adrenal cortex regulates glucocorticoid (GC, primarily cortisol for fish and most mammalian species; corticosterone for birds, reptiles, amphibians, and rodents) and adrenal androgen secretion by the zona fasciculata and reticularis, respectively, and participates in the control of aldosterone secretion by the zona glomerulosa (Charmandari *et al.*, 2005; Nicolaides *et al.*, 2015; Tsigos *et al.*, 2020). Under normal (unstressed) conditions, GC secretion shows pronounced temporal regulation, with both pulsatile or ultradian and circadian rhythmicity (Dickmeis, 2009). The circadian peak in GC release is linked to the start of animals' activity phase, that is, in the early morning for diurnal and the early night for nocturnal animals (Cheifetz, 1971; Irvine & Alexander, 1994; Klemcke *et al.*, 1989; Lefcourt *et al.*, 1993; Perlow *et al.*, 1981; Van Cauter, 1990). At the same time, the ultradian pulses occur with a frequency of about one to two per hour (Loudon *et al.*, 1994; Sarnyai *et al.*, 1995; Tapp *et al.*, 1984; Weitzman *et al.*, 1971; Windle *et al.*, 1998). Diurnal secretory patterns of GCs can be disrupted by changes in lighting, feeding schedule, and activity, as well as in response to a homeostasis-threatening stimulus (Charmandari *et al.*, 2005).

Glucocorticoids, the end-products of the HPA axis, are steroids that induce tissue-specific genomic responses throughout the body by binding the ubiquitously expressed glucocorticoid receptor (GR) and the more tissue-restricted mineralocorticoid receptor (Charmandari *et al.*, 2005; Coffman, 2020; Tsigos *et al.*, 2020). A significant function of GC signalling is to regulate metabolism through primarily catabolic actions, for example, lipolysis in white adipose tissue and protein degradation in skeletal muscle, to increase the availability of gluconeogenetic substrates (Chourpiliadis & Aeddula, 2022; Coffman, 2020; Tsigos *et al.*, 2020). Furthermore, GCs antagonise the anabolic actions of growth hormone (GH), insulin, and sex steroids on their target organs or tissues (Tsigos *et al.*, 2020). Another primary function of GC signalling is to regulate the immune system through potent anti-inflammatory and immunosuppressant activities (Chourpiliadis & Aeddula, 2022). A third primary function of GC signalling is to control the basal activity of the HPA axis and terminate the stress response by inhibiting CRH and ACTH secretion via GR-mediated negative feedback on the hypothalamus and the pituitary gland (Coffman, 2020; Karin *et al.*, 2020). These dynamics are a functionally important aspect of the HPA axis response to stress, as well as circadian and ultradian rhythms (Karin *et al.*, 2020), and limit the duration of the total tissue exposure to GCs (Tsigos *et al.*, 2020).

The sympathetic nervous system (SNS) constitutes the other effector component of the stress system, regulating the cardiovascular, respiratory, GI, renal, endocrine, and other vital systems as part of the 'fight-or-flight response' (Nicolaides *et al.*, 2015; Romero & Wingfield, 2015). Sympathetic innervation of peripheral organs originates from the preganglionic fibres (axons), whose cell bodies are located in the intermediolateral column of the spinal cord. The axons synapse with postganglionic neurons inside the sympathetic ganglia, and, from there, postganglionic axons leave the ganglia and project onto visceral effectors (Charmandari *et al.*, 2005; Tsigos *et al.*, 2020). To transmit the neural signal, the preganglionic and postganglionic

axon terminals release the well-known neurotransmitters acetylcholine and NE, respectively (Nicolaides *et al.*, 2015). The SNS also offers a humoral contribution consisting of circulating epinephrine (E) and, to a lesser extent, NE by the adrenal medulla and NE by the LC/NE system (Chu *et al.*, 2021). The parasympathetic system antagonises or assists sympathetic functions by increasing or withdrawing its activity, respectively (Tsigos *et al.*, 2020).

When homeostasis is threatened or perceived as so, the stress system is activated through two waves of serial hormonal secretion providing the adaptive response. The first wave of the stress response begins within seconds. It involves: (i) increased release of E and NE from the SNS, (ii) secretion of hypothalamic CRH into the hypophyseal portal system and subsequent enhanced release of ACTH, (iii) decreased release of hypothalamic gonadotropin-releasing hormone followed by decreased secretion of follicle-stimulating hormone and luteinising hormone from the anterior pituitary, and (iv) enhanced secretion of pituitary-derived prolactin and GH, and increased release of glycogen from the pancreas (Sapolsky *et al.*, 2000). The second wave of stress-induced hormonal secretion is more gradual and involves the GCs (Romero & Wingfield, 2015).

Activation of the stress system leads to behavioural and physical adaptations that acutely increase the chances of survival against the imposed stressor(s) (Nicolaides *et al.*, 2015). Behavioural adaptation during acute stress includes increased alertness, arousal, vigilance, improved cognition, focused attention, euphoria, enhanced analgesia, suppression of feeding, and inhibition of reproductive function (Charmandari *et al.*, 2005; Nicolaides *et al.*, 2015; Tsigos *et al.*, 2020). Physical adaptation mediates an adaptive redirection of oxygen and nutrients to the CNS and the stressed body site(s) through increased cardiovascular tone and respiratory rate and a shift of intermediary metabolism towards catabolism. Moreover, organisms activate detoxification processes for any unnecessary metabolic products from the

stress-related reactions, while nonadaptive functions in the short-term (e.g., digestive, reproduction, growth, and immunity) are inhibited.

Homeostasis is effectively achieved through the regular basal activity of the stress system and the appropriate magnitude and duration of the stress response (Nicolaides *et al.*, 2015). The optimally functioning stress system is a dynamic equilibrium between feedforward and feedback regulation (Coffman, 2020) such that the adaptive stress response is both of magnitude to maintain or re-establish homeostasis and of time-limited duration, rendering the associated catabolic, anti-reproductive, anti-growth, and immunosuppressive effects transient and in favour of survival (Kyrou & Tsigos, 2009; Tsigos *et al.*, 2020). However, following chronic or repeated exposure to stressors, homeostasis can become disrupted, and the dynamic regulation of the system is compromised (Tsigos *et al.*, 2020).

If an animal is chronically or repeatedly exposed to stressors, elevated GC levels may not be effectively downregulated by the negative feedback loops, resulting in hypercortisolism and additional HPA axis dysregulation (Guilliams & Edwards, 2010; Romero & Wingfield, 2015). The feedback and feedforward regulators that mediate optimally dynamic functionality adaptively reduce system responsivity to protect the individual from chronically elevated GCs that threaten long-term survival (Coffman, 2020). For example, chronic stress induces hypertrophy of the inner zona fasciculata and medulla and hyperplasia limited to the outer zona fasciculata (Ulrich-Lai *et al.*, 2006). The larger adrenal functional mass leads to elevated GC levels, but in adapted (that is, baseline) levels of ACTH and blunted ACTH and, ultimately, GC responses (Karin *et al.*, 2020).

Inappropriate (low or high) basal activity and responsiveness to homeostasis-threatening stimuli, in terms of both magnitude and duration, can have detrimental effects on several vital physiologic functions (e.g., growth, reproduction, metabolism, and immunocompetence) as

well as behaviour, contributing to the development of acute or chronic pathologic conditions (Chrousos, 2009; Kyrou & Tsigos, 2009; Tsigos *et al.*, 2020). The development and severity of these conditions depend on the genetic, epigenetic, and constitutional vulnerability or resilience of the individual to stress, their exposure to stressors during critical periods of development, the presence of concurrent adverse or protective environmental factors as well as the timing, magnitude, and duration of stress (Charmandari *et al.*, 2005; Chrousos, 2009; Tsigos *et al.*, 2020).

1.1.3 Behavioural and Physiological Indicators of Stress and Welfare

As discussed above, the adaptive response of each individual to stress is determined by a multiplicity of genetic, environmental, and developmental factors (Charmandari *et al.*, 2005; Chrousos, 2009; Tsigos *et al.*, 2020). For many animals, captivity can be a source of psychological stress, inducing the associated behavioural and physiological adaptations (Mason, 2010). For example, the cheetah's reported susceptibility to stress-related welfare concerns (Munson *et al.*, 2005; Terio *et al.*, 2004; see section 1.1.1 for more information on the captive welfare of the cheetah). However, stress is multilevel, emergent, and depends on individual and environmental context, making it inherently complex to measure. Cross-validating different outcome-based welfare measures is considered best practice to be holistic in interpreting these data and inferences drawn.

For this thesis, behavioural observations were performed to record species-specific behaviours linked with positive and negative welfare states. To offer mechanistic insights potentially inaccessible from ethology-based assessment, faecal glucocorticoid metabolite (fGCM) concentration analysis was performed as an integrated measure of adrenocortical activity in addition to faecal consistency scoring as a preliminary indicator of animals' GI

health. Furthermore, the potential of biologging technology for measuring stress in the cheetah was explored by recording concurrent body temperature (T_b), heart rate (HR), and locomotor activity (LA).

1.1.3.1 Behavioural Observations

Since its conceptualisation, the scientific discipline of animal welfare has mainly focused on animal behaviour, i.e., ethology (Binding *et al.*, 2020; Marchant-Forde, 2015). Behaviour is the observable response of an individual to stimuli originating internally as part of their physiology or from the external environment (Manning & Dawkins, 2012). Complex and essential animals survive by constantly, and often instinctively, reacting to stimuli, culminating in behavioural expression with adaptive value (Manning & Dawkins, 2012; Rose & Riley, 2021).

Justification for the supremacy of animal behaviour as the most-oft employed diagnostic tool in treating welfare concerns is multifactorial. Direct observation and study of behaviour offer insight into animals' responses to the environment, allowing quickly gained inferences on their requirements, preferences, dislikes, and welfare status (Mench, 1998). Moreover, as an outcome-based approach, behaviour is noticeable, measurable, and can be manipulated (Rose & Riley, 2021). Behavioural observations are also non-invasive and do not require special equipment, making them inexpensive compared to other disciplines (Watters *et al.*, 2021). Further pragmatic is the various behavioural methodologies and theories available and how observational techniques can be integrated into broader research programs (Rose & Riley, 2021).

From the early stages of the development of the discipline, there has been a focus bias toward negative welfare indicators (Melfi, 2009; Watters *et al.*, 2021), possibly attributed to the relative ease of observing when an animal was performing abnormal or repetitive,

unvarying, and apparently functionless behaviour, i.e., stereotypies (Mason, 1991). Stereotypical behaviour is rare in wild animals and is thought to arise from frustrated attempts to perform intrinsically motivated behaviours or forebrain dysfunction impeding normal behavioural inhibition (Latham & Mason, 2010). For example, natural ranging behaviours and activity patterns reportedly predict carnivore stereotypy levels (Clubb & Mason, 2003; 2007). Stereotypic pacing is common among captive felids (Clubb & Mason, 2007; Clubb & Vickery, 2006; Livingston, 2009; Mason *et al.*, 2007), including the cheetah (Quirke & O'Riordan, 2011a; 2011b; Quirke *et al.*, 2012). Stereotypies are frequently attended by elevated levels of GCs (Carlstead, 1998; Mason, 1991), i.e., the end-products of the HPA axis stress response. However, it is crucial not to infer welfare based only on stereotypical behaviour or negative welfare indicators. Rather than having minimum care standards, enhancing animals' quality of life requires positive welfare indicators.

The shift away from negative to positive welfare indicators (Mellor, 2016; Mellor & Beausoleil, 2015) has led to a burgeoning observational focus on animals' engagement in species-appropriate behaviours from their natural repertoire (Allard & Bashaw, 2019; Basset & Buchanan-Smith, 2007; Buchanan-Smith & Badihi, 2012) as well as both momentary and cumulative affective states, or emotions (Watters *et al.*, 2021). There are few validated measures of positive animal welfare or affect, but behavioural diversity is a potential positive welfare indicator (Clark & Melfi, 2012; Collins *et al.*, 2016; Razal *et al.*, 2016). Behavioural diversity can be defined as the richness of behaviour (number of behaviours) and evenness (frequency of each behaviour). It is theorised that when behavioural diversity is high, it is possible that the individual's behavioural needs are being met (Miller *et al.*, 2020). In contrast, when low, an animal is likely stereotyping or lethargic, which could indicate compromised welfare (Mason & Latham, 2004). The growing body of evidence supporting behavioural

diversity as a positive welfare indicator includes a reportedly inverse relationship with stereotypical behaviour (Dantzer, 1986; Gunn & Morton, 1995) and fGCMs (Miller *et al.*, 2016). Additionally, animals are observed as having higher behavioural diversity following management practices thought to enhance welfare (e.g., environmental enrichment [EE], habitat complexity, appropriate social groups, and animal training) (Shepherdson *et al.*, 1993).

Though the remit of animal welfare research has expanded in scope and types over the years, direct observation of animal behaviour remains a consistent focus. However, bias, confounding, and validity issues may arise when using behavioural observations to measure stress and animal welfare (Dawkins, 2007). Ethologists have long been aware that their presence can influence animals' behaviour (Carpenter, 1934; Schneirla, 1950), in particular when they perceive humans as predators (Caro, 1999) or are naturally secretive or elusive (Chapman, 1927; Maffei *et al.*, 2005). Furthermore, human perception can be selective and biased, predisposing direct observation and interpretation to expectations about behavioural outcomes and assumptions and possibly confounding the subjective assessment of welfare (Altmann, 1974; Tuyttens *et al.*, 2014). Successful diagnosis of welfare issues requires accurate interpretation of behaviours observed so that interventions are not ineffective, unwarranted, or harmful (Watters *et al.*, 2021). As such, robust research that has been planned, implemented, and analysed appropriately so that interpretations and inferences are valid, is necessary to enact meaningful change in animal management and care (Rose & Riley, 2021). Considering the individuality of behaviours expressed (Rose & Riley, 2021), it is also recommended that observations be handled by researchers with knowledge of inter- and intraspecific behavioural response patterns to safeguard against misinterpretation. Another consideration is that welfare is a multifaceted construct. Collaboration with physiological indicators could enable a more

holistic understanding of behaviour's cause, outcome, and feelings associated with its expression (Watters *et al.*, 2021).

1.1.3.2 Faecal Glucocorticoid Metabolite Concentrations

Scientists have increasingly employed physiological parameters to gain insight into animals' physical condition, psychological health, and overall welfare status (Dantzer *et al.*, 2014). Glucocorticoids, culminating from the hormonal cascade along the neuroendocrine HPA axis, are essential regulators of basal and stress-related homeostasis and influence a wide array of genes in almost every organ and tissue (Charmandari *et al.*, 2005; Coffman, 2020; Tsigos *et al.*, 2020). Specifically, GCs increase the availability of resources to meet the energetic demands of the stressor and maintain or re-establish homeostasis while inhibiting functions nonessential to adaptation (Charmandari *et al.*, 2005; Nicolaides *et al.*, 2015; Tsigos *et al.*, 2020). As key regulators of the physiological adaptation to stress (Munck *et al.*, 1984; Sapolsky *et al.*, 2000), GCs enhance survival (Nicolaides *et al.*, 2015). However, following chronic or repeated exposure to stressors, the dynamic equilibrium by which the HPA axis is regulated may be disrupted (Tsigos *et al.*, 2020), having detrimental effects (e.g., reproductive problems, growth reduction, muscle wasting, immune deficiency, and impaired neurological function) (Wielebnowski, 2003). Considering their physiological importance, GC hormones and their metabolites have become a standard in animal welfare studies to quantify adrenocortical activity and assist in identifying stressors (Millspaugh & Washburn, 2004; Mormède *et al.*, 2007; Möstl *et al.*, 1999; Palme, 2012; Sheriff *et al.*, 2011; Wielebnowski & Watters, 2007).

Traditionally, GCs were measured in plasma or serum samples. However, handling animals during blood collection exposes them to additional stress, particularly for individuals not accustomed to humans. Consequently, interpretation challenges may arise from the invasive

nature of the blood sampling procedure itself and the associated collection bias (Palme *et al.*, 2005). Furthermore, a single sample does not accurately reflect HPA activity within an individual; instead, it offers a point-in-time snapshot of hormone concentration, elucidating the necessity for repeated blood draws (Otovic & Hutchinson, 2015). While several studies have reported peak concentrations of GCs, some research suggests that the duration of release or the total amount is most indicative of HPA axis activity (Romero, 2004; Sapolsky, 1990). Whether to analyse free or total GCs is an additional source of error (Alexander & Irvine, 1998). Another issue with making inferences from single samples is that GCs, like many other hormones, are secreted in diurnal secretory patterns (Dickmeis, 2009). To circumvent the potential limitations of blood-based analysis, non-invasive and feedback-free sampling methods such as the collection of faeces have increased in popularity.

Before their excretion, GCs are primarily metabolised in the liver, though many other organs contribute to the process (Taylor, 1971). The resulting metabolites are excreted via the urine or bile predominantly as conjugates with glucuronides or sulfates to increase water solubility (Palme *et al.*, 1996). Intestinal bacteria in the gut then deconjugate most of the metabolites in bile (Devendran *et al.*, 2018; Macdonald *et al.*, 1983; Möstl *et al.*, 2005), which are then either reabsorbed into the blood or excreted in the faeces after a species-specific time delay (Palme *et al.*, 1996). The measurement of GC metabolites in faeces has been validated as an indicator of adrenocortical activity in carnivores (Malmkvist *et al.*, 2011; Monfort *et al.*, 1998; Schatz & Palme, 2001; Young *et al.*, 2004), including the cheetah (Terio *et al.*, 1999; 2004; Wells *et al.*, 2004; Wielebnowski *et al.*, 2002b).

In contrast to other matrices, faecal specimens are naturally collected without capturing and handling and can be sampled repeatedly with minimal risk of influencing the GC response (Palme, 2019; Touma & Palme, 2005). In addition to being non-invasive, fGCM analysis is

further advantageous for its long-term character. Faecal glucocorticoid metabolites represent an integrated measure of adrenocortical activity and reflect the cumulative hormone secretion over several hours (6–24 hours, depending on species-specific defaecation rate), thereby attenuating fluctuations due to ultradian and circadian rhythmicity (Palme, 2019; Palme *et al.*, 2005; Shepherdson *et al.*, 2013; Sheriff *et al.*, 2011). Repeated faeces sampling can be used as a diagnostic tool in longitudinal studies for an individual or group to measure animal stress (Hein *et al.*, 2020).

Using physiological indicators related to the stress response is encouraged because they are quantitative and not subjective. However, several researchers have described the challenges and limitations of using GCs to measure stress and animal welfare (Dantzer *et al.*, 2014; Dickens & Romero, 2013; Wielebnowski, 2003). Firstly, the absence of elevated GC concentrations does not infer ensured welfare. Many welfare issues lead to attempts to cope not involving GCs and, instead, to other negative welfare indicators (e.g., stereotypical behaviour) (Broom, 2017; Mormède *et al.*, 2007). Secondly, stress is not inherently harmful. It is crucial to remember that the HPA axis may be repeatedly activated by beneficial stimuli that do not negatively impact welfare over the long term, such as stressors during the breeding season (Whitham & Wielebnowski, 2013; Wielebnowski, 2003). Indeed, HPA activation occurs even in response to positive stimuli, such as exercise (Whitham & Wielebnowski, 2013). In the short-term, stress can have psychological and physiological benefits (e.g., enhanced cognitive and mental performance, immune functions, and resilience to subsequent stressors) conditional on animals' ability to respond effectively to the stressor. It is only when stress is of a nature, magnitude, or duration that is beyond the adaptive resources of an individual that its welfare becomes compromised (Charmandari *et al.*, 2005; Tsigos *et al.*, 2020). It can be challenging to differentiate between an adaptive and maladaptive stress response

(Wielebnowski, 2003). Furthermore, as discussed above, low or high basal HPA axis activity can be linked to pathologic conditions and poor welfare (Dickens & Romero, 2013; Miller *et al.*, 2007). To be a robust physiological welfare indicator, GC measurement is best when integrated into a holistic approach with other methodologies that reflect individual arousal levels and, if possible, the valence (positive or negative) of that arousal. Finally, GC concentrations may also be influenced by a variety of other factors, including season, sex, age, and reproductive condition (Dantzer *et al.*, 2014; Romero, 2002).

Specific to faecal-based analysis, sampling issues, assay artefacts, and particular biological variables must be considered (for review, see Millspaugh & Washburn, 2004). Since fGCM concentration may change at different rates post-defecation (Millspaugh & Washburn, 2004; Palme *et al.*, 2005), it is recommended to validate stability concerning species, method, and environmental conditions to interpret respective hormone values reliably (Palme *et al.*, 2013). Assay validation is essential to ensure its applicability for the species-specific hormone matrix of interest and as a reliable quantification of respective GC metabolites (Ganswindt *et al.*, 2012). Immunoassays are a commonly used approach to measure fGCM concentration noninvasively and have been applied to a diverse range of species (Wasser *et al.*, 2000), including the cheetah; for which both versions, i.e., radioimmunoassay (RIA) (Terio *et al.*, 1999; 2004; Wells *et al.*, 2004; Wielebnowski *et al.*, 2002b) and enzyme immunoassay (EIA) (Ludwig *et al.*, 2013; Valk, 2012; Young *et al.*, 2004), have been validated. Enzyme immunoassays have been employed more frequently than RIAs in recent years because they do not require special permits, are relatively safe to use, and involve less expensive equipment (Kersey & Dehnhard, 2014; Palme, 2019). Such validations also provide information about the time delay between the activation of the HPA axis and the appearance of GC metabolites in

excreta (Palme, 2019; Touma *et al.*, 2003). Individual differences (e.g., sex, age, reproductive status, and diet) and species can influence fGCM levels or GI transit time (Palme, 2005).

1.1.3.3 Faecal Consistency Scores

The mammalian gut harbours a complex and diverse microbial assemblage colonised by bacteria, archaea, viruses (including bacteriophages), fungi, and protozoa (Desselberger, 2018). Symbiotic gut microbes serve various essential functions in animals, ranging from nutrition to disease mediation and influencing behaviour and speciation (Wasimuddin *et al.*, 2017). Much work has been done in this century on defining the role of the gut microbiome in host physiology and the aetiopathogenesis of dysbiotic states (Shreiner *et al.*, 2015).

Intrinsic host traits (e.g., sex, age, genetic constitution) and external environmental factors (e.g., diet and habitat heterogeneity) are shown to influence host-bacteria relationships and shape interindividual variation in the gut microbiome composition (Benson *et al.*, 2010). Disturbance-related deviation in the microbial diversity and abundance pattern beyond a natural range can advance pathophysiology and affect host health. Compositional and functional gut dysbiosis in humans and laboratory species has been linked to increased susceptibility to infections, the development of inflammatory and metabolic disorders, as well as cardiovascular, respiratory, and liver diseases (Carding *et al.*, 2015; Ellis *et al.*, 2013; McKenna *et al.*, 2008; Turnbaugh *et al.*, 2007; Yoshimoto *et al.*, 2013).

Reflecting its growing importance in other research fields, the gut microbiome has been proposed as a mediator of the host condition in captivity (Bahrndorff *et al.*, 2016). A healthy gut microbiome is, in most cases, characterised by high microbial richness and diversity, thought to reflect a stable and resilient microbial ecosystem (Cho & Blaser, 2012; Lozupone *et al.*, 2012). Captivity-related changes likely to affect microbial communities include changes or restrictions in the diet, reduced habitat heterogeneity and interspecific interactions, antibiotics

and other veterinary interventions, and exposure to human-associated microbes and those prevalent in a built environment (McKenzie *et al.*, 2017). Activation of the neuroendocrine stress system may also be associated with alterations in gut microbiota (Accarie & Vanuytsel, 2020; Farzi *et al.*, 2018). The HPA axis is the endocrine effector of the stress response, with a central role for CRH, secreted in the hypothalamus and locally in the GI tract (Bunnett, 2005). The CRH is the principal hypothalamic regulator of the ACTH, which, in turn, regulates the secretion of GCs (Charmandari *et al.*, 2005; Nicolaides *et al.*, 2015; Tsigos *et al.*, 2020), critical regulators of the physiological adaptation to stress (Munck *et al.*, 1984; Sapolsky *et al.*, 2000). That CRH receptors are also found to be expressed in both the GI tract and the CNS suggests a crucial role for this factor in the stress-related disruption of gut homeostasis following chronic or repeated exposure to stressors (Taché & Perdue, 2004), including transit (Martínez *et al.*, 2004; 2002; Yin *et al.*, 2008), visceral sensitivity (Larauche *et al.*, 2009; Million *et al.*, 2006; Trimble *et al.*, 2007), intestinal permeability (Barreau *et al.*, 2004; la Fleur *et al.*, 2005), and gastric inflammation (Hagiwara *et al.*, 2018).

Many studies comparing captive to wild counterparts within a species have suggested a trend toward decreased symbiotic microbial diversity in captivity (Clayton *et al.*, 2016; Kohl & Dearing, 2014; Metcalf *et al.*, 2017; Wienemann *et al.*, 2011). However, some studies have reported no difference in microbiome composition (Gibson *et al.*, 2019; Oliveira *et al.*, 2020) and even increased gut diversity in captive animals (Nelson *et al.*, 2013; Tsukayama *et al.*, 2018; Xenoulis *et al.*, 2010). That is, there is tremendous variation in how species' gut microbes respond to the captive environment, i.e., microbial plasticity (Diaz & Reese, 2021). Presently, the extent, predictability, and drivers of microbiome changes under captivity remain unclear, limiting the utility of microbial interventions to alleviate captivity-related welfare concerns,

such as the high incidence of pathogenic bacterial infections (Wasimuddin *et al.*, 2017) and GI diseases in captive cheetahs (Terio *et al.*, 2005).

Downstream analyses of the functional interactions between a host and its microbiome have attracted the attention of researchers to offer mechanistic insights into these host-microbe relationships (Shreiner *et al.*, 2015). For pragmatic reasons (e.g., convenient and repeatable sampling, non-invasive, sufficient biomass for analysis, and inexpensive), faecal specimens are the samples for most intestinal microbiota studies (Tang *et al.*, 2020). Intestinal colonic transit time is a crucial determinant of the gut microbial ecosystem (Lewis & Heaton, 1997), but the direct measurement is often impractical or mildly invasive. As an alternative, studies have used cheaper surrogate markers of gut transit time with minimal participant burden, such as faecal consistency (Lewis & Heaton, 1997; Vandeputte *et al.*, 2016). As a proxy for gut transit time, faecal consistency measured with the Bristol Stool Scale or faecal moisture has been linked to gut microbiota composition in humans (Tigchelaar *et al.*, 2016; Vandeputte *et al.*, 2016); emphasising the importance of considering faecal consistency as a potential confounding factor in microbiota analyses.

Faecal consistency scoring has been used to monitor GI health in carnivore species (Nery *et al.*, 2010; 2012; Vester *et al.*, 2008), including the cheetah (Whitehouse-Tedd *et al.*, 2015). However, several challenges and limitations must be considered before faecal consistency scoring can be a robust diagnostic tool and interventional strategy in epidemiological studies. Firstly, faecal consistency scoring lacks specificity as a stand-alone measure of GI health. Other measurements of microbiome health effects (e.g., digestibility, pH, incidence of vomiting or diarrhoea, veterinary diagnosis of GI disease, fermentation by-products, faecal frequency, dry weight percentage, or short-chain fatty acids) are required to construct validity. Secondly, due to its association with GI transit time, faecal consistency scoring is subject to interindividual

differences in interpretation (Lewis & Heaton, 1997), linked to dietary intake and stress (Lemay *et al.*, 2021). Faecal consistency scoring and the resultant mitigation strategies will likely need to be handled on an interspecific basis performed by researchers with knowledge of the animal's biology. However, at the time of writing, a universally standardised scoring system by which facilities could assess their resident cheetahs' faecal consistency and set a benchmark for which to aim was unavailable (AZA Tiger Species Survival Plan®, 2016; Whitehouse-Tedd *et al.*, 2015).

1.1.3.4 Biologging: Body Temperature, Heart Rate, and Locomotor Activity

Technological innovations have historically spurred exponential growth in scientific understanding across disciplines. Recent methodological advancements for data acquisition have transformed animal research (Cooke *et al.*, 2004; Ropert-Coudert & Wilson, 2005; Wilmers *et al.*, 2015). The technology-based approach to studying animals has been termed biologging (Naito, 2004), "the use of miniaturized animal-attached tags for logging and/or relaying data about an animal's movements, behaviour, physiology and/or environment" (Rutz & Hays, 2009, p. 289).

In the last couple of decades, biologging devices have become increasingly miniaturised and sophisticated, resulting in smaller, lightweight electronic units with improved transmission capabilities and storage that support diverse quantities and types of onboard sensors (Evans *et al.*, 2013). Biologging technology has also become more affordable and accessible than in past years (Diana *et al.*, 2021). As a result, today, researchers have access to a wide array of sensors that can remotely and continuously monitor most aspects of animals' states and simultaneously the conditions of their environment (Evans *et al.*, 2013). Biologging, mainly as it records data independent of vagaries and confounding influences of the human researcher (Hetem *et al.*, 2019), offers a degree of accuracy once inaccessible for quantifying animal movement,

behaviour, and physiology in context with environmental variables. Additionally, compared to traditional methods confined to a single-point sample collected over a short period, biologgers can be deployed for extended periods and log complementary lines of evidence up to multiple times per second (Menzies, 2020). Biologging devices that concurrently measure several variables reveal how various functions interact (Hetem *et al.*, 2019). The deployment of animal-borne data loggers has enabled new and unprecedented insight into already extensively studied species (Brown *et al.*, 2013; Evans *et al.*, 2013). Moreover, remote sensing is advancing our ability to study cryptic or wide-ranging species that have previously evaded investigation (Rutz & Hays, 2009; Wilmers *et al.*, 2015).

Locomotor activity, HR, and T_b represent three functional traits that can currently be readily logged. Firstly, movement is the fundamental behavioural response to intrinsic motivation and the external environment (Mench, 1998). As arguably, the most crucial way individuals interact with, respond to, and control their surroundings, behaviour has been a consistent focus of animal research. Recently, scientists have recognised the potential of accelerometers in animal-borne data loggers to circumvent the limitations of direct behavioural observations (e.g., observer effect and evasive species) (Brown *et al.*, 2013; Wilson *et al.*, 2008; Yoda *et al.*, 1999). An accelerometer sensor is a tool for measuring fine-scale movement data with a high temporal resolution, unlimited by animal visibility, observer bias, terrain, climate, or geographical scale (Brown *et al.*, 2013). Logging three different dimensions of motion, i.e., surge, heave, and sway (tri-axial), is the most accurate and precise way of measuring the change in body posture and movement from its static state (Brown *et al.*, 2013; Nathan *et al.*, 2012; Shepard *et al.*, 2008). Initial applications of animal-borne accelerometers have primarily focused on marine species (e.g., seabirds; Arai *et al.*, 2000; Ropert-Coudert *et al.*, 2004; Weimerskirch *et al.*, 2006; Yoda *et al.*, 1999; seals; Mitani *et al.*, 2004; Sato *et al.*, 2003; turtles;

Fossette *et al.*, 2010; 2012; and fish; Gleiss *et al.*, 2011; Tsuda *et al.*, 2006; Whitney *et al.*, 2010), for whom few other behavioural observation methods were possible. With the advancement of biologging technology, researchers have been able to extend the application of accelerometers to diverse species in captivity and the wild (Byrnes *et al.*, 2011; Duriez *et al.*, 2014; Halsey *et al.*, 2009; McClune *et al.*, 2014; Nathan *et al.*, 2012; Williams *et al.*, 2014), including the cheetah (Grünewälder *et al.*, 2012; Hetem *et al.*, 2019; Wilson *et al.*, 2013). In all the research described, accelerometry allowed for detailed measurements of the multifaceted nature of movement (Eikelboom *et al.*, 2020) that, before its development, were seldom attainable outside of the laboratory setting and without the influence of the researchers' presence (Brown *et al.*, 2013). However, there are significant methodological challenges to getting the most out of accelerometer data linked to the acquisition and analysis (Brown *et al.*, 2013).

Heart rate and T_b are vital physiological parameters that provide essential insights into studying animal behaviour, physiology, or responses to environmental change. Major effector components of the stress response are the autonomic nervous system (ANS) and the neuroendocrine system (Moberg, 2000). The ANS has two divisions: the sympathetic, i.e., 'fight-or-flight response,' and parasympathetic, i.e., 'rest-and-digest response,' which exert antagonistic effects on the heart. Sympathetic innervation of the heart is mediated by the release of the catecholamines hormones E and NE (Gordan *et al.*, 2015). The activation of beta1 adrenergic receptors by the release of these hormones has the following positive effects on the heart: chronotropic (increase in heart rate via the predominant pacemaker of the heart, the sinoatrial node), inotropic (increase in myocardial contractibility), and dromotropic (increase in atrioventricular node conduction velocity) (Gordan *et al.*, 2015). Heart rate can reflect how animals perceive and respond to specific stimuli accurately and at a high temporal resolution

(Wascher, 2021), even without observable changes in behaviour (Beerda *et al.*, 1998; Moraes *et al.*, 2021). Changes in HR and heart rate variability (HRV), the natural variation in time between heartbeats) (Malik *et al.*, 1996), therefore, enable precise identification of stressors and are often thought to constitute more immediate and detailed stress indicators than HPA measures (Kovács *et al.*, 2014; Mialon *et al.*, 2012; Stewart *et al.*, 2008). Furthermore, as a reliable proxy of energy expenditure (Halsey *et al.*, 2019), HR responses monitored during stressful conditions allow the assessment of relative costs associated with different stimuli (Ellenberg *et al.*, 2013). Regarding their sensitivity, researchers now employ other technologies for HR measurements (e.g., implanted loggers or transmitters, wearable HR belts, skin surface-mounted transmitters, and artificial eggs) (for review, see Wascher, 2021). That HR variation, like several other physiological parameters, is subject to sex, age, and circadian rhythm must be considered, particularly with short-term measurements (Sammito & Böckelmann, 2016).

The core body temperature (T_c) fluctuates within a narrow range depending on several variables, such as muscle activity, time- and feeding-related metabolism, as well as physiological (e.g., sex, age, and body size) and environmental factors (Garami & Székely, 2014). Thermoregulation is critical to survival as it allows organs and bodily systems to work effectively and plays an adaptive role in the body's response to stressors (Osilla *et al.*, 2022). Activation of the LC/NE and PVN/CRH systems by stressors increases T_c (Charmandari *et al.*, 2005; Tsigos *et al.*, 2020). Intracerebroventricular administration of both NE and CRH results in temperature elevation, possibly through prostanoid-mediated actions on the septal and hypothalamic temperature-regulating centre (Diamant & de Wied, 1991; Mora *et al.*, 1983). Additionally, CRH has been shown to mediate the pyrogenic effects of the inflammatory cytokines partly, tumor necrosis factor-alpha(α), interleukin(IL)-1, and IL-6, following stimulation by lipopolysaccharide (endotoxin, a potent exogenous pyrogen) (Chrousos, 1998;

2007). Systemic inflammation-induced increases in T_c, i.e., fever, are an integral component of the host response to interoceptive stress, such as invading infectious agents (Mackowiak, 1998; Scholtz, 2003). Distinct from fever, psychological stress also significantly affects the central thermoregulatory system, inducing increases in T_c through activation of thermoregulatory sympathetic premotor neurons in the medullary raphe region (Lkhagvasuren *et al.*, 2011). This stress-induced hyperthermia (SIH) (Oka *et al.*, 2001) is proportional to both the magnitude and duration of the underlying psychological stressor (Bouwknecht *et al.*, 2007; Oka & Oka, 2007). A variety of techniques are available to the researcher for measuring internal (e.g., thermometry, surgically implanted loggers or transmitters, GI or non-surgically placed devices) and peripheral (e.g., passive integrated transponder tags, skin surface-mounted loggers and transmitters, or infrared thermography) temperature in animals (for review, see McCafferty *et al.*, 2015). However, for any T_b measurement study, it is essential to understand the considerable spatial and temporal variation of temperature (McCafferty *et al.*, 2015).

Advances in biologging technology over the past decades have enabled the long-term collection of previously inaccessible physiological data, including measurements of HR (Chaise *et al.*, 2017; Clark *et al.*, 2010; Laske *et al.*, 2018; Madliger *et al.*, 2018; Moraes *et al.*, 2021) and T_b (Adam *et al.*, 2016; Friend *et al.*, 2020; Hetem *et al.*, 2019; 2012; Meyer *et al.*, 2008; Trethowan *et al.*, 2017). Activation of the physiological stress response has been proposed to have both adaptive and maladaptive aspects (Selye, 1973). More research is needed to investigate in which contexts arousal and activation of the physiological stress response are adaptive and help an animal cope with stressors and in which they are maladaptive and have deleterious effects (Proudfoot & Habing, 2015). Long-term monitoring of HR and T_b would allow us to differentiate short-term arousal levels from long-term activation of the physiological stress response, which is not well understood yet (Wascher, 2021).

Biologging science is a fast-moving field containing many desirable features of animal welfare research, such as inter-disciplinarity. However, as with any emerging technological advance, three significant limitations are associated with the application of animal-borne sensors. First and foremost, capture and handling for device attachment or implantation and retrieval are not without consequences for the animals carrying them (Bodey *et al.*, 2017; Thorstad *et al.*, 2001; Vandenabeele *et al.*, 2014; Wilson *et al.*, 2019). Technological development must evolve ethical standards to safeguard animal welfare (Wilmers *et al.*, 2015). Secondly, biologgers store rather than transmit data, so the advantages of collecting information on a wide array of variables must be balanced against challenges with device recovery, linked with the third limitation of biologging, expense. Though more affordable than in past years, biologging continues to be expensive, limiting the number of devices that can be deployed (Ropert-Coudert & Wilson, 2005; Rutz & Hays, 2009). Threatening the statistical reliability, mainly where the unpredictability of study animals reduces recovery rates. Further development of biologging technology (e.g., reduced impact of devices on animal carriers, novel attachment or implantation and retrieval methods, and remote data transmission) is crucial to advance animal-borne sensors. It will only be achieved through multidisciplinary collaborations (Williams *et al.*, 2020).

1.1.4 Significance of the Thesis

A growing concern in wildlife conservation is the apparent tendency for threatened species to have a reduced ability to adapt to variable environmental conditions, predisposing them to the deleterious outcomes of chronic stress. To understand inter- and intraspecific differences in resilience, one must better measure it. Foremost, the significant contribution of this thesis was the assessment of different methods for measuring behavioural and physiological

responses to psychological stressors, including biologging technology to capture ecologically relevant data beyond traditional methods.

Moreover, for the full potential of cheetah conservation *ex situ* to be realised, considering the species' reported susceptibility to captivity-induced stress, a greater understanding of the conditions inducing a response and why and the methodological advances used to quantify these reactions is necessary. The thesis potentially also provided a basis on which appropriate management and husbandry protocols may be developed for cheetahs that better accommodate their requirements.

1.1.5 Objectives of the Research

In this thesis, I simulated scenarios depicting conditions considered to be potential psychological stressors for captive cheetahs, namely (i) a more naturalistic reduced feeding days schedule, (ii) the provision of EE, (iii) participation in animal-visitor interactions (AVI), and (iv) the close proximity to other large predators. Using emerging biologging technology and more traditional measures of stress-related behavioural and physiological responses, variations in T_b, HR, LA, behaviour, fGCM concentration, and faecal consistency score (FCS) were documented. The objectives, and corresponding hypotheses, for each of the subsequent chapters, are as follows:

Chapter 2 –

Objective: An epidemiological relationship between unnatural diet composition and feeding regimes and the various diseases prevalent in captive cheetahs has been suggested. This study investigated the response of captive-born (hand-reared) cheetahs (n = 5) to a more naturalistic reduced feeding days schedule characterised by offering larger quantities of food less frequently for three weeks.

Hypothesis: I hypothesised that the more natural feeding pattern would influence the GI environment, including microbial parameters and/or GI physiology or functional traits. Additionally, I hypothesised that reducing feeding days would induce an acute stress response.

Chapter 3 –

Objective: Environmental enrichment has now been integrated as a fundamental principle of captive animal husbandry to provide species-appropriate challenges, opportunities, and stimulation. This study investigated the response of captive-born (hand-reared) cheetahs (n = 5) to the provision of EE for three weeks.

Hypothesis: I hypothesised that EE would encourage natural active behaviours and induce arousal and activation of the physiological stress response.

Chapter 4 –

Objective: This study investigated the response of captive-born (hand-reared) cheetahs (n = 5) to participation in AVIs for three weeks.

Hypothesis: I hypothesised that up-close and, at times, hands-on contact with visitors would exacerbate the cheetah's stress response to captivity.

Chapter 5 –

Objective: Even closely related species, such as varied felids, can be averse to the vicinity of other similar species in captivity. This study investigated the response of captive-born (hand-reared) cheetahs (n = 5) to the close proximity of species, i.e., a leopard (*Panthera pardus*) and lions (*Panthera leo*), that generally compete with and/or predate the cheetah in the wild for three weeks.

Hypothesis: I hypothesised that olfactory, auditory, and/or visual contact with other large predators would exacerbate the cheetah's stress response to captivity.

1.1.6 Research Methodology

1.1.6.1 Study Site and Animals

The experimental trials occurred between April and September 2019 at the Cango Wildlife Ranch and Conservation Centre (33°33'S, 22°12'E) 4 km north of Oudtshoorn, in the Western Cape of South Africa. Oudtshoorn is a semi-arid region with three distinct plant biomes: succulent karoo, cape thicket, and fynbos. Study months (autumn and predominantly winter) were characterised by short photoperiods and cold air temperatures (T_a), ranging from 13 to 17 °C.

Three male (CH-2205, -2206, and -2271) and three female (CH-2207, -2276, and -2277) resident adult cheetahs (Table 1.1) habituated to human presence and interacting daily with the facility's caretakers in the absence of restraint were allocated to the research. The study cheetahs were kept either on-exhibit at the Cango Wildlife Ranch or off-exhibit at the Jill Bryden-Fayers Reserve neighbouring the Ranch, where they were held in outdoor enclosures ranging in size from 400 to 1350 m^2. The topography of the enclosures was varied and naturalistic, made up of a dirt substrate as well as vantage points and marking areas (e.g., rocks, tree stumps), sufficient vegetation to hide, and a wooden shed for shelter. Enclosures were cleaned once or twice daily.

Table 1.1. Demographic information of the study cheetahs.

Group	Identification Number	Origin	Housing	DOB (YY-MM-DD)	BM1 (kg)	BM2 (kg)	Sex
1	CH-2205	Captive-born (hand-reared)	Paired (with CH-2206)	2016-06-09	45.0	45.0	M
	CH-2206	Captive-born (hand-reared)	Paired (with CH-2205)	2016-06-09	47.15	47.65	M
	CH-2207	Captive-born (hand-reared)	Single	2016-06-09	36.6	37.1	F
2	CH-2271	Captive-born (hand-reared)	Single	2017-08-28	37.65	41.75	M
	CH-2276	Captive-born (hand-reared)	Paired (with CH-2277)	2017-09-16	39.9	39.35	F
	CH-2277	Captive-born (hand-reared)	Paired (with CH-2276)	2017-09-16	43.05	43.55	F

DOB: date of birth, BM1: body mass at the start of the experimental trials, BM2: body mass at the end of the experimental trials.

At the Cango Wildlife Ranch, cheetahs are fed on a variable-time schedule between enclosures a supplemented (Predator powder, V-Tech Pty Ltd, Midrand, South Africa and Glycine, WildCat Nutrition Pty Ltd, Pretoria, South Africa) horse-based muscle meat diet prepared onsite. Bones are offered randomly once a week in place of the day's meals to maintain variety and provide periodontal stimulation. The three-year-old study cheetahs (CH-2205, -2206, and -2207) were fed 1.8 kg portioned into two daily rations six days a week, and the two-year-old study cheetahs (CH-2271, -2276, and -2277) were fed 1.6 kg portioned into two daily rations six days a week. Weekly fasting days were allocated to Sundays. In the event of paired housing, the caretakers separated individuals to reduce competition for food. Leftover food was removed, weighed, recorded, and discarded. Water was available *ad-libitum*.

1.1.6.2 Body Temperature, Heart Rate, and Locomotor Activity Measurement Intervals

A single cardiac-, temperature- and movement-sensitive biologger (DST centi-HRT ACT, Star-Oddi, Gardabaer, Iceland) with the dimensions of 46 mm x 15 mm x 15 cm and weighing approximately 19 g was implanted in each of the study cheetahs. The biologgers were calibrated individually against a high-accuracy thermometer (Hart 1504, Fluke, Utah, US). Calibrated accuracy was better than 0.1 °C. Before surgical implantation, the biologgers were

set to record tri-axial LA, i.e., heave, surge, sway, every minute for the overall dynamic body acceleration (ODBA) (Wilson *et al.*, 2020) and T_b (°C) and leadless single-channel electrocardiogram (ECG)-derived HR (beats per minute; bpm) every 5 minutes at 200 Hz. Representative traces of raw ECG recordings were saved every 24 hours for validating the HR measurements' Quality Index (QI) (where QI_0 was the highest quality and QI_1, QI_2, and QI_3 were of progressively reduced quality). Afterwards, the biologging devices were sterilised using ethylene oxide.

1.1.6.3 Surgical Procedures

On the day of surgery, each of the study cheetahs (while in their enclosures) received a combination of 0.03 mg/kg of medetomidine (Medetomidine 20-mg/mL, Kyron Laboratories, Johannesburg, South Africa) and 0.8 mg/kg of tiletamine zolazepam (Zoletil, Virbac Animal Health, Johannesburg, South Africa), administered intramuscularly (IM) by hand injection. Once recumbent, the study cheetahs were transported to an onsite clinic where they were weighed, intubated with an endotracheal tube of appropriate size, and maintained under anaesthesia with 2–3% isoflurane (Forane, Abbott, Weltevreden Park, South Africa), administered in 100% oxygen. The depth of anaesthesia and standard anaesthetic parameters were monitored throughout the procedure. In addition, the study cheetahs received lactated Ringer's solution (injected gradually via cannula, inserted subcutaneously) to account for any fluid loss during surgery.

At the commencement of surgical procedures, 5 mL of blood was collected from the jugular vein of each cheetah. Blood samples were divided between lithium heparin and EDTA tubes. Following this, a comprehensive haematological evaluation (using an onsite HM5 analyser; Abaxis Veterinary Diagnostics, California, US) and plasma biochemistry profile (using an onsite VetScan VS2 analyser; Abaxis Veterinary Diagnostics, California, US) were conducted

to determine the health status of the study cheetahs before the onset of the field research. Furthermore, urine samples were collected via a 6 FG dog urinary catheter and evaluated using a standard multiparameter dipstick and refractometer (Jorgensen Laboratories, Colorado, US).

The study cheetahs were placed in a left lateral recumbent position, and a thoracic incision site was shaved and sterilised with chlorhexidine gluconate (Hibitane, Zeneca, Johannesburg, South Africa). A 50 mm cranial-caudal incision was made in the skin, and individual biologgers were implanted IM between the deep and superficial pectoralis major without tethering. Wounds were closed with continuous subcutaneous and intradermal sutures (5/0 Monosyn, B-Braun, Barcelona, Spain) and treated with a topical antiseptic and ectoparasiticide spray (F10 Germicidal Wound Spray, Health and Hygiene Pty Ltd, Roodepoort, South Africa).

Anaesthesia was reversed with 0.1 mg/kg atipamezole (Antisedan, Zoetis, Sandton, South Africa) IM. The study cheetahs were placed in individual crates to recover from the anaesthetic before being transported back to their enclosures. Surgical sites were monitored to ensure there were no signs of postoperative complications.

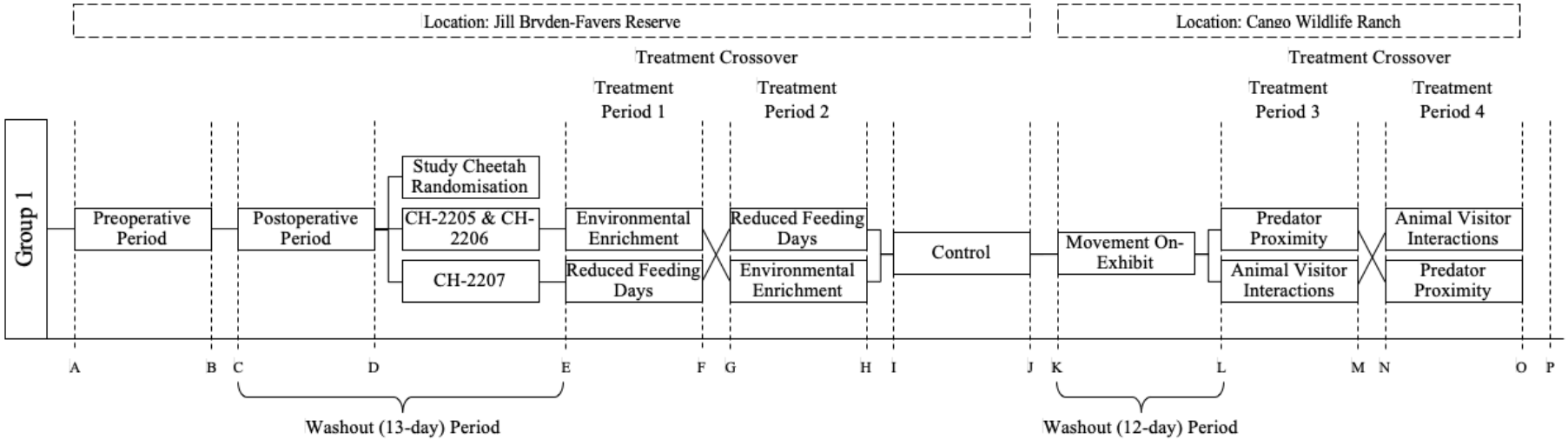
Upon completion of the experimental trials, the same anaesthesia-surgical protocols were used to remove the biologging devices.

1.1.6.4 Experimental Design

Experimental trials commenced following a ≥12-day washout period after surgical implantation of the biologgers and were conducted using a longitudinal crossover design, whereby the study cheetahs served as their control. Grouped by their respective ages (Table 1.1), the study cheetahs received the following succession of four different treatments and control at random:

Group 1 (Fig. 1.1) –

While off-exhibit at the Jill Bryden-Fayers Reserve, CH-2205, CH-2206, and CH-2207 were assigned to either EE or reduced feeding days in the first 3-week period (2019-04-23 to 2019-05-13) and the alternative in the second period (2019-05-15 to 2019-06-04) of equal duration, with a washout period of 24 hours between interventions. They were followed by a 3-week control period (2019-06-06 to 2019-06-26), against which the effects of the treatments were measured. Afterwards, the study cheetahs were placed in crates to which they were habituated and transported by vehicle to the Cango Wildlife Ranch (±500 m). After movement on-exhibit (2019-06-27), a 12-day washout period was introduced for the study cheetahs to habituate to the associated increase in human presence. The assignment of treatments on-exhibit was either AVIs or predator proximity in the first 3-week period (2019-07-09 to 2019-07-29) and the alternative in the second period (2019-07-31 to 2019-08-20) of equal duration, with a washout period of 24 hours between interventions. The study cheetahs' enclosures were rotated accordingly (Fig. 1.2).



Denotation	Date (YY-MM-DD)	Study Phase	Comment
A	2019-04-04	Start of Pre-operative Period	
B	2019-04-08	End of Pre-operative Period	
C	2019-04-10	Start of Post-operative Period	Surgical implantation of data loggers
D	2019-04-20	End of Post-operative Period	
E	2019-04-23	Start of Treatment Period 1	
F	2019-05-13	End of Treatment Period 1	
G	2019-05-15	Start of Treatment Period 2	
H	2019-06-04	End of Treatment Period 2	
I	2019-06-06	Start of Control Period	
J	2019-06-26	End of Control Period	
K	2019-06-27		Moved to Cango Wildlife Ranch (on-exhibit)
L	2019-07-09	Start of Treatment Period 3	
M	2019-07-29	End of Treatment Period 3	
N	2019-07-31	Start of Treatment Period 4	
O	2019-08-20	End of Treatment Period 4	
P	2019-09-12		Surgical removal of data loggers

Figure 1.1. Group 1 experimental design.

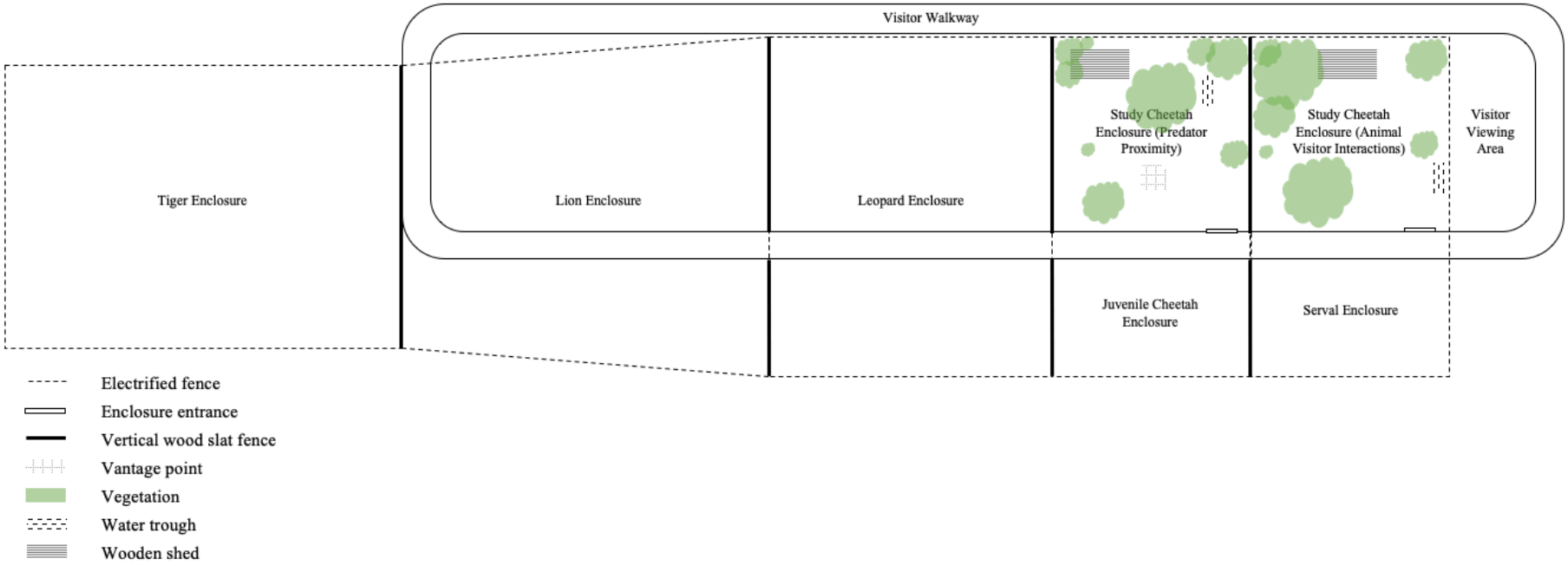
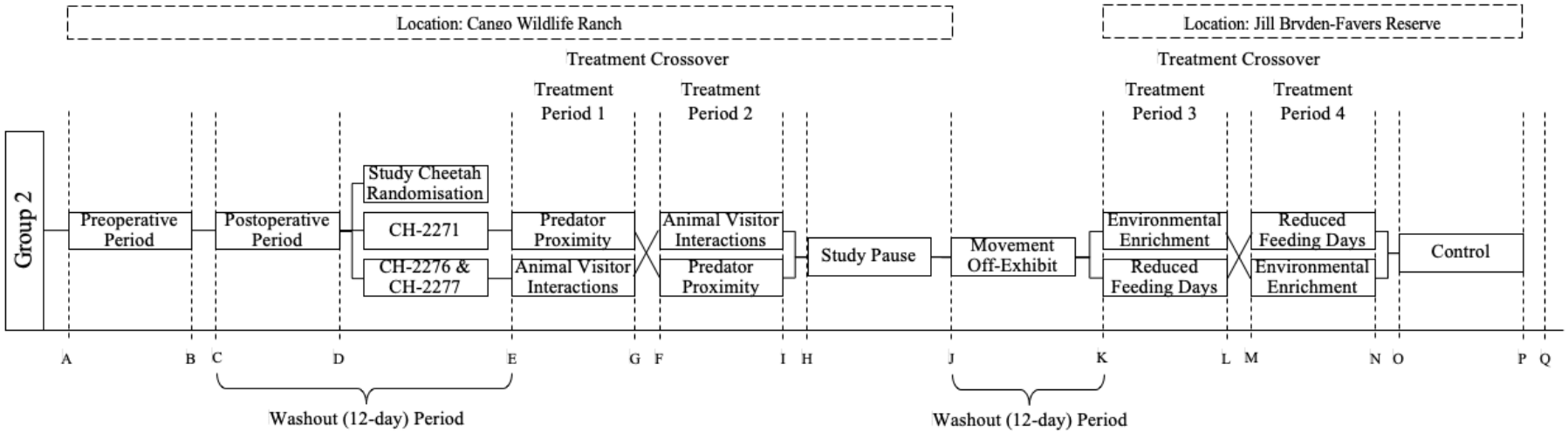


Figure 1.2. Enclosure layout on-exhibit at the Cango Wildlife Ranch and Conservation Centre.

Group 2 (Fig. 1.3) –

While on-exhibit at the Cango Wildlife Ranch, CH-2271, CH-2276, and CH-2277 were assigned to either AVIs or predator proximity in the first 3-week period (2019-04-23 to 2019-05-13) and the alternative in the second period (2019-05-15 to 2019-06-04) of equal duration, with a washout period of 24 hours between interventions. The study cheetahs' enclosures were rotated accordingly (Fig. 1.2). To accommodate the high tourist season and the inherent requirement for cheetahs on-exhibit to participate in AVIs, a study pause was incorporated from 2019-06-05 to 2019-06-27. After that, they were placed in crates to which they were habituated and transported by vehicle to the Jill Bryden-Fayers Reserve (±500 m). After movement (2019-06-27), a 12-day washout period was introduced for the study cheetahs to habituate to the associated decrease in human presence. The assignment of treatments off-exhibit was either EE or reduced feeding days in the first 3-week period (2019-07-09 to 2019-07-29) and the alternative in the second period (2019-07-31 to 2019-08-20) of equal duration, with a washout period of 24 hours between interventions. They were followed by a 3-week control period (2019-08-22 to 2019-09-11), against which the effects of the treatments were measured.



Denotation	Date (YY-MM-DD)	Study Phase	Comment
A	2019-04-04	Start of Pre-operative Period	
B	2019-04-08	End of Pre-operative Period	
C	2019-04-11	Start of Post-operative Period	Surgical implantation of data loggers
D	2019-04-21	End of Post-operative Period	
E	2019-04-23	Start of Treatment Period 1	
F	2019-05-13	End of Treatment Period 1	
G	2019-05-15	Start of Treatment Period 2	
H	2019-06-04	End of Treatment Period 2	
I	2019-06-06		Study paused to accommodate tourism high season
J	2019-06-27		Moved to Jill Bryden-Fayers Reserve (off-exhibit)
K	2019-07-09	Start of Treatment Period 3	
L	2019-07-29	End of Treatment Period 3	
M	2019-07-31	Start of Treatment Period 4	
N	2019-08-20	End of Treatment Period 4	
O	2019-08-22	Start of Control Period	
P	2019-09-11	End of Control Period	
Q	2019-09-13		Surgical removal of data loggers

Figure 1.3. Group 2 experimental design.

The duration of each period, i.e., treatment, control, and washout, was selected to best accommodate the inverse relationship between the number of T_b, HR, and LA recordings made by the biologgers and the lifespan of their batteries.

1.1.6.5 Behavioural Data Collection

Instantaneous scan sampling (Altmann, 1974) with a 5-minute inter-scan interval was used (Quirke & O'Riordan, 2011a; 2011b). During five weekly 60-minute observation sessions, the researcher (KLB) carried out 12 instantaneous scan samples on focal animals, one enclosure at a time and in a randomised order to prevent time-of-day effects. For the study cheetahs housed in pairs (Table 1.1), behavioural data were collected simultaneously using physical characteristics to identify individuals. Observations were performed between 0700 and 1700 based on the operating hours of the Cango Wildlife Ranch and Conservation Centre.

Fifteen behaviours, categorised into inactive, active, and not observed, were recorded (Table 1.2). Preliminary observation of the study cheetahs and the literature on felids' behaviour (Altman *et al.*, 2005; Quirke & O'Riordan, 2011a; 2011b; Regaiolli *et al.*, 2019) informed the ethogram used. Time spent out of sight (hiding or staying away from the human observer) was recorded as its performance may be indicative of a psychological stress response (Carlstead *et al.*, 1993; Davey, 2006; Hosey, 2013; Morgan & Tromborg, 2007; Sellinger & Ha, 2005).

Table 1.2. Ethogram of behaviours recorded and their definitions.

Inactive	
Inactive	Laying/asleep, laying/awake, sitting (stationary in a bipedal position)
Active	
Individual Behaviour	
Appetitive behaviour	Feeding, food anticipatory activity, stalking
Attention	Staring at one area or paying attention to any visual or auditory stimulus
Auto-grooming	Licking or scratching of the own body
Environmental Enrichment	Interacting with an enrichment device by biting, dragging, scratching, or carrying it in the mouth
Locomotion	Jumping, running, solitary play, walking
Maintenance	Drinking, defecating/urinating, yawning
Olfactory exploration	Sniffing the air, an object, or the substrate; performing flehmen.
Scent-marking	Marking substrates or objects in the enclosure by urine-spraying (releasing urine backwards against a vertical surface or object while standing with tail raised vertically), rolling and rubbing (leaving scents on the substrate or on any object, respectively)
Standing	Stationary in a quadrupedal position
Stereotypical	Pacing (repetitive, apparently functionless locomotory movement along a given route uninterrupted by other behaviours)
Vocalisation	Chirping, growling, purring, stutter-barking, or yowling
Social Behaviour	
Affiliative behaviour*	Social play (play-fight, chasing, or playing together with an enrichment item), pawing or rubbing on a conspecific, social grooming (licking a conspecific or being licked), paying attention to conspecifics by observing them with interest, and interacting with human caretakers
Agnostic behaviour*	Aggression, dominance mount, threat display
Interspecific behaviour	Paying attention to another species' presence
Not Observed	
Out of sight	Focal animal is not visible from the point of observation/behaviour unknown

*Include actions performed or received by the focal animal. Preliminary observation of the study cheetahs and the literature on felids' behaviour (Altman *et al.*, 2005; Quirke & O'Riordan, 2011a; 2011b; Regaiolli *et al.*, 2019) informed the ethogram.

A total of 900 (AVIs: n = 180, EE: n = 180, reduced feeding days: n = 180, predator proximity: n = 180, and control: n = 180) scan samples per cheetah were collected across the 15-week study period.

1.1.6.6 Faecal Sample Collection and Consistency Scoring

To differentiate between individual faecal samples in the event of paired housing (Table 1.1), 1 tbsp of uncooked rice was thoroughly mixed into the diet of study cheetahs CH-2206 and CH-2277 once per day. The caretakers monitored the study cheetahs during feeding times to ensure the sufficient consumption of rice and prevent meal sharing. Only faeces found to have uncooked rice were considered to have originated from those individuals fed rice. Due to the operating hours of the Cango Wildlife Ranch and Conservation Centre, the enclosures could not be entered between 1800–0800 hours. As such, once daily between 0800–1000 hours, faeces from the previous night were collected from each enclosure within 16 hours of defecation (4–10 °C T_a) (Ludwig *et al.*, 2013).

Following sample collection, the researcher (KLB) assigned FCS as per a five-point grading system, where two points (grade 4: firm and dry and grade 5: firm) were considered to be 'normal,' and three points (grades 1–3: liquid, soft without shape, and soft with shape) were considered to be 'suboptimal' (adapted from AZA Tiger Species Survival Plan®, 2016; Whitehouse-Tedd *et al.*, 2015). Afterwards, the samples were deposited into appropriately labelled (sample collection date and study cheetah and sample identification [ID] numbers) 50 mL polypropylene specimen containers and frozen at –20 °C.

In total, 574 (AVIs: n = 121, EE: n = 116, reduced feeding days: n = 105, predator proximity: n = 124, and control: n = 108) faecal samples were collected from the study cheetahs across the 15-week study period.

1.1.6.7 Faecal Steroid Extraction and Quantification

Following completion of the experimental trials, faecal samples were transported frozen to the Endocrine Research Laboratory, University of Pretoria, South Africa. Faecal steroids were extracted and subsequently analysed for fGCM concentration.

Frozen faecal samples were lyophilised, and the resultant dry faeces were pulverised and sieved through a mesh strainer to remove fibrous material (Fieß *et al.*, 1999). Between 0.050–0.055 g of faecal powder was weighed per sample and extracted using 3 mL of 80% ethanol. The suspensions were vortexed for 15 minutes and centrifuged at 1500 x g for 10 minutes (Ganswindt *et al.*, 2010). Supernatants were decanted into 1.5 mL safe-lock microcentrifuge tubes, labelled, and stored frozen at –20 °C until further analysis.

Immunoreactive fGCM concentrations were quantified using a corticosterone-3-CMO EIA (Ludwig *et al.*, 2013; Palme & Möstl, 1997) according to procedures described by Ganswindt *et al.* (2002). Detailed assay characteristics, including full descriptions of the assay components and cross-reactivities, are provided by Palme & Möstl (1997). The parallelism between the standard curve and serial dilutions of pooled faecal extracts was performed by Ludwig *et al.* (2013). The sensitivity of the EIA used at 90% binding was 3.6 ng/g faecal dry weight (DW). Inter-assay coefficients of variation (CV), determined by repeated measurements of low- and high-quality controls, were 11,74% and 12.91%, respectively, and intra-assay CV were 5.59% and 6.61%, respectively.

Faecal steroid concentrations are presented as µg/g faecal DW.

1.1.6.8 Ethical Statement

All of the experimental procedures involving the study cheetahs were approved by the University of Pretoria's Animal (clearance number V075-18) and Research Ethics Committees (clearance number REC069-18).

1.1.6.9 Data Preparation

1.1.6.9.1 Behavioural Observations

The frequency of each behaviour performed by each of the study cheetahs during each observation session was expressed as a proportion of the total number of scan samples recorded

during the observation session (Quirke & O'Riordan, 2011a; 2011b). The resultant data indicated the proportion of scan samples in which each behaviour was observed during the treatment and control for each of the study cheetahs.

1.1.6.9.2 Faecal Glucocorticoid Metabolite Concentrations and Consistency Scores

Diurnal secretory patterns of GCs are largely attenuated in faeces (Keay *et al.*, 2006). However, as discussed above, species and individual differences can influence fGCM levels or GI transit time (Palme, 2005). After ACTH injection, Terio *et al.* (1999) observed peak GC metabolite concentrations in the first faecal sample collected from cheetahs, similar to the faecal cortisol excretion rate in domestic cats (Graham & Brown, 1996). Therefore, to accommodate the 24-hour gut passage rate and excretory pattern specific to the cheetah, fGCM concentration and FCS were presumed to represent the previous day's intervention. The crossover design used is such that the study cheetahs served as their control, eliminating interindividual variability (Maclure, 1991).

1.1.6.9.3 Body Temperature, Heart Rate, and Locomotor Activity Recordings

Battery malfunction of the biologgers implanted in four of the six study cheetahs (CH-2207, -2271, -2276, and -2277) impeded the acquisition of complete T_b, HR, and LA data sets for those individuals. Due to the catastrophic failure of CH-2207's biologging device battery shortly after implantation (>15 days), it was decided for this animal to be excluded from the research.

The initial examination of the raw HR data for the study cheetahs with functional biologgers (CH-2205 and -2206), in addition to partial data for CH-2271, CH-2276, and CH-2277, revealed values ranging from 0 to 1005 bpm, the extremes of which were likely due to incomplete, low-quality readings or implant movement within the pectoral muscle when the study cheetahs were active. To remove erroneous measurements, ensuring only plausible

values were included in the analyses, upper and lower thresholds were created. At the low end of the range, all readings of 0 bpm were removed, creating a new minimum of 30 bpm. For the upper end, where most unlikely measurements occurred, HR values were filtered informed by the literature on ECG data in the cheetah (Button *et al.*, 1981; Schumacher *et al.*, 2003), in addition to those to which QI_3 was assigned (see section 1.1.6.2 for more information of ECG-derived validation of HR measurements). Once filtered, all HR recordings (n = 226564/250820, which represented over 90% of the raw data initially captured) fell within the 30- and 200-bpm thresholds set. As T_b recordings were not sensitive to implant movement within the pectoral muscle and the lack of extreme values, they were not filtered.

To investigate the circadian patterns of T_b, HR, and LA, data were categorised into six time periods based on when they were recorded, namely (i) early morning (0000–0400 hours), (ii) morning (0400–0800 hours), (iii) late morning (0800–1200), (iv) afternoon (1200–1600 hours), (v) evening (1600–2000 hours), and (vi) night (2000–0000 hours).

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Chapter 2: Responses to Reduced Feeding Days in Captive-Born Cheetahs (*Acinonyx jubatus*): Implications for Behavioural and Physiological Stress and Gastrointestinal Health

ABSTRACT. An epidemiological relationship between unnatural diet composition and feeding regimes and the various diseases prevalent in captive cheetahs has been suggested. This study investigated the response of captive-born (hand-reared) cheetahs (n = 5) to a more naturalistic reduced feeding days schedule characterised by offering larger quantities of food less frequently for three weeks. Using emerging biologging technology and more traditional measures of stress-related behavioural and physiological responses, variations in body temperature, heart rate (HR), locomotor activity (LA), behaviour, faecal glucocorticoid metabolite concentration, and faecal consistency score (FCS) were documented. Reduced feeding days elicited in the study cheetahs higher FCS ($p < .01$). The more natural feeding pattern resulted in lower inactivity levels and HR ($p < .0001$) and higher LA ($p < .0001$), consistent with the widespread use of food-based enrichment. Sex and age group affected the study cheetahs' activity levels and physiological responses, which may be attributable to the study cheetahs all experiencing some level of arousal and/or activation of the physiological stress response. Faecal glucocorticoid metabolite concentration and LA appeared to demonstrate habituation. This study's findings encourage a more naturalistic reduced feeding days schedule to mediate the unnatural composition of horsemeat-based diets routinely fed to cheetahs in captivity and as an effective environmental enrichment strategy.

2.1 INTRODUCTION

Depending on prey abundance and vulnerability, free-ranging cheetahs satisfy their energetic demands by eating a diverse range of vertebrate species, from birds and hares to adult ungulates (Marker *et al.*, 2003). They seldom eat daily or at a fixed interval but are opportunistic hunters, specialising in rapid pursuits (Mills & Harvey, 2001). Other morphological features, such as dental adaptations (Steenkamp *et al.*, 2017) and wide nares, enable the cheetah to consume a large amount of food (>10 kg) in less than 2 hours (Mills & Bester, 2005; Phillips, 1993; Schaller, 1968). In contrast to the wild, captive cheetahs are fed a

nonvarying diet of skinned muscle meat from livestock species, commercially prepared carnivore diets, carcass parts, or a mixture of these (Tordiffe *et al.*, 2016; Whitehouse-Tedd *et al.*, 2015), offered at fixed intervals once or twice daily, or with one fast day per week. The species' naturally varied source of nutrients and the feeding behaviours involved in their acquisition are, understandably, difficult to replicate. However, unnatural diet composition and feeding regimes in captivity have been suggested as aetiological agents in the high incidence of GI and metabolic diseases (Lane *et al.*, 2012; Whitehouse-Tedd *et al.*, 2015).

Crude protein is anticipated to be high in all carnivorous diets. Still, the frequency with which it is fed in captivity and its quality may influence GI health by changing the amount of protein reaching the intestine. Once there, bacterial fermentation of the excess dietary protein modifies microbiota composition in favour of proteolytic bacteria, some of which can be pathogenic in high concentrations (Becker *et al.*, 2014; Wasimuddin *et al.*, 2017) and produce putrefactive compounds (e.g., ammonia, indoles, phenols) linked with several disease states (Depauw *et al.*, 2012a; 2013; Rochus *et al.*, 2013).

Chronic gastritis, associated with gut bacteria of the genus *Helicobacter* (Eaton *et al.*, 1993; Terio *et al.*, 2005), is the basis of significant morbidity and mortality in geographically distinct populations of captive cheetahs (Munson, 1993; Munson *et al.*, 1999). However, despite colonisation by abundant spiral bacteria, some cheetahs in captivity and most of their wild counterparts show no clinical manifestations of gastritis, indicating the disease aetiopathogenesis is multifactorial. Using nutritional epidemiology, Whitehouse-Tedd *et al.* (2015) identified feeding horsemeat as a significant risk factor for gastritis in cheetahs, which could be related to its protein content (Lee *et al.*, 2007) and digestibility (Vester *et al.*, 2010). Since horse is routinely fed as muscle meat without low to non-digestible (glyco)protein-rich matter (e.g., bone, tendons, cartilage), its relative lack of 'animal fibre' may further increase

putrefaction of digesta in the intestine. This was confirmed by studies in which cheetahs were shown to produce faeces with lower concentrations of putrefactive compounds and improved consistency following a dietary change from raw meat to whole rabbit carcasses (Depauw *et al.*, 2012b; 2013). In addition, animal fibre decreases GI inflammation (Depauw *et al.*, 2014).

Transforming gut bacteria-derived putrefactants into toxic metabolites negatively affects multiple organ systems and metabolic pathways. Uraemic toxicity of indoxyl sulphate is associated with the progression of chronic kidney failure (Niwa *et al.*, 2010), a significant cause of death in captive cheetahs (Bolton & Munson, 1999; Munson, 1993; Munson *et al.*, 1999; Papendick *et al.*, 1997). The renal lesion in cheetahs resembles diabetic glomerulopathy in humans and chronic progressive nephropathy in rats (Bolton & Munson, 1999). Since high-protein diets, particularly when fed *ad-libitum* and continually, accelerate glomerulosclerosis in rats (Bertani *et al.*, 1989; Brenner *et al.*, 1982; Olson & Heptinstall, 1988), the high incidence in captive cheetahs may have comparable dietary risk factors.

A growing body of evidence supports feeding restrictions potentially shaping the gut ecosystem, function, and interaction with the host. Intermittent fasting (IF) has beneficial regulatory effects on immune homeostasis and intestinal microbiota composition in humans and rodent models (Li *et al.*, 2020; 2017; Maifeld *et al.*, 2021; Patterson & Sears, 2017). Moreover, IF attenuates the inflammatory response and oxidative stress in colon tissues (Li *et al.*, 2020; Zhang *et al.*, 2020). Following the adaption of captive lions from a conventional zoo feeding programme of predictable, fixed, small daily meals to a more natural gorge and fast feeding schedule of larger, more infrequent meals, Altman *et al.* (2005) reported improved digestibility of a horsemeat-based diet. Considering the species' natural feeding ecology, fasting conditions could have similar digestive health benefits for the cheetah as they did for the lion.

This study's overall aim was to investigate the response of captive-born (hand-reared) cheetahs to a more naturalistic reduced feeding days schedule characterised by offering larger quantities of food less frequently. I hypothesised that the more natural feeding pattern would influence the GI environment, including microbial parameters and/or GI physiology or functional traits. Faecal consistency scoring was applied as a preliminary indicator of the study cheetahs' GI health (Whitehouse-Tedd *et al.*, 2015). Additionally, I hypothesised that reducing feeding days would induce an acute stress response. As established stress-related markers, behavioural observations (Quirke *et al.*, 2012) and fGCM concentration analysis (Terio *et al.*, 1999) were performed. A secondary objective was to explore the potential of biologging technology for measuring stress in the cheetah by recording concurrent T_b, HR, and LA. I predicted higher fGCM concentration, T_b, and HR, consistent with a physiological stress response.

2.2 MATERIALS AND METHODS

2.2.1 Study Site and Animals

The experimental trials occurred between April and September 2019 at the Cango Wildlife Ranch and Conservation Centre (33°33'S, 22°12'E) 4 km north of Oudtshoorn, in the Western Cape of South Africa (see section 1.1.6.1 for more information on the study site and animals). Three male (CH-2205, -2206, and -2271) and two female (CH-2276 and -2277) resident adult cheetahs (Table 1.1) were allocated to this study.

2.2.2 Body Temperature, Heart Rate, and Locomotor Activity Measurement Intervals

A single cardiac-, temperature- and movement-sensitive biologger (DST centi-HRT ACT, Star-Oddi, Gardabaer, Iceland) with the dimensions of 46 mm x 15 mm x 15 cm and weighing approximately 19 g was implanted in each of the study cheetahs. The biologgers were calibrated individually against a high-accuracy thermometer (Hart 1504, Fluke, Utah, US). Calibrated accuracy was better than 0.1 °C. Before surgical implantation, the biologgers were set to record tri-axial LA, i.e., heave, surge, sway, every minute for the ODBA and T_b (°C) and leadless single-channel ECG-derived HR (bpm) every 5 minutes at 200 Hz. Representative traces of raw ECG recordings were saved every 24 hours for validating the HR measurements' QI (where QI_0 was the highest quality and QI_1, QI_2, and QI_3 were of progressively reduced quality). Afterwards, the biologging devices were sterilised using ethylene oxide and implanted (see section 1.1.6.3 in the introduction for more information on the surgical procedure).

2.2.3 Experimental Design

The study cheetahs housed individually (CH-2271) or in pairs (CH-2205 and -2206, and CH-2276 and -2277, respectively) were assigned randomly to either the treatment, i.e., a more

naturalistic reduced feeding days schedule or the control in an initial 3-week period at variable times and the alternative in a period following of equal duration (see section 1.1.6.4 for more information on the experimental design). During the treatment, the study cheetahs were housed off-exhibit at the Jill Bryden-Fayers Reserve neighbouring the Ranch. They were fed on a more naturalistic schedule of four once-daily meals per week. Following the introduction of additional fast days, larger than regular meals were offered on feed days, not to be contrary to the study cheetahs' welfare. The three-year-old study cheetahs (CH-2205, -2206, and -2207) were fed 2.7 kg per meal four days a week, and the two-year-old study cheetahs (CH-2271, -2276, and -2277) were fed 2.5 kg per meal four days a week. Weekly fasting days were allocated to Wednesday, Saturday, and Sunday.

During the control, the three-year-old study cheetahs were fed 1.8 kg portioned into two daily rations six days a week, and the two-year-old study cheetahs were fed 1.6 kg portioned into two daily rations six days a week. Weekly fasting days were allocated to Sundays. Other than the specific intervention being investigated, i.e., a more naturalistic reduced feeding days schedule, the study cheetahs' environment, housing, and management (as described in section 1.1.6.1) were maintained across the treatment and control, including bones offered randomly once a week in place of the day's meals.

Throughout the treatment and control, the study cheetahs were monitored regarding (i) behaviour, (ii) fGCM concentration, and (iii) FCS, as well as (iv) T_b, HR, and LA concurrently recorded by implanted data loggers.

2.2.4 Behavioural Data Collection

Instantaneous scan sampling (Altmann, 1974) with a 5-minute inter-scan interval was used in this study (Quirke & O'Riordan, 2011a; 2011b). During five weekly 60-minute observation

sessions, the researcher (KLB) carried out 12 instantaneous scan samples on focal animals, one enclosure at a time and in a randomised order to prevent time-of-day effects. For the study cheetahs housed in pairs (Table 1.1), behavioural data were collected simultaneously using physical characteristics to identify individuals. Observations were performed between 0700 and 1700 based on the operating hours of the Cango Wildlife Ranch and Conservation Centre.

Fifteen behaviours, categorised into inactive, active, and not observed, were recorded (Table 1.2). Preliminary observation of the study cheetahs and the literature on felids' behaviour (Altman *et al.*, 2005; Quirke & O'Riordan, 2011a; 2011b; Regaiolli *et al.*, 2019) informed the ethogram used. Time spent out of sight (hiding or staying away from the human observer) was recorded as its performance may be indicative of a psychological stress response (Carlstead *et al.*, 1993; Davey, 2006; Hosey, 2013; Morgan & Tromborg, 2007; Sellinger & Ha, 2005).

2.2.5 Faecal Sample Collection and Consistency Scoring

To differentiate between individual faecal samples in the event of paired housing (Table 1.1), 1 tbsp of uncooked rice was thoroughly mixed into the diet of study cheetahs CH-2206 and CH-2277 once per day. The caretakers monitored the study cheetahs during feeding times to ensure the sufficient consumption of rice and prevent meal sharing. Only faeces found to have uncooked rice were considered to have originated from those individuals fed rice. Due to the operating hours of the Cango Wildlife Ranch and Conservation Centre, the enclosures could not be entered between 1800–0800 hours. As such, once daily between 0800–1000 hours, faeces from the previous night were collected from each enclosure within 16 hours of defecation (4–10 °C T_a) (Ludwig *et al.*, 2013).

Following sample collection, the researcher (KLB) assigned FCS as per a five-point grading system, where two points (grade 4: firm and dry and grade 5: firm) were considered to be 'normal,' and three points (grades 1–3: liquid, soft without shape, and soft with shape) were considered to be 'suboptimal' (adapted from AZA Tiger Species Survival Plan®, 2016; Whitehouse-Tedd *et al.*, 2015). Afterwards, the samples were deposited into appropriately labelled (sample collection date and study cheetah and sample ID numbers) 50 mL polypropylene specimen containers and frozen at –20 °C.

2.2.6 Faecal Steroid Extraction and Quantification

Following completion of the experimental trials, faecal samples were transported frozen to the Endocrine Research Laboratory, University of Pretoria, South Africa. Faecal steroids were extracted and analysed for fGCM concentration as described in section 1.1.6.7.

2.2.7 Ethical Statement

All of the experimental procedures involving the study cheetahs were approved by the University of Pretoria's Animal (clearance number V075-18) and Research Ethics Committees (clearance number REC069-18).

2.2.8 Statistical Analysis

Statistical analysis was performed using Microsoft Excel (version 16.0) and JMP Pro software (version 16.0) for Windows, developed by SAS Institute Inc (North Carolina, US). Raw data was manipulated before detailed analyses (see section 1.1.6.9 for more information on the data preparation). The data were screened for univariate outliers greater than three interquartile ranges away from the 99.5th or 0.05th percentiles (LA: n = 40), which were subsequently excluded from descriptive statistics and analyses. Normal distribution and

homogeneity of variance were verified using Anderson-Darling and Levene's tests, respectively. Box-Cox transformations were used to more closely satisfy the assumption of normality and homogeneity in the case of departure. To maintain statistical integrity, the data were back-transformed for descriptive statistics and visual representation. A mixed model for repeated measures (MMRM) analysis was conducted to investigate the following:

- The random effect of the study cheetah, the independent fixed effect of the study period, and the interaction fixed effects of the study period and (i) feed versus starve day, (ii) sex, and (iii) age group on each behaviour.
- The random effect of the study cheetah and the fixed effect of control versus treatment week (one, two, or three) on each behaviour.
- The random effect of the study cheetah, the independent fixed effect of the study period, and the interaction fixed effects of the study period and (i) feed versus starve day, (ii) sex, and (iii) age group on fGCM concentration and FCS.
- The random effect of the study cheetah and the fixed effect of control versus treatment week (one, two, or three) on fGCM concentration and FCS.
- The random effect of the study cheetah and the fixed effects of hours within the day and part of the day on T_b, HR, and LA.
- The random effect of the study cheetah, the independent fixed effect of the study period, and the interaction fixed effects of the study period and (i) feed versus starve day, (ii) sex, and (iii) age group on T_b, HR, and LA.
- The random effect of the study cheetah and the fixed effect of control versus treatment week (one, two, or three) on T_b, HR, and LA.
- The random effect of the study cheetah and the independent and interaction fixed effects of mealtime and part of the day on T_b, HR, and LA.

Tukey's honestly significant difference (HSD) post hoc tests were performed for multiple pair-wise comparisons. The treatment effect size was calculated using Cohen's d. Descriptive statistics were reported as median ± median absolute deviation (MAD), and the significance level, α, was set at 0.05.

2.3 RESULTS

This study's overall aim was to investigate the response of captive-born (hand-reared) cheetahs to a more naturalistic reduced feeding days schedule characterised by offering larger quantities of food less frequently. It investigated whether demographic factors (sex and age group) and habituation affected this response.

2.3.1 Behavioural Observations

Behavioural data were Box-Cox transformed to more closely satisfy the assumption of normality and homogeneity. The study cheetahs spent the majority of their time inactive (for the control: 47% and the treatment: 41%) (Fig. 2.1). Inactivity levels were lower during the treatment than in the control (~6%) in addition to greater displays of attention (~3%) and locomotion (~5%). The MMRM analysis revealed that these numerical differences failed to achieve statistical significance. Post hoc comparisons using Tukey's HSD test revealed that interspecific behaviour was significantly higher on treatment feed days than on treatment starve days ($t_{66.4}$ = 3.40, p = .0062). Post hoc comparisons using Tukey's HSD test revealed that the female study cheetahs showed significantly higher auto-grooming during the treatment than in the control ($t_{15.0}$ = -3.00, p = .0400). Post hoc comparisons using Tukey's HSD test revealed that the two-year-old study cheetahs showed significantly higher auto-grooming during the treatment than in the control ($t_{15.0}$ = -3.72, p = .0100).

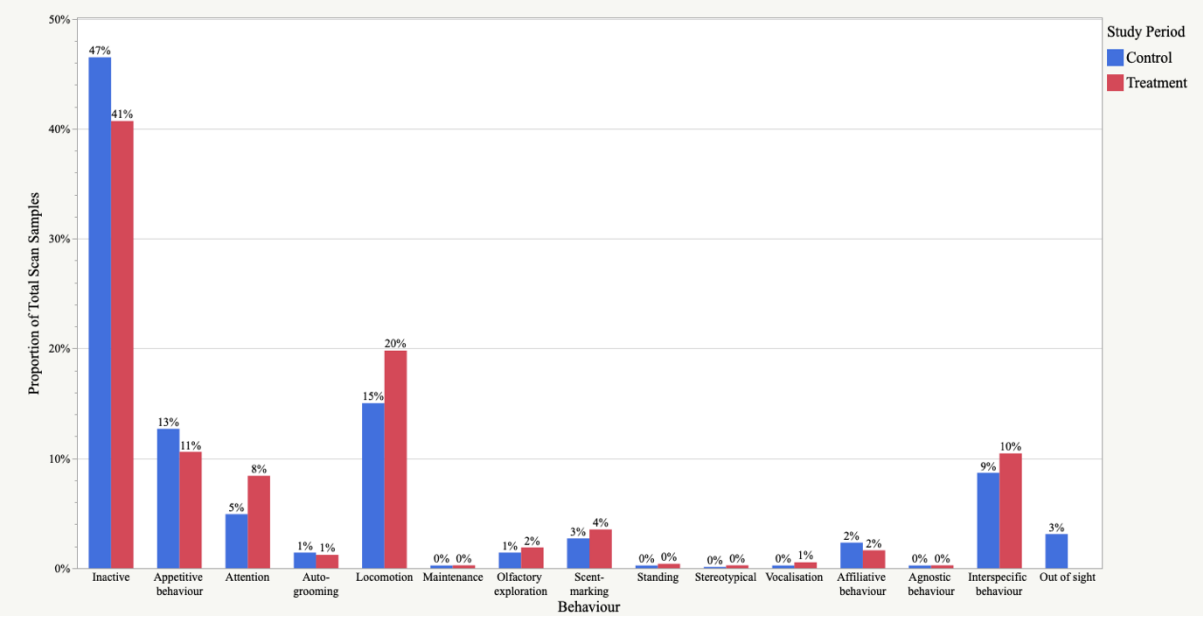

Figure 2.1. Bar chart of each behaviour for the study cheetahs (CH-2205, -2206, -2271, -2276, and -2277) during the control and treatment. Statistics were performed by a mixed model for repeated measures. The number above the bar indicates the proportion of total scan samples study cheetahs were observed performing each behaviour during the control and treatment, respectively.

No significant effect.

2.3.2 Faecal Glucocorticoid Metabolite Concentrations

Faecal glucocorticoid metabolite concentration data were Box-Cox transformed to more closely satisfy the assumption of normality and homogeneity. The median fGCM concentration was higher during the treatment (1.09 ± 0.30 µg/g DW) than in the control (0.90 ± 0.21 µg/g DW). The MMRM analysis revealed that this numerical difference failed to achieve statistical significance. Post hoc comparisons using Tukey's HSD test revealed that fGCM concentration was significantly higher on treatment feed days (1.13 ± 0.28 µg/g DW) than on control feed days (0.90 ± 0.22 µg/g DW; $t_{167.7}$ = -2.84, p = .0256) (Fig. 2.2i). Post hoc comparisons using Tukey's HSD test revealed that the fGCM concentration of the male study cheetahs was significantly higher during the treatment (1.19 ± 0.21 µg/g DW) than in the control (0.95 ±

0.27 µg/g DW; $t_{167.3}$ = -2.94, p = .0193) (Fig. 2.3i). Post hoc comparisons using Tukey's HSD test revealed that the fGCM concentration of the three-year-old study cheetahs was significantly higher during the treatment (1.18 ± 0.21 µg/g DW) than in the control (0.93 ± 0.30 µg/g DW; $t_{167.6}$ = -2.83, p = .0267) (Fig. 2.4i).

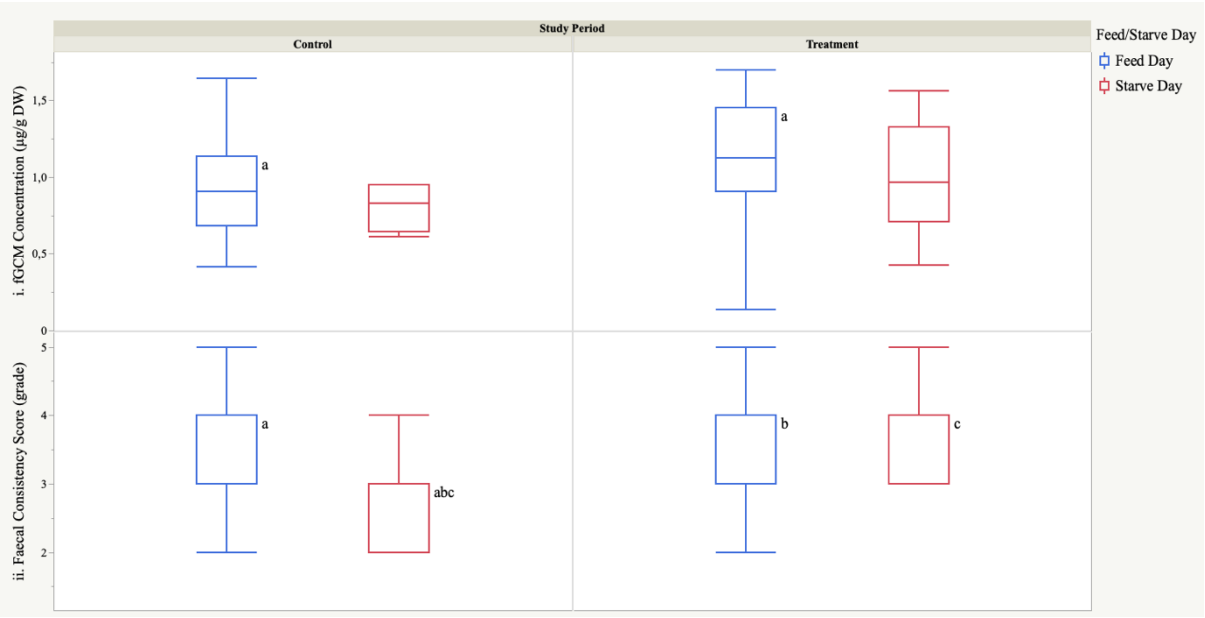

Figure 2.2. Box and whisker plot of (i) faecal glucocorticoid metabolite (fGCM) concentration (µg/g dry weight [DW]) and (ii) faecal consistency score (grade) for the study cheetahs (CH-2205, -2206, -2271, -2276, and -2277). Effect of feed versus starve day during the control and treatment. Statistics were performed by a mixed model for repeated measures and Tukey's HSD post hoc test.

(i) a: p = .0256. (ii) a: p = .0030, b: p = .0013, and c: p = .0025.

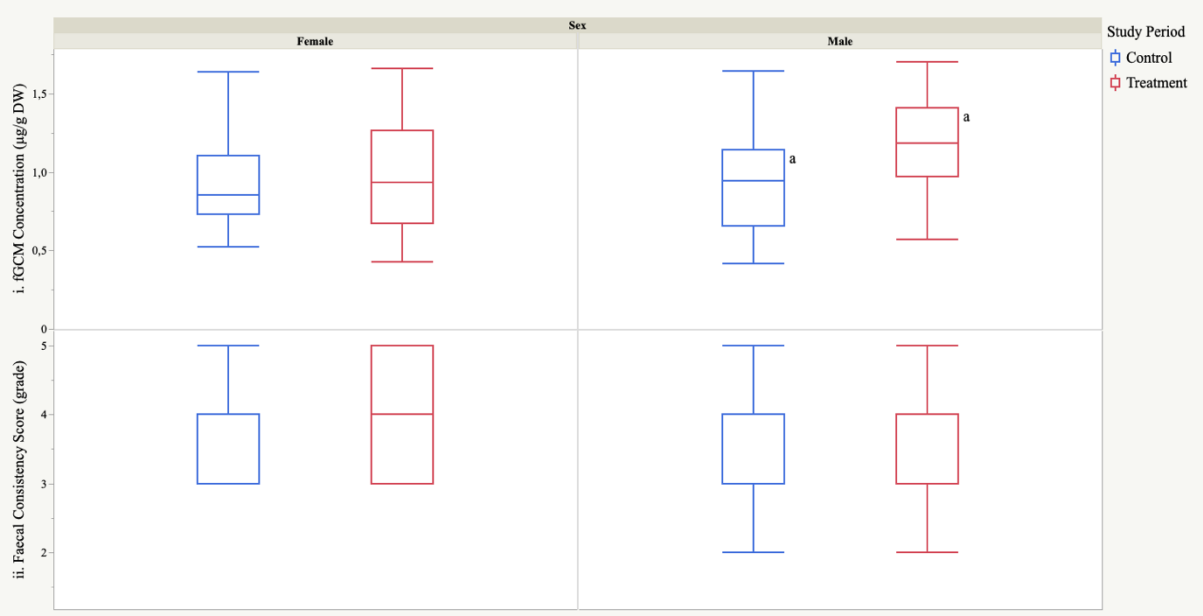

Figure 2.3. Box and whisker plot of (i) faecal glucocorticoid metabolite (fGCM) concentration (µg/g dry weight [DW]) and (ii) faecal consistency score (grade) for the study cheetahs (CH-2205, -2206, -2271, -2276, and -2277). Effect of sex (female and male) during the control and treatment. Statistics were performed by a mixed model for repeated measures and Tukey's HSD post hoc test.

(i) a: $p = .0193$.

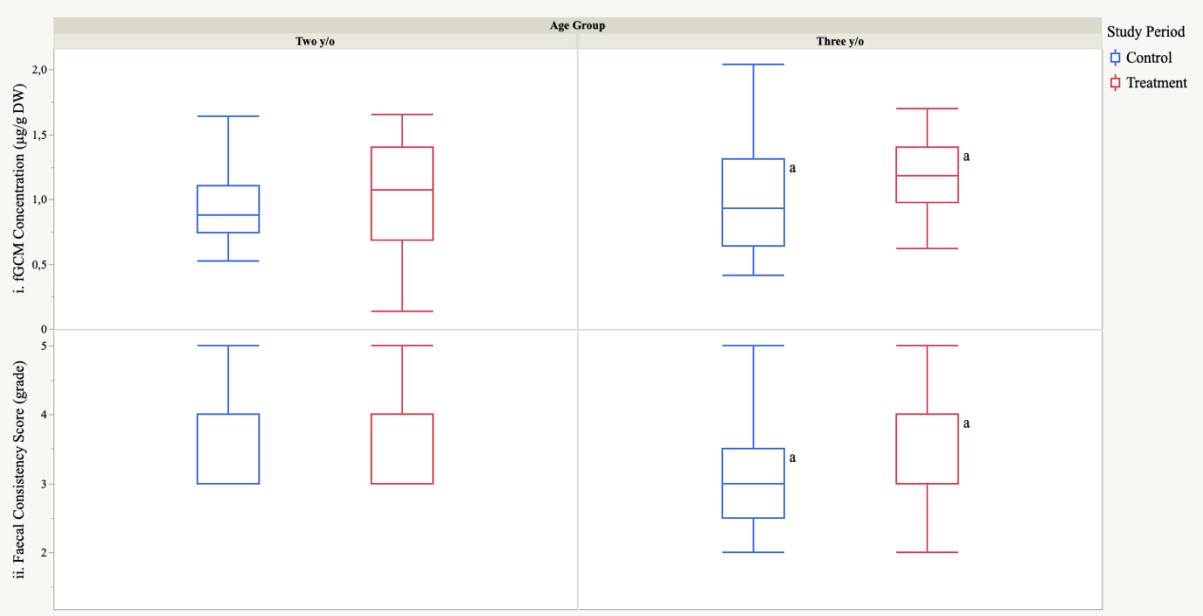

Figure 2.4. Box and whisker plot of (i) faecal glucocorticoid metabolite (fGCM) concentration (µg/g dry weight [DW]) and (ii) faecal consistency score (grade) for the study cheetahs (CH-2205, -2206, -2271, -2276, and -2277). Effect of age group (two and three years old [y/o]) during the control and treatment. Statistics were performed by a mixed model for repeated measures and Tukey's HSD post hoc test.

(i) a: $p = .0267$. (ii) a: $p = .0309$.

Post hoc comparisons using Tukey's HSD test revealed that fGCM concentration was significantly higher during week two of the treatment (1.17 ± 0.29 µg/g DW) than in the control (0.90 ± 0.21 µg/g DW; $t_{166.3} = -2.86$, $p = .0248$) (Fig. 2.5i).

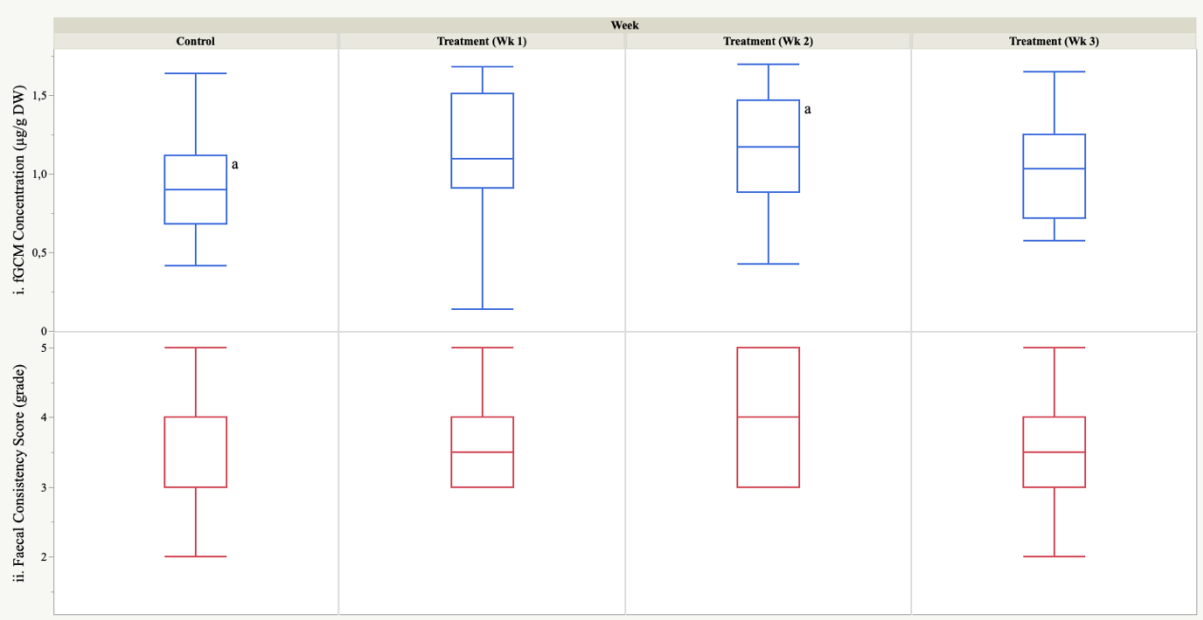

Figure 2.5. Box and whisker plot of (i) faecal glucocorticoid metabolite (fGCM) concentration (µg/g dry weight [DW]) and (ii) faecal consistency score (grade) for the study cheetahs (CH-2205, -2206, -2271, -2276, and -2277). Effect of control versus treatment week (Wk; one, two, or three). Statistics were performed by a mixed model for repeated measures and Tukey's HSD post hoc test.

(i) a: $p = .0248$.

Cohen's d revealed a trivial effect size ($d = 0.17$; $t_{172} = 2.52$, $p = .0127$) on fGCM concentration (Table 2.1).

Table 2.1. Treatment effect size on behaviour, faecal glucocorticoid metabolite (fGCM) concentration (µg/g dry weight [DW]), faecal consistency score, body temperature (^{0}C), heart rate (beats per minute [bpm]), and locomotor activity (overall dynamic body acceleration [ODBA]) for the study cheetahs (CH-2205, -2206, -2271, -2276, and -2277). Calculations were performed using Cohen's d.

df: degrees of freedom, CI: confidence interval.

	t	*p*	df	Cohen's d	95% CI for Cohen's d	
					Lower	Upper
Behaviour						
Inactive	0.93	.3556	112	0.17	-0.19	0.54
Appetitive behaviour	0.34	.7377	45	0.10	-0.48	0.67
Attention	1.10	.2781	52	0.30	-0.24	0.85
Auto-grooming	1.06	.3051	17	0.49	-0.44	1.39
Locomotion	1.09	.2783	86	0.23	-0.19	0.65
Olfactory exploration	0.84	.4109	18	0.38	-0.52	1.28
Scent-marking	0.57	.5738	34	0.19	-0.47	0.85
Vocalisation	1.34	.2722	3	1.22	-0.87	3.17
Affiliative behaviour	1.89	.0715	23	0.81	-0.07	1.67
Interspecific behaviour	0.06	.9493	70	0.02	-0.45	0.48
fGCM Concentration (µg/g DW)	2.52	.0127	172	0.17	0.04	0.30
Faecal Consistency Score (grade)	1.47	.1428	172	0.22	-0.08	0.52
Body Temperature (°C)	2.89	.0039	33441	0.03	0.01	0.05
Heart Rate (bpm)	8.44	< .0001	30223	0.10	0.07	0.12
Locomotor Activity (ODBA)	18.20	< .0001	33401	0.20	0.18	0.22

2.3.3 Faecal Consistency Scores

Faecal consistency score data were Box-Cox transformed to more closely satisfy the assumption of normality and homogeneity. The soft with shape faecal grade was the most frequently recorded in the study cheetahs (for the control: 45.5% and the treatment: 45.3%) (Fig. 2.6). During the treatment, the 'normal' grades of firm (~0.4%) and firm and dry (~7.6%) were higher and the 'suboptimal' grade of soft without shape was lower (~7.9%) than in the control. The MMRM analysis revealed that FCS was significantly higher during the treatment (4 ± 1) than in the control (3 ± 1; $F_{1,166.8}$ = 12.79, p = .0005). Post hoc comparisons using Tukey's HSD test revealed that FCS was significantly lower on control starve days (2 ± 0) than on control feed days (3 ± 1; $t_{167.1}$ = 3.53, p = .0030), treatment feed days (4 ± 1; $t_{167.1}$ = -3.76, p = .0013), and treatment starve days (4 ± 1; $t_{167.1}$ = -3.58, p = .0025) (Fig. 2.2ii). Post hoc

comparisons using Tukey's HSD test revealed that the FCS of the three-year-old study cheetahs was significantly higher during the treatment (3 ± 0) than in the control (3 ± 0; $t_{168.0} = -2.78$, $p = .0309$) (Fig. 2.4ii).

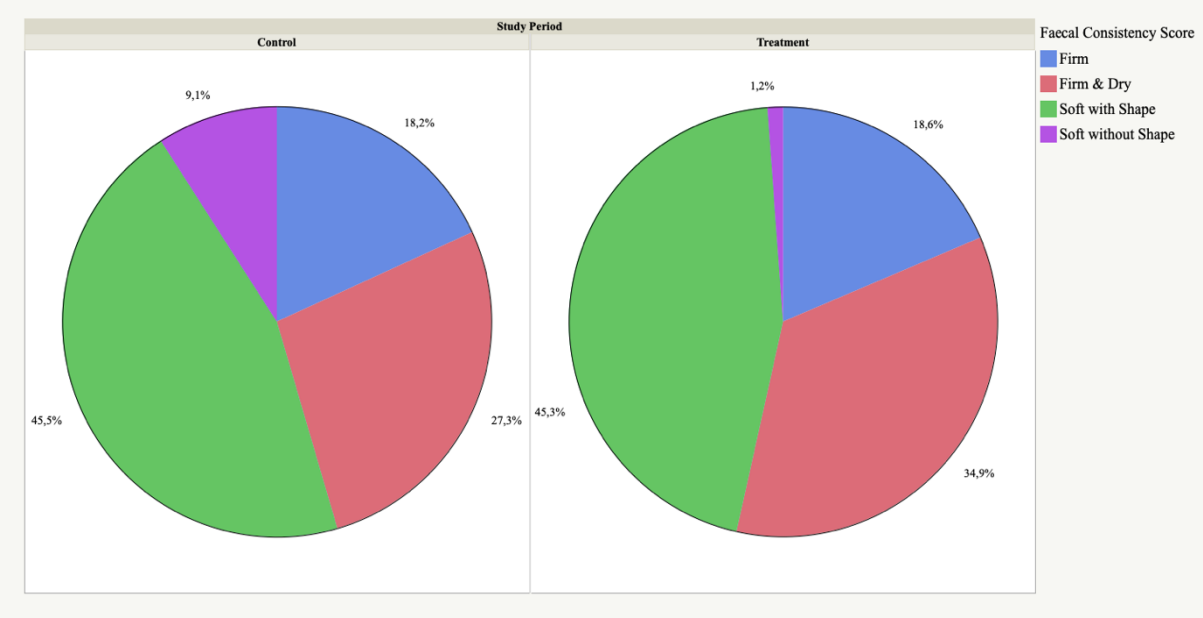

Figure 2.6. Pie chart of faecal consistency score for the study cheetahs (CH-2205, -2206, -2271, -2276, and -2277) during the control and treatment, where firm and firm and dry were considered to be 'normal' and liquid, soft without shape, and soft with shape were considered to be 'suboptimal' (adapted from AZA Tiger Species Survival Plan®, 2016; Whitehouse-Tedd *et al.*, 2015). The number above the slice indicates the proportion of total faecal samples collected for each score.

2.3.4 Body Temperature, Heart Rate, and Locomotor Activity Recordings

Battery malfunction of the biologgers implanted in the study cheetahs CH-2271, CH-2276, and CH-2277 impeded the acquisition of complete T_b, HR, and LA data sets for those individuals. The initial examination of the raw HR data for the study cheetahs with functional data loggers (CH-2205 and -2206), in addition to partial data for CH-2276, revealed values ranging from 0 to 1005 bpm, the extremes of which were likely because of incomplete, low-quality readings or implant movement within the pectoral muscle when the study cheetahs were

active. To remove erroneous measurements, ensuring only plausible values were included in the analyses, upper and lower thresholds were created (see section 1.1.6.9.3 for more information on the data preparation of T_b, HR, and LA recordings). Once filtered, all HR recordings (n = 30225/33443, which represented over 90% of raw data initially obtained from the loggers) fell within the 30- and 200-bpm thresholds set.

Body temperature data were Box-Cox transformed to more closely satisfy the assumption of normality and homogeneity. The circadian rhythm of T_b fluctuated significantly during the 24-hour cycle (for part of the day: $F_{5,33417} = 2886.93$, $p < .0001$; and for hours within the day: $F_{18,33417} = 171.90$, $p < .0001$) (Fig. 2.7i). Throughout the study, i.e., in the treatment and control, the median T_b was higher during the evening (38.0 ± 0.2 ^{0}C) between 1600–1659 hours (38.1 ± 0.1 ^{0}C).

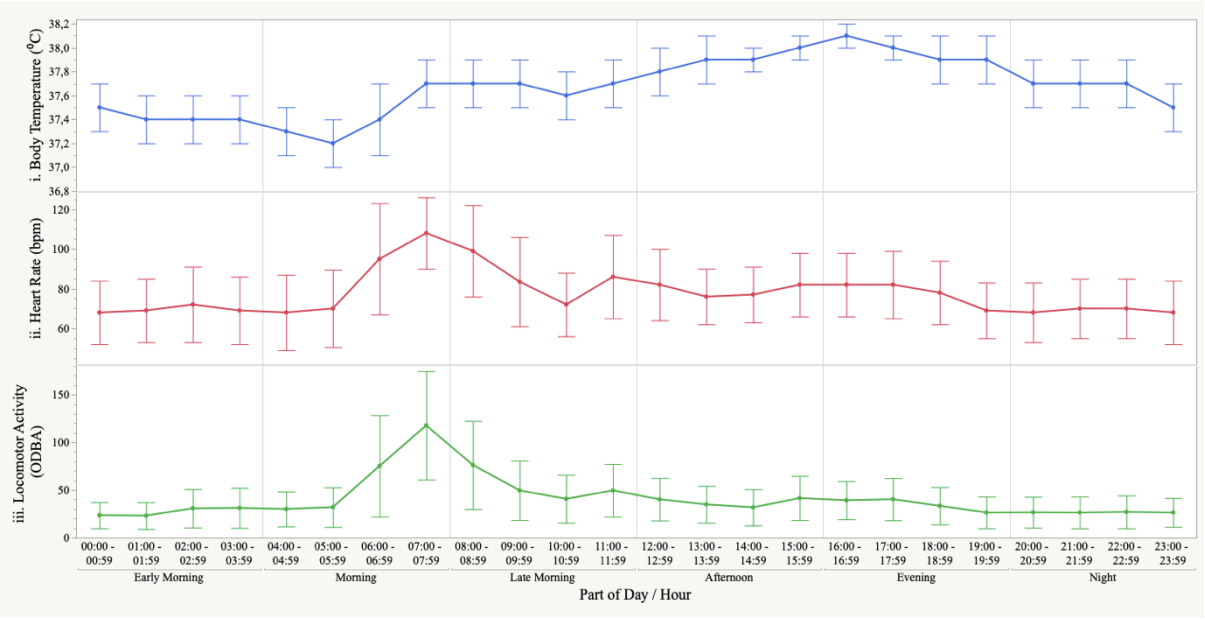

Figure 2.7. Circadian rhythm of (i) body temperature (^{0}C), (ii) heart rate (beats per minute [bpm]), and (iii) locomotor activity (overall dynamic body acceleration [ODBA]) (median, median absolute deviation) for the study cheetahs (CH-2205, -2206, and -2276) during the control and treatment.

The median T_b was higher during the treatment (37.7 ± 0.3 ^{0}C) than in the control (37.7 ± 0.3 ^{0}C). The MMRM analysis revealed that this numerical difference failed to achieve statistical significance. Post hoc comparisons using Tukey's HSD test revealed that T_b was significantly higher on treatment feed days (37.7 ± 0.3 ^{0}C) than on control feed days (37.7 ± 0.3 ^{0}C; t_{33437} = -6.30, p < .0001) and treatment starve days (37.7 ± 0.3 ^{0}C; t_{33437} = 7.36, p < .0001) (Fig. 2.8i). Post hoc comparisons using Tukey's HSD test revealed that the T_b of the female study cheetahs was significantly higher during the treatment (37.7 ± 0.3 ^{0}C) than in the control (37.7 ± 0.3 ^{0}C; t_{33438} = -9.18, p < .0001) (Fig. 2.9i). The T_b of the male study cheetahs was significantly lower during the treatment (37.7 ± 0.2 ^{0}C) than in the control (37.7 ± 0.2 ^{0}C; t_{33438} = 2.61, p = .0446). Post hoc comparisons using Tukey's HSD test revealed that the T_b of the two-year-old study cheetahs was significantly higher during the treatment (37.7 ± 0.3 ^{0}C) than in the control (37.7 ± 0.3 ^{0}C; t_{33438} = -9.18, p < .0001) (Fig. 2.10i). The T_b of the three-year-old study cheetahs was significantly lower during the treatment (37.7 ± 0.2 ^{0}C) than in the control (37.7 ± 0.2 ^{0}C; t_{33438} = 2.61, p = .0446).

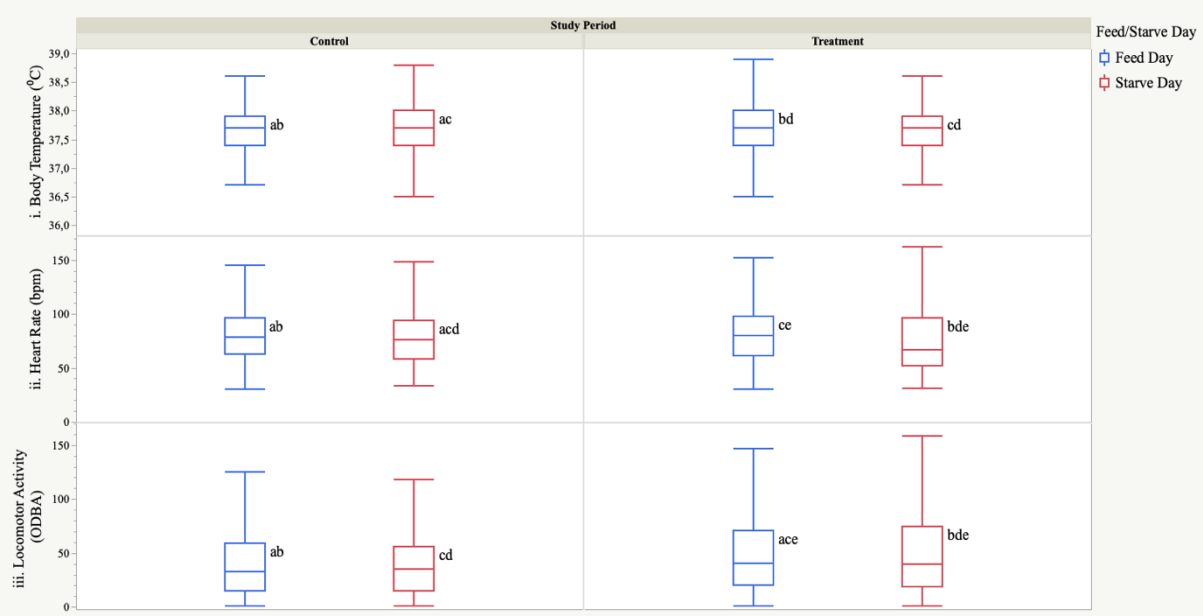

Figure 2.8. Box and whisker plot of (i) body temperature (^{0}C), (ii) heart rate (beats per minute [bpm]), and (iii) locomotor activity (overall dynamic body acceleration [ODBA]) for study cheetahs (CH-2205, -2206, and -2276). Effect of feed versus starve day during the control and treatment. Statistics were performed by a mixed model for repeated measures and Tukey's HSD post hoc test.

(i) a: $p = .0002$ and b, c, d: $p < .0001$. (ii) a: $p = .0006$; b, d, e: $p < .0001$; and c: $p = .0065$. (iii) a, b, c, d, e: $p < .0001$.

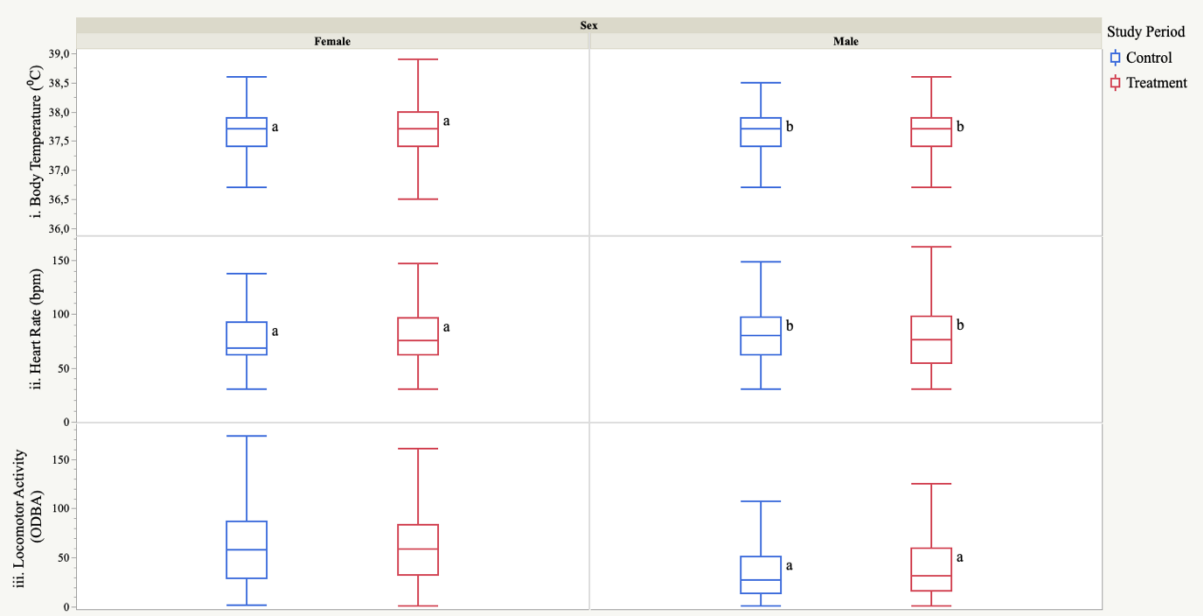

Figure 2.9. Box and whisker plot of (i) body temperature (^{0}C), (ii) heart rate (beats per minute [bpm]), and (iii) locomotor activity (overall dynamic body acceleration [ODBA]) for the study cheetahs (CH-2205, -2206, and -2276). Effect of sex (female and male) during the control and treatment. Statistics were performed by a mixed model for repeated measures and Tukey's HSD post hoc test.

(i) a: $p < .0001$ and b: $p = .0446$. (ii) a: $p = .0001$ and b: $p < .0001$. (iii) a: $p < .0001$.

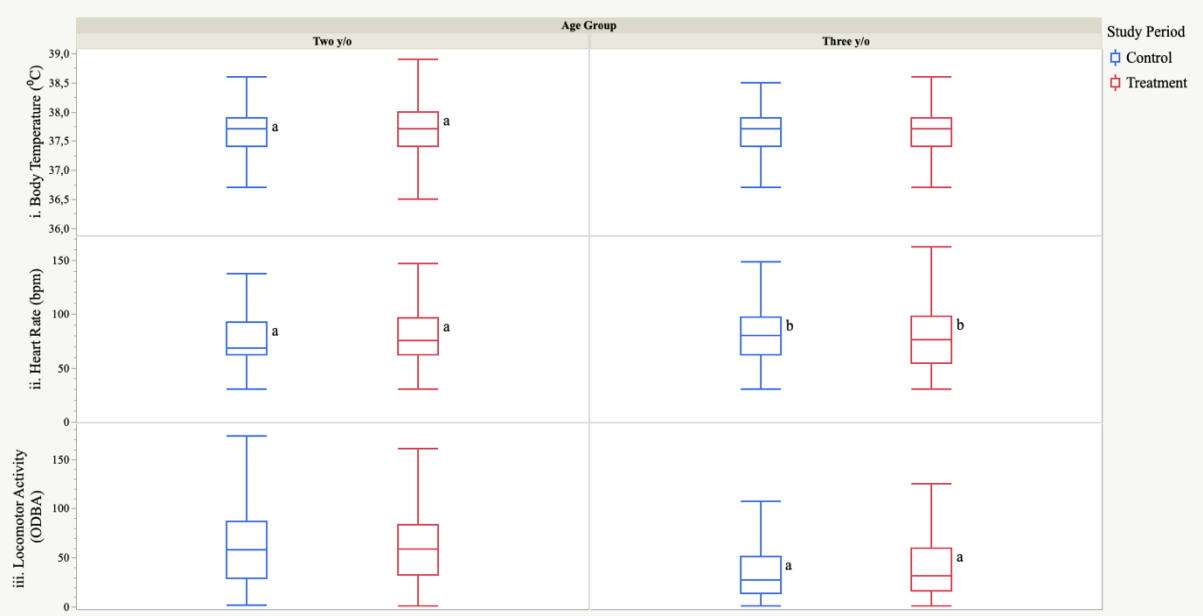

Figure 2.10. Box and whisker plot of (i) body temperature (^{0}C), (ii) heart rate (beats per minute [bpm]), and (iii) locomotor activity (overall dynamic body acceleration [ODBA]) for the study cheetahs (CH-2205, -2206, and -2276). Effect of age group (two and three years old [y/o]) during the control and treatment. Statistics were performed by a mixed model for repeated measures and Tukey's HSD post hoc test.

(i) a: $p < .0001$ and b: $p = .0446$. (ii) a: $p = .0001$ and b: $p < .0001$. (iii) a: $p < .0001$.

Post hoc comparisons using Tukey's HSD test revealed that T_b was significantly higher during week three of the treatment (37.7 ± 0.3 ^{0}C) than in the control (37.7 ± 0.3 ^{0}C; $t_{33418} = -6.24$, $p < .0001$) and weeks one (37.7 ± 0.3 ^{0}C; $t_{33418} = -4.37$, $p < .0001$) and two (37.7 ± 0.3 ^{0}C; $t_{33418} = -6.94$, $p < .0001$) (Fig. 2.11i).

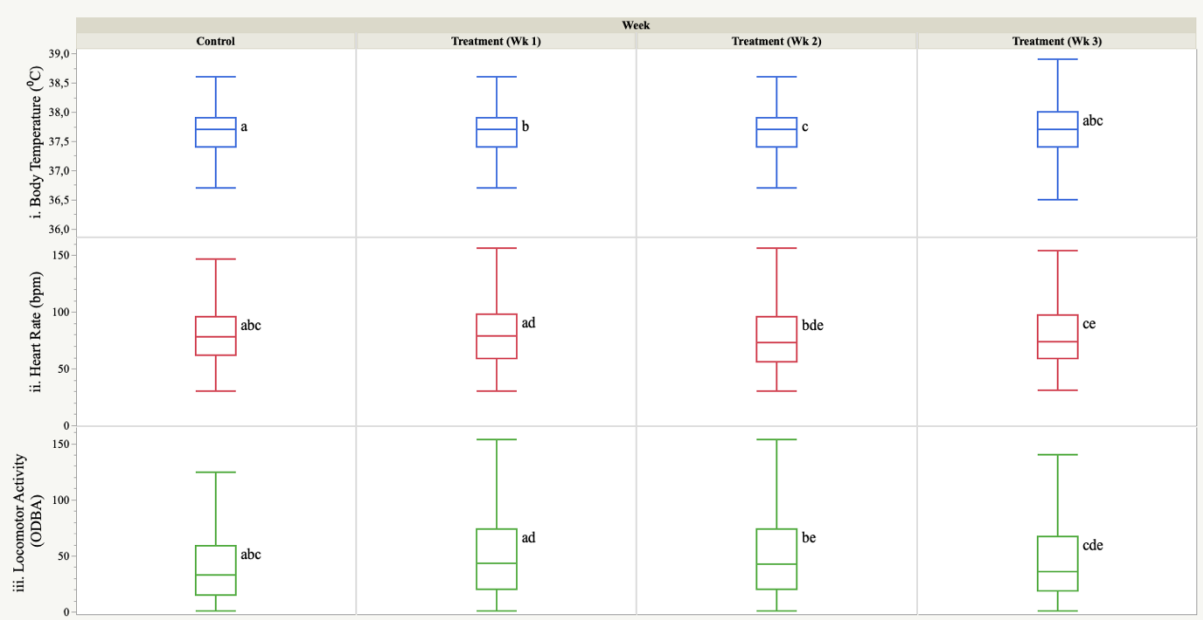

Figure 2.11. Box and whisker plot of (i) body temperature (^{0}C), (ii) heart rate (beats per minute [bpm]), and (iii) locomotor activity (overall dynamic body acceleration [ODBA]) for the study cheetahs (CH-2205, -2206, and -2276). Effect of control versus treatment week (Wk; one, two, or three). Statistics were performed by a mixed model for repeated measures and Tukey's HSD post hoc test.

(i) a, b, c: $p < .0001$. (ii) a: $p = .0041$ and b, c, d, e: $p < .0001$. (iii) a, b, c, d, e: $p < .0001$.

The MMRM analysis revealed that T_b was significantly higher at mealtime (37.7 ± 0.3 ^{0}C) than when the study cheetahs were not feeding (37.9 ± 0.2 ^{0}C; $F_{1,16698} = 30.41$, $p < .0001$). Post hoc comparisons using Tukey's HSD test revealed that T_b was significantly higher during the afternoon at mealtime (38.0 ± 0.2 ^{0}C) than when the study cheetahs were not feeding (37.9 ± 0.2 ^{0}C; $t_{16697} = -3.26$, $p = .0141$) and significantly higher during the evening at mealtime (38.2 ± 0.1 ^{0}C) than when the study cheetahs were not feeding (38.0 ± 0.2 ^{0}C; $t_{16697} = -4.53$, $p < .0001$) (Fig. 2.12i).

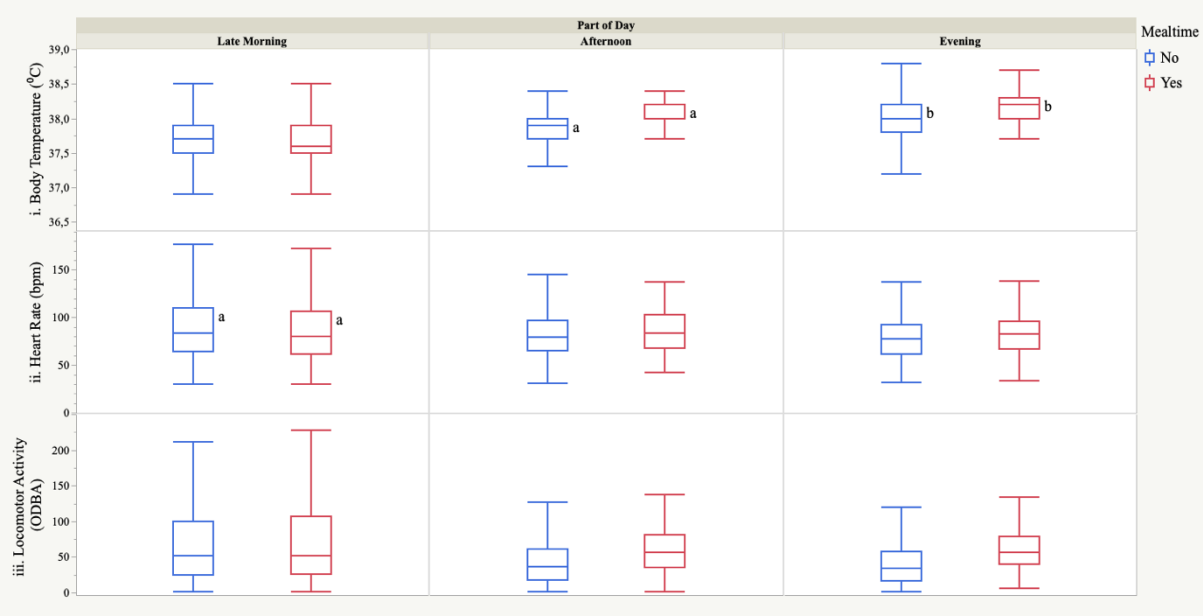

Figure 2.12. Box and whisker plot of (i) body temperature (^{o}C), (ii) heart rate (beats per minute [bpm]), and (iii) locomotor activity (overall dynamic body acceleration [ODBA]) for the study cheetahs (CH-2205, -2206, and -2276). Effect of mealtime by a part of the day. Statistics were performed by a mixed model for repeated measures and Tukey's HSD post hoc test.

(i) a: $p = .0141$ and b: $p < .0001$. (ii) a: $p = .0031$.

Cohen's d revealed a trivial effect size ($d = 0.03$; $t_{33441} = 2.89$, $p = .0039$) on T_b (Table 2.1).

Heart rate data were Box-Cox transformed to more closely satisfy the assumption of normality and homogeneity. The circadian rhythm of HR fluctuated significantly during the 24-hour cycle (for part of the day: $F_{5,30199} = 300.96$, $p < .0001$; and for hours within the day: $F_{18,30199} = 81.77$, $p < .0001$) (Fig. 2.7ii). Throughout the study, i.e., in the treatment and control, the median HR was higher during the morning (85 ± 27 bpm) between 0700–0759 hours (108 ± 18 bpm).

The MMRM analysis revealed that HR was significantly lower during the treatment (76 ± 19 bpm) than in the control (78 ± 16 bpm; $F_{1,30210} = 56.85$, $p < .0001$). Post hoc comparisons using Tukey's HSD test revealed that HR was significantly higher on control feed days (78 ± 16 bpm) than on control starve days (76 ± 18 bpm; $t_{30219} = 3.89$, $p = .0006$) and significantly

higher on treatment feed days (80 ± 18 bpm) than on treatment starve days (67 ± 19 bpm; t_{30219} = 17.94, p < .0001) (Fig. 2.8ii). Post hoc comparisons using Tukey's HSD test revealed that the HR of the female study cheetahs was significantly higher during the treatment (75 ± 15 bpm) than in the control (68 ± 10 bpm; t_{30220} = -4.23, p = .0001) (Fig. 2.9ii). The HR of the male study cheetahs was significantly lower during the treatment (76 ± 22 bpm) than in the control (80 ± 17 bpm; t_{30220} = 13.12, p < .0001). Post hoc comparisons using Tukey's HSD test revealed that the HR of the two-year-old study cheetahs was significantly higher during the treatment (75 ± 15 bpm) than in the control (68 ± 10 bpm; t_{30220} = -4.23, p = .0001) (Fig. 2.10ii). The HR of the three-year-old study cheetahs was significantly lower during the treatment (76 ± 22 bpm) than in the control (80 ± 17 bpm; t_{30220} = 13.12, p < .0001).

Post hoc comparisons using Tukey's HSD test revealed that HR was significantly lower during weeks one (79 ± 19 bpm; t_{30218} = 3.37, p = .0041), two (73 ± 19 bpm; t_{30218} = 10.85, p < .0001), and three of the treatment (74 ± 18 bpm; t_{30218} = 5.57, p < .0001) than in control (78 ± 16 bpm), significantly higher during week one of the treatment than in week two (t_{30218} = 6.28, p < .0001), and significantly lower during week two of the treatment than in week three (t_{30218} = -4.44, p < .0001) (Fig. 2.11ii).

The MMRM analysis revealed that HR was significantly higher at mealtime (81 ± 19 bpm) than when the study cheetahs were not feeding (80 ± 17 bpm; $F_{1,14832}$ = 6.57, p = .0104). Post hoc comparisons using Tukey's HSD test revealed that HR was significantly lower during the late morning at mealtime (80 ± 20 bpm) than when the study cheetahs were not feeding (84 ± 22 bpm; t_{14833} = 3.69, p = .00031) (Fig. 2.12ii).

Cohen's d revealed a trivial effect size (d = 0.10; t_{30223} = 8.44, p < .0001) on HR (Table 2.1).

Locomotor activity data were Box-Cox transformed to more closely satisfy the assumption of normality and homogeneity. The circadian rhythm of LA fluctuated significantly during the 24-hour cycle (for part of the day: $F_{5,33377} = 723.70$, $p < .0001$; and for hours within the day: $F_{18,33377} = 138.37$, $p < .0001$) (Fig. 2.7iii). Throughout the study, i.e., in the treatment and control, the median LA was higher during the morning (55.2 ± 39.6 ODBA) between 0700–0759 hours (117.7 ± 56.9 ODBA).

The MMRM analysis revealed that LA was significantly higher during the treatment (40.6 ± 23.8 ODBA) than in the control (33.2 ± 20.2 ODBA; $F_{1,33397} = 115.50$, $p < .0001$). Post hoc comparisons using Tukey's HSD test revealed that LA was significantly higher on treatment starve days (39.8 ± 24.2 ODBA) than on control feed days (33 ± 20 ODBA; $t_{33397} = -13.18$, $p < .0001$), control starve days (35.4 ± 20.7 ODBA; $t_{33397} = -7.85$, $p < .0001$), and treatment feed days (40.8 ± 23.4 ODBA; $t_{33397} = -5.99$, $p < .0001$) (Fig. 2.8iii). Post hoc comparisons using Tukey's HSD test revealed that the LA of the male study cheetahs was significantly higher during the treatment (31.2 ± 19 ODBA) than in the control (27.2 ± 17.2 ODBA; $t_{33398} = -14.01$, $p < .0001$) (Fig. 2.9iii). Post hoc comparisons using Tukey's HSD test revealed that the LA of the three-year-old study cheetahs was significantly higher during the treatment (31.2 ± 19 ODBA) than in the control (27.2 ± 17.2 ODBA; $t_{33398} = -14.01$, $p < .0001$) (Fig. 2.10iii).

Post hoc comparisons using Tukey's HSD test revealed that LA was significantly higher during weeks one (43.4 ± 24.6 ODBA; $t_{33397} = -13.02$, $p < .0001$), two (42.8 ± 25 ODBA; $t_{33397} = -10.30$, $p < .0001$), and three of the treatment (35.8 ± 21 ODBA; $t_{33397} = -4.41$, $p < .0001$) than in the control (33.2 ± 20.2 ODBA) and significantly higher during weeks one ($t_{33397} = 7.22$, $p < .0001$) and two of the treatment ($t_{33397} = 4.94$, $p < .0001$) than in week three (Fig. 2.11iii).

The MMRM analysis revealed that LA was significantly higher at mealtime (52.7 ± 30.4 ODBA) than when the study cheetahs were not feeding (40 ± 23.2 ODBA; $F_{1,16685} = 9.38$, $p = .0022$).

Cohen's d revealed a small effect size ($d = 0.20$; $t_{33401} = 18.20$, $p < .0001$) on LA (Table 2.1).

2.4 DISCUSSION

Unnatural diet composition and feeding regimes have been suggested in the aetiology of the various diseases prevalent in captive cheetahs (Bechert *et al.*, 2002; Depauw *et al.*, 2012b; Lane *et al.*, 2012; Whitehouse-Tedd *et al.*, 2015). This study mainly concerns the potential for the maldigestion of high dietary protein concentrations routinely fed to cheetahs in captivity to disrupt gut homeostasis. Symbiotic gut microbes harboured in animals' GI tracts serve various essential functions (Wasimuddin *et al.*, 2017). However, disturbance-related deviation in the microbial diversity and abundance pattern beyond a natural range, i.e., gut dysbiosis, can advance pathophysiology and affect host health (Shreiner *et al.*, 2015). Here, a more naturalistic reduced feeding days schedule characterised by offering larger quantities of food less frequently elicited in the study cheetahs significantly higher FCS. Faecal consistency is purportedly linked to intestinal microbiota composition (Tigchelaar *et al.*, 2016; Vandeputte *et al.*, 2016) and could indicate GI health (Whitehouse-Tedd *et al.*, 2015). The results of this analysis support, to some extent, the researcher's hypothesis that the more natural feeding pattern would influence the GI environment, including microbial parameters and/or GI physiology or functional traits.

Alternatively, chronic or repeated exposure to stressors has been identified to disrupt gut homeostasis (Accarie & Vanuytsel, 2020; Farzi *et al.*, 2018). Therefore, the higher FCS may be attributable to the stress-reducing effects of the more natural feeding pattern as a potential form of EE. The animal husbandry principle of EE is widely used to provide species-appropriate challenges to stimulus-poor captive animals, encouraging them to engage actively with their environments, reducing stress and, subsequently, stereotypical behaviour (Carlstead & Shepherdson, 2000; Swaisgood & Shepherdson, 2005). One of the most common types is

food-based enrichment, including for the cheetah (Bond & Lindburg, 1990; Quirke & O'Riordan, 2011a; 2011b; Quirke *et al.*, 2012; Skibiel *et al.*, 2007).

Absent the opportunity to hunt, predictable, fixed, and small daily meals can exacerbate carnivores' propensity to be inactive in captivity, leading to obesity and, subsequently, compromised welfare (Depauw *et al.*, 2012b). The study cheetahs spent the majority of their time inactive. This is consistent with findings described by other authors on captive cheetahs (Skibiel *et al.*, 2007) as well as many other felids (Acaralp-Rehnberg *et al.*, 2020; Bashaw *et al.*, 2007; De Souza Resende *et al.*, 2014; Macri & Patterson-Kane, 2011; Moreira *et al.*, 2007; Shepherdson *et al.*, 1993; Weller & Bennet, 2001). Reduced feeding days resulted in numerically lower inactivity levels and significantly higher LA. Following the adaption from a conventional zoo feeding programme to a more natural gorge and fast feeding schedule, increased activity was similarly reported in captive lions (Altman *et al.*, 2005). Alternatively, that the study cheetahs were more active may infer behavioural agitation. Cheetahs and other carnivores in the wild spend a significant portion of their activity budget resting (Siegel, 2005). While reducing the risk of obesity, the motivation for increased activity levels, possibly hunger, could have negative welfare implications. Significantly higher LA on treatment starve days further suggests a lack of satiety during fasting. These findings emphasise the importance of offering sufficiently large meals if additional fast days are introduced not to be contrary to animal welfare.

Stress reduction by EE extends to sympathoadrenal responses. The two branches of the neuroendocrine stress system are the SNS and HPA axis (see section 1.1.2 for more information on the adaptive stress response). Both branches originate in the hypothalamus and converge on the adrenal gland, working together to maintain or re-establish homeostasis by orchestrating behavioural and physiological adaptations to the stressor (Coffman, 2020). The SNS provides

the immediate first wave of the stress response, mediating the rapid release of the catecholamine hormones E and NE from the adrenal medulla (Romero & Wingfield, 2015). The second wave is more gradual and involves the GCs, the end-products of the hormonal cascade along the HPA axis (Sapolsky *et al.*, 2000). Environmental enrichment blunts E release (Moncek *et al.*, 2004) and, consequently, stress-related increases in HR (Azar *et al.*, 2012; Marashi *et al.*, 2003; Ravenelle *et al.*, 2014; Sharp *et al.*, 2014; 2002). Significantly lower HR was observed in response to reduced feeding days, indicating stress reduction (Wascher *et al.*, 2021).

Multiple hypotheses have been proposed to explain the stress-reducing effects of EE, some of which are based on the contention that EE itself acts as a mild stressor (Smail *et al.*, 2020). By providing challenges appropriate to an animal's sensory, physical, and cognitive capacities (Meehan & Mench, 2007), EE is thought to enable arousal and activation of the physiological stress response without pushing the animal into high, maladaptive levels of stress (Ashokan *et al.*, 2016; Liu *et al.*, 2018). In this manner, EE is adaptive, improving animals' capacity to cope with stress, or resilience (Crofton *et al.*, 2015; Lyons *et al.*, 2009; Parker & Maestripieri, 2011). For example, regular exposure to acute stress can optimise animals' responsiveness to environmental stimuli (Carlstead & Shepherdson, 1994). The study cheetahs showed significantly higher interspecific behaviour on treatment feed days when the feeding vehicle was likely in close proximity to their enclosure on the Jill Bryden-Fayers Reserve than on treatment starve days. Increased attention directed at the feeding vehicle and the associated olfactory and/or auditory cues could be considered stimulating for the study cheetahs.

Physiologically, enrichment-induced arousal was attended by significantly higher fGCM concentration on treatment feed days than on control feed days, significantly higher T_b, and numerically higher HR (salient features of the SNS-mediated reaction to stress). Body

temperature and HR were significantly higher at mealtime. Stress-induced hyperthermia has been demonstrated to some extent by a study in which the increase in T_b of free-ranging cheetahs after a successful hunt was shown to be higher than that of an unsuccessful hunt (mean ± standard deviation [SD]; 1.38 ± 0.28 ^{0}C versus 0.58 ± 0.18 ^{0}C), despite similar levels of physical activity (Hetem *et al.*, 2019; 2013). The authors attributed the hyperthermia experienced by hunting cheetahs to the stress associated with vulnerability to attack and kleptoparasitism by more dominant intraguild predators (Hetem *et al.*, 2019; 2013). The study cheetahs were fed in a space free of interference competition. Therefore, the thermal and cardiac response at times related to feeding suggests psychological excitation to food consumption rather than a predator-induced stress response. As it corresponded with significantly higher LA, the higher T_b and HR at mealtime could have been caused by the demands of increased physical activity. During physical activity, requirements for oxygen and gluconeogenetic substrates in skeletal muscle are increased, as are the removal of metabolites and carbon dioxide (Burton *et al.*, 2004). The cardiac output is increased to meet the demand for blood flow by contracting muscles, attributed to sympathetically-mediated increases in HR and stroke volume. Increased T_b follows an increase in HR due to heat generated during the conversion of nutrients to muscular work (Burton *et al.*, 2004).

All animals require food to survive and, as such, are motivated to interact with food-related stimuli. The demographic factors, sex and age group, affected the study cheetahs' activity levels and physiological (fGCM concentration, T_b, and HR) responses, possibly explained by the study cheetahs all experiencing some level of arousal and/or activation of the physiological stress response to reduced feeding days.

Novel stimuli lose their effect over time due to habituation (Kuczaj *et al.*, 2002), whereby animals' responsiveness to a stimulus diminishes with repeated exposure to that stimulus

(McFarland, 2021). The temporal profiles of fGCM concentration and LA appeared to demonstrate habituation, with values increasing during week one of the treatment, significantly higher during week two, and decreasing in week three.

This study's findings encourage a more naturalistic reduced feeding days schedule to mediate the unnatural composition of horsemeat-based diets routinely fed to cheetahs in captivity and as an effective EE strategy. However, as faecal consistency scoring lacks specificity as a stand-alone measure of GI health, further scientific research is necessary for determining the optimal feeding regime(s) to modulate cheetahs' intestinal microbiota composition beneficially. I recommend that future studies validate FCS against other measurements of microbiome health effects (e.g., digestibility, pH, the incidence of vomiting or diarrhoea, veterinary diagnosis of GI disease, fermentation by-products, faecal frequency, dry weight percentage, or short-chain fatty acids), as well as consider cheetahs living under different dietary and husbandry regimes.

Furthermore, I demonstrated that biologging technology could be used to record concurrent T_b, HR, and LA and, in addition to behavioural and physiological metrics, has tremendous potential as a tool to measure stress in the cheetah. However, a significant limitation of the present study was the battery malfunction of the biologgers implanted in the study cheetahs CH-2271, CH-2276, and CH-2277, impeding the acquisition of complete T_b, HR, and LA data sets for those individuals. For the technology to be routinely utilised in animal welfare studies, there is still a need to develop more reliable devices capable of remote data transmission to avoid repeated capture and handling.

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Chapter 3: Responses to Environmental Enrichment in Captive-Born Cheetahs (*Acinonyx jubatus*): Implications for Behavioural and Physiological Stress and Gastrointestinal Health

ABSTRACT. Environmental enrichment (EE) has now been integrated as a fundamental principle of captive animal husbandry to provide species-appropriate challenges, opportunities, and stimulation. This study investigated the response of captive-born (hand-reared) cheetahs (n = 5) to the provision of EE (physical, social, cognitive, sensory, and nutritional) for three weeks. Using emerging biologging technology and more traditional measures of stress-related behavioural and physiological responses, variations in body temperature, heart rate (HR), locomotor activity (LA), behaviour, faecal glucocorticoid metabolite concentration, and faecal consistency score were documented. Providing EE elicited in the study cheetahs lower inactivity levels ($p < .01$), auto-grooming ($p \leq .05$), and higher LA ($p < .01$). Environmental enrichment resulted in lower HR ($p < .0001$) and higher affiliative behaviour ($p \leq .05$), consistent with its stress-reducing effects. Sex and age group affected the study cheetahs' activity levels and physiological responses, which may be attributable to the sensitivity of the female cheetahs and those two years old to EE. Locomotor activity appeared to demonstrate that EE was sufficiently dynamic to provide prolonged stimulation. This study's findings encourage the provision of EE to enhance the welfare of captive cheetahs, particularly cognitive, sensory, and nutritional enrichment, while cautioning against social enrichment as an effective EE strategy.

3.1 INTRODUCTION

Animals in the wild face challenges as a result of biotic interactions (e.g., competition, predation, and mating) and abiotic environmental change (e.g., weather, illness, starvation, and dehydration). However, they use competencies, genetically inherited or learned through interactions with the environment, to cope with novel challenges when they arise (Špinka & Wemelsfelder, 2011). Agency is the intrinsic tendency of an animal to engage actively with the environment to gain information and build competencies (Špinka & Wemelsfelder, 2011). Significant facets of agency and competence are the behaviours of problem-solving, exploration, and play, which can be positively reinforcing for animals independent of any

functional outcome they may have (Špinka & Wemelsfelder, 2011). For example, contra-freeloading; that is, the observed behaviour whereby an animal will make an effort to work for a reward even if the reward is also available freely (Hessle *et al.*, 2008; Inglis *et al*., 1997; de Jonge *et al.*, 2008; Lindqvist & Jensen, 2008). Therefore, (novel) challenge is a crucial factor in an animal's life.

In contrast, captive animals often still live in simple, monotonous, and predictable environments where they are challenged infrequently or not at all. Confinement and the absence of various stimuli in captivity remove the opportunity and/or motivation for animals to express agency and, as such, frustrate the intrinsic need and rewards to do so (Špinka & Wemelsfelder, 2011). This could negatively impact animals' affective states and decrease their coping abilities to novelty and, subsequently, lead to fear, anxiety, stress, and the development of abnormal or repetitive, unvarying, and apparently functionless behaviour, i.e., stereotypies (Mason, 1991). There is growing attention to the value of expressing agency, building competencies, and appropriate levels of challenge as essential contributors to the welfare of captive animals (Browning & Veit, 2021; Špinka, 2019; Špinka & Wemelsfelder, 2011).

The animal husbandry principle of EE "seeks to enhance the quality of captive animal care by identifying and providing the environmental stimuli necessary for optimal psychological and physiological well-being" (Shepherdson *et al.*, 1998, p. 1). Environmental enrichment is a tool that includes a range of techniques (e.g., naturalistic enclosures, novel stimuli) widely used to provide animals with the opportunity and motivation to engage actively with their environments, reducing stress and, subsequently, stereotypical behaviour (Carlstead & Shepherdson, 2000; Swaisgood & Shepherdson, 2005). Multiple hypotheses have been proposed to explain the stress-reducing effects of EE, some of which are based on the contention that EE itself acts as a mild stressor (Smail *et al.*, 2020). In the short term, stress can

have psychological and physiological benefits conditional on animals' ability to respond to the stressor effectively. It is only when stress is of a nature, magnitude, or duration that is beyond the adaptive resources of an individual that its welfare becomes compromised (Charmandari *et al.*, 2005; Tsigos *et al.*, 2020). By providing challenges appropriate to an animal's sensory, physical, and cognitive capacities (Meehan & Mench, 2007), EE is thought to enable arousal and activation of the physiological stress response without pushing the animal into high, maladaptive levels of stress (Ashokan *et al.*, 2016; Liu *et al.*, 2018). In this manner, EE is adaptive, improving animals' capacity to cope with stress, or resilience (Crofton *et al.*, 2015; Lyons *et al.*, 2009; Parker & Maestripieri, 2011). These benefits act prophylactically in preparation for future stressors and ameliorate the effects of previously experienced stressors (Smail *et al.*, 2020).

Enrichment techniques can be classified in many ways, including physical enrichment (changes to the structure or contents of enclosures), sensory enrichment (stimulation of senses), cognitive enrichment (stimulation of intellectual capacities), social enrichment (inter- or intraspecific interaction), and nutritional enrichment (changes to food availability) (Dominquez, 2008).

In addition to several other species for which it is known to be beneficial (Swaisgood & Shepherdson, 2005), EE has become an essential part of captive felid husbandry (Quirke & O'Riordan, 2011a; Yu *et al.*, 2009). Stereotypic pacing is common among captive felids (Clubb & Mason, 2007; Clubb & Vickery, 2006; Livingston, 2009; Mason *et al.*, 2007), including the cheetah (Quirke & O'Riordan, 2011a; 2011b; Quirke *et al.*, 2012). Enriched and challenging environments are reported to decrease pacing and increase activity and alertness in various felids (Jenny & Schmid, 2002; Quirke & O'Riordan, 2011a; Yu *et al.*, 2009). These findings

are encouraging, considering the propensity for carnivores in captivity to become obese from lack of exercise and experience physical injuries (Hope & Deem, 2006; Law *et al.*, 1997).

However, felids are among the most challenging species to develop EE programmes (Mellen *et al.*, 1998) due to the complexity of behaviours required for predation and exploration in these species (Quirke *et al.*, 2013; Skibiel *et al.*, 2007). Time and resources (Hoy *et al.*, 2010; Mellor *et al.*, 2015), a lack of variety of effective enrichment types for individual species, the need for methodological control and evaluation, and habituation to the stimuli used (Tarou & Bashaw, 2007), are all factors affecting the success of EE (Damasceno *et al.*, 2017). Habituation has been defined as "response decrement as a result of repeated stimulation" (Harris, 1943, p. 385). This is an evolutionary strategy related to exploration and environmental adaptations (Thompson & Spencer, 1966; Wong *et al.*, 2010). However, concerning EE, habituation can eventually lessen the effectiveness of a particular enrichment (Tarou & Bashaw, 2007). Environmental enrichment programmes for felids should be dynamic and constantly modified (Mellen *et al.*, 1998).

This study's overall aim was to investigate the response of captive-born (hand-reared) cheetahs to the provision of EE. I hypothesised that EE would encourage natural active behaviours and induce arousal and activation of the physiological stress response. As established stress-related markers, behavioural observations (Quirke *et al.*, 2012) and fGCM concentration analysis (Terio *et al.*, 1999) were performed, in addition to faecal consistency scoring as a preliminary indicator of the study cheetahs' GI health (Whitehouse-Tedd *et al.*, 2015). A secondary objective was to explore the potential of biologging technology for measuring stress in the cheetah by recording concurrent T_b, HR, and LA. I hypothesised lower inactivity levels and higher LA. Moreover, I predicted higher fGCM concentration, T_b, and HR consistent with a physiological stress response.

3.2 MATERIALS AND METHODS

3.2.1 Study Site and Animals

The experimental trials occurred between April and September 2019 at the Cango Wildlife Ranch and Conservation Centre (33°33'S, 22°12'E) 4 km north of Oudtshoorn, in the Western Cape of South Africa (see section 1.1.6.1 for more information on the study site and animals). Three male (CH-2205, -2206, and -2271) and two female (CH-2276 and -2277) resident adult cheetahs (Table 1.1) were allocated to this study.

3.2.2 Body Temperature, Heart Rate, and Locomotor Activity Measurement Intervals

A single cardiac-, temperature- and movement-sensitive biologger (DST centi-HRT ACT, Star-Oddi, Gardabaer, Iceland) with the dimensions of 46 mm x 15 mm x 15 cm and weighing approximately 19 g was implanted in each of the study cheetahs. The biologgers were calibrated individually against a high-accuracy thermometer (Hart 1504, Fluke, Utah, US). Calibrated accuracy was better than 0.1 °C. Before surgical implantation, the biologgers were set to record tri-axial LA, i.e., heave, surge, sway, every minute for the ODBA and T_b (°C) and leadless single-channel ECG-derived HR (bpm) every 5 minutes at 200 Hz. Representative traces of raw ECG recordings were saved every 24 hours for validating the HR measurements' QI (where QI_0 was the highest quality and QI_1, QI_2, and QI_3 were of progressively reduced quality). Afterwards, the biologging devices were sterilised using ethylene oxide and implanted (see section 1.1.6.3 in the introduction for more information on the surgical procedure).

3.2.3 Experimental Design

The study cheetahs housed individually (CH-2271) or in pairs (CH-2205 and -2206, and CH-2276 and -2277, respectively) were assigned randomly to either the treatment, i.e., the

provision of EE or the control in an initial 3-week period at variable times and the alternative in a period following of equal duration (see section 1.1.6.4 for more information on the experimental design). During the treatment, the study cheetahs were housed off-exhibit at the Jill Bryden-Fayers Reserve neighbouring the Ranch. They were provided with a succession of seventeen enrichment items, categorised into physical, social, cognitive, sensory, and nutritional enrichment, as shown in Table 3.1. The literature on captive felids informed the enrichment items selected (Grötting, 2017; Mellen & Shepherdson, 1997; Quirke & O'Riordan, 2011a; Skibiel *et al.*, 2007). Individual or pair-housed study cheetah(s) were enriched at the same time each day.

Table 3.1. Enrichment items as per their type and description of use.

Physical Enrichment	
Large branches	Placed inside the enclosure for study cheetahs' to interact with for approximately 24 hours. Removed from the enclosure before provisioning of the next day's enrichment item.
Pile of dead leaves	Placed inside the enclosure for study cheetahs' to interact with for approximately 24 hours. Removed from the enclosure before provisioning of the next day's enrichment item.
Social Enrichment	
Big mirror	Placed outside the enclosure for study cheetahs' to interact with for approximately one hour.
Animal sounds	Played from outside the enclosure for study cheetahs' to interact for approximately one hour. Included sounds from competitor and/or predator species (e.g., lion, hyena, leopard, jackal, baboon, and vulture) and prey species (antelope, warthog, and guineafowl).
Cognitive Enrichment	
Feather lure	Played with study cheetahs for approximately one hour. Left inside the enclosure for study cheetahs' to interact with for approximately 24 hours. Removed from the enclosure before provisioning of the next day's enrichment item.
Jolly egg	Played with study cheetahs for approximately one hour. Left inside the enclosure for study cheetahs' to interact with for approximately 24 hours. Removed from the enclosure before provisioning of the next day's enrichment item.

Paper jets	Played with study cheetahs for approximately one hour. Left inside the enclosure for study cheetahs' to interact with for approximately 24 hours. Removed from the enclosure before provisioning of the next day's enrichment item.
Boxes	Placed inside the enclosure for study cheetahs' to interact with for approximately 24 hours. Removed from the enclosure before provisioning of the next day's enrichment item.
Sensory Enrichment	
Snakeskin	Placed inside the enclosure for study cheetahs' to interact with for approximately 24 hours. Removed from the enclosure before provisioning of the next day's enrichment item.
Goat/sheep scent (straw from goat/sheep pen)	Placed inside the enclosure for study cheetahs' to interact with for approximately 24 hours. Removed from the enclosure before provisioning of the next day's enrichment item.
Duiker scent (faeces)	Placed inside the enclosure for study cheetahs' to interact with for approximately 24 hours. Removed from the enclosure before provisioning of the next day's enrichment item.
Fruit essences	Sprinkled on dirt and rocks inside the enclosure for study cheetahs' to interact with for approximately 24 hours.
Bubbles	Played with study cheetahs for approximately one hour.
Horse blood trail	Sprinkled on dirt and rocks inside the enclosure for study cheetahs' to interact with for approximately 24 hours.
Nutritional Enrichment	
Horse hoof	Placed inside the enclosure for study cheetahs' to interact with for approximately 24 hours. Weight deducted from study cheetahs' daily food ration. Remainders removed from the enclosure before provisioning of the next day's enrichment item.
50 g of horsemeat mince hidden in straw	Placed inside the enclosure for study cheetahs' to interact with for approximately 24 hours. Weight deducted from study cheetahs' daily food ration. Remainders removed from the enclosure before provisioning of the next day's enrichment item.
Meat-based ice lollies/jelly-cakes	Placed inside the enclosure for study cheetahs' to interact with for approximately 24 hours. Remainders removed from the enclosure before provisioning of the next day's enrichment item.
50 g of horsemeat mince hidden in straw	Placed inside the enclosure for study cheetahs' to interact with for approximately 24 hours. Weight deducted from study cheetahs' daily food ration. Remainders removed from the enclosure before provisioning of the next day's enrichment item.
Horse hoof	Placed inside the enclosure for study cheetahs' to interact with for approximately 24 hours. Weight deducted from study cheetahs' daily food ration. Remainders removed from the enclosure before provisioning of the next day's enrichment item.

Meat-based ice lollies/jelly-cakes	Placed inside the enclosure for study cheetahs' to interact with for approximately 24 hours. Remainders removed from the enclosure before provisioning of the next day's enrichment item.
50 g of horsemeat mince hidden in straw	Placed inside the enclosure for study cheetahs' to interact with for approximately 24 hours. Weight deducted from study cheetahs' daily food ration. Remainders removed from the enclosure before provisioning of the next day's enrichment item.

During the control, the study cheetahs were not provided EE. Other than the specific intervention being investigated, i.e., the provision of EE, the study cheetahs' environment, housing, and management (as described in section 1.1.6.1) were maintained across the treatment and control, including vantage points and marking areas (e.g., rocks, tree stumps) and bones offered randomly once a week in place of the day's meals.

Throughout the treatment and control, the study cheetahs were monitored regarding (i) behaviour, (ii) fGCM concentration, and (iii) FCS, as well as (iv) T_b, HR, and LA concurrently recorded by implanted data loggers.

3.2.4 Behavioural Data Collection

Instantaneous scan sampling (Altmann, 1974) with a 5-minute inter-scan interval was used in this study (Quirke & O'Riordan, 2011a; 2011b). During five weekly 60-minute observation sessions, the researcher (KLB) carried out 12 instantaneous scan samples on focal animals, one enclosure at a time and in a randomised order to prevent time-of-day effects. For the study cheetahs housed in pairs (Table 1.1), behavioural data were collected simultaneously using physical characteristics to identify individuals. Observations were performed between 0700 and 1700 based on the operating hours of the Cango Wildlife Ranch and Conservation Centre. An instantaneous behavioural sample was recorded when different enrichment items were provided.

Fifteen behaviours, categorised into inactive, active, and not observed, were recorded (Table 1.2). Preliminary observation of the study cheetahs and the literature on felids' behaviour (Altman *et al.*, 2005; Quirke & O'Riordan, 2011a; 2011b; Regaiolli *et al.*, 2019) informed the ethogram used. Time spent out of sight (hiding or staying away from the human observer) was recorded as its performance may be indicative of a psychological stress response (Carlstead *et al.*, 1993; Davey, 2006; Hosey, 2013; Morgan & Tromborg, 2007; Sellinger & Ha, 2005).

3.2.5 Faecal Sample Collection and Consistency Scoring

To differentiate between individual faecal samples in the event of paired housing (Table 1.1), 1 tbsp of uncooked rice was thoroughly mixed into the diet of study cheetahs CH-2206 and CH-2277 once per day. The caretakers monitored the study cheetahs during feeding times to ensure the sufficient consumption of rice and prevent meal sharing. Only faeces found to have uncooked rice were considered to have originated from those individuals fed rice. Due to the operating hours of the Cango Wildlife Ranch and Conservation Centre, the enclosures could not be entered between 1800–0800 hours. As such, once daily between 0800–1000 hours, faeces from the previous night were collected from each enclosure within 16 hours of defecation (4–10 °C T_a) (Ludwig *et al.*, 2013).

Following sample collection, the researcher (KLB) assigned FCS as per a five-point grading system, where two points (grade 4: firm and dry and grade 5: firm) were considered to be 'normal,' and three points (grades 1–3: liquid, soft without shape, and soft with shape) were considered to be 'suboptimal' (adapted from AZA Tiger Species Survival Plan®, 2016; Whitehouse-Tedd *et al.*, 2015). Afterwards, the samples were deposited into appropriately

labelled (sample collection date and study cheetah and sample ID numbers) 50 mL polypropylene specimen containers and frozen at –20 °C.

3.2.6 Faecal Steroid Extraction and Quantification

Following completion of the experimental trials, faecal samples were transported frozen to the Endocrine Research Laboratory, University of Pretoria, South Africa. Faecal steroids were extracted and analysed for fGCM concentration as described in section 1.1.6.7.

3.2.7 Ethical Statement

All of the experimental procedures involving the study cheetahs were approved by the University of Pretoria's Animal (clearance number V075-18) and Research Ethics Committees (clearance number REC069-18).

3.2.8 Statistical Analysis

Statistical analysis was performed using Microsoft Excel (version 16.0) and JMP Pro software (version 16.0) for Windows, developed by SAS Institute Inc (North Carolina, US). Raw data was manipulated before detailed analyses (see section 1.1.6.9 for more information on the data preparation). The data were screened for univariate outliers greater than three interquartile ranges away from the 99.5th or 0.05th percentiles (LA: n = 51), which were subsequently excluded from descriptive statistics and analyses. Normal distribution and homogeneity of variance were verified using Anderson-Darling and Levene's tests, respectively. Box-Cox transformations were used to more closely satisfy the assumption of normality and homogeneity in the case of departure. To maintain statistical integrity, the data were back-transformed for descriptive statistics and visual representation. An MMRM analysis was conducted to investigate the following:

- The random effect of the study cheetah, the independent fixed effect of the study period and the interaction fixed effects of the study period and (i) sex and (ii) age group on each behaviour.
- The random effect of the study cheetah and the fixed effects of control versus (i) enrichment category and (ii) enrichment item within the category on each behaviour.
- The random effect of the study cheetah and the fixed effect of control versus treatment week (one, two, or three) on each behaviour.
- The random effect of the study cheetah, the independent fixed effect of the study period, and the interaction fixed effects of the study period and (i) sex and (ii) age group on fGCM concentration and FCS.
- The random effect of the study cheetah and the fixed effects of control versus (i) enrichment category and (ii) enrichment item within the category on fGCM concentration and FCS.
- The random effect of the study cheetah and the fixed effect of control versus treatment week (one, two, or three) on fGCM concentration and FCS.
- The random effect of the study cheetah and the fixed effects of hours within the day and part of the day on T_b, HR, and LA.
- The random effect of the study cheetah, the independent fixed effect of the study period, and the interaction fixed effects of the study period and (i) sex and (ii) age group on T_b, HR, and LA.
- The random effect of the study cheetah and the fixed effects of control versus (i) enrichment category and (ii) enrichment item within the category on T_b, HR, and LA.
- The random effect of the study cheetah and the fixed effect of control versus treatment week (one, two, or three) on T_b, HR, and LA.
- The random effect of the study cheetah and the independent and interaction fixed effects of enrichment time and part of the day on T_b, HR, and LA.

Tukey's HSD post hoc tests were performed for multiple pair-wise comparisons. The treatment effect size was calculated using Cohen's d. Descriptive statistics were reported as median ± MAD and the significance level, α, was set at 0.05.

3.3 RESULTS

The overall aim of this study was to investigate the response of captive-born (hand-reared) cheetahs to the provision of EE. It investigated whether demographic factors (sex and age group) and habituation affected this response.

3.3.1 Behavioural Observations

Behavioural data were Box-Cox transformed to more closely satisfy the assumption of normality and homogeneity. The study cheetahs spent the majority of their time inactive (for the control: 47% and the treatment: 35%) (Fig. 3.1). The MMRM analysis revealed that inactivity levels ($F_{1,105.6} = 7.26$, $p = .0082$) and auto-grooming ($F_{1,9.83} = 5.22$, $p = .0459$) were significantly lower during the treatment than in the control in addition to greater displays scent-marking ($F_{1,35} = 5.93$, $p = .0201$) and affiliative behaviour ($F_{1,27.0} = 6.45$, $p = .0172$). Post hoc comparisons using Tukey's HSD test revealed that the inactivity levels of the female study cheetahs were significantly lower during the treatment than in the control ($t_{104.0} = 3.61$, $p = .0026$). Post hoc comparisons using Tukey's HSD test revealed that the inactivity levels of the two-year-old study cheetahs were significantly lower during the treatment than in the control ($t_{103.6} = 3.83$, $p = .0012$). Post hoc comparisons using Tukey's HSD test revealed that the three-year-old study cheetahs showed significantly higher auto-grooming during the treatment than in the control ($t_{7.9} = -3.33$, $p = .0426$).

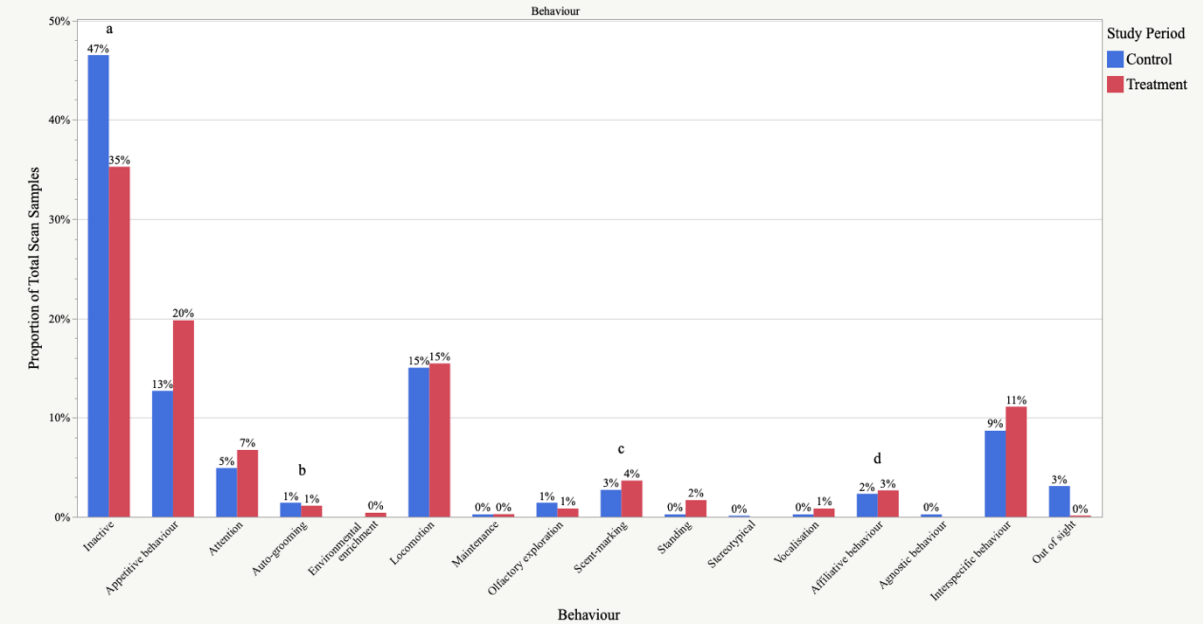

Figure 3.1. Bar chart of each behaviour for the study cheetahs (CH-2205, -2206, -2271, -2276, and -2277) during the control and treatment. Statistics were performed by a mixed model for repeated measures. The number above the bar indicates the proportion of total scan samples study cheetahs showed each behaviour during the control and treatment, respectively.

A: p = .0082, b: p = .0459, c: p = .0201, and d: p = .0172.

Post hoc comparisons using Tukey's HSD test revealed that affiliative behaviour was significantly higher during the treatment of sensory enrichment than in the control ($t_{20.9}$ = -3.79, p = .0055), most significantly in response to snakeskin ($t_{20.5}$ = -3.39, p = .0292).

Post hoc comparisons using Tukey's HSD test revealed that inactivity levels were significantly lower during week two of the treatment than in the control ($t_{104.1}$ = 2.64, p = .0469). Standing was significantly higher during week one of the treatment than in weeks two ($t_{3.8}$ = 7382.5, p < .0001) and three ($t_{3.8}$ = 6905.7, p < .0001). Affiliative behaviour was significantly higher during week two of the treatment than in the control ($t_{22.5}$ = -3.25, p = .0174).

Cohen's d revealed a large effect size on auto-grooming (d = 1.31; t_{13} = 2.39, p = .0327) and affiliative behaviour (d = 1.09; t_{27} = 2.88, p = .0077) and a medium effect size (d = 0.51; t_{108} = 2.66, p = .0090) on inactivity levels (Table 3.2).

Table 3.2. Treatment effect size on behaviour, faecal glucocorticoid metabolite (fGCM) concentration (µg/g dry weight [DW]), faecal consistency score, body temperature (^{0}C), heart rate (beats per minute [bpm]), and locomotor activity (overall dynamic body acceleration [ODBA]) for the study cheetahs (CH-2205, -2206, -2271, -2276, and -2277). Calculations were performed using Cohen's d.

df: degrees of freedom, CI: confidence interval.

	t	*p*	df	Cohen's d	95% CI for Cohen's d	
					Lower	Upper
Behaviour						
Inactive	2.66	.0090	108	0.51	0.13	0.89
Appetitive behaviour	0.21	.8368	63	0.05	-0.44	0.55
Attention	0.09	.9278	49	0.03	-0.53	0.58
Auto-grooming	2.39	.0327	13	1.31	0.10	2.47
Locomotion	1.55	.1244	90	0.32	-0.09	0.74
Olfactory exploration	1.31	.2149	12	0.71	-0.40	1.79
Scent-marking	1.38	.1762	35	0.46	-0.21	1.12
Standing	0.68	.5113	9	0.53	-1.03	2.07
Vocalisation	1.46	.2031	5	1.12	-0.57	2.72
Affiliative behaviour	2.88	.0077	27	1.09	0.28	1.87
Interspecific behaviour	1.12	.2676	67	0.27	-0.21	0.74
Out of sight	1.43	.2124	5	1.54	-0.83	3.79
fGCM Concentration (µg/g DW)	2.53	.0121	181	0.38	0.08	0.67
Faecal Consistency Score (grade)	0.85	.3985	181	0.13	-0.17	0.42
Body Temperature (°C)	10.51	< .0001	33441	0.12	0.09	0.14
Heart Rate (bpm)	5.81	< .0001	30449	0.07	0.04	0.09
Locomotor Activity (ODBA)	8.56	< .0001	33390	0.09	0.07	0.12

The study cheetahs interacted with cognitive enrichment the most and physical enrichment the least (Fig. 3.2). Sensory enrichment elicited greater displays of olfactory exploration. Social enrichment elicited greater displays of stereotypic pacing and agnostic behaviour.

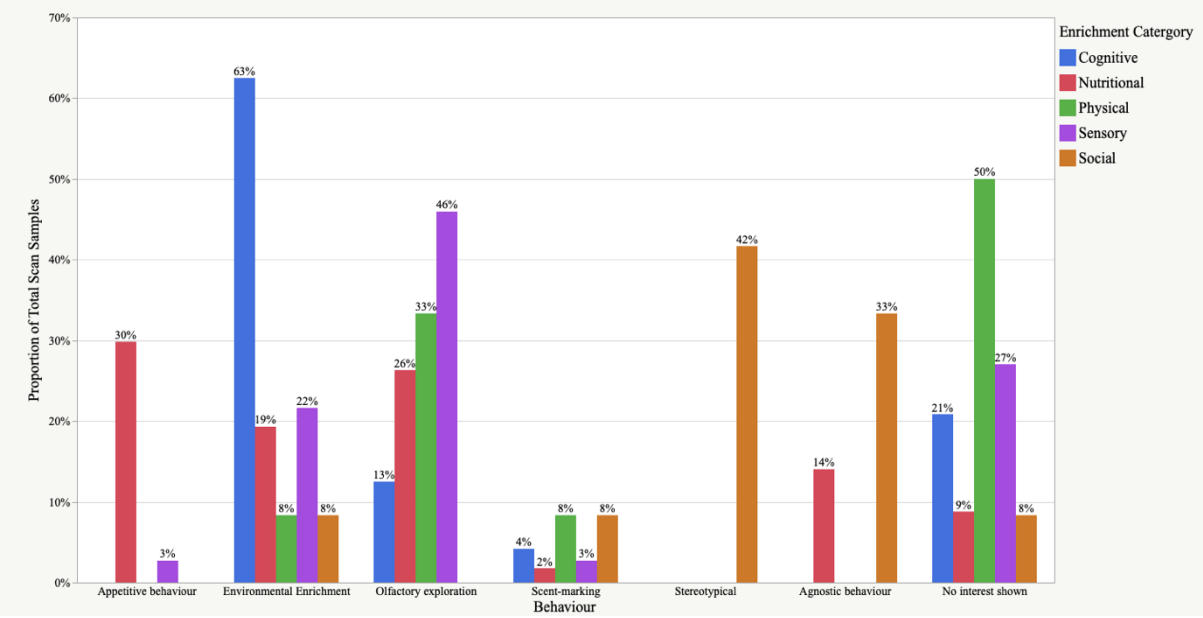

Figure 3.2. Bar chart of each behaviour for the study cheetahs (CH-2205, -2206, -2271, -2276, and -2277). Effect of enrichment category at enrichment time. The number above the bar indicates the proportion of total scan samples study cheetahs showed each behaviour at the time enrichment was provided.

3.3.2 Faecal Glucocorticoid Metabolite Concentrations

Faecal glucocorticoid metabolite concentration data were Box-Cox transformed to more closely satisfy the assumption of normality and homogeneity. The MMRM analysis revealed that fGCM concentration was significantly higher during the treatment (1.08 ± 0.25 µg/g DW) than in the control (0.90 ± 0.21 µg/g DW; $F_{1,177.2} = 7.36$, $p = .0073$). Post hoc comparisons using Tukey's HSD test revealed that the fGCM concentration of the female study cheetahs was significantly higher during the treatment (1.17 ± 0.26 µg/g DW) than in the control (0.85 ± 0.18 µg/g DW; $t_{176.1} = -2.76$, $p = .0318$) (Fig. 3.3i).

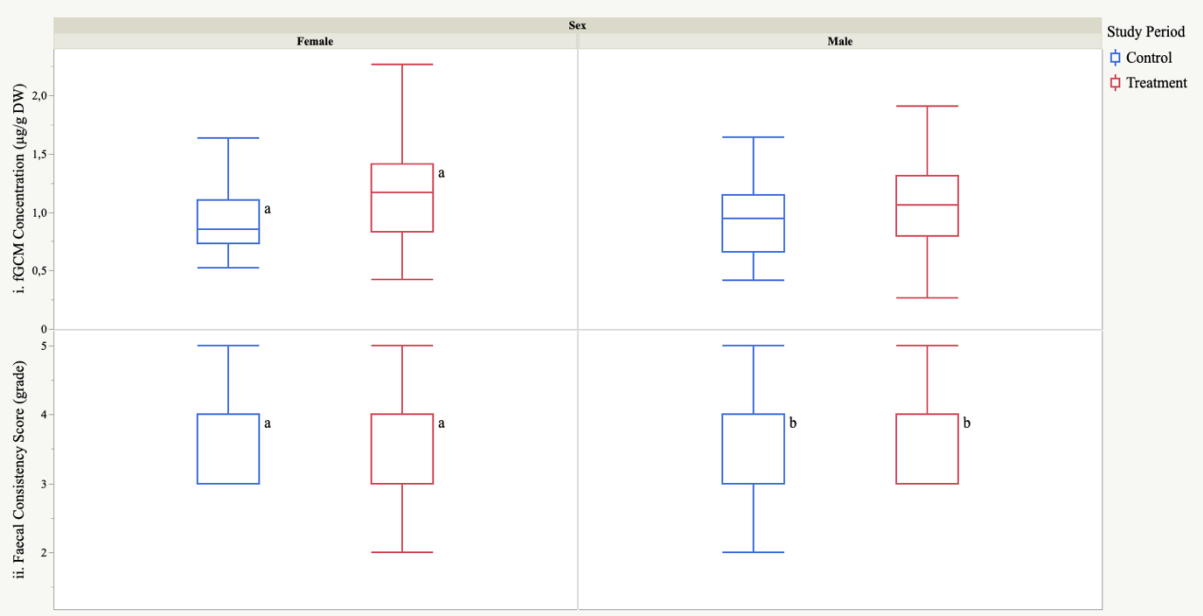

Figure 3.3. Box and whisker plot of (i) faecal glucocorticoid metabolite (fGCM) concentration (µg/g dry weight [DW]) and (ii) faecal consistency score (grade) for the study cheetahs (CH-2205, -2206, -2271, -2276, and -2277). Effect of sex (female and male) during the control and treatment. Statistics were performed by a mixed model for repeated measures and Tukey's HSD post hoc test.

(3) a: $p = .0318$. (ii) a: $p = .0267$ and b: $p = .0046$.

Post hoc comparisons using Tukey's HSD test revealed that fGCM concentration was significantly higher during the treatment of sensory enrichment (1.13 ± 0.18 µg/g DW) than in the control (0.90 ± 0.21 µg/g DW; $t_{161.2} = -2.88$, $p = .0499$) (Table 3.3).

Table 3.3. Median ± median absolute deviation (MAD) of faecal glucocorticoid metabolite (fGCM) concentration (µg/g dry weight [DW]), faecal consistency score (grade), body temperature (°C), heart rate (beats per minute [bpm]), and locomotor activity (overall dynamic body acceleration [ODBA]) for the study cheetahs (CH-2205, -2206, -2271, -2276, and -2277) during the control and treatment. Effect of enrichment category and enrichment item within the category.

Enrichment Category	fGCM Concentration (µg/g DW)	Faecal Consistency Score (grade)	Body Temperature (°C)	Heart Rate (bpm)	Locomotor Activity (ODBA)
	median ± MAD	median ± MAD	median ± MAD	median ± MAD	median ± MAD
Control	0.90 ± 0.21	3 ± 1	37.7 ± 0.3	78 ± 16	33.2 ± 20.2
Physical	1.13 ± 0.14	4 ± 0	37.8 ± 0.3	78 ± 18	33.4 ± 20.8
Large branches	1.33 ± 0.29	3 ± 0	37.9 ± 0.2	78 ± 18	34.1 ± 22.3
Dead leaves	1.08 ± 0.09	4 ± 0	37.7 ± 0.2	78 ± 18	33.2 ± 19.8
Social	1.32 ± 0.07	4 ± 1	37.8 ± 0.3	78 ± 18	31.2 ± 17.8
Big mirror	1.31 ± 0.06	4 ± 0	37.8 ± 0.3	78 ± 19	29.8 ± 16.4
Animal sounds	1.34 ± 0.22	4 ± 1	37.8 ± 0.2	79 ± 18	33 ± 19.4
Cognitive	1.04 ± 0.24	3 ± 0	37.7 ± 0.3	77 ± 17	37.4 ± 22.4
Feathers	0.83 ± 0.17	3 ± 1	37.7 ± 0.2	77 ± 17	36.2 ± 20.1
Jolly egg	1.06 ± 0.14	4 ± 0	37.7 ± 0.3	85 ± 19	40.2 ± 21.9
Paper jets	1.23 ± 0.22	3 ± 0	37.5 ± 0.2	70 ± 18	39 ± 25
Boxes	1.13 ± 0.73	4 ± 0.5	37.9 ± 0.2	76 ± 16	36 ± 22.9
Sensory	1.13 ± 0.18	3 ± 0	37.7 ± 0.2	77 ± 17	29.6 ± 18.4
Snakeskin	1.34 ± 0.28	3 ± 0	37.7 ± 0.2	77 ± 16	28 ± 17
Goat/sheep scent	0.96 ± 0.20	4 ± 1	37.7 ± 0.2	75 ± 16	30.9 ± 21.9
Duiker scent	1.13 ± 0.52	3 ± 0	37.8 ± 0.2	73 ± 15	29.6 ± 21.2
Fruit essences	1.14 ± 0.35	3 ± 0	37.7 ± 0.2	77 ± 16	30.1 ± 15.1
Bubbles	1.09 ± 0.23	4 ± 1	37.7 ± 0.3	77 ± 25	30 ± 18.2
Blood trail	1.30 ± 0.00	3 ± 0	37.8 ± 0.3	80 ± 18	29.3 ± 16.7
Nutritional	0.97 ± 0.26	4 ± 1	37.7 ± 0.3	77 ± 18	42.1 ± 22.2
Hooves	1.32 ± 0.25	4 ± 0	37.6 ± 0.2	82 ± 19	44.3 ± 21.1
50 g mince in straw	0.97 ± 0.13	4 ± 1	37.7 ± 0.3	78 ± 17	42 ± 25.4
Ice lollies/Jelly cakes	0.91 ± 0.28	4 ± 1	37.6 ± 0.3	71 ± 17	40.1 ± 20.3

Post hoc comparisons using Tukey's HSD test revealed that fGCM concentration was significantly higher during week two of the treatment (1.14 ± 0.21 µg/g DW) than in the control (0.90 ± 0.21 µg/g DW; $t_{175.1}$ = -2.93, p = .0201) (Fig. 3.4i).

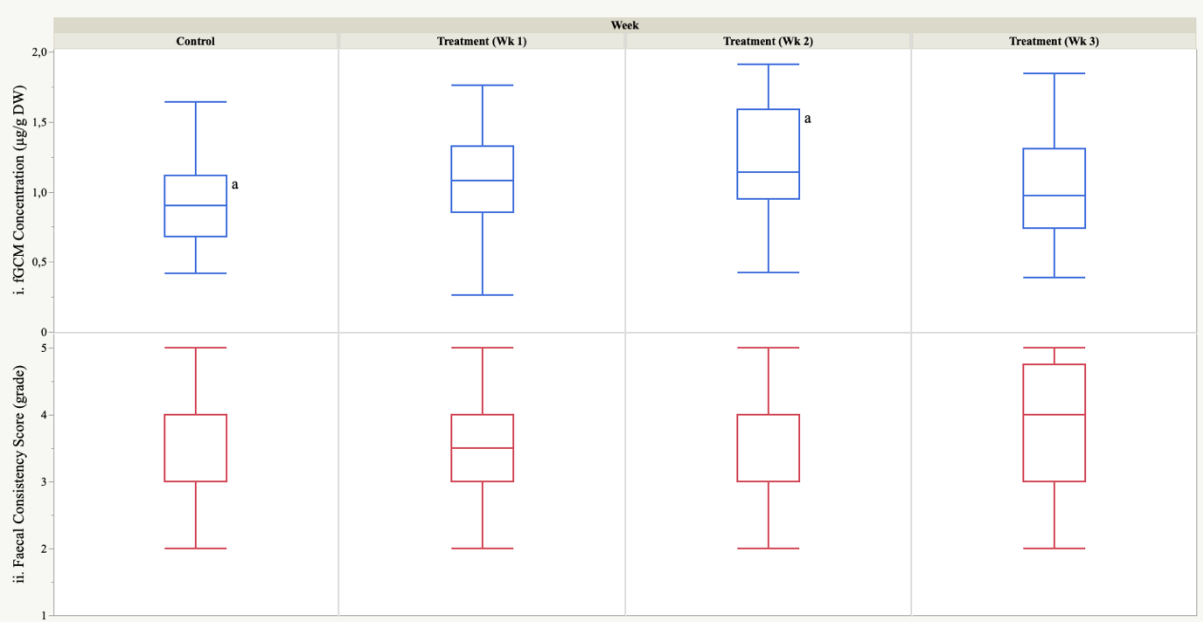

Figure 3.4. Box and whisker plot of (i) faecal glucocorticoid metabolite (fGCM) concentration (µg/g dry weight [DW]) and (ii) faecal consistency score (grade) for the study cheetahs (CH-2205, -2206, -2271, -2276, and -2277). Effect of control versus treatment week (Wk; one, two, or three). Statistics were performed by a mixed model for repeated measures and Tukey's HSD post hoc test.

(i) a: $p = .0201$.

Cohen's d revealed a small effect size ($d = 0.38$; $t_{181} = 2.53$, $p = .0121$) on fGCM concentration (Table 3.2).

3.3.3 Faecal Consistency Scores

Faecal consistency score data were Box-Cox transformed to more closely satisfy the assumption of normality and homogeneity. The soft with shape faecal grade was the most frequently recorded in the study cheetahs (for the control: 45.5% and the treatment: 45.3%) (Fig. 3.5). During the treatment, the 'normal' grade of firm and dry was higher (~9.5%) and the 'normal' grade of firm (~3.5%) and the 'suboptimal' grade of soft without shape (~5.9%) were lower than in the control. The median FCS was higher during the treatment (4 ± 1) than in the control (3 ± 1). The MMRM analysis revealed that this numerical difference failed to

achieve statistical significance. Post hoc comparisons using Tukey's HSD test revealed that the FCS of the female study cheetahs was significantly lower during the treatment (3 ± 0) than in the control (4 ± 1; $t_{176.1} = 2.83$, $p = .0267$) (Fig. 3.3ii). The FCS of the male study cheetahs was significantly higher during the treatment (4 ± 1) than in the control (3 ± 1; $t_{176.1} = -3.40$, $p = .0046$). Post hoc comparisons using Tukey's HSD test revealed that the FCS of the three-year-old study cheetahs was significantly higher during the treatment (4 ± 0) than in the control (3 ± 0; $t_{176.2} = -4.43$, $p < .0001$) (Fig. 3.6ii).

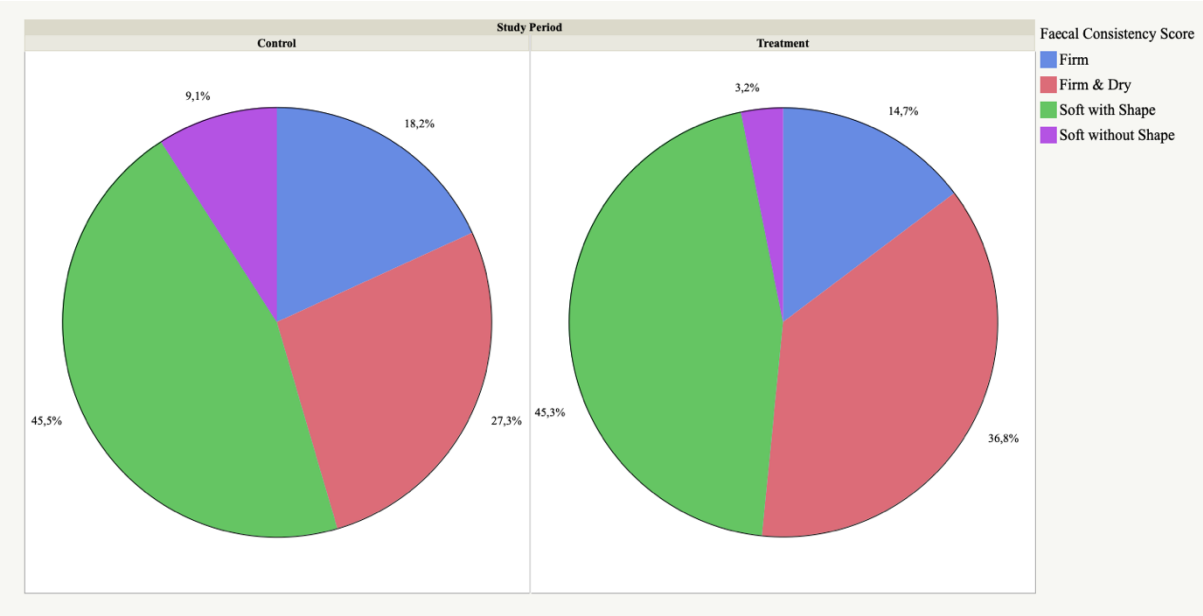

Figure 3.5. Pie chart of faecal consistency score for the study cheetahs (CH-2205, -2206, -2271, -2276, and -2277) during the control and treatment, where firm and firm and dry were considered to be 'normal' and liquid, soft without shape, and soft with shape were considered to be 'suboptimal' (adapted from AZA Tiger Species Survival Plan®, 2016; Whitehouse-Tedd *et al.*, 2015). The number above the slice indicates the proportion of total faecal samples collected for each score.

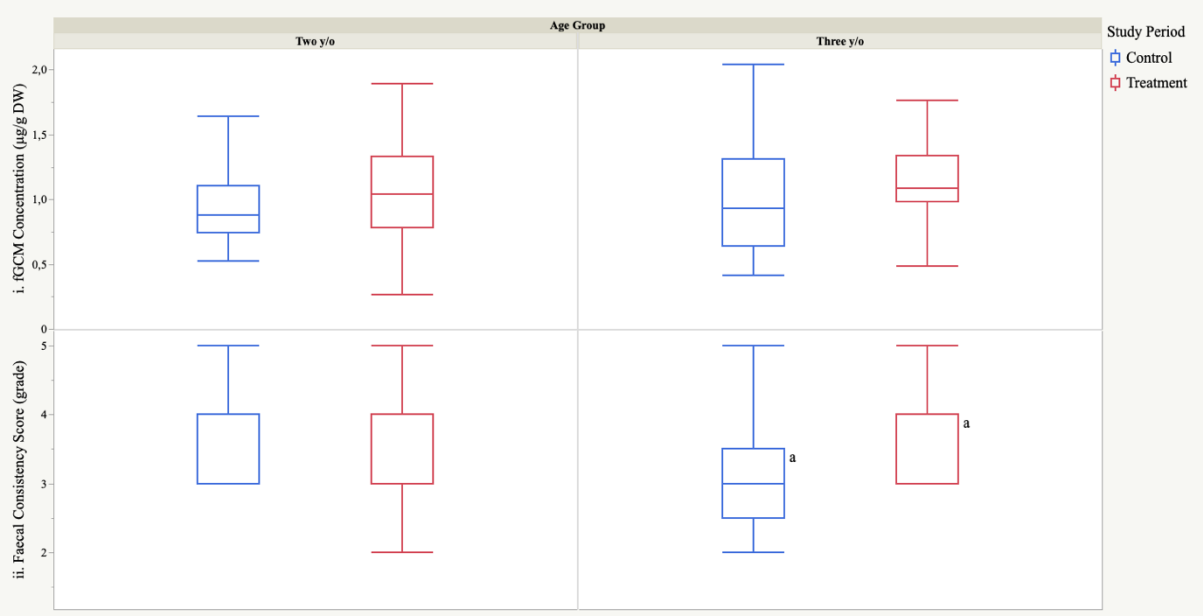

Figure 3.6. Box and whisker plot of (i) faecal glucocorticoid metabolite (fGCM) concentration (µg/g dry weight [DW]) and (ii) faecal consistency score (grade) for the study cheetahs (CH-2205, -2206, -2271, -2276, and -2277). Effect of age group (two and three years old [y/o]) during the control and treatment. Statistics were performed by a mixed model for repeated measures and Tukey's HSD post hoc test.

(ii) a: $p < .0001$.

3.3.4 Body Temperature, Heart Rate, and Locomotor Activity Recordings

Battery malfunction of the biologgers implanted in the study cheetahs CH-2271, CH-2276, and CH-2277 impeded the acquisition of complete T_b, HR, and LA data sets for those individuals. The initial examination of the raw HR data for the study cheetahs with functional data loggers (CH-2205 and -2206), in addition to partial data for CH-2276, revealed values ranging from 0 to 1005 bpm, the extremes of which were likely because of incomplete, low-quality readings or implant movement within the pectoral muscle when the study cheetahs were active. To remove erroneous measurements, ensuring only plausible values were included in the analyses, upper and lower thresholds were created (see section 1.1.6.9.3 for more information on the data preparation of T_b, HR, and LA recordings). Once filtered, all HR

recordings (n = 30451/33443, which represented over 90% of raw data initially obtained from the loggers) fell within the 30- and 200-bpm thresholds set.

Body temperature data were Box-Cox transformed to more closely satisfy the assumption of normality and homogeneity. The circadian rhythm of T_b fluctuated significantly during the 24-hour cycle (for part of the day: $F_{5,33417} = 3017.45$, $p < .0001$; and for hours within the day: $F_{18,33417} = 173.34$, $p < .0001$) (Fig. 3.7i). Throughout the study, i.e., in the treatment and control, the median T_b was higher during the evening (38.0 ± 0.2 ^{0}C) between 1600–1659 hours (38.0 ± 0.2 ^{0}C).

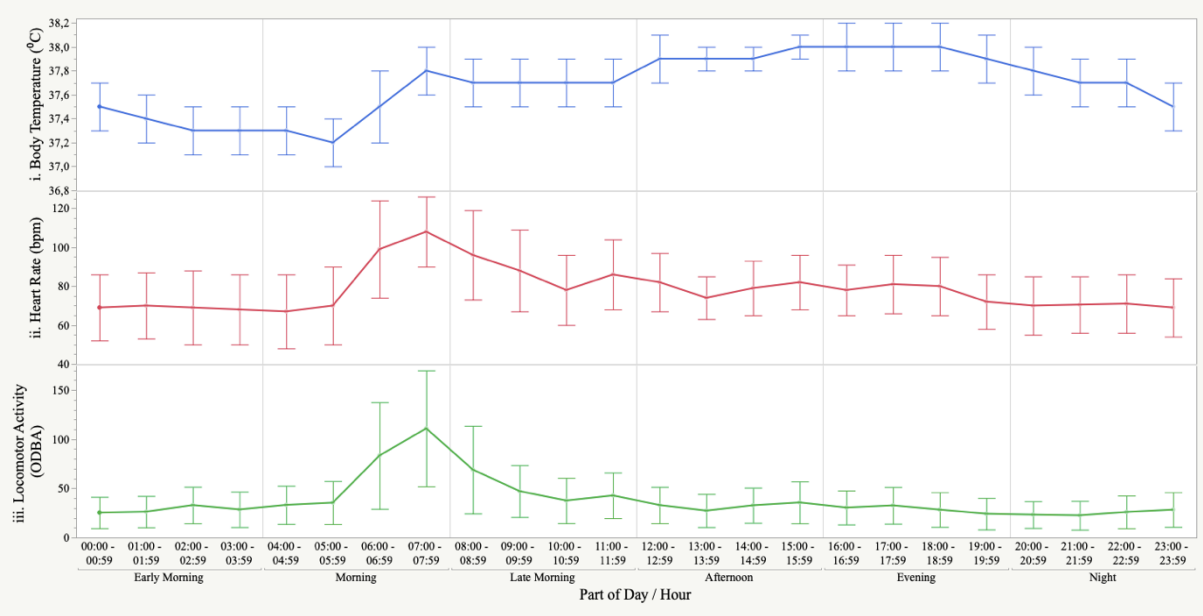

Figure 3.7. Circadian rhythm of (i) body temperature (^{0}C), (ii) heart rate (beats per minute [bpm]), and (iii) locomotor activity (overall dynamic body acceleration [ODBA]) (median, median absolute deviation) for the study cheetahs (CH-2205, -2206, and -2276) during the control and treatment.

The MMRM analysis revealed that T_b was significantly higher during the treatment (37.7 ± 0.3 ^{0}C) than in the control (37.7 ± 0.3 ^{0}C; $F_{1,33441} = 48.29$, $p < .0001$). Post hoc comparisons using Tukey's HSD test revealed that the T_b of the female study cheetahs was significantly higher during the treatment (37.9 ± 0.3 ^{0}C) than in the control (37.7 ± 0.3 ^{0}C; $t_{33438} = -27.30$, p

< .0001) (Fig. 3.8i). The T_b of the male study cheetahs was significantly lower during the treatment (37.7 ± 0.2 ^{0}C) than in the control (37.7 ± 0.2 ^{0}C; t_{33438} = 7.92, p < .0001). Post hoc comparisons using Tukey's HSD test revealed that the T_b of the two-year-old study cheetahs was significantly higher during the treatment (37.9 ± 0.3 ^{0}C) than in the control (37.7 ± 0.3 ^{0}C; t_{33438} = -27.30, p < .0001) (Fig. 3.9i). The T_b of the three-year-old study cheetahs was significantly lower during the treatment (37.7 ± 0.2 ^{0}C) than in the control (37.7 ± 0.2 ^{0}C; t_{33438} = 7.92, p < .0001).

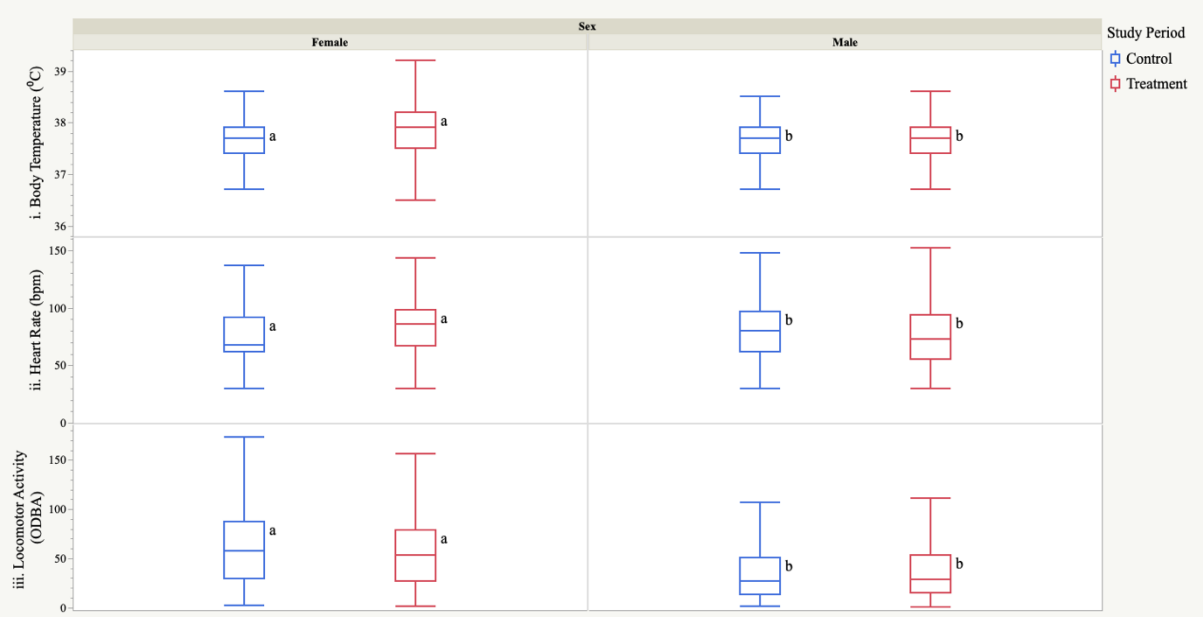

Figure 3.8. Box and whisker plot of (i) body temperature (^{0}C), (ii) heart rate (beats per minute [bpm]), and (iii) locomotor activity (overall dynamic body acceleration [ODBA]) for the study cheetahs (CH-2205, -2206, and -2276). Effect of sex (female and male) during the control and treatment. Statistics were performed by a mixed model for repeated measures and Tukey's HSD post hoc test.

(i) a: p < .0001 and b: p < .0001. (ii) a: p < .0001 and b: p < .0001. (iii) a: p < .0001 and b: p < .0001.

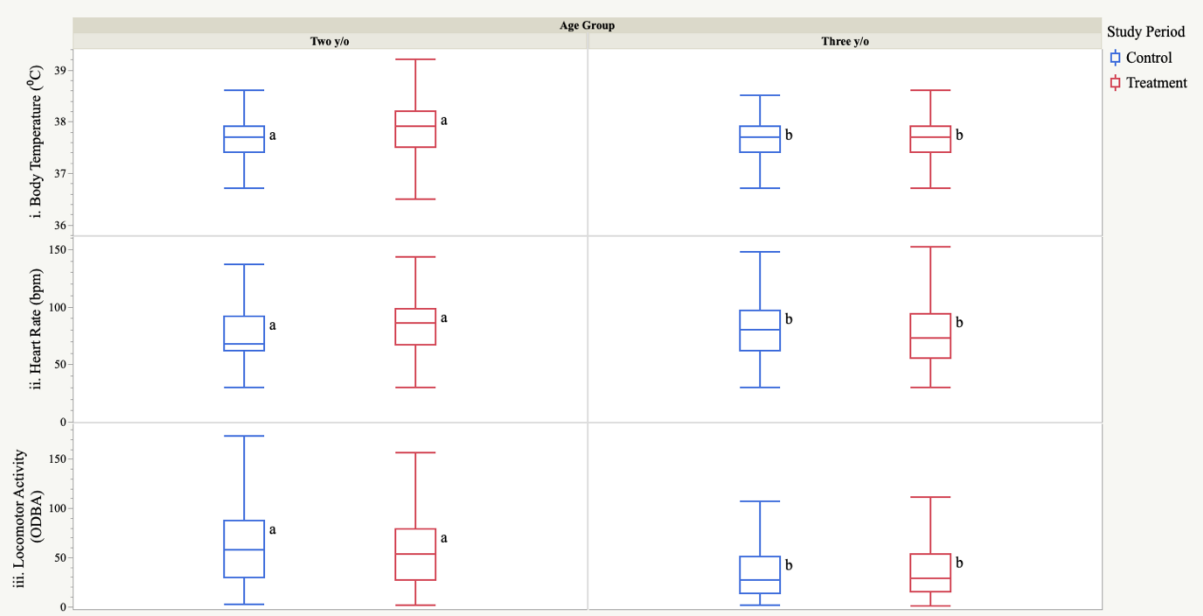

Figure 3.9. Box and whisker plot of (i) body temperature (^{0}C), (ii) heart rate (beats per minute [bpm]), and (iii) locomotor activity (overall dynamic body acceleration [ODBA]) for the study cheetahs (CH-2205, -2206, and -2276). Effect of age group (two and three years old [y/o]) during the control and treatment. Statistics were performed by a mixed model for repeated measures and Tukey's HSD post hoc test.

(i) a: $p < .0001$ and b: $p < .0001$. (ii) a: $p < .0001$ and b: $p < .0001$. (iii) a: $p < .0001$ and b: $p < .0001$.

Post hoc comparisons using Tukey's HSD test revealed that T_b was significantly higher during the treatment of physical (37.8 ± 0.3 ^{0}C; $t_{33424} = -6.05$, $p < .0001$), social (37.8 ± 0.3 ^{0}C; $t_{33424} = -5.81$, $p < .0001$), sensory (37.7 ± 0.2 ^{0}C; $t_{33424} = -10.64$, $p < .0001$), and nutritional enrichment (37.7 ± 0.3 ^{0}C; $t_{33424} = 2.88$, $p = .0455$) than in the control (37.7 ± 0.3 ^{0}C) (Table 3.3).

Post hoc comparisons using Tukey's HSD test revealed that T_b was significantly higher during weeks one (37.7 ± 0.3 ^{0}C; $t_{33438} = -6.47$, $p < .0001$) and two of the treatment (37.7 ± 0.3 ^{0}C; $t_{33438} = -10.30$, $p < .0001$) than in the control (37.7 ± 0.3 ^{0}C), significantly higher during week two of the treatment than in weeks one ($t_{33438} = -3.07$, $p = .0114$) and three (37.7 ± 0.3

^{0}C; t_{33438} = 9.72, p < .0001), and significantly higher during week one of the treatment than in week three (t_{33438} = 6.65, p < .0001) (Fig. 3.10i).

Figure 3.10. Box and whisker plot of (i) body temperature (^{0}C), (ii) heart rate (beats per minute [bpm]), and (iii) locomotor activity (overall dynamic body acceleration [ODBA]) for the study cheetahs (CH-2205, -2206, and -2276). Effect of control versus treatment week (one, two, or three). Statistics were performed by a mixed model for repeated measures and Tukey's HSD post hoc test.

(i) a, b, d, e: p < .0001 and c: p = .0114. (ii) a, b, c: p < .0001. (iii) a, b, c, d, e: p < .0001.

Cohen's d revealed a trivial effect size (d = 0.12; t_{33441} = 10.51, p < .0001) on T_b (Table 3.2).

Heart rate data were Box-Cox transformed to more closely satisfy the assumption of normality and homogeneity. The circadian rhythm of HR fluctuated significantly during the 24-hour cycle (for part of the day: $F_{5,30425}$ = 359.06, p < .0001; and for hours within the day: $F_{18,30425}$ = 86.08, p < .0001) (Fig. 3.7ii). Throughout the study, i.e., in the treatment and control, the median HR was higher during the late morning (86 ± 21 bpm) and higher between 0700–0759 hours (108 ± 18 bpm).

The MMRM analysis revealed that HR was significantly lower during the treatment (77 ± 17 bpm) than in the control (78 ± 16 bpm; $F_{1,30448} = 65.28$, $p < .0001$). Post hoc comparisons using Tukey's HSD test revealed that the HR of the female study cheetahs was significantly higher during the treatment (86 ± 17 bpm) than in the control (68 ± 10 bpm; $t_{30446} = -13.07$, $p < .0001$) (Fig. 3.8ii). The HR of the male study cheetahs was significantly lower during the treatment (73 ± 19 bpm) than in the control (80 ± 17 bpm; $t_{30446} = 17.25$, $p < .0001$). Post hoc comparisons using Tukey's HSD test revealed that the HR of the two-year-old study cheetahs was significantly higher during the treatment (86 ± 17 bpm) than in the control (68 ± 10 bpm; $t_{30446} = -13.07$, $p < .0001$) (Fig. 3.9ii). The HR of the three-year-old study cheetahs was significantly lower during the treatment (73 ± 19 bpm) than in the control (80 ± 17 bpm; $t_{30446} = 17.25$, $p < .0001$).

Post hoc comparisons using Tukey's HSD test revealed that HR was significantly lower during the treatment of cognitive (77 ± 17 bpm; $t_{30432} = 5.03$, $p < .0001$), sensory (77 ± 17 bpm; $t_{30432} = 6.47$, $p < .0001$), and nutritional enrichment (77 ± 18 bpm; $t_{30432} = 6.85$, $p < .0001$) than in the control (78 ± 16 bpm) (Table 3.3).

Post hoc comparisons using Tukey's HSD test revealed that HR was significantly lower during weeks one (78 ± 18 bpm; $t_{30446} = 4.82$, $p < .0001$), two (77 ± 17 bpm; $t_{30446} = 6.23$, $p < .0001$), and three of the treatment (77 ± 18 bpm; $t_{30446} = 6.56$, $p < .0001$) than in the control (78 ± 16 bpm) (Fig. 3.10ii).

The MMRM analysis revealed that HR was significantly higher at enrichment time (97 ± 17 bpm) than when the study cheetahs were not provided EE (83 ± 16 bpm; $F_{1,5488} = 4.47$, $p = .0345$). Post hoc comparisons using Tukey's HSD test revealed that HR was significantly higher during the afternoon at enrichment time (102.5 ± 15 bpm) than when the study cheetahs were not provided EE (79 ± 13 bpm; $t_{5489.2} = -4.15$, $p = .0002$) (Fig. 3.11ii).

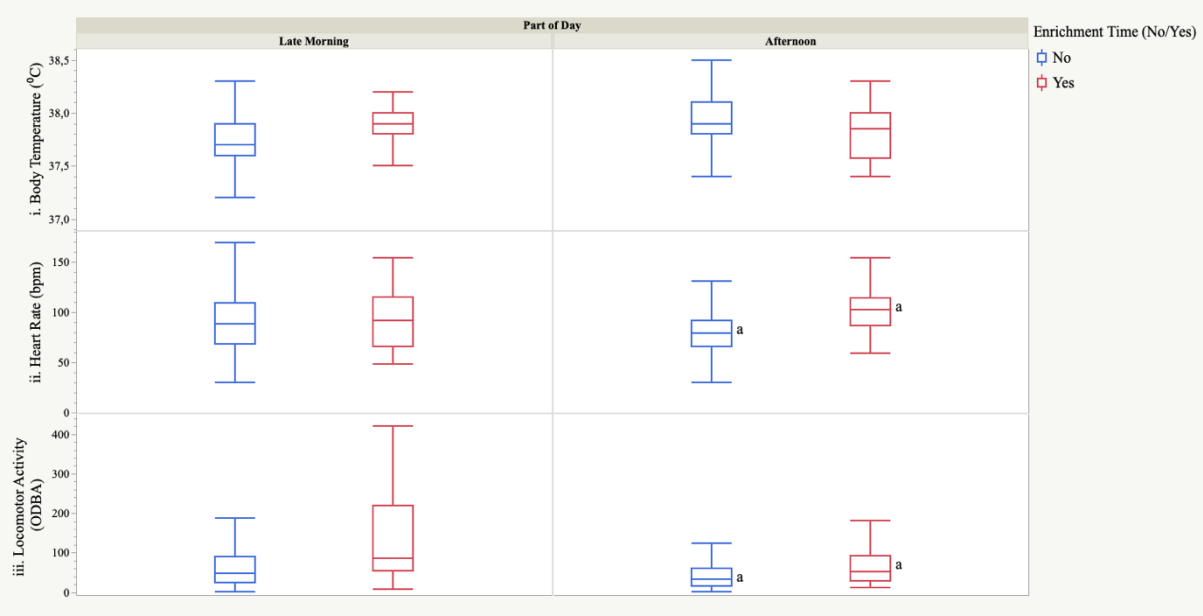

Figure 3.11. Box and whisker plot of (i) body temperature (^{0}C), (ii) heart rate (beats per minute [bpm]), and (iii) locomotor activity (overall dynamic body acceleration [ODBA]) for the study cheetahs (CH-2205, -2206, and -2276). Effect of enrichment time by a part of the day. Statistics were performed by a mixed model for repeated measures and Tukey's HSD post hoc test.

(ii) a: $p = .0002$. (iii) a: $p < .0001$.

Cohen's d revealed a trivial effect size ($d = 0.07$; $t_{30449} = 5.81$, $p < .0001$) on HR (Table 3.2).

Locomotor activity data were Box-Cox transformed to more closely satisfy the assumption of normality and homogeneity. The circadian rhythm of LA fluctuated significantly during the 24-hour cycle (for part of the day: $F_{5,33366} = 772.19$, $p < .0001$; and for hours within the day: $F_{18,33366} = 113.19$, $p < .0001$) (Fig. 3.7iii). Throughout the study, i.e., in the treatment and control, the median LA was higher during the morning (57.6 ± 40.4 ODBA) between 0700–0759 hours (111 ± 59 ODBA).

The MMRM analysis revealed that LA was significantly higher during the treatment (35.4 ± 21.6 ODBA) than in the control (33.2 ± 20.2 ODBA; $F_{1,33389} = 7.52$, $p = .0061$). Post hoc comparisons using Tukey's HSD test revealed that the LA of the female study cheetahs was significantly lower during the treatment (53.2 ± 26 ODBA) than in the control (57.6 ± 28.8

ODBA; t_{33387} = 6.12, p < .0001) (Fig. 3.8iii). The LA of the male study cheetahs was significantly higher during the treatment (28.8 ± 17 ODBA) than in the control (27.2 ± 17.2 ODBA; t_{33387} = -6.78, p < .0001). Post hoc comparisons using Tukey's HSD test revealed that the LA of the two-year-old study cheetahs was significantly lower during the treatment (53.2 ± 26 ODBA) than in the control (57.6 ± 28.8 ODBA; t_{33387} = 6.12, p < .0001) (Fig. 3.9iii). The LA of the three-year-old study cheetahs was significantly higher during the treatment (28.8 ± 17 ODBA) than in the control (27.2 ± 17.2 ODBA; t_{33387} = -6.78, p < .0001).

Post hoc comparisons using Tukey's HSD test revealed that LA was significantly lower during the treatment of sensory enrichment (29.6 ± 18.4 ODBA) than in the control (33.2 ± 20.2 ODBA; t_{33372} = 5.42, p < .0001) and significantly higher during the treatment of nutritional enrichment (42.1 ± 22.2 ODBA) than in the control (t_{33372} = -9.72, p < .0001) (Table 3.3).

Post hoc comparisons using Tukey's HSD test revealed that LA was significantly higher during weeks two (30.2 ± 18.8 ODBA; t_{33386} = 4.91, p < .0001) and three of the treatment (42.1 ± 22.2 ODBA; t_{33386} = -9.54, p < .0001) than in the control (33.2 ± 20.2 ODBA), significantly higher during week one of the treatment (35 ± 21 ODBA) than in week two (t_{33386} = 5.26, p < .0001), and significantly higher during week three of the treatment than in weeks one (t_{33386} = -6.87, p < .0001) and two (t_{33386} = -12.13, p < .0001) (Fig. 3.10iii).

The MMRM analysis revealed that LA was significantly higher at enrichment time (59 ± 31.9 ODBA) than when the study cheetahs were not provided EE (38.8 ± 23.2 ODBA; $F_{1,6028}$ = 4.47, p < .0001). Post hoc comparisons using Tukey's HSD test revealed that LA was significantly higher during the afternoon at enrichment time (52.5 ± 25 ODBA) than when the study cheetahs were not provided EE (32.6 ± 18.8 ODBA; $t_{6028.3}$ = -5.34, p < .0001) (Fig. 3.11iii).

Cohen's d revealed a trivial effect size (d = 0.09; t_{33390} = 8.56, p < .0001) on LA (Table 3.2).

3.4 DISCUSSION

Modern zoological institutions attempt to ameliorate confinement problems by providing animals in their care with enriched and challenging environments. However, it is necessary to determine what types of EE are effective for individual species in captivity. Considering enrichment behaviourally irrelevant to a species or an individual will not enhance their well-being, animals' behavioural responses are fundamental to assessing the efficiency of any EE programme (Mench, 1998; Newberry, 1995). The study cheetahs spent the majority of their time inactive. This is consistent with findings described by other authors on captive cheetahs (Skibiel *et al.*, 2007) as well as many other felids (Acaralp-Rehnberg *et al.*, 2020; Bashaw *et al.*, 2007; De Souza Resende *et al.*, 2014; Macri & Patterson-Kane, 2011; Moreira *et al.*, 2007; Shepherdson *et al.*, 1993; Weller & Bennet, 2001). Here, the provision of EE elicited in the study cheetahs significantly lower inactivity levels and higher LA, most significantly in response to nutritional enrichment. The territorial behaviour, scent-marking was significantly higher and, in response to sensory enrichment, the study cheetahs showed greater displays of olfactory exploration (Quirke & O'Riordan, 2015; Tarou & Bashaw, 2007; Wells, 2009; Wells & Egli, 2004). The results of these analyses support the researcher's hypothesis that EE would encourage natural active behaviours.

Following enrichment, increased active behaviour is a recurrent observation among captive carnivores, such as cheetahs (Quirke & O'Riordan, 2011a; 2011b), African wild dogs (*Lycaon pictus*; Rafacz & Santymire, 2014), lions (Bashaw *et al.*, 2003; Powell, 1995), Amur leopards (*Panthera pardus orientalis*; Yu *et al.*, 2009), tigers (*Panthera tigris*; Jenny & Schmid, 2002), and black-footed cats (*Felis nigripes*; Wells & Egli, 2004).

Environmental enrichment is promoted to reduce captivity-induced stress and animal stereotypies (Carlstead & Shepherdson, 2000; Swaisgood & Shepherdson, 2005) by providing

species-appropriate challenges (Meehan & Mench, 2007). Though felids are well-known meticulous groomers, if performed excessively, auto-grooming can become stereotypical and self-injurious rather than a normal body care behaviour pattern (Smith & Wettlaufer, 2009). Desirably so, then, auto-grooming was significantly lower in response to EE.

Stress reduction by EE extends to sympathoadrenal responses. The two branches of the neuroendocrine stress system are the SNS and HPA axis (see section 1.1.2 for more information on the adaptive stress response). Both branches originate in the hypothalamus and converge on the adrenal gland, working together to maintain or re-establish homeostasis by orchestrating behavioural and physiological adaptations to the stressor (Coffman, 2020). The SNS provides the immediate first wave of the stress response, mediating the rapid release of the catecholamine hormones E and NE from the adrenal medulla (Romero & Wingfield, 2015). The second wave is more gradual and involves the GCs, the end-products of the hormonal cascade along the HPA axis (Sapolsky *et al.*, 2000). Environmental enrichment blunts E release (Moncek *et al.*, 2004) and, consequently, stress-related increases in HR (Azar *et al.*, 2012; Marashi *et al.*, 2003; Ravenelle *et al.*, 2014; Sharp *et al.*, 2014; 2002). Lower HR was observed, most significantly in response to cognitive, sensory, and nutritional enrichment, indicating stress reduction (Wascher *et al.*, 2021). The study cheetahs showed higher affiliative behaviour, most significantly in response to sensory enrichment. Affiliative behaviour can itself have a stress-reducing effect; grooming performed by a familiar human decreased HR in laboratory-kept rhesus monkeys (*Macaca mulatta*; Grandi & Ischida, 2015), dairy cows (Schmied *et al.*, 2008), and lambs (Coulon *et al.*, 2015).

Based on the proposed contention that EE acts as a mild stressor (Smail *et al.*, 2020), the researcher hypothesised that providing EE would induce arousal and activate the physiological stress response. In support of this hypothesis, fGCM concentration and T_b (a salient feature of

the SNS-mediated reaction to stress) were higher, most significantly in response to sensory enrichment and physical, social, sensory, and nutritional enrichment, respectively. Enrichment-induced arousal was further attended by significantly higher HR at enrichment time, which, as it corresponded with significantly higher LA, could have been caused by the demands of increased physical activity. During physical activity, requirements for oxygen and gluconeogenetic substrates in skeletal muscle are increased, as are the removal of metabolites and carbon dioxide (Burton *et al.*, 2004). The cardiac output is increased to meet the demand for blood flow by contracting muscles, attributed to sympathetically-mediated increases in HR and stroke volume.

Despite the benefits of EE, zoos should impose mild stressors with trepidation. It is necessary to ensure that the intended arousal and resilience are achieved while avoiding unintended frustrations or the development of negative affective states in animals (Learmonth, 2019). Undesirable behaviours in captive animals include self-injurious actions like excessive grooming and stereotypical and aggressive behaviours (Smith & Wettlaufer, 2009). Environmental enrichment is often used to increase activity and decrease stereotypies and aggression in captive felids (Bashaw *et al.*, 2003; Bloomsmith *et al.*, 1988; McPhee, 2002; Powell, 1995; Shepherdson *et al.*, 1993). Contrastingly, the study cheetahs showed greater displays of stereotypical pacing and agnostic behaviours in response to social enrichment (big mirror and animal sounds), cautioning against it as an effective EE strategy for the cheetah. If the study cheetahs perceived their reflection as an enemy (competitor or predator), they could have felt intimidated. Pacing and agnostic behaviours may have resulted from the frustration of attempts to perform intrinsically motivated behaviour in response to auditory and/or visual predator and prey cues (Mason, 2006).

Environmental enrichment actions vary between the sexes and by the age of the animal, with females (Belz *et al.*, 2003; Girbovan & Plamondon, 2013; Hendershott *et al.*, 2016; Mármol *et al.*, 2015) and adolescents (O'Leary *et al.*, 2019; Peña *et al.*, 2009; Segovia *et al.*, 2009) showing more pronounced effects. The demographic factors, sex and age group, affected the study cheetahs' activity levels and physiological (fGCM concentration, T_b, and HR) responses, possibly explained by the sensitivity of the female study cheetahs and those two years old to EE.

Novel stimuli lose their effect over time due to habituation (Kuczaj *et al.*, 2002), whereby animals' responsiveness to a stimulus diminishes with repeated exposure to that stimulus (McFarland, 2021). The study cheetahs showed significantly lower activity levels and higher affiliative behaviour during week two of the treatment. The temporal profiles of fGCM concentration and T_b appeared to demonstrate habituation, with values increasing during week one of the treatment, significantly higher during week two, and decreasing in week three. Alternatively, sensory enrichment was provided during week two of the treatment, to which the study cheetahs had significant affiliative behaviour, fGCM concentration, and T_b responses. Locomotor activity was significantly higher during week three of the treatment, suggesting that EE was sufficiently dynamic to provide prolonged stimulation to the study cheetahs.

The study cheetahs' FCS was significantly higher during week three of the treatment. Symbiotic gut microbes harboured in animals' GI tracts serve various essential functions (Wasimuddin *et al.*, 2017). However, disturbance-related deviation in the microbial diversity and abundance pattern beyond a natural range, i.e., gut dysbiosis, can advance pathophysiology and affect host health (Shreiner *et al.*, 2015). Faecal consistency is purportedly linked to intestinal microbiota composition (Tigchelaar *et al.*, 2016; Vandeputte *et al.*, 2016) and could indicate GI health (Whitehouse-Tedd *et al.*, 2015). Chronic or repeated exposure to stressors

has been identified to disrupt gut homeostasis (Accarie & Vanuytsel, 2020; Farzi *et al.*, 2018). Therefore, the higher FCS may be attributable to the stress-reducing effects of EE (Smail *et al.*, 2020).

This study's findings encourage the provision of inexpensive, easy-to-administer EE to enhance the welfare of captive cheetahs, particularly cognitive, sensory, and nutritional enrichment. However, it is essential to utilise effective types of enrichment to sustain their future use (Quirke & O'Riordan, 2011b). A particular enrichment used continuously in a regular, predictable schedule may reduce its effectiveness. As such, I support the current recommendation of using a randomised EE schedule rather than a sequential one like in the present study to provide a greater degree of novelty and ensure the cheetahs do not habituate to any single enrichment type or item (Quirke & O'Riordan, 2011a; 2011b). To ensure that the study cheetahs were enriched for the duration of the 3-week treatment period, different numbers of items were used to represent each type of EE, and nutritional enrichment items were provided two (horse hoof and ice lollies/jelly cakes) or three (50 g of mince in straw) times on nonconsecutive days. It must be acknowledged that this could have biased the results.

Furthermore, I demonstrated that biologging technology could be used to record concurrent T_b, HR, and LA and, in addition to behavioural and physiological metrics, has tremendous potential as a tool to measure stress in the cheetah. However, a significant limitation of the present study was the battery malfunction of the biologgers implanted in the study cheetahs CH-2271, CH-2276, and CH-2277, impeding the acquisition of complete T_b, HR, and LA data sets for those individuals. For the technology to be routinely utilised in animal welfare studies, there is still a need to develop more reliable devices capable of remote data transmission to avoid repeated capture and handling.

3.5 LITERATURE CITED

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Chapter 4: Responses to Animal-Visitor Interactions in Captive-Born Cheetahs (*Acinonyx jubatus*): Implications for Behavioural and Physiological Stress and Gastrointestinal Health

ABSTRACT. Considering the current prevalence of various animal-visitor interactions (AVI) offered at zoos and aquaria worldwide, more research is required on interactive experiences regarding their impact on animal welfare. This study investigated the response of captive-born (hand-reared) cheetahs (n = 5) to participation in AVIs for three weeks. Using emerging biologging technology and more traditional measures of stress-related behavioural and physiological responses, variations in body temperature (T_b), heart rate (HR), locomotor activity (LA), behaviour, faecal glucocorticoid metabolite (fGCM) concentration, and faecal consistency score (FCS) were documented. Up-close and, at times, hands-on contact with visitors elicited in the study cheetahs higher fGCM concentration ($p < .01$), T_b ($p < .01$), and HR ($p < .0001$). Animal-visitor interactions resulted in higher LA ($p < .0001$) and FCS ($p \leq .05$) and, at interaction time, lower T_b ($p < .0001$), HR, and LA ($p < .0001$), consistent with their purportedly enriching effects. Sex and age group affect the study cheetahs' activity levels and physiological response, which may be attributable to the sensitivity of the female cheetahs and those two years old to EE or the visitors if perceived to be competitors or predators. Body temperature and HR appeared to demonstrate habituation. However, the temporal profile of LA suggested AVIs were sufficiently dynamic to provide prolonged stimulation. This study's findings reiterate the ambiguous impact of AVIs on the animals involved, including whether they are positive, neutral, or negative.

4.1 INTRODUCTION

Attracting around 700 million visits annually (Gusset & Dick, 2011), modern zoos and aquaria worldwide are uniquely positioned to connect people with nature. One way zoos can facilitate emotional connections may be through up-close interactions with a live animal (Anderson *et al.*, 2003; Luebke *et al.*, 2016; Powell & Bullock, 2015). Over 75% of institutions affiliated with the World Association of Zoos and Aquariums (WAZA) offer at least one type of AVI experience (D'Cruze *et al.*, 2019). Animal-visitor interactions occurring at zoological institutions do so on the presumption that they contribute to influencing visitors' attitudes,

emotions, knowledge, and behaviour toward conserving the species they interacted with and the natural world as a whole (Clayton *et al.*, 2017; Kreger & Mench, 1995; Skibins & Powell, 2013; WAZA, 2020). Additionally, they support conservation efforts by generating much-needed monetary funds (Ward & Sherwen, 2018). Despite the popularity of AVIs, the available evidence of their impact on the animal (or the visitor), including whether they are positive, neutral, or negative, is often equivocal (Hosey, 2008; Hosey & Melfi, 2015; WAZA, 2020).

The nature of AVIs offered by zoos and aquaria varies widely, as do the taxa involved (D'Cruze *et al.*, 2019). Animal-visitor interactions can be either indirect (e.g., non-hand feeding, drive-through safaris, animal shows or performances) or indirect experiences (e.g., hand-feeding, petting, riding, walking or swimming with animals) (D'Cruze *et al.*, 2019). Animal Ambassador Encounters involve one-to-one close contact between visitors and specially trained animals, acting as ambassadors for their species or a conservation cause (Spooner *et al.*, 2021).

Initial research has focused on the effects of visitor presence more than AVIs (e.g., Fernandez *et al.*, 2009; Kuhar, 2008; Quadros *et al.*, 2014; Sherwen *et al.*, 2015). Visitors are proposed to have three potential effects on the welfare of captive animals: a neutral effect, an enriching effect, or a stressful effect (Hosey, 2000). Some studies demonstrate the neutral effect, where visitor number or intensity was not associated with changes in behaviour (Choo *et al.*, 2011; Górecki *et al.*, 2012; Margulis *et al.*, 2003; O'Donovan *et al.*, 1993; Sherwen *et al.*, 2014), and others suggest animals potentially experience positive emotions associated with visitor presence (Bloomfield *et al.*, 2015; Claxton, 2011; Nimon & Dalziel, 1992). However, most published visitor effect studies support the stressful hypothesis. Mallapur & Chellam (2002) observed visitor presence to decrease activity in leopards. The presence of visitors has also been shown to cause increased stereotypies and agnostic behaviour and decreased

affiliative behaviour in many species (Carder & Semple, 2008; Chamove *et al.*, 1988; Hosey, 2000; Kuhar, 2008; Mallapur *et al.*, 2005; Sellinger & Ha, 2005; Sherwen *et al.*, 2015; Wells, 2005). Wielebnowski *et al.* (2002a) found that fGCM concentrations were higher in clouded leopards (*Neofelis nebulosa*) on public display than in individuals housed off-exhibit. Similarly, cortisol concentrations were positively correlated with the number of visitors in capuchins (*Cebinae*; Sherwen *et al.*, 2015), spider monkeys (*Ateles*; Davis *et al.*, 2005), and black rhinos (*Diceros bicornis*; Carlstead & Brown, 2005).

While the studies mentioned above investigated the effects of passive visitors on exhibited animals, the impact of interactive visitor experiences on animal welfare has rarely been considered. Of the few studies, most reported a net positive or neutral impact of up-close AVIs (Collins *et al.*, 2017; Jones *et al.*, 2016; Learmonth *et al.*, 2021), though intense, longer-lasting interactions may lead to the reduced welfare of captive animals (Baird *et al.*, 2016; Spooner *et al.*, 2021).

Moreover, felids are under-represented in research, despite an increasing number of zoos offering visitor experiences with the naturally solitary and elusive species (Szokalski *et al.*, 2013). One study found increased stereotypic pacing in captive lions and a tiger participating in direct interactive experiences, which authors suggested was associated with the animals being fed during the interaction rather than the stress caused by close contact with visitors (Szokalski *et al.*, 2013). Contrastingly, Szokalski *et al.* (2013) observed affiliative behaviour by cheetahs towards both keepers and visitors during interactive walks, suggesting that it may have been a positive affective experience for the cheetahs (Szokalski *et al.*, 2013). A variable effect of interactive programmes was reported in tigers, where programme animals had higher overall fGCM concentrations at one institution than non-participating animals. However, the opposite was observed at a second zoo (Narayan *et al.*, 2013).

This variation in how animals respond to AVIs depends not only on the nature and intensity of the interaction (indirect or direct experiences) but also species-specific differences, enclosure design, individual animal traits (e.g., temperament and motivational state, as well as previous experiences and familiarity with humans), and visitor characteristics (Sherwen & Hemsworth, 2019). Its "nonaggressive temperament and ease of trainability and handling when raised by experienced and qualified handlers" (Rapp *et al.*, 2018, p. 404) make the cheetah a model candidate for ambassador programmes. Considering the widespread occurrence of AVIs in zoos and their popular role as programme animals, studies investigating the welfare impacts on captive cheetahs are needed to justify the continued participation of this species in interactions ethically. Such research would optimise the care and welfare of individual animals and contribute to our understanding of AVIs in felids.

This study's overall aim was to investigate the response of captive-born (hand-reared) cheetahs to participation in AVIs. Considering the reported susceptibility of the cheetah to captivity-induced stress (Munson *et al.*, 2005; Terio *et al.*, 2004), I hypothesised that up-close and, at times, hands-on contact with visitors would exacerbate this response. As established stress-related markers, behavioural observations (Quirke *et al.*, 2012) and fGCM concentration analysis (Terio *et al.*, 1999) were performed, in addition to faecal consistency scoring as a preliminary indicator of the study cheetahs' GI health (Whitehouse-Tedd *et al.*, 2015). A secondary objective was to explore the potential of biologging technology for measuring stress in the cheetah by recording concurrent T_b, HR, and LA. I predicted greater displays of stereotypic pacing and agnostic behaviour and higher fGCM concentration, T_b, and HR consistent with a behavioural and physiological stress response.

4.2 MATERIALS AND METHODS

4.2.1 Study Site and Animals

The experimental trials occurred between April and September 2019 at the Cango Wildlife Ranch and Conservation Centre (33°33'S, 22°12'E) 4 km north of Oudtshoorn, in the Western Cape of South Africa (see section 1.1.6.1 for more information on the study site and animals). Three male (CH-2205, -2206, and -2271) and two female (CH-2276 and -2277) resident adult cheetahs (Table 1.1) hand-reared and conditioned to daily interactions with the facility's caretakers and visitors were allocated to this study.

4.2.2 Body Temperature, Heart Rate, and Locomotor Activity Measurement Intervals

A single cardiac-, temperature- and movement-sensitive biologger (DST centi-HRT ACT, Star-Oddi, Gardabaer, Iceland) with the dimensions of 46 mm x 15 mm x 15 cm and weighing approximately 19 g was implanted in each of the study cheetahs. The biologgers were calibrated individually against a high-accuracy thermometer (Hart 1504, Fluke, Utah, US). Calibrated accuracy was better than 0.1 °C. Before surgical implantation, the biologgers were set to record tri-axial LA, i.e., heave, surge, sway, every minute for the ODBA and T_b (°C) and leadless single-channel ECG-derived HR (bpm) every 5 minutes at 200 Hz. Representative traces of raw ECG recordings were saved every 24 hours for validating the HR measurements' QI (where QI_0 was the highest quality and QI_1, QI_2, and QI_3 were of progressively reduced quality). Afterwards, the biologging devices were sterilised using ethylene oxide and implanted (see section 1.1.6.3 in the introduction for more information on the surgical procedure).

4.2.3 Experimental Design

The study cheetahs housed individually (CH-2271) or in pairs (CH-2205 and -2206, and CH-2276 and -2277, respectively) were assigned randomly to either the treatment, i.e., participation in AVIs or the control in an initial 3-week period at variable times and the alternative in a period following of equal duration (see section 1.1.6.4 for more information on the experimental design). During the treatment, the study cheetahs were housed on-exhibit (Fig. 1.2). They participated in visitor interactions conducted in a highly controlled environment by experienced caretakers, with stringent safety protocols and the highest welfare standards adhered to. The Cango Wildlife Ranch prioritises natural encounters, whereby it is the animals' choice whether or not to participate in interactions. On introducing the visitor(s) to the enclosure, caretakers assess the study cheetahs' psychological state and, if deemed motivated to interact, supervise up-close and, at times, hands-on contact with visitors whereby they may pet and stroke the cheetah on top of its head and flank. For the protection of animals and humans alike, encounters are limited by visitor age, health status, and the number of visitors per interaction, i.e., a maximum of four.

Encounters took place between 0900–1800 hours. Depending on visitor demand and the study cheetahs' motivational state, interactive experiences varied daily regarding the number of interactions and visitors interacted per day and the duration of interactions (Table 4.1). In the event of paired housing (Table 1.1), visitor presence in the enclosure was assigned to both individuals, regardless of which cheetah participated in the interaction directly.

Table 4.1. Animal-visitor interaction statistics for the treatment period.

Identification Number	Number of Interactions Per Day			Number of Visitors Interacted with Per Day			Interaction Duration (HH:MM:SS; mean ± SD)
	Min	Max	Mean ± SD	Min	Max	Mean ± SD	
CH-2205 & -2206	0	7	2.57 ± 1.91	0	20	7.57 ± 5.96	00:12:58,9 ± 0:00:04
CH-2271	0	20	11.86 ± 5.42	0	68	37.10 ± 19.59	00:08:45,5 ± 0:00:05
CH-2276 & -2277	0	19	10.76 ± 5.32	0	60	31 ± 17.59	00:10:41,7 ± 0:00:05

Min: minimum, Max: maximum, SD: standard deviation.

During the control, the study cheetahs were housed off-exhibit at the Jill Bryden-Fayers Reserve neighbouring the Ranch, where no AVIs were conducted. After movement on- or off-exhibit, the study cheetahs were habituated to the associated increase or decrease in the human presence (see section 1.1.6.4 for more information on the experimental design). Other than the specific intervention being investigated, i.e., participation in AVIs, the study cheetahs' environment, housing, and management (as described in section 1.1.6.1) were maintained across the treatment and control, including daily interactions with the facility's caretakers.

Throughout the treatment and control, the study cheetahs were monitored regarding (i) behaviour, (ii) fGCM concentration, and (iii) FCS, as well as (iv) T_b, HR, and LA concurrently recorded by implanted data loggers.

4.2.4 Behavioural Data Collection

Instantaneous scan sampling (Altmann, 1974) with a 5-minute inter-scan interval was used in this study (Quirke & O'Riordan, 2011a; 2011b). During five weekly 60-minute observation sessions, the researcher (KLB) carried out 12 instantaneous scan samples on focal animals, one enclosure at a time and in a randomised order to prevent time-of-day effects. For the study cheetahs housed in pairs (Table 1.1), behavioural data were collected simultaneously using

physical characteristics to identify individuals. Observations were performed between 0700 and 1700 based on the operating hours of the Cango Wildlife Ranch and Conservation Centre.

Fifteen behaviours, categorised into inactive, active, and not observed, were recorded (Table 1.2). Preliminary observation of the study cheetahs and the literature on felids' behaviour (Altman *et al.*, 2005; Quirke & O'Riordan, 2011a; 2011b; Regaiolli *et al.*, 2019) informed the ethogram used. Time spent out of sight (hiding or staying away from the human observer) was recorded as its performance may be indicative of a psychological stress response (Carlstead *et al.*, 1993; Davey, 2006; Hosey, 2013; Morgan & Tromborg, 2007; Sellinger & Ha, 2005).

4.2.5 Faecal Sample Collection and Consistency Scoring

To differentiate between individual faecal samples in the event of paired housing (Table 1.1), 1 tbsp of uncooked rice was thoroughly mixed into the diet of study cheetahs CH-2206 and CH-2277 once per day. The caretakers monitored the study cheetahs during feeding times to ensure the sufficient consumption of rice and prevent meal sharing. Only faeces found to have uncooked rice were considered to have originated from those individuals fed rice. Due to the operating hours of the Cango Wildlife Ranch and Conservation Centre, the enclosures could not be entered between 1800–0800 hours. As such, once daily between 0800–1000 hours, faeces from the previous night were collected from each enclosure within 16 hours of defecation (4–10 °C T_a) (Ludwig *et al.*, 2013).

Following sample collection, the researcher (KLB) assigned FCS as per a five-point grading system, where two points (grade 4: firm and dry and grade 5: firm) were considered to be 'normal,' and three points (grades 1–3: liquid, soft without shape, and soft with shape) were considered to be 'suboptimal' (adapted from AZA Tiger Species Survival Plan®, 2016;

Whitehouse-Tedd *et al.*, 2015). Afterwards, the samples were deposited into appropriately labelled (sample collection date and study cheetah and sample ID numbers) 50 mL polypropylene specimen containers and frozen at –20 °C.

4.2.6 Faecal Steroid Extraction and Quantification

Following completion of the experimental trials, faecal samples were transported frozen to the Endocrine Research Laboratory, University of Pretoria, South Africa. Faecal steroids were extracted and analysed for fGCM concentration as described in section 1.1.6.7.

4.2.7 Ethical Statement

All of the experimental procedures involving the study cheetahs were approved by the University of Pretoria's Animal (clearance number V075-18) and Research Ethics Committees (clearance number REC069-18).

4.2.8 Statistical Analysis

Statistical analysis was performed using Microsoft Excel (version 16.0) and JMP Pro software (version 16.0) for Windows, developed by SAS Institute Inc (North Carolina, US). Raw data was manipulated before detailed analyses (see section 1.1.6.9 for more information on the data preparation). The data were screened for univariate outliers greater than three interquartile ranges away from the 99.5th or 0.05th percentiles (LA: n = 66), which were subsequently excluded from descriptive statistics and analyses. Normal distribution and homogeneity of variance were verified using Anderson-Darling and Levene's tests, respectively. Box-Cox transformations were used to more closely satisfy the assumption of normality and homogeneity in the case of departure. To maintain statistical integrity, the data

were back-transformed for descriptive statistics and visual representation. An MMRM analysis was conducted to investigate the following:

- The random effect of the study cheetah, the independent fixed effect of the study period, and the interaction fixed effects of the study period and (i) sex and (ii) age group on each behaviour.
- The random effect of the study cheetah and the fixed effect of control versus treatment week (one, two, or three) on each behaviour.
- The random effect of the study cheetah, the independent fixed effect of the study period, and the interaction fixed effects of the study period and (i) sex and (ii) age group on fGCM concentration and FCS.
- The random effect of the study cheetah and the fixed effect of control versus treatment week (one, two, or three) on fGCM concentration and FCS.
- The random effect of the study cheetah and the fixed effects of hours within the day and part of the day on T_b, HR, and LA.
- The random effect of the study cheetah, the independent fixed effect of the study period, and the interaction fixed effects of the study period and (i) sex and (ii) age group on T_b, HR, and LA.
- The random effect of the study cheetah and the fixed effect of control versus treatment week (one, two, or three) on T_b, HR, and LA.
- The random effect of the study cheetah and the independent and interaction fixed effects of interaction time and part of the day on T_b, HR, and LA.

Tukey's HSD post hoc tests were performed for multiple pair-wise comparisons. The treatment effect size was calculated using Cohen's d. Pearson's correlation coefficient (r) was conducted to explore the relationship between the number of daily interactions and behaviour, fGCM concentration, FCS, T_b, HR, and LA, followed by simple linear regression. Descriptive statistics were reported as median ± MAD and the significance level, α, was set at 0.05.

4.3 RESULTS

This study's overall aim was to investigate the response of captive-born (hand-reared) cheetahs to participation in AVIs. It investigated whether demographic factors (sex and age group) and habituation affected this response.

4.3.1 Behavioural Observations

Behavioural data were Box-Cox transformed to more closely satisfy the assumption of normality and homogeneity. The study cheetahs spent the greatest proportion of their time inactive (for the control: 47% and the treatment: 49%) (Fig. 4.1). The MMRM analysis revealed that auto-grooming ($F_{1,20.76} = 7.12$, $p = .0145$) and affiliative behaviour ($F_{1,33.81} = 35.25$, $p < .0001$) were significantly higher during the treatment than in the control in addition to lower interspecific behaviour ($F_{1,42.4} = 4.81$, $p = .0338$). Post hoc comparisons using Tukey's HSD test revealed that the inactivity levels of the female study cheetahs were significantly lower during the treatment than in the control ($t_{101.4} = 4.00$, $p = .0007$). Post hoc comparisons using Tukey's HSD test revealed that the male study cheetahs showed significantly higher auto-grooming during the treatment than in the control ($t_{19.0} = -3.83$, $p = .0057$). Post hoc comparisons using Tukey's HSD test revealed that the female study cheetahs showed significantly higher affiliative behaviour during the treatment than in the control ($t_{31.2} = -5.47$, $p < .0001$). Post hoc comparisons using Tukey's HSD test revealed that the inactivity levels of the two-year-old study cheetahs were significantly lower during the treatment than in the control ($t_{101.8} = 4.20$, $p = .0003$). The inactivity levels of the three-year-old study cheetahs were significantly higher during the treatment than in the control ($t_{101.8} = -4.68$, $p < .0001$). Post hoc comparisons using Tukey's HSD test revealed that the three-year-old study cheetahs showed significantly lower locomotion during the treatment than in the control ($t_{74.5} = 2.94$, $p = .0224$).

Post hoc comparisons using Tukey's HSD test revealed that the two-year-old study cheetahs showed significantly lower interspecific behaviour during the treatment than in the control ($t_{46.0}$ = 3.67, p = .0035).

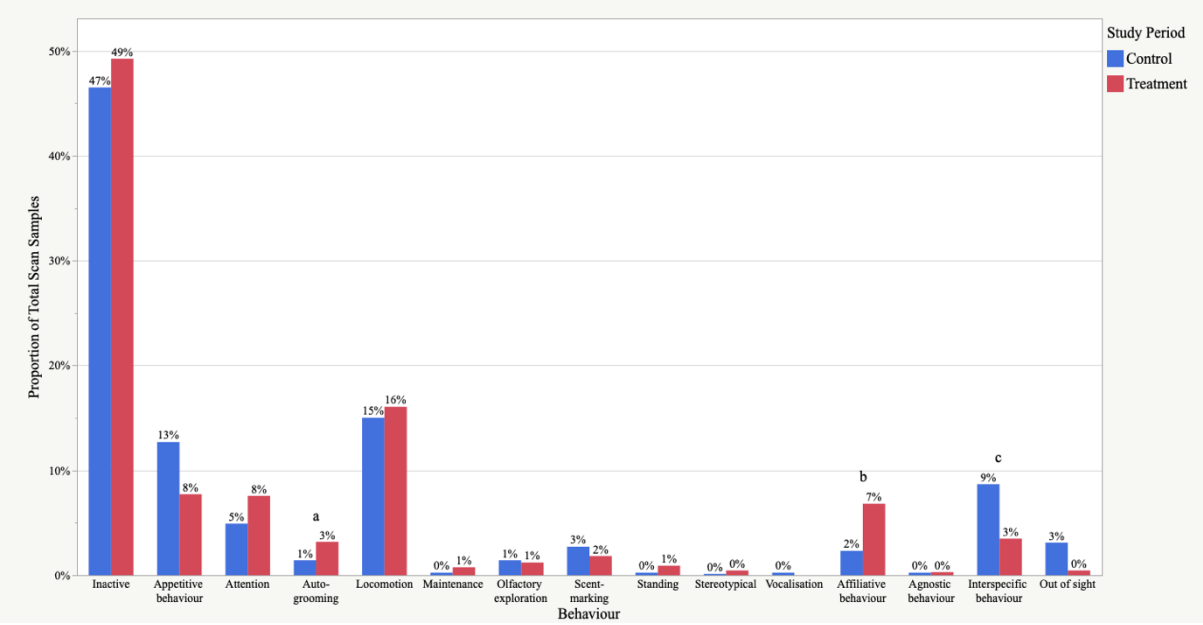

Figure 4.1. Bar chart of each behaviour for the study cheetahs (CH-2205, -2206, -2271, -2276, and -2277) during the control and treatment. Statistics were performed by a mixed model for repeated measures. The number above the bar indicates the proportion of total scan samples study cheetahs showed each behaviour during the control and treatment, respectively.

a: p = .0145, b: p < .0001, and c: p = .0338.

The MMRM analysis revealed that treatment week significantly affected standing ($F_{3,0.395}$ = 7456540, p = .0327). Standing was higher during week three of the treatment than in the control and weeks one and two. Post hoc comparisons using Tukey's HSD test could not be computed. Post hoc comparisons using Tukey's HSD test revealed that affiliative behaviour was significantly higher during weeks one ($t_{30.4}$ = -4.58, p = .0004), two ($t_{30.4}$ = -4.56, p = .0004), and three of the treatment ($t_{30.4}$ = -3.38, p = .0103) than in the control.

Cohen's d revealed a large effect size on auto-grooming (d = 1.06; t_{21} = 2.52, p = .0199) and affiliative behaviour (d = 2.07; t_{34} = 6.21, p < .0001) and medium effect size (d = 0.74; t_{48} = 2.43, p = .0187) on interspecific behaviour (Table 4.2).

Table 4.2. Treatment effect size on behaviour, faecal glucocorticoid metabolite (fGCM) concentration (µg/g dry weight [DW]), faecal consistency score, body temperature (^{0}C), heart rate (beats per minute [bpm]), and locomotor activity (overall dynamic body acceleration [ODBA]) for the study cheetahs (CH-2205, -2206, -2271, -2276, and -2277). Calculations were performed using Cohen's d.

df: degrees of freedom, CI: confidence interval.

	t	*p*	df	Cohen's d	95% CI for Cohen's d	
					Lower	Upper
Behaviour						
Inactive	0.25	.8001	106	0.05	-0.33	0.43
Appetitive behaviour	2.02	.0493	45	0.59	0.00	1.18
Attention	0.74	.4633	47	0.21	-0.35	0.78
Auto-grooming	2.52	.0199	21	1.06	0.17	1.93
Locomotion	0.12	.9086	78	0.03	-0.41	0.47
Maintenance	0.67	.5415	4	0.58	-1.20	2.29
Olfactory exploration	0.21	.8403	12	0.11	-0.95	1.17
Scent-marking	1.03	.3136	23	0.42	-0.39	1.23
Standing	1.15	.3125	4	1.00	-0.88	2.77
Affiliative behaviour	6.21	< .0001	34	2.07	1.24	2.88
Interspecific behaviour	2.43	.0187	48	0.74	0.12	1.35
Out of sight	0.35	.7392	5	0.38	-1.77	2.49
fGCM Concentration (µg/g DW)	2.66	.0084	186	0.39	0.10	0.68
Faecal Consistency Score (grade)	1.87	.063	186	0.27	-0.01	0.56
Body Temperature (°C)	9.43	< .0001	45537	0.09	0.07	0.11
Heart Rate (bpm)	19.30	< .0001	40969	0.20	0.18	0.22
Locomotor Activity (ODBA)	20.47	< .0001	45471	0.20	0.18	0.22

Pearson's correlation coefficient and simple linear regression revealed a negative relationship between the number of daily interactions and inactivity levels (r_2 = 0.23, p = .0004) and a positive relationship between the number of daily interactions and olfactory exploration (r_2 = 0.81, p = .0142).

4.3.2 Faecal Glucocorticoid Metabolite Concentrations

Faecal glucocorticoid metabolite concentration data were Box-Cox transformed to more closely satisfy the assumption of normality and homogeneity. The MMRM analysis revealed that fGCM concentration was significantly higher during the treatment (1.05 ± 0.21 µg/g DW) than in the control (0.90 ± 0.21 µg/g DW; $F_{1,182.2} = 7.56$, $p = .0066$). Post hoc comparisons using Tukey's HSD test revealed that the fGCM concentration of the female study cheetahs was significantly higher during the treatment (1.22 ± 0.16 µg/g DW) than in the control (0.85 ± 0.18 µg/g DW; $t_{181.1} = -3.93$, $p = .0007$) (Fig. 4.2i). Post hoc comparisons using Tukey's HSD test revealed that the fGCM concentration of the two-year-old study cheetahs was significantly higher during the treatment (1.19 ± 0.21 µg/g DW) than in the control (0.88 ± 0.18 µg/g DW; $t_{181.1} = -3.13$, $p = .0108$) (Fig. 4.3i).

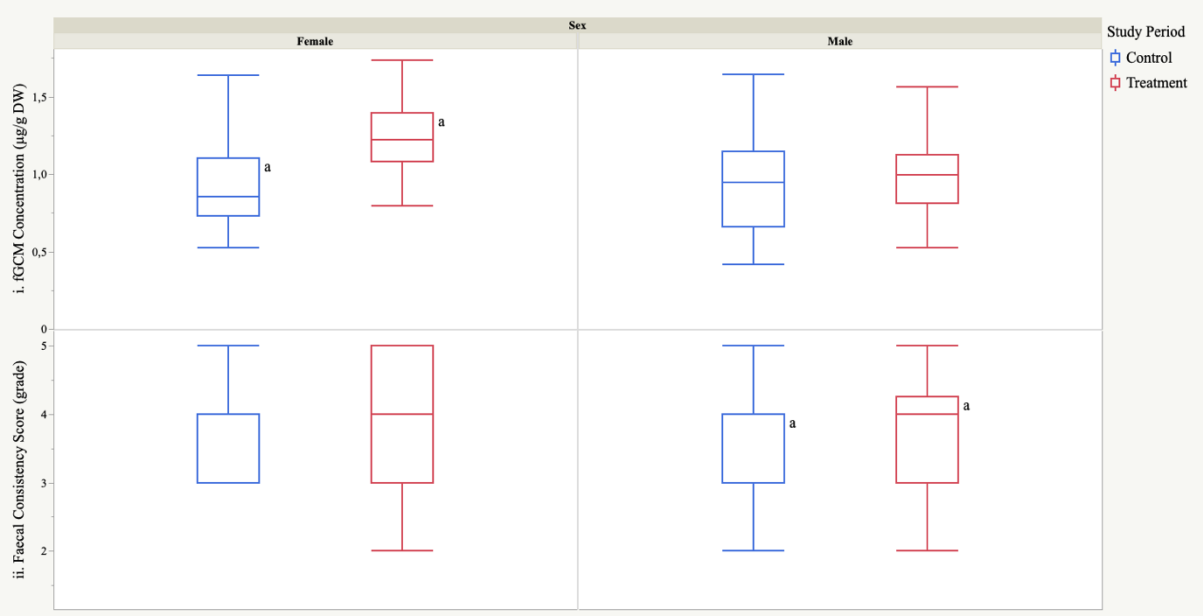

Figure 4.2. Box and whisker plot of (i) faecal glucocorticoid metabolite (fGCM) concentration (µg/g dry weight [DW]) and (ii) faecal consistency score (grade) for the study cheetahs (CH-2205, -2206, -2271, -2276, and -2277). Effect of sex (female and male) during the control and treatment. Statistics were performed by a mixed model for repeated measures and Tukey's HSD post hoc test.

(i) a: $p = .0007$. (ii) a: $p = .0420$.

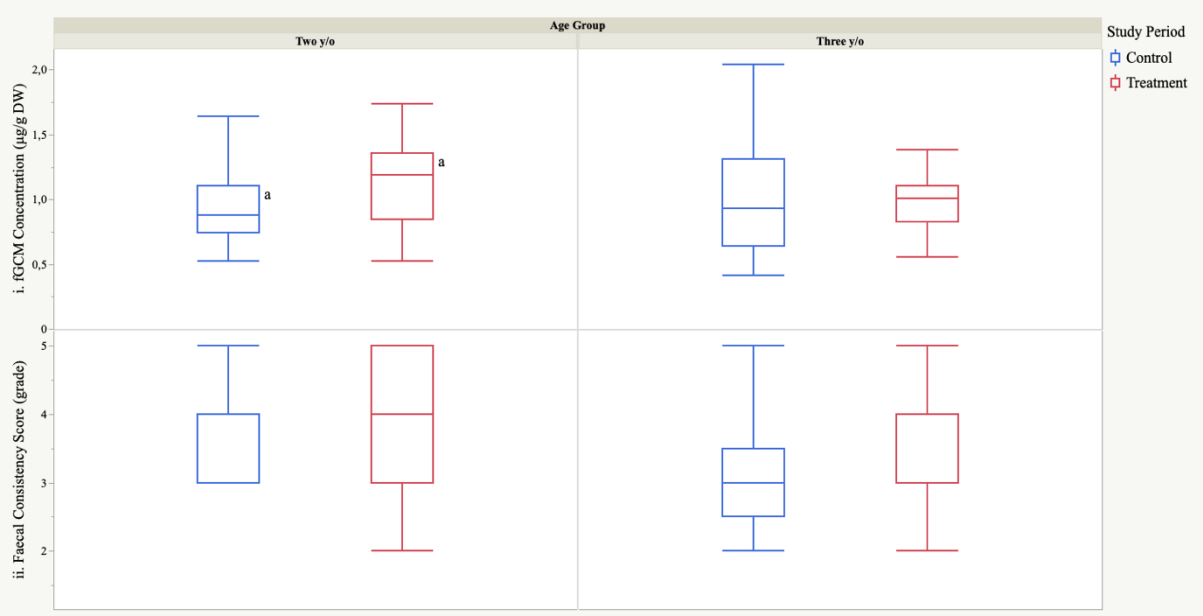

Figure 4.3. Box and whisker plot of (i) faecal glucocorticoid metabolite (fGCM) concentration (µg/g dry weight [DW]) and (ii) faecal consistency score (grade) for the study cheetahs (CH-2205, -2206, -2271, -2276, and -2277). Effect of age group (two and three years old [y/o]) during the control and treatment. Statistics were performed by a mixed model for repeated measures and Tukey's HSD post hoc test.

(i) a: $p = .0108$.

Cohen's d revealed a small effect size ($d = 0.39$; $t_{186} = 2.66$, $p = .0084$) on fGCM concentration (Table 4.2).

4.3.3 Faecal Consistency Scores

Faecal consistency score data were Box-Cox transformed to more closely satisfy the assumption of normality and homogeneity. The soft with shape faecal grade was the most frequently recorded in the study cheetahs (for the control: 45.5% and the treatment: 42.0%) (Fig. 4.4). During the treatment, the 'normal' grades of firm (~7.8%) and firm and dry (~1.7%) were higher and the 'suboptimal' grade of soft without shape was lower (~6.1%) than in the control. The MMRM analysis revealed that FCS was significantly higher during the treatment (4 ± 1) than in the control (3 ± 1; $F_{1,182.1} = 5.32$, $p = .0221$). Post hoc comparisons using Tukey's

HSD test revealed that the FCS of the male study cheetahs was significantly higher during the treatment (4 ± 1) than in the control (3 ± 1; $t_{181.0}$ = -2.66, p = .0420) (Fig. 4.2ii).

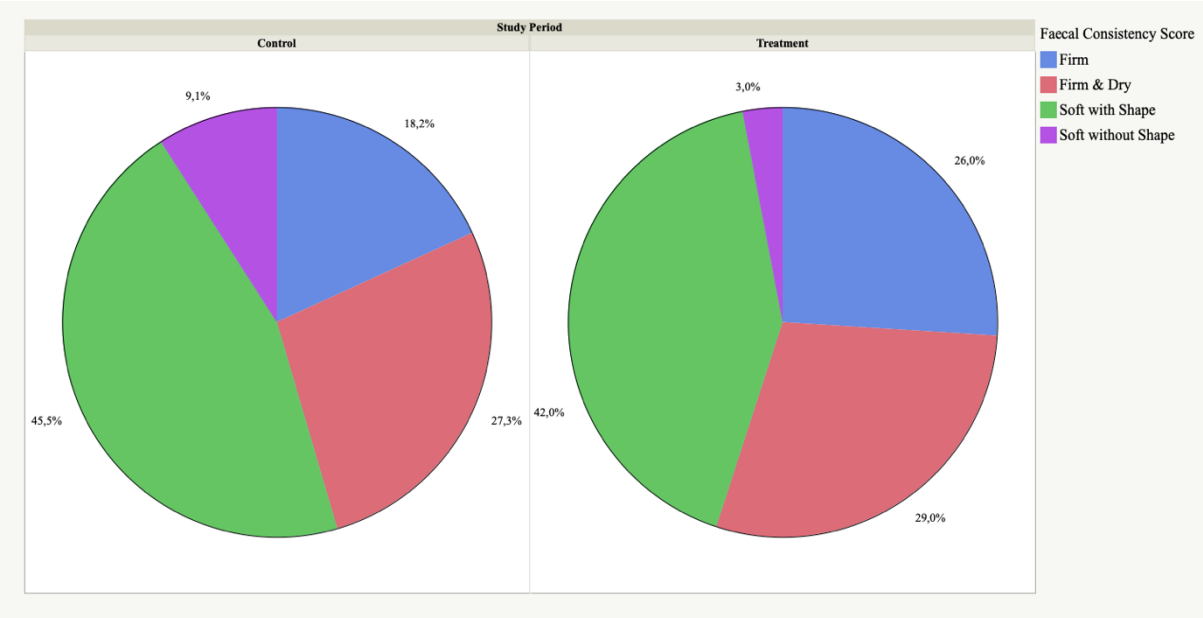

Figure 4.4. Pie chart of faecal consistency score for the study cheetahs (CH-2205, -2206, -2271, -2276, and -2277) during the control and treatment, where firm and firm and dry were considered to be 'normal' and liquid, soft without shape, and soft with shape were considered to be 'suboptimal' (adapted from AZA Tiger Species Survival Plan®, 2016; Whitehouse-Tedd *et al.*, 2015). The number above the slice indicates the proportion of total faecal samples collected for each score.

Cohen's d revealed a small effect size (d = 0.27; t_{186} = 1.87, p = .063) on FCS (Table 4.2).

Pearson's correlation coefficient and simple linear regression revealed a positive relationship between the number of daily interactions and FCS (r_2 = 0.08, p = .0051).

4.3.4 Body Temperature, Heart Rate, and Locomotor Activity Recordings

Battery malfunction of the biologgers implanted in the study cheetahs CH-2271, CH-2276, and CH-2277 impeded the acquisition of complete T_b, HR, and LA data sets for those individuals. The initial examination of the raw HR data for the study cheetahs with functional data loggers (CH-2205 and -2206), in addition to partial data for CH-2271, CH-2276, and CH-

2277), revealed values ranging from 0 to 1005 bpm, the extremes of which were likely because of incomplete, low-quality readings or implant movement within the pectoral muscle when the cheetahs were active. To remove erroneous measurements, ensuring only plausible values were included in the analyses, upper and lower thresholds were created (see section 1.1.6.9.3 for more information on the data preparation of T_b, HR, and LA recordings). Once filtered, all HR recordings (n = 40971/45539, which represented over 90% of raw data initially obtained from the loggers) fell within the 30- and 200-bpm thresholds set.

Body temperature data were Box-Cox transformed to more closely satisfy the assumption of normality and homogeneity. The circadian rhythm of T_b fluctuated significantly during the 24-hour cycle (for part of the day: $F_{5,45511} = 4283.67$, $p < .0001$; and for hours within the day: $F_{18,45511} = 460.60$, $p < .0001$) (Fig. 4.5i). Throughout the study, i.e., in the treatment and control, the median T_b was higher during the evening (37.9 ± 0.1 ^{0}C) between 1800–1859 hours (38.0 ± 0.1 ^{0}C).

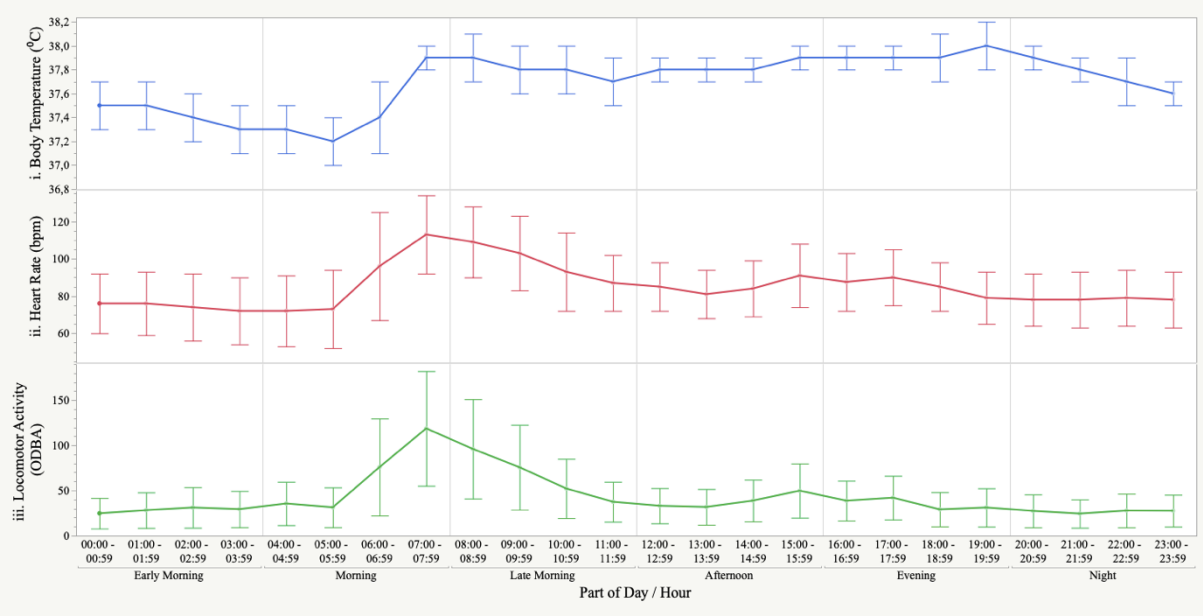

Figure 4.5. Circadian rhythm of (i) body temperature (^{0}C), (ii) heart rate (beats per minute [bpm]), and (iii) locomotor activity (overall dynamic body acceleration [ODBA]) (median, median absolute deviation) for the study cheetahs (CH-2205, -2206, -2271, -2276, and -2277) during the control and treatment.

The MMRM analysis revealed that T_b was significantly higher during the treatment (37.7 ± 0.2 ^{0}C) than in the control (37.7 ± 0.3 ^{0}C; $F_{1,45326} = 8.84$, $p = .0029$). Post hoc comparisons using Tukey's HSD test revealed that the T_b of the female study cheetahs was significantly higher during the treatment (37.8 ± 0.2 ^{0}C) than in the control (37.7 ± 0.3 ^{0}C; $t_{44658} = -23.89$, $p < .0001$) (Fig. 4.6i). The T_b of the male study cheetahs was significantly lower during the treatment (37.7 ± 0.2 ^{0}C) than in the control (37.7 ± 0.2 ^{0}C; $t_{44658} = 17.53$, $p < .0001$). Post hoc comparisons using Tukey's HSD test revealed that the T_b of the two-year-old study cheetahs was significantly higher during the treatment (37.8 ± 0.2 ^{0}C) than in the control (37.7 ± 0.3 ^{0}C; $t_{44627} = -23.85$, $p < .0001$) (Fig. 4.7i). The T_b of the three-year-old study cheetahs was significantly lower during the treatment (37.7 ± 0.2 ^{0}C) than in the control (37.7 ± 0.2 ^{0}C; $t_{44627} = 17.59$, $p < .0001$).

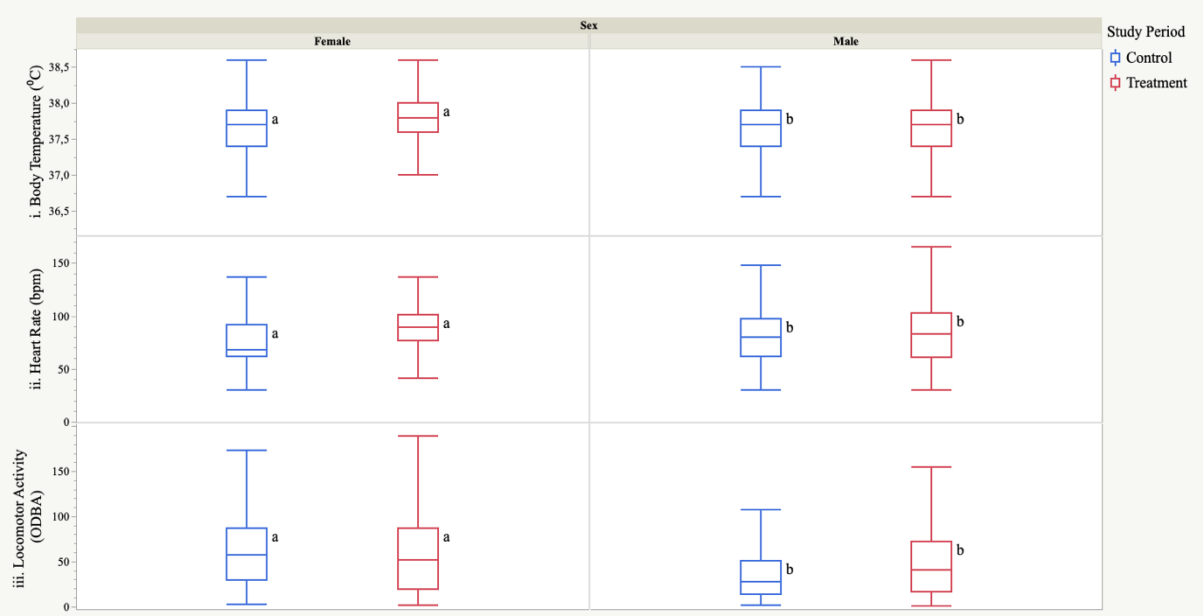

Figure 4.6. Box and whisker plot of (i) body temperature (^{0}C), (ii) heart rate (beats per minute [bpm]), and (iii) locomotor activity (overall dynamic body acceleration [ODBA]) for the study cheetahs (CH-2205, -2206, -2271, -2276, and -2277). Effect of sex (female and male) during the control and treatment. Statistics were performed by a mixed model for repeated measures and Tukey's HSD post hoc test.

(i) a, b: $p < .0001$. (ii) a, b: $p < .0001$. (iii) a, b: $p < .0001$.

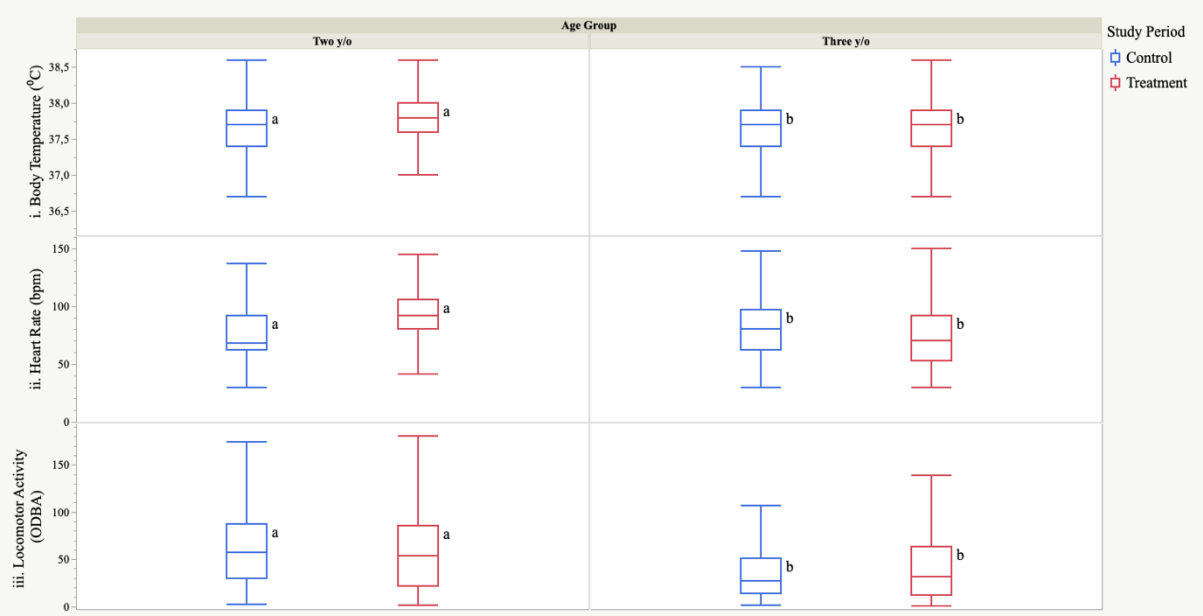

Figure 4.7. Box and whisker plot of (i) body temperature (^{0}C), (ii) heart rate (beats per minute [bpm]), and (iii) locomotor activity (overall dynamic body acceleration [ODBA]) for the study cheetahs (CH-2205, -2206, -2271, -2276, and -2277). Effect of age group (two and three years old [y/o]) during the control and treatment. Statistics were performed by a mixed model for repeated measures and Tukey's HSD post hoc test.

(i) a, b: $p < .0001$. (ii) a, b: $p < .0001$. (iii) a, b: $p < .0001$.

Post hoc comparisons using Tukey's HSD test revealed that T_b was significantly higher during week two of the treatment (37.7 ± 0.2 ^{0}C) than in the control (37.7 ± 0.3 ^{0}C; $t_{45498} = 5.51$, $p < .0001$) and weeks one (37.8 ± 0.2 ^{0}C; $t_{45498} = 5.24$, $p < .0001$) and three (37.7 ± 0.2 ^{0}C; $t_{45498} = -3.86$, $p = .0007$) (Fig. 4.8i).

Figure 4.8. Box and whisker plot of (i) body temperature (^{0}C), (ii) heart rate (beats per minute [bpm]), and (iii) locomotor activity (overall dynamic body acceleration [ODBA]) for the study cheetahs (CH-2205, -2206, -2271, -2276, and -2277). Effect of control versus treatment week (Wk; one, two, or three). Statistics were performed by a mixed model for repeated measures and Tukey's HSD post hoc test.

(i) a, b: $p < .0001$; and c: $p = .0007$. (ii) a, b, c: $p < .0001$. (iii) a, b, c: $p < .0001$; and d: $p = .0036$.

The MMRM analysis revealed that T_b was significantly lower at interaction time (37.8 ± 0.1 ^{0}C) than when the study cheetahs were not participating in AVIs (37.9 ± 0.1 ^{0}C; $F_{1,15113} = 207.36$, $p < .0001$). Post hoc comparisons using Tukey's HSD test revealed that T_b was significantly lower during the late morning at interaction time (37.8 ± 0.2 ^{0}C) than when the study cheetahs were not participating in AVIs (37.9 ± 0.2 ^{0}C; $t_{15110} = 10.37$, $p < .0001$), significantly lower during the afternoon at interaction time (37.8 ± 0.1 ^{0}C) than when the study cheetahs were not participating in AVIs (37.8 ± 0.1 ^{0}C; $t_{15110} = 4.20$, $p < .0001$), and significantly lower during the evening at interaction time (37.8 ± 0.1 ^{0}C) than when the study cheetahs were not participating in AVIs (37.9 ± 0.1 ^{0}C; $t_{15110} = 9.70$, $p < .0001$) (Fig. 4.9i).

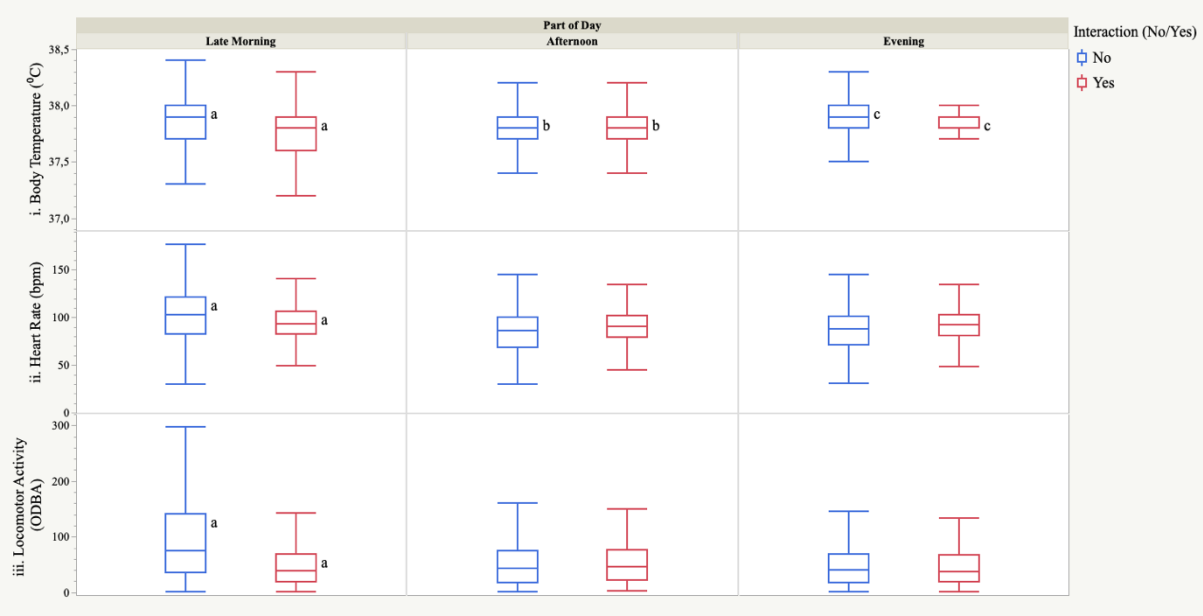

Figure 4.9. Box and whisker plot of (i) body temperature (^{0}C), (ii) heart rate (beats per minute [bpm]), and (iii) locomotor activity (overall dynamic body acceleration [ODBA]) for the study cheetahs (CH-2205, -2206, and -2276). Effect of interaction time by a part of the day. Statistics were performed by a mixed model for repeated measures and Tukey's HSD post hoc test.

(i) a, b, c: $p < .0001$. (ii) a: $p < .0001$. (iii) a: $p < .0001$.

Cohen's d revealed a trivial effect size ($d = 0.09$; $t_{45537} = 9.43$, $p < .0001$) on T_b (Table 4.2).

Pearson's correlation coefficient and simple linear regression revealed a positive relationship between the number of daily interactions and T_b ($r_2 = 0.03$, $p < .0001$).

Heart rate data were Box-Cox transformed to more closely satisfy the assumption of normality and homogeneity. The circadian rhythm of HR fluctuated significantly during the 24-hour cycle (for part of the day: $F_{5,40943} = 644.04$, $p < .0001$; and for hours within the day: $F_{18,40943} = 146.48$, $p < .0001$) (Fig. 4.5ii). Throughout the study, i.e., in the treatment and control, the median HR was higher during the late morning (97 ± 21 bpm) and higher between 0700–0759 hours (113 ± 21 bpm).

The MMRM analysis revealed that HR was significantly higher during the treatment (86 ± 18 bpm) than in the control (78 ± 16 bpm; $F_{1,40966} = 118.90$, $p < .0001$). Post hoc comparisons

using Tukey's HSD test revealed that the HR of the female study cheetahs was significantly higher during the treatment (89 ± 12 bpm) than in the control (68 ± 10 bpm; t_{40966} = -17.58, p < .0001) (Fig. 4.6ii). The HR of the male study cheetahs was significantly higher during the treatment (83 ± 21 bpm) than in the control (80 ± 17 bpm; t_{40966} = 23.22, p < .0001). Post hoc comparisons using Tukey's HSD test revealed that the HR of the two-year-old study cheetahs was significantly higher during the treatment (92 ± 13 bpm) than in the control (68 ± 10 bpm; t_{40634} = -17.63, p < .0001) (Fig. 4.7ii). The HR of the three-year-old study cheetahs was significantly lower during the treatment (70 ± 18 bpm) than in the control (80 ± 17 bpm; t_{40634} = 23.24, p < .0001).

Post hoc comparisons using Tukey's HSD test revealed that HR was significantly higher during weeks one (88 ± 19 bpm; t_{40965} = 8.07, p < .0001), two (86 ± 17 bpm; t_{40965} = 9.27, p < .0001), and three of the treatment (86 ± 17 bpm; t_{40965} = 9.00, p < .0001) than in the control (78 ± 16 bpm) (Fig. 4.8ii).

Post hoc comparisons using Tukey's HSD test revealed that HR was significantly lower during the late morning at interaction time (93 ± 11 bpm) than when the study cheetahs were not participating in AVIs (103 ± 19 bpm; t_{13363} = 7.94, p < .0001) (Fig. 4.9ii).

Cohen's d revealed a small effect size (d = 0.20; t_{40969} = 19.30, p < .0001) on HR (Table 4.2).

Pearson's correlation coefficient and simple linear regression revealed a positive relationship between the number of daily interactions and HR (r_2 = 0.08, p < .0001).

Locomotor activity data were Box-Cox transformed to more closely satisfy the assumption of normality and homogeneity. The circadian rhythm of LA fluctuated significantly during the 24-hour cycle (for part of the day: $F_{5,45445}$ = 1158.77, p < .0001; and for hours within the day: $F_{18,45445}$ = 257.48, p < .0001) (Fig. 4.5iii). Throughout the study, i.e., in the treatment and

control, the median LA was higher during the late morning (61 ± 38.8 ODBA) and higher between 0700–0759 hours (118.7 ± 63.5 ODBA).

The MMRM analysis revealed that LA was significantly higher during the treatment (44.6 ± 29.6 ODBA) than in the control (33.2 ± 20.2 ODBA; $F_{1,45469} = 15.30$, $p < .0001$). Post hoc comparisons using Tukey's HSD test revealed that the LA of the female study cheetahs was significantly lower during the treatment (52 ± 33.8 ODBA) than in the control (57.6 ± 28.8 ODBA; $t_{45467} = 22.05$, $p < .0001$) (Fig. 4.6iii). The LA of the male study cheetahs was significantly higher during the treatment (40.6 ± 26.2 ODBA) than in the control (27.2 ± 17.2 ODBA; $t_{45467} = -8.39$, $p < .0001$). Post hoc comparisons using Tukey's HSD test revealed that the LA of the two-year-old study cheetahs was significantly lower during the treatment (53.6 ± 31.8 ODBA) than in the control (57.6 ± 28.8 ODBA; $t_{45464} = 22.05$, $p < .0001$) (Fig. 4.7iii). The LA of the three-year-old study cheetahs was significantly higher during the treatment (31.2 ± 22.4 ODBA) than in the control (27.2 ± 17.2 ODBA; $t_{45464} = -8.38$, $p < .0001$).

Post hoc comparisons using Tukey's HSD test revealed that LA was significantly higher during week one of the treatment (40 ± 26.4 ODBA) than in the control (33.2 ± 20.2 ODBA; $t_{45467} = 9.38$, $p < .0001$), significantly lower during week one of the treatment than in weeks two (44.8 ± 30 ODBA; $t_{45467} = -7.40$, $p < .0001$) and three (49.1 ± 32.5 ODBA; $t_{45467} = -10.81$, $p < .0001$), and significantly lower during week two of the treatment than in week three ($t_{45467} = -3.41$, $p = .0036$) (Fig. 4.8iii).

The MMRM analysis revealed that LA was significantly lower at interaction time (42.2 ± 25 ODBA) than when the study cheetahs were not participating in AVIs (50.8 ± 32.2 ODBA; $F_{1,15083} = 130.47$, $p < .0001$). Post hoc comparisons using Tukey's HSD test revealed that LA was significantly lower during the late morning at interaction time (38.4 ± 22.2 ODBA) than

when the study cheetahs were not participating in AVIs (75.2 ± 46.6 ODBA; $t_{15082} = 15.61$, $p < .0001$) (Fig. 4.9iii).

Cohen's d revealed a small effect size ($d = 0.20$; $t_{45471} = 20.47$, $p < .0001$) on LA (Table 4.2).

Pearson's correlation coefficient and simple linear regression revealed a positive relationship between the number of daily interactions and LA ($r_2 = 0.01$, $p < .0001$).

4.4 DISCUSSION

Despite equivocal evidence, animal-visitor interactive experiences are offered on the assumption that they are enriching for the animals involved, educational for the visitors, and financially beneficial for the zoo. However, advances in understanding animal welfare have expanded on some preexisting concerns regarding the potential for AVIs to be a disturbing and negative source of distress for animal participants (Mellor, 2015; Moorhouse *et al.*, 2015; Schmidt-Burbach *et al.*, 2015). The two branches of the neuroendocrine stress system are the SNS and HPA axis (see section 1.1.2 for more information on the adaptive stress response). Both branches originate in the hypothalamus and converge on the adrenal gland, working together to maintain or re-establish homeostasis by orchestrating behavioural and physiological adaptations to the stressor (Coffman, 2020). The SNS provides the immediate first wave of the stress response, mediating the rapid release of the catecholamine hormones E and NE from the adrenal medulla (Romero & Wingfield, 2015). The second wave is more gradual and involves the GCs, the end-products of the hormonal cascade along the HPA axis (Sapolsky *et al.*, 2000). Here, participation in AVIs elicited in the study cheetahs significantly higher fGCM concentration and T_b and HR (salient features of the SNS-mediated reaction to stress). Body temperature and HR were positively correlated with the number of daily interactions. The results of these analyses support the researcher's hypothesis that up-close and, at times, hands-on contact with visitors would exacerbate the cheetah's physiological stress response to captivity.

However, it is difficult to determine if AVIs per se were responsible for the results. During the treatment, the study cheetahs were housed in an on-exhibit enclosure smaller than the off-exhibit enclosure in which they were kept during the control (see section 1.1.6.1 for more information on the study site and animals). In the wild, cheetahs are solitary, have large home

ranges, and avoid human contact (Caro, 1994), so it may not be surprising that a zoo exhibit environment is related to stress in this species. Housing in public exhibits has been associated with behavioural and physiological stress in cheetahs (Wells *et al.*, 2004) and other elusive cats (Mallapur & Chellam, 2002; Sellinger & Ha, 2005; Wielebnowski *et al.*, 2002a). Interestingly, the study cheetahs showed significantly lower interspecific behaviour while on-exhibit, suggesting a lack of interest in the proximity of humans and the other species housed at the Cango Wildlife Ranch and Conservation Centre.

Some research indicates that certain AVIs, when conducted in a manner that prioritises animal welfare, can be rewarding and a positive source of enrichment for the animals involved (Claxton, 2011; Hosey, 2008; Sherwen & Hemsworth, 2019). The animal husbandry principle of EE is widely used to provide species-appropriate challenges to stimulus-poor captive animals, encouraging them to engage actively with their environments, reducing stress and, subsequently, stereotypical behaviour (Carlstead & Shepherdson, 2000; Swaisgood & Shepherdson, 2005). The enriching effect of AVIs has been demonstrated to some extent by a study in which affiliative behaviour by cheetahs was observed towards both keepers and visitors during interactive walks (Szokalski *et al.*, 2013). The authors attributed affiliative behaviour to the interaction being a positive affective experience for the cheetahs (Szokalski *et al.*, 2013). Similarly, the study cheetahs showed significantly higher affiliative behaviour in response to AVIs. Affiliative behaviour can itself have a stress-reducing effect; grooming performed by a familiar human decreased HR in laboratory-kept rhesus monkeys (Grandi & Ischida, 2015), dairy cows (Schmied *et al.*, 2008), and lambs (Coulon *et al.*, 2015). Furthermore, olfactory exploration was positively correlated with the number of daily interactions, recurrently found in captive animals following enrichment (Quirke & O'Riordan, 2015; Tarou & Bashaw, 2007; Wells, 2009; Wells & Egli, 2004).

Visitor effect studies have often used fear- or stress-related behaviours, such as aggression, avoidance, and stereotypies, as negative indicators of welfare states (Davey, 2007; Mallapur *et al.*, 2005). However, changes in normal behaviours (e.g., resting and auto-grooming) can also indicate stress (Wells, 2005). Though felids are well-known meticulous groomers, if performed excessively, auto-grooming can become stereotypical and self-injurious rather than a normal body care behaviour pattern (Smith & Wettlaufer, 2009). Undesirably so, then, auto-grooming was significantly higher in response to AVIs.

The study cheetahs spent the majority of their time inactive. This is consistent with findings described by other authors on captive cheetahs (Skibiel *et al.*, 2007) as well as many other felids (Acaralp-Rehnberg *et al.*, 2020; Bashaw *et al.*, 2007; De Souza Resende *et al.*, 2014; Macri & Patterson-Kane, 2011; Moreira *et al.*, 2007; Shepherdson *et al.*, 1993; Weller & Bennet, 2001). Animal-visitor interactions resulted in significantly higher LA and a positive and negative correlation between LA and inactivity levels, respectively, and the number of daily interactions. This is an encouraging finding considering the propensity for captive carnivores to become obese from lack of exercise and experience physical injuries (Hope & Deem, 2006; Law *et al.*, 1997). Alternatively, that the study cheetahs were more active may infer behavioural agitation. Cheetahs and other carnivores in the wild spend a significant portion of their activity budget resting (Siegel, 2005). While reducing the risk of obesity, the motivation for increased activity levels, possibly human avoidance, could have negative welfare implications.

However, LA was significantly lower at interaction time when the study cheetahs were in close and, at times, hands-on contact with visitors, suggesting they were more relaxed while interacting (Whitehouse-Tedd *et al.*, 2018). Moreover, T_b and HR were significantly lower at

interaction time, possibly explained by stroking having a soothing or calming effect, as in domestic cats (Bradshaw *et al.*, 2012).

The study cheetahs' FCS was significantly higher in response to AVIs. Symbiotic gut microbes harboured in animals' GI tracts serve various essential functions (Wasimuddin *et al.*, 2017). However, disturbance-related deviation in the microbial diversity and abundance pattern beyond a natural range, i.e., gut dysbiosis, can advance pathophysiology and affect host health (Shreiner *et al.*, 2015). Faecal consistency is purportedly linked to intestinal microbiota composition (Tigchelaar *et al.*, 2016; Vandeputte *et al.*, 2016) and could indicate GI health (Whitehouse-Tedd *et al.*, 2015). Chronic or repeated exposure to stressors has been identified to disrupt gut homeostasis (Accarie & Vanuytsel, 2020; Farzi *et al.*, 2018). Therefore, the higher FCS may be attributable to the stress-reducing and potentially enriching effects of AVIs (Smail *et al.*, 2020). A positive correlation between FCS and the number of daily interactions was observed.

Environmental enrichment actions vary between the sexes and by the age of the animal, with females (Belz *et al.*, 2003; Girbovan & Plamondon, 2013; Hendershott *et al.*, 2016; Mármol *et al.*, 2015) and adolescents (O'Leary *et al.*, 2019; Peña *et al.*, 2009; Segovia *et al.*, 2009) showing more pronounced effects. The demographic factors, sex and age group, affected the study cheetahs' activity levels and physiological (fGCM concentration, T_b, and HR) responses, possibly explained by the sensitivity of the female study cheetahs and those two years old to EE; alternatively, if the study cheetahs perceived (unfamiliar) humans as competitors or predators, the (smaller) female study cheetahs and those two years old could have felt intimidated by the visitors.

Novel stimuli lose their effect over time due to habituation (Kuczaj *et al.*, 2002), whereby animals' responsiveness to stimulation diminishes with repeated exposure to that stimulus

(McFarland, 2021). The temporal profile of T_b appeared to demonstrate habituation, with values significantly higher during week two of the treatment and significantly lower during week three. Similarly, HR was significantly higher during week one of the treatment and, after that, numerically lower during weeks two and three. However, LA increased during weeks one and two of the treatment and was significantly higher during week three, suggesting that AVIs were sufficiently dynamic to provide prolonged stimulation to the study cheetahs.

This study's findings encourage, to some extent, participation in AVIs as a potential form of EE for cheetahs in captivity. More so, they reiterate the ambiguous impact of AVIs on the animals involved, including whether they are positive, neutral, or negative. Further scientific research is needed as to what may constitute positive AVIs, both ethically and educationally, and ways of conducting such interactions without leading to unintended frustrations or the development of negative affective states in animals. I recommend that future studies investigate the factors that may play a role in cheetahs' variable responses to AVIs (e.g., indirect or direct experiences, enclosure design, individual animal traits, and visitor characteristics). Since animals' motivation to participate in visitor interactions can vary daily (Learmonth, 2019), I support the current recommendation that all AVIs must be assessed by applying relevant and ongoing welfare assessment and monitoring (Hosey, 2008; WAZA, 2020). It must also be acknowledged that the present study was conducted on hand-reared cheetahs, conditioned to daily interactions with the facility's caretakers and visitors. Therefore, the findings here should not be generalised to all captive cheetahs.

Furthermore, I demonstrated that biologging technology could be used to record concurrent T_b, HR, and LA and, in addition to behavioural and physiological metrics, has tremendous potential as a tool to measure stress in the cheetah. However, a significant limitation of the present study was the battery malfunction of the biologgers implanted in the study cheetahs

CH-2271, CH-2276, and CH-2277, impeding the acquisition of complete T_b, HR, and LA data sets for those individuals. For the technology to be routinely utilised in animal welfare studies, there is still a need to develop more reliable devices capable of remote data transmission to avoid repeated capture and handling.

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Chapter 5: Responses to Predator Proximity in Captive-Born Cheetahs (*Acinonyx jubatus*): Implications for Behavioural and Physiological Stress and Gastrointestinal Health

ABSTRACT. Even closely related species, such as varied felids, can be averse to the vicinity of other similar species in captivity. This study investigated the response of captive-born (hand-reared) cheetahs ($n = 5$) to the close proximity of species, i.e., leopards and lions, that generally compete with and predate the cheetah in the wild for three weeks. Using emerging biologging technology and more traditional measures of stress-related behavioural and physiological responses, variations in body temperature (T_b), heart rate (HR), locomotor activity (LA), behaviour, faecal glucocorticoid metabolite concentration, and faecal consistency score (FCS) were documented. Olfactory, auditory, and/or visual contact with other large predators elicited in the study cheetahs higher T_b ($p < .0001$) and HR ($p < .0001$). Predator proximity resulted in lower inactivity levels ($p < .0001$) and higher LA ($p < .0001$), consistent with its purported use as environmental enrichment. Sex and age group affected the study cheetahs' activity levels and physiological responses, which may be attributable to the threat-sensitivity of the female study cheetahs and those two years old to predators. Body temperature and HR appeared to demonstrate habituation. However, after prolonged predator proximity, higher stereotypic pacing was observed in the study cheetahs and lower appetitive behaviour ($p \leq .05$) and FCS ($p \leq .05$). This study's findings highlight the importance of trepidation when imposing minimally harmful negative events not to be contrary to the welfare of cheetahs in captivity.

5.1 INTRODUCTION

Within the natural world, predation has a profound effect on the structure of ecological communities (Sih *et al.*, 1985), and the role apex carnivores play in shaping ecosystems extends beyond that of their prey species to mesocarnivores, i.e., middle trophic level predators (Prugh *et al.*, 2009). Through direct predation, kleptoparasitism (the theft of prey), and harassment, dominant guild members can influence subordinate species' distribution and population dynamics (Prugh *et al.*, 2009; Ritchie & Johnson, 2009). However, mesocarnivores can minimise the risks of these aggressive interactions, generally referred to as interference

competition, and coexist with apex carnivores through various avoidance strategies. One example of pervasive interference competition is among Africa's large predator guild (Caro & Stoner, 2003).

Having evolved as a rapid pursuit specialist, the cheetah's cursorial adaptations make it a successful hunter, but they also present a disadvantage in the predator hierarchy. Due to their smaller body size and solitary nature, cheetahs are competitively inferior to larger predatory species, including lions, leopards, jackals (*Canis aureus*), and hyenas (*Hyaenidae*) (Caro, 1994; Marker *et al.*, 2018). In areas with these large competitors, cheetahs are estimated to lose between 2.2% and 11.8% of kills through kleptoparasitism (Bissett & Bernard, 2006; Hunter *et al.*, 2007; Mills & Mills, 2013; Mills *et al.*, 2017). Kleptoparasitism may force cheetahs to hunt repeatedly, increasing their daily energy expenditure (Scantlebury *et al.*, 2014) and the probability of injuries sustained through hunting. In addition to hunting pressures, cheetahs are vulnerable to predation by other large carnivores that may try to kill an adult cheetah or its young to reduce competition for prey and territory (Caro, 1994; Durant, 1998, 2000a; Laurenson *et al.*, 1995; but see Mills & Mills, 2013). In this manner, the abundance of competitively superior predators directly impacts the cheetah's feeding ecology, behaviour, reproductive success, and survival.

Cheetahs have been described as fugitive species, ranging widely and persisting only in marginal areas with low lion, spotted hyena (*Crocuta crocuta*), and prey densities, or competition refuges, to avoid interference competition (Caro & Stoner, 2003; Chauvenet *et al.*, 2011; Durant, 1998; 2000a; Laurenson, 1994; 1995; Saleni, 2007). However, the large-scale displacement of subordinate species from their preferred habitats and the resources within could result in a costly loss of feeding opportunities. Recent studies suggest that cheetahs' use of space is a hierarchical process, driven primarily by resource acquisition and fine-tuned by

predator avoidance (Broekhuis *et al.*, 2013; Vanak *et al.*, 2013). That is, rather than long-term spatial avoidance of habitats and high-risk areas for aggressive encounters, i.e., a predictive response, cheetahs reactively alter habitat use and activity patterns, responding to more immediately and spatially reliable cues, such as visual or auditory detection (Durant, 2000a; 2000b). Such fine-scale spatiotemporal avoidance may facilitate the successful coexistence of cheetahs with other large carnivores (Broekhuis *et al.*, 2013; Swanson *et al.*, 2016; 2014).

In captivity, solitary felids are routinely housed in pairs and, at times, kept in the vicinity of their natural competitors and predators (Mellen, 1991). Continual exposure to olfactory, auditory, visual, and/or tactile predator cues absent the opportunity to respond appropriately could confer a significant source of stress for captive animals (Morgan & Tromborg, 2007). When moved to barren enclosures near where large predators were housed (e.g., lions and tigers), leopard cats (*Felis bengalensis*) were reported as having elevated urinary cortisol concentrations and increased stereotypic pacing (Carlstead, 1998). After the leopard cats' enclosures were enriched with hiding places, urinary cortisol concentrations and stereotypic pacing decreased while exploratory behaviour increased (Carlstead, 1998). Similarly, Wielebnowski *et al.* (2002a) found that fGCM concentrations were higher in clouded leopards on public display or in visual contact with other large predators than in individuals housed off-exhibit or without predator visibility.

This study's overall aim was to investigate the response of captive-born (hand-reared) cheetahs to the close proximity of species, i.e., a leopard and lions, that generally compete with and/or predate the cheetah in the wild. Considering the reported susceptibility of the cheetah to captivity-induced stress (Munson *et al.*, 2005; Terio *et al.*, 2004), I hypothesised that olfactory, auditory, and/or visual contact with other large predators would exacerbate this response. As established stress-related markers, behavioural observations (Quirke *et al.*, 2012) and fGCM

concentration analysis (Terio *et al.*, 1999) were performed, in addition to faecal consistency scoring as a preliminary indicator of the study cheetahs' GI health (Whitehouse-Tedd *et al.*, 2015). A secondary objective was to explore the potential of biologging technology for measuring stress in the cheetah by recording concurrent T_b, HR, and LA. I predicted more significant displays of stereotypic pacing and agnostic behaviour and higher fGCM concentration, T_b, and HR, consistent with a behavioural and physiological stress response.

5.2 MATERIALS AND METHOD

5.2.1 Study Site and Animals

The experimental trials occurred between April and September 2019 at the Cango Wildlife Ranch and Conservation Centre (33°33'S, 22°12'E) 4 km north of Oudtshoorn, in the Western Cape of South Africa (see section 1.1.6.1 for more information on the study site and animals). Three male (CH-2205, -2206, and -2271) and two female (CH-2276 and -2277) resident adult cheetahs (Table 1.1) were allocated to this study.

5.2.2 Body Temperature, Heart Rate, and Locomotor Activity Measurement Intervals

A single cardiac-, temperature- and movement-sensitive biologger (DST centi-HRT ACT, Star-Oddi, Gardabaer, Iceland) with the dimensions of 46 mm x 15 mm x 15 cm and weighing approximately 19 g was implanted in each of the study cheetahs. The biologgers were calibrated individually against a high-accuracy thermometer (Hart 1504, Fluke, Utah, US). Calibrated accuracy was better than 0.1 °C. Before surgical implantation, the biologgers were set to record tri-axial LA, i.e., heave, surge, sway, every minute for the ODBA and T_b (°C) and leadless single-channel ECG-derived HR (bpm) every 5 minutes at 200 Hz. Representative traces of raw ECG recordings were saved every 24 hours for validating the HR measurements' QI (where QI_0 was the highest quality and QI_1, QI_2, and QI_3 were of progressively reduced quality). Afterwards, the biologging devices were sterilised using ethylene oxide and implanted (see section 1.1.6.3 in the introduction for more information on the surgical procedure).

5.2.3 Experimental Design

The study cheetahs housed individually (CH-2271) or in pairs (CH-2205 and -2206, and CH-2276 and -2277, respectively) were assigned randomly to either the treatment, i.e., the

close proximity to other large predators or the control in an initial 3-week period at variable times and the alternative in a period following of equal duration (see section 1.1.6.4 for more information on the experimental design). During the treatment, the study cheetahs were housed in an on-exhibit enclosure (Fig. 1.2). They had olfactory, auditory, and/or visual contact with other large predators, including an adult female leopard and a male and female adult lion (in order of proximity). During the control, the study cheetahs were housed off-exhibit at the Jill Bryden-Fayers Reserve neighbouring the Ranch, at which only conspecifics were kept. After movement on- or off-exhibit, the study cheetahs were habituated to the associated increase or decrease in the human presence (see section 1.1.6.4 for more information on the experimental design). Other than the specific intervention being investigated, i.e., the close proximity to other large predators, the study cheetahs' environment, housing, and management (as described in section 1.1.6.1) were maintained across the treatment and control, including sufficient vegetation to hide and a wooden shed for shelter.

Throughout the treatment and control, the study cheetahs were monitored regarding (i) behaviour, (ii) fGCM concentration, and (iii) FCS, as well as (iv) T_b, HR, and LA concurrently recorded by implanted data loggers.

5.2.4 Behavioural Data Collection

Instantaneous scan sampling (Altmann, 1974) with a 5-minute inter-scan interval was used in this study (Quirke & O'Riordan, 2011a; 2011b). During five weekly 60-minute observation sessions, the researcher (KLB) carried out 12 instantaneous scan samples on focal animals, one enclosure at a time and in a randomised order to prevent time-of-day effects. For the study cheetahs housed in pairs (Table 1.1), behavioural data were collected simultaneously using

physical characteristics to identify individuals. Observations were performed between 0700 and 1700 based on the operating hours of the Cango Wildlife Ranch and Conservation Centre.

Fifteen behaviours, categorised into inactive, active, and not observed, were recorded (Table 1.2). Preliminary observation of the study cheetahs and the literature on felids' behaviour (Altman *et al.*, 2005; Quirke & O'Riordan, 2011a; 2011b; Regaiolli *et al.*, 2019) informed the ethogram used. Time spent out of sight (hiding or staying away from the human observer) was recorded as its performance may be indicative of a psychological stress response (Carlstead *et al.*, 1993; Davey, 2006; Hosey, 2013; Morgan & Tromborg, 2007; Sellinger & Ha, 2005).

5.2.5 Faecal Sample Collection and Consistency Scoring

To differentiate between individual faecal samples in the event of paired housing (Table 1.1), 1 tbsp of uncooked rice was thoroughly mixed into the diet of study cheetahs CH-2206 and CH-2277 once per day. The caretakers monitored the study cheetahs during feeding times to ensure the sufficient consumption of rice and prevent meal sharing. Only faeces found to have uncooked rice were considered to have originated from those individuals fed rice. Due to the operating hours of the Cango Wildlife Ranch and Conservation Centre, the enclosures could not be entered between 1800–0800 hours. As such, once daily between 0800–1000 hours, faeces from the previous night were collected from each enclosure within 16 hours of defecation (4–10 °C T_a) (Ludwig *et al.*, 2013).

Following sample collection, the researcher (KLB) assigned FCS as per a five-point grading system, where two points (grade 4: firm and dry and grade 5: firm) were considered to be 'normal,' and three points (grades 1–3: liquid, soft without shape, and soft with shape) were considered to be 'suboptimal' (adapted from AZA Tiger Species Survival Plan®, 2016;

Whitehouse-Tedd *et al.*, 2015). Afterwards, the samples were deposited into appropriately labelled (sample collection date and study cheetah and sample ID numbers) 50 mL polypropylene specimen containers and frozen at –20 °C.

5.2.6 Faecal Steroid Extraction and Quantification

Following completion of the experimental trials, faecal samples were transported frozen to the Endocrine Research Laboratory, University of Pretoria, South Africa. Faecal steroids were extracted and analysed for fGCM concentration as described in section 1.1.6.7.

5.2.7 Ethical Statement

All of the experimental procedures involving the study cheetahs were approved by the University of Pretoria's Animal (clearance number V075-18) and Research Ethics Committees (clearance number REC069-18).

5.2.8 Statistical Analysis

Statistical analysis was performed using Microsoft Excel (version 16.0) and JMP Pro software (version 16.0) for Windows, developed by SAS Institute Inc (North Carolina, US). Raw data was manipulated before detailed analyses (see section 1.1.6.9 for more information on the data preparation). The data were screened for univariate outliers greater than three interquartile ranges away from the 99.5th or 0.05th percentiles (LA: n = 50), which were subsequently excluded from descriptive statistics and analyses. Normal distribution and homogeneity of variance were verified using Anderson-Darling and Levene's tests, respectively. Box-Cox transformations were used to more closely satisfy the assumption of normality and homogeneity in the case of departure. To maintain statistical integrity, the data

were back-transformed for descriptive statistics and visual representation. An MMRM analysis was conducted to investigate the following:

- The random effect of the study cheetah, the independent fixed effect of the study period, and the interaction fixed effects of the study period and (i) sex and (ii) age group on each behaviour.
- The random effect of the study cheetah and the fixed effect of control versus treatment week (one, two, or three) on each behaviour.
- The random effect of the study cheetah, the independent fixed effect of the study period, and the interaction fixed effects of the study period and (i) sex and (ii) age group on fGCM concentration and FCS.
- The random effect of the study cheetah and the fixed effect of control versus treatment week (one, two, or three) on fGCM concentration and FCS.
- The random effect of the study cheetah and the fixed effects of hours within the day and part of the day on T_b, HR, and LA.
- The random effect of the study cheetah, the independent fixed effect of the study period, and the interaction fixed effects of the study period and (i) sex and (ii) age group on T_b, HR, and LA.
- The random effect of the study cheetah and the fixed effect of control versus treatment week (one, two, or three) on T_b, HR, and LA.

Tukey's HSD post hoc tests were performed for multiple pair-wise comparisons. The treatment effect size was calculated using Cohen's d. Descriptive statistics were reported as median ± MAD and the significance level, α, was set at 0.05.

5.3 RESULTS

This study's overall aim was to investigate the response of captive-born (hand-reared) cheetahs to the close proximity of species, i.e., a leopard and lions, that generally compete with and/or predate the cheetah in the wild. It investigated whether demographic factors (sex and age group) and habituation affected this response.

5.3.1 Behavioural Observations

Behavioural data were Box-Cox transformed to more closely satisfy the assumption of normality and homogeneity. The study cheetahs spent the majority of their time inactive (for the control: 47% and the treatment: 28%) (Fig. 5.1). The MMRM analysis revealed that inactivity levels were significantly lower during the treatment than in the control ($F_{1,111.6}$ = 16.54, $p < .0001$). Post hoc comparisons using Tukey's HSD test revealed that the inactivity levels of the female study cheetahs were significantly lower during the treatment than in the control ($t_{110.2} = 5.00$, $p < .0001$). Post hoc comparisons using Tukey's HSD test revealed that the inactivity levels of the two-year-old study cheetahs were significantly lower during the treatment than in the control ($t_{109.2} = 6.15$, $p < .0001$).

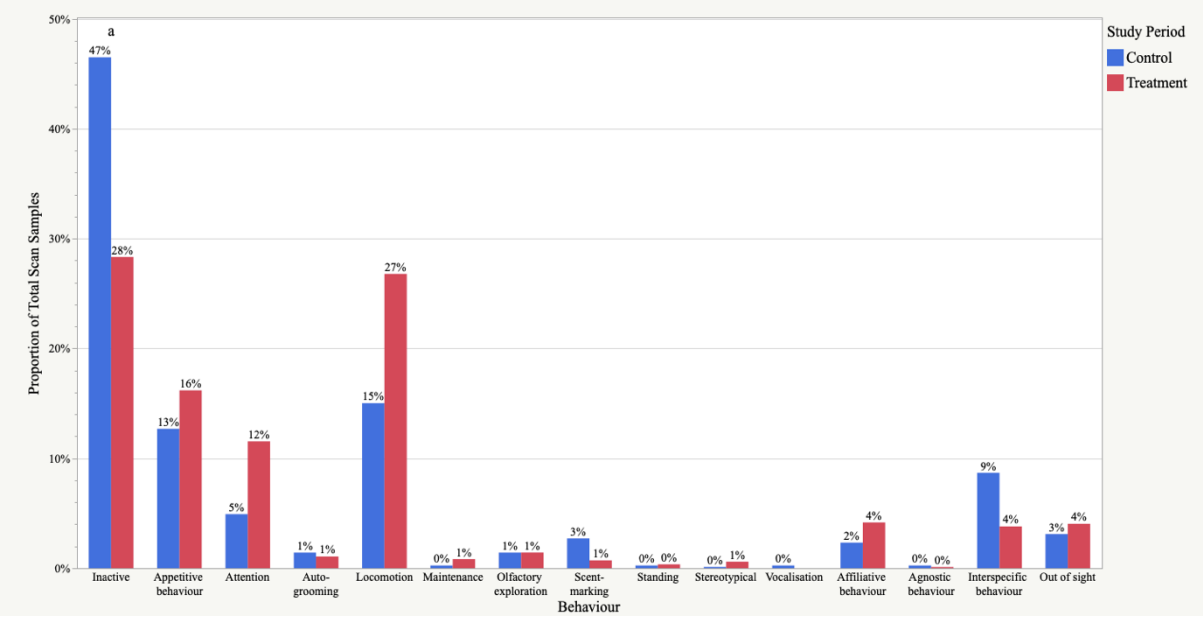

Figure 5.1. Bar chart of each behaviour for the study cheetahs (CH-2205, -2206, -2271, -2276, and -2277) during the control and treatment. Statistics were performed by a mixed model for repeated measures. The bar above indicates the proportion of total scan samples study cheetahs showed each behaviour during the control and treatment, respectively.

a: $p < .0001$.

Post hoc comparisons using Tukey's HSD test revealed that inactivity levels were significantly lower during weeks two ($t_{109.2} = 2.81$, $p = .0295$) and three of the treatment ($t_{109.2} = 4.21$, $p = .0003$) than in the control. Post hoc comparisons using Tukey's HSD test revealed that appetitive behaviour was significantly lower during week three of the treatment than in the control ($t_{44.9} = 2.74$, $p = .0426$) and weeks one ($t_{44.9} = 2.85$, $p = .0320$) and two ($t_{44.9} = 3.41$, $p = .0074$). Post hoc comparisons using Tukey's HSD test revealed that locomotion was significantly higher during week three of the treatment than in the control ($t_{94.4} = -3.72$, $p = .0019$) and week two ($t_{94.4} = -3.99$, $p = .0007$). The MMRM analysis revealed that treatment week significantly affected stereotypic pacing ($F_{2,0.667} = 8388607$, $p = .0034$). Stereotypic pacing was higher during week three of the treatment than in the control and weeks one and two. Post hoc comparisons using Tukey's HSD test could not be computed.

Cohen's d revealed a medium effect size on inactivity levels (d = 0.73; t_{113} = 3.93, p = .0001) and interspecific behaviour (d = 0.60; t_{52} = 2.12, p = .0385) (Table 5.1).

Table 5.1. Treatment effect size on behaviour, faecal glucocorticoid metabolite (fGCM) concentration (µg/g dry weight [DW]), faecal consistency score, body temperature (^{0}C), heart rate (beats per minute [bpm]), and locomotor activity (overall dynamic body acceleration [ODBA]) for the study cheetahs (CH-2205, -2206, -2271, -2276, and -2277). Calculations were performed using Cohen's d.

	t	*p*	df	Cohen's d	95% CI for Cohen's d	
					Lower	Upper
Behaviour						
Inactive	3.93	.0001	113	0.73	0.35	1.11
Appetitive behaviour	0.61	.542	63	0.16	-0.34	0.65
Attention	1.47	.147	62	0.39	-0.14	0.91
Auto-grooming	1.13	.2764	16	0.53	-0.42	1.47
Locomotion	1.86	.0665	99	0.37	-0.03	0.77
Olfactory exploration	0.67	.5144	16	0.32	-0.62	1.25
Scent-marking	0.78	.4436	18	0.40	-0.62	1.42
Stereotypical	0.45	.685	3	0.50	-1.76	2.69
Affiliative behaviour	1.71	.0943	39	0.54	-0.09	1.17
Interspecific behaviour	2.12	.0385	52	0.60	0.03	1.16
Out of sight	0.73	.4757	14	0.38	-0.65	1.39
fGCM Concentration (µg/g DW)	1.56	.1193	189	0.23	-0.06	0.51
Faecal Consistency Score (grade)	1.06	.2925	189	0.15	-0.13	0.44
Body Temperature (°C)	15.78	< .0001	42866	0.16	0.14	0.18
Heart Rate (bpm)	19.26	< .0001	39072	0.20	0.18	0.22
Locomotor Activity (ODBA)	25.96	< .0001	42816	0.26	0.24	0.28

df: degrees of freedom, CI: confidence interval.

5.3.2 Faecal Glucocorticoid Metabolite Concentrations

Faecal glucocorticoid metabolite concentration data were Box-Cox transformed to more closely satisfy the assumption of normality and homogeneity. The median fGCM concentration was higher during the treatment (0.98 ± 0.24 µg/g DW) than in the control (0.90 ± 0.21 µg/g DW). The MMRM analysis revealed that this numerical difference failed to achieve statistical significance.

5.3.3 Faecal Consistency Scores

Faecal consistency score data were Box-Cox transformed to more closely satisfy the assumption of normality and homogeneity. The soft with shape faecal grade was the most frequently recorded in the study cheetahs (for the control: 45.5% and the treatment: 46.6%) (Fig. 5.2). During the treatment, the 'normal' grade of firm was higher (~6.1%) and the 'normal' grade of firm and dry (~3.0%) and the 'suboptimal' grade of soft without shape (~4.2%) were lower than in the control. There was no numerical difference in the median FCS between the control (3 ± 1) and treatment (3 ± 1).

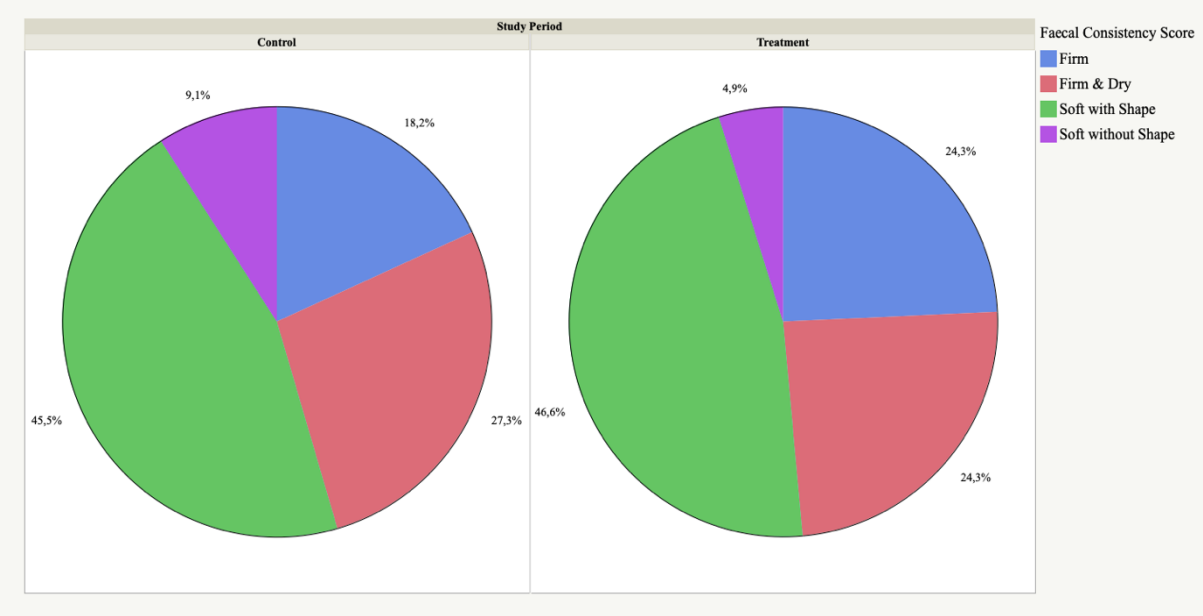

Figure 5.2. Pie chart of faecal consistency score for the study cheetahs (CH-2205, -2206, -2271, -2276, and -2277) during the control and treatment, where firm and firm and dry were considered to be 'normal' and liquid, soft without shape, and soft with shape were considered to be 'suboptimal' (adapted from AZA Tiger Species Survival Plan®, 2016; Whitehouse-Tedd *et al.*, 2015). The number above the slice indicates the proportion of total faecal samples collected for each score.

Post hoc comparisons using Tukey's HSD test revealed that FCS was significantly lower during week three of the treatment (3 ± 0) than in week one (4 ± 1; $t_{183.0} = 2.59$, $p = .0498$) (Fig. 5.3ii).

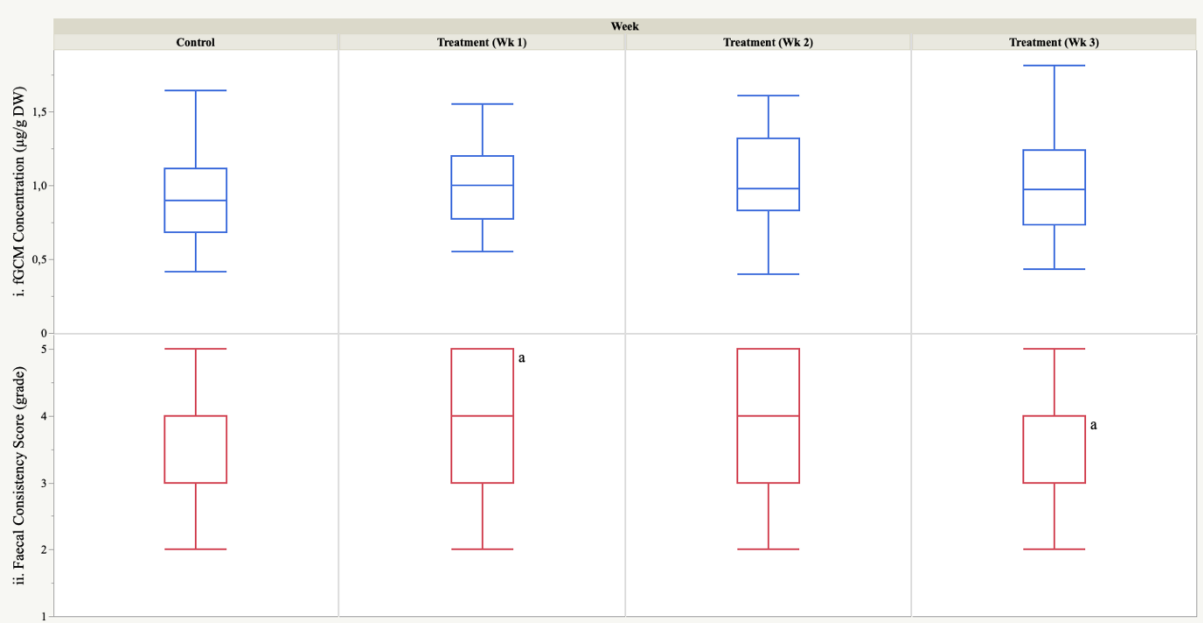

Figure 5.3. Box and whisker plot of (i) faecal glucocorticoid metabolite (fGCM) concentration (µg/g dry weight [DW]) and (ii) faecal consistency score (grade) for the study cheetahs (CH-2205, -2206, -2271, -2276, and -2277). Effect of control versus treatment week (Wk; one, two, or three). Statistics were performed by a mixed model for repeated measures and Tukey's HSD post hoc test.

(i) a: $p = .0498$.

5.3.4 Body Temperature, Heart Rate, and Locomotor Activity Recordings

Battery malfunction of the biologgers implanted in the study cheetahs CH-2271, CH-2276, and CH-2277 impeded the acquisition of complete T_b, HR, and LA data sets for those individuals. The initial examination of the raw HR data for the study cheetahs with functional data loggers (CH-2205 and -2206), in addition to partial data for CH-2271, CH-2276, and CH-2277, revealed values ranging from 0 to 1005 bpm, the extremes of which were likely because of incomplete, low-quality readings or implant movement within the pectoral muscle when the

study cheetahs were active. To remove erroneous measurements, ensuring only plausible values were included in the analyses, upper and lower thresholds were created (see section 1.1.6.9.3 for more information on the data preparation of T_b, HR, and LA recordings). Once filtered, all HR recordings (n = 39074/42868, which represented over 90% of raw data initially obtained from the loggers) fell within the 30- and 200-bpm thresholds set.

Body temperature data were Box-Cox transformed to more closely satisfy the assumption of normality and homogeneity. The circadian rhythm of T_b fluctuated significantly during the 24-hour cycle (for part of the day: $F_{5,42840} = 5512.22$, $p < .0001$; and for hours within the day: $F_{18,42480} = 373.37$, $p < .0001$) (Fig. 5.4i). Throughout the study, i.e., in the treatment and control, the median T_b was higher during the evening (38.0 ± 0.1 ^{0}C) between 1600–1659 hours (38.0 ± 0.1 ^{0}C).

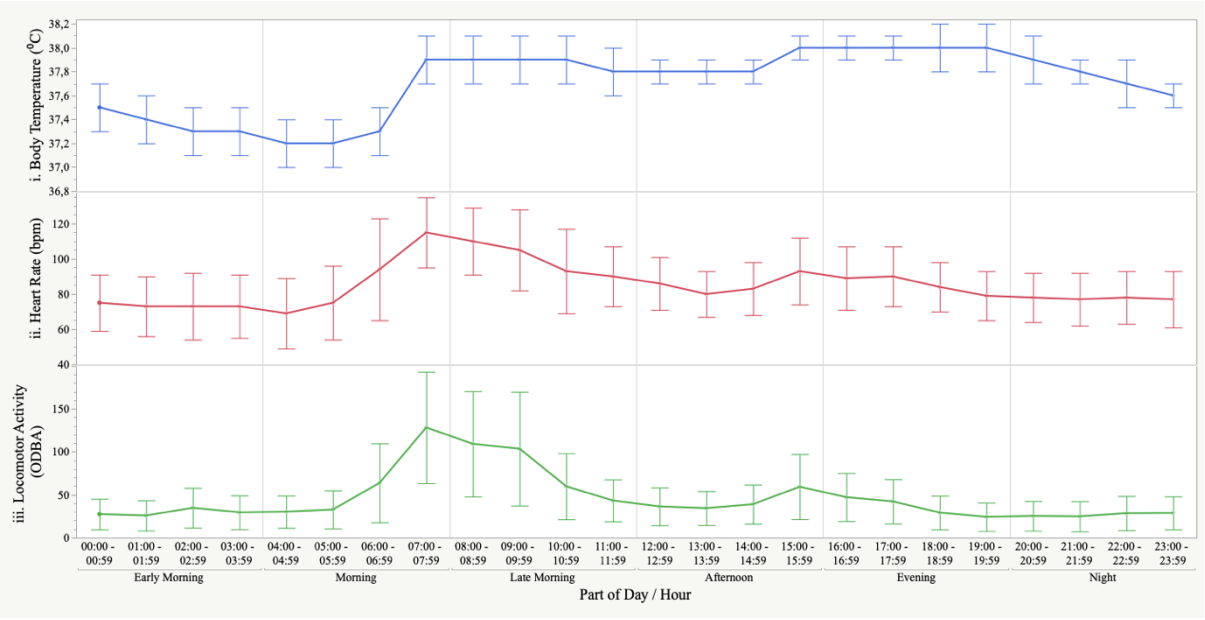

Figure 5.4. Circadian rhythm of (i) body temperature (^{0}C), (ii) heart rate (beats per minute [bpm]), and (iii) locomotor activity (overall dynamic body acceleration [ODBA]) (median, median absolute deviation) for the study cheetahs (CH-2205, -2206, -2271, -2276, and -2277) during the control and treatment.

The MMRM analysis revealed that T_b was significantly higher during the treatment (37.8 ± 0.2 ^{0}C) than in the control (37.7 ± 0.3 ^{0}C; $F_{1,42613} = 23.53$, $p < .0001$). Post hoc comparisons using Tukey's HSD test revealed that the T_b of the female study cheetahs was significantly higher during the treatment (37.7 ± 0.2 ^{0}C) than in the control (37.7 ± 0.3 ^{0}C; $t_{42713} = -7.63$, $p < .0001$) (Fig. 5.5i). Post hoc comparisons using Tukey's HSD test revealed that the T_b of the two-year-old study cheetahs was significantly higher during the treatment (37.8 ± 0.2 ^{0}C) than in the control (37.7 ± 0.3 ^{0}C; $t_{42482} = -7.66$, $p < .0001$) (Fig. 5.6i).

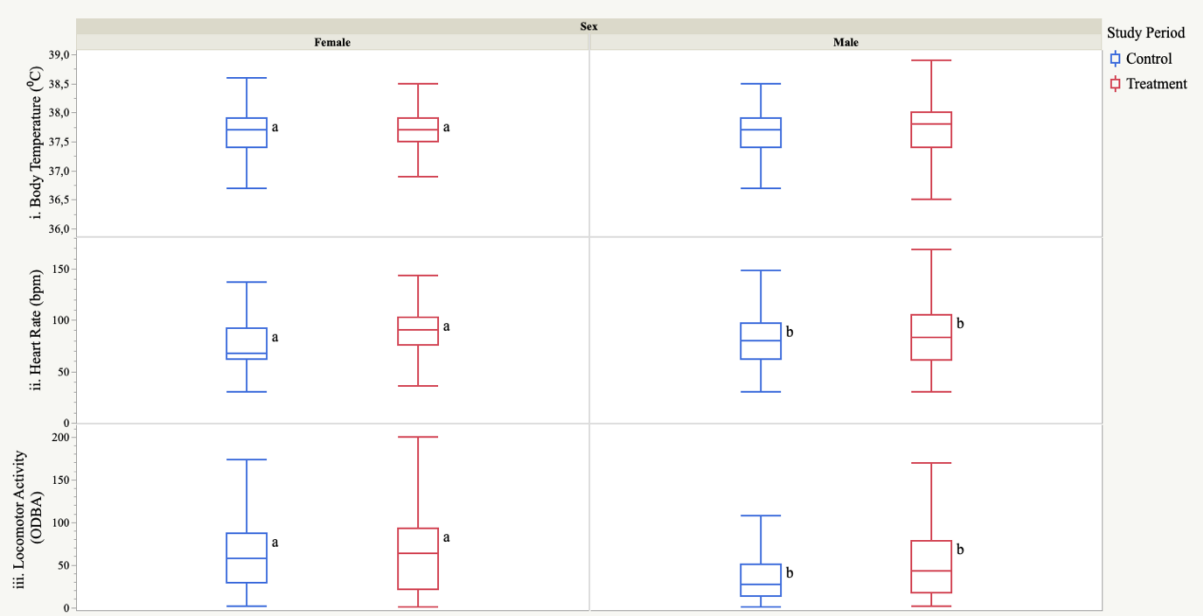

Figure 5.5. Box and whisker plot of (i) body temperature (^{0}C), (ii) heart rate (beats per minute [bpm]), and (iii) locomotor activity (overall dynamic body acceleration [ODBA]) for the study cheetahs (CH-2205, -2206, -2271, -2276, and -2277). Effect of sex (female and male) during the control and treatment. Statistics were performed by a mixed model for repeated measures and Tukey's HSD post hoc test.

(i) a: $p < .0001$. (ii) a, b: $p < .0001$. (iii) a, b: $p < .0001$.

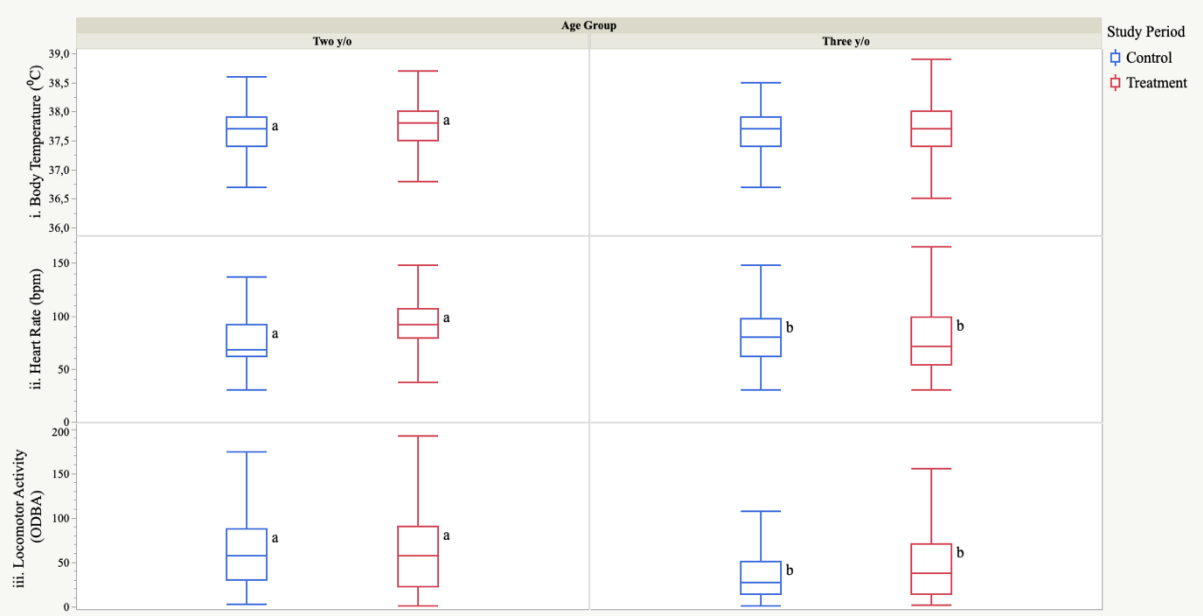

Figure 5.6. Box and whisker plot of (i) body temperature (^{0}C), (ii) heart rate (beats per minute [bpm]), and (iii) locomotor activity (overall dynamic body acceleration [ODBA]) for the study cheetahs (CH-2205, -2206, -2271, -2276, and -2277). Effect of age group (two and three years old [y/o]) during the control and treatment. Statistics were performed by a mixed model for repeated measures and Tukey's HSD post hoc test.

(i) a: $p < .0001$. (ii) a, b: $p < .0001$. (iii) a, b: $p < .0001$.

Post hoc comparisons using Tukey's HSD test revealed that T_b was significantly higher during weeks one (37.8 ± 0.2 ^{0}C; $t_{42770} = -7.96$, $p < .0001$) and two of the treatment (37.8 ± 0.2 ^{0}C; $t_{42770} = -2.65$, $p = .0404$) than in control (37.7 ± 0.3 ^{0}C) and significantly higher during week one of the treatment than in weeks two ($t_{42770} = 5.17$, $p < .0001$) and three (37.7 ± 0.2 ^{0}C; $t_{42770} = 6.36$, $p < .0001$) (Fig. 5.7i).

Figure 5.7. Box and whisker plot of (i) body temperature (^{0}C), (ii) heart rate (beats per minute [bpm]), and (iii) locomotor activity (overall dynamic body acceleration [ODBA]) for the study cheetahs (CH-2205, -2206, -2271, -2276, and -2277). Effect of control versus treatment week (Wk; one, two, or three). Statistics were performed by a mixed model for repeated measures and Tukey's HSD post hoc test.

(i) a, c, d: $p < .0001$ and b: $p = .0404$. (ii) a, b, c, d: $p < .0001$ and e: $p = .0351$. (iii) a, b, d: $p < .0001$ and c: $p = .0119$.

Cohen's d revealed a trivial effect size ($d = 0.16$; $t_{42866} = 15.78$, $p < .0001$) on T_b (Table 5.1).

Heart rate data were Box-Cox transformed to more closely satisfy the assumption of normality and homogeneity. The circadian rhythm of HR fluctuated significantly during the 24-hour cycle (for part of the day: $F_{5,39046} = 670.58$, $p < .0001$; and for hours within the day: $F_{18,39046} = 136.37$, $p < .0001$) (Fig. 5.4ii). Throughout the study, i.e., in the treatment and control, the median HR was higher during the late morning (99 ± 23 bpm) and higher between 0700–0759 hours (115 ± 20 bpm).

The MMRM analysis revealed that HR was significantly higher during the treatment (87 ± 19 bpm) than in the control (78 ± 16 bpm; $F_{1,39066} = 35.43$, $p < .0001$). Post hoc comparisons

using Tukey's HSD test revealed that the HR of the female study cheetahs was significantly higher during the treatment (90 ± 13 bpm) than in the control (68 ± 10 bpm; t_{39066} = -16.39, p < .0001) (Fig. 5.5ii). The HR of the male study cheetahs was significantly higher during the treatment (83 ± 22 bpm) than in the control (80 ± 17 bpm; t_{39066} = 16.71, p < .0001). Post hoc comparisons using Tukey's HSD test revealed that the HR of the two-year-old study cheetahs was significantly higher during the treatment (92 ± 14 bpm) than in the control (68 ± 10 bpm; t_{38677} = -16.44, p < .0001) (Fig. 5.6ii). The HR of the three-year-old study cheetahs was significantly lower during the treatment (71 ± 20 bpm) than in the control (80 ± 17 bpm; t_{38677} = 16.73, p < .0001).

Post hoc comparisons using Tukey's HSD test revealed that HR was significantly higher during weeks two (87 ± 19 bpm; t_{39067} = 5.26, p < .0001) and three of the treatment (84 ± 21 bpm; t_{39067} = 8.02, p < .0001) than in the control (78 ± 16 bpm), significantly higher during week one of the treatment (89 ± 19 bpm) than in weeks two (t_{39067} = 4.48, p < .0001) and three (t_{39067} = 6.85, p < .0001), and significantly higher during week two of the treatment than in week three (t_{39067} = 2.70, p = .0351) (Fig. 5.7ii).

Cohen's d revealed a small effect size (d = 0.20; t_{39072} = 19.26, p < .0001) on HR (Table 5.1).

Locomotor activity data were Box-Cox transformed to more closely satisfy the assumption of normality and homogeneity. The circadian rhythm of LA fluctuated significantly during the 24-hour cycle (for part of the day: $F_{5,42790}$ = 1241.34, p < .0001; and for hours within the day: $F_{18,42790}$ = 286.63, p < .0001) (Fig. 5.4iii). Throughout the study, i.e., in the treatment and control, the median LA was higher during the late morning (69 ± 46 ODBA) and higher between 0700–0759 hours (128.4 ± 65 ODBA).

The MMRM analysis revealed that LA was significantly higher during the treatment (48 ± 32.2 ODBA) than in the control (33.2 ± 20.2 ODBA; $F_{1,42815}$ = 27.40, p < .0001). Post hoc comparisons using Tukey's HSD test revealed that the LA of the female study cheetahs was significantly higher during the treatment (63 ± 36.8 ODBA) than in the control (57.6 ± 28.8 ODBA; t_{42813} = 21.14, p < .0001) (Fig. 5.5iii). The LA of the male study cheetahs was significantly higher during the treatment (42.6 ± 27.6 ODBA) than in the control (27.2 ± 17.2 ODBA; t_{42813} = -18.55, p < .0001). Post hoc comparisons using Tukey's HSD test revealed that the LA of the two-year-old study cheetahs was significantly lower during the treatment (57.4 ± 34.4 ODBA) than in the control (57.6 ± 28.8 ODBA; t_{42813} = 21.14, p < .0001) (Fig. 5.6iii). The LA of the three-year-old study cheetahs was significantly higher during the treatment (37.8 ± 26.2 ODBA) than in the control (27.2 ± 17.2 ODBA; t_{42813} = -18.54, p < .0001).

Post hoc comparisons using Tukey's HSD test revealed that LA was significantly higher during weeks two (50.4 ± 33.2 ODBA; t_{42813} = -4.59, p < .0001) and three of the treatment (44.4 ± 29 ODBA; t_{42813} = -6.27, p < .0001) than in the control (33.2 ± 20.2 ODBA)and significantly higher during week one of the treatment (48.6 ± 33.6 ODBA) than in weeks two (t_{42813} = -3.06, p = .0019) and three (t_{42813} = -4.53, p < .0001) (Fig. 5.7iii).

Cohen's d revealed a small effect size (d = 0.26; t_{42816} = 25.96, p < .0001) on LA (Table 5.1).

5.4 DISCUSSION

Fine-scale spatiotemporal avoidance is believed to be one of the primary mechanisms by which the competitively subordinate cheetah minimises the risk of interference competition with and predation by more dominant large carnivores (Broekhuis *et al.*, 2013; Swanson *et al.*, 2016; 2014). Captive cheetahs are sometimes kept in the vicinity of other large predators, which, absent the opportunity to respond appropriately, could confer a significant source of stress. The two branches of the neuroendocrine stress system are the SNS and HPA axis (see section 1.1.2 for more information on the adaptive stress response). Both branches originate in the hypothalamus and converge on the adrenal gland, working together to maintain or re-establish homeostasis by orchestrating behavioural and physiological adaptations to the stressor (Coffman, 2020). The SNS provides the immediate first wave of the stress response, mediating the rapid release of the catecholamine hormones E and NE from the adrenal medulla (Romero & Wingfield, 2015). The second wave is more gradual and involves the GCs, the end-products of the hormonal cascade along the HPA axis (Sapolsky *et al.*, 2000). Here, the close proximity to species, i.e., a leopard and lions, that generally compete with and/or predate the cheetah in the wild, elicited in the study cheetahs significantly higher T_b and HR (salient features of the SNS-mediated reaction to stress). The results of these analyses support the researcher's hypothesis that olfactory, auditory, and/or visual contact with other large predators would exacerbate the cheetah's physiological stress response to captivity.

Predator cues have recurrently been observed to induce a sympathoadrenal response in various species (e.g., wapiti [*Cervus elaphus canadensis*]; Chabot *et al.*, 1996; domestic fowls [*Gallus gallus domesticus*]; Beauchamp, 2019; salmon; Johnsson *et al.*, 2001; Sundström *et al.*, 2005; house mouse [*Mus musculus*]; Lecorps *et al.*, 2019; rats; Dielenberg *et al.*, 2001). To my knowledge, predator-induced stress on the sympathetic tone of the heart is unstudied in the

cheetah. However, SIH has been demonstrated to some extent by a study in which the increase in T_b of free-ranging cheetahs after a successful hunt was shown to be higher than that of an unsuccessful hunt (mean ± SD; 1.38 ± 0.28 ^{0}C versus 0.58 ± 0.18 ^{0}C), despite similar levels of physical activity (Hetem *et al.*, 2019; 2013). The authors attributed the hyperthermia experienced by hunting cheetahs to the stress associated with vulnerability to attack and kleptoparasitism by more dominant intraguild predators (Hetem *et al.*, 2019; 2013). Though a statistically significant difference in T_b and HR was observed, the effect of predator proximity on either parameter was likely not of biological relevance to the study cheetahs.

Recently, many zoos have recognised the potential of imposing harmless or minimally harmful negative events (Learmonth, 2019). Standard practices for the imposition of minimally harmful negative events include olfactory, auditory, and/or visual proximity between predator-prey or dominant-subordinate species (Maple & Perdue, 2013; Sherwen *et al.*, 2018). These circumstances are thought to confer some resilience to an animal through arousal and activation of the physiological stress response, which can be adaptive and beneficial if experienced acutely (Crofton *et al.*, 2015; Lyons *et al.*, 2009; Parker & Maestripieri, 2011). Indeed, some of the hypotheses proposed to explain the stress-reducing effects of the widely used animal husbandry principle of EE are based on the contention that EE itself acts as a mild stressor (Smail *et al.*, 2020). Despite the potential benefits, zoos should impose minimally harmful negative events with trepidation. It is necessary to ensure that the intended arousal and resilience are achieved while avoiding unintended frustrations or the development of negative affective states in animals (Learmonth, 2019).

The study cheetahs spent the majority of their time inactive. This is consistent with findings described by other authors on captive cheetahs (Skibiel *et al.*, 2007) as well as many other felids (Acaralp-Rehnberg *et al.*, 2020; Bashaw *et al.*, 2007; De Souza Resende *et al.*, 2014;

Macri & Patterson-Kane, 2011; Moreira *et al.*, 2007; Shepherdson *et al.*, 1993; Weller & Bennet, 2001). Predator proximity resulted in significantly lower inactivity levels and significantly higher LA. This is an encouraging finding considering the propensity for captive carnivores to become obese from lack of exercise and experience physical injuries (Hope & Deem, 2006; Law *et al.*, 1997). Alternatively, that the study cheetahs were more active may infer behavioural agitation. Cheetahs and other carnivores in the wild spend a significant portion of their activity budget resting (Siegel, 2005). While reducing the risk of obesity, the motivation for increased activity levels, possibly predator avoidance, could have negative welfare implications.

Not all individuals are equally vulnerable to predation (Pettorelli *et al.*, 2011), affecting their response to predator-related threats. Interindividual differences in vulnerability may arise from intrinsic (e.g., age, sex, social grouping, habitat use, and body size) or extrinsic sources (e.g., climatic conditions, vegetation structure and cover) (Morin *et al.*, 2021). For example, female cheetahs are more vulnerable to predators as a consequence of being solitary and smaller than males (Hunter *et al.*, 2007). The demographic factors, sex and age group, affected the study cheetahs' activity levels and physiological (T_b and HR) responses, possibly explained by the threat sensitivity of the female study cheetahs and those two years old to predators.

Novel stimuli lose their effect over time due to habituation (Kuczaj *et al.*, 2002), whereby animals' responsiveness to a stimulus diminishes with repeated exposure to that stimulus (McFarland, 2021). The temporal profiles of T_b and HR appeared to demonstrate habituation, with values significantly higher during week one of the treatment and significantly lower during weeks two and three. As it corresponded with significantly higher LA during week one of the treatment and significantly lower LA during weeks two and three, the higher T_b and HR could have been caused by the demands of increased physical activity and lower T_b and HR

caused by decreased physical activity. During physical activity, requirements for oxygen and gluconeogenetic substrates in skeletal muscle are increased, as are the removal of metabolites and carbon dioxide (Burton *et al.*, 2004). The cardiac output is increased to meet the demand for blood flow by contracting muscles, attributed to sympathetically-mediated increases in HR and stroke volume. Increased T_b follows an increase in HR due to heat generated during the conversion of nutrients to muscular work (Burton *et al.*, 2004).

Stereotypic pacing is common among captive felids (Clubb & Mason, 2007; Clubb & Vickery, 2006; Livingston, 2009; Mason *et al.*, 2007), including the cheetah (Quirke & O'Riordan, 2011a; 2011b; Quirke *et al.*, 2012). The study cheetahs showed numerically higher stereotypical pacing during week three of the treatment. This may be attributable to the frustration of attempts to perform intrinsically motivated, anti-predator behaviours (Mason, 2006). Significantly lower appetitive behaviour during week three of the treatment (or other non-stereotypical, active behaviour), as has been demonstrated by free-ranging cheetahs to prioritise vigilance when primarily concerned by predation (Hilborn *et al.*, 2018), was further undesirable.

The study cheetahs' FCS was significantly lower during week three of the treatment. Symbiotic gut microbes harboured in animals' GI tracts serve various essential functions (Wasimuddin *et al.*, 2017). However, disturbance-related deviation in the microbial diversity and abundance pattern beyond a natural range, i.e., gut dysbiosis, can advance pathophysiology and affect host health (Shreiner *et al.*, 2015). Faecal consistency is purportedly linked to intestinal microbiota composition (Tigchelaar *et al.*, 2016; Vandeputte *et al.*, 2016) and could indicate GI health (Whitehouse-Tedd *et al.*, 2015). Chronic or repeated exposure to stressors has been identified to disrupt gut homeostasis (Accarie & Vanuytsel, 2020; Farzi *et al.*, 2018).

Therefore, the lower FCS may be attributable to the stress of prolonged predator proximity (Smail *et al.*, 2020).

This study's findings encourage, to some extent, the close proximity to species, i.e., a leopard and lions, that generally compete with and/or predate the cheetah in the wild as a potential form of EE for cheetahs in captivity. More so, they highlight the importance of trepidation when imposing minimally harmful negative events. These circumstances must be mild and transient, as chronic or repeated exposure to stressors (and the attending activation of the stress response) can be detrimental to the welfare of captive cheetahs.

However, it is difficult to determine if predator proximity per se was responsible for the results. During the treatment, the study cheetahs were housed in an on-exhibit enclosure smaller than the off-exhibit enclosure in which they were kept during the control (see section 1.1.6.1 for more information on the study site and animals). In the wild, cheetahs are solitary, have large home ranges, and avoid human contact (Caro, 1994), so it may not be surprising that a zoo exhibit environment is related to stress in this species. Housing in public exhibits has been associated with behavioural and physiological stress in cheetahs (Wells *et al.*, 2004) and other elusive cats (Mallapur & Chellam, 2002; Sellinger & Ha, 2005; Wielebnowski *et al.*, 2002a). I would recommend further scientific research on the overall welfare implications of predator cues as a minimally harmful negative event, considering intrinsic (e.g., age, sex, social grouping, and body size) and extrinsic (e.g., enclosure design) sources of variation in threat sensitivity between individual animals. It must also be acknowledged that the present study was conducted on captive-born (hand-reared) cheetahs, so the findings here should not be generalised to all captive cheetahs. I recommend additional caution if housing wild-caught cheetahs (potentially with previous experience of interference competition) where they may have olfactory, auditory, and/or visual contact with other large predators.

Furthermore, I demonstrated that biologging technology could be used to record concurrent T_b, HR, and LA and, in addition to behavioural and physiological metrics, has tremendous potential as a tool to measure stress in the cheetah. However, a significant limitation of the present study was the battery malfunction of the biologgers implanted in the study cheetahs CH-2271, CH-2276, and CH-2277, impeding the acquisition of complete T_b, HR, and LA data sets for those individuals. For the technology to be routinely utilised in animal welfare studies, there is still a need to develop more reliable devices capable of remote data transmission to avoid repeated capture and handling.

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Chapter 6: General Discussion and Conclusions

Free-ranging cheetah populations are in decline today, and a holistic approach to conservation, combining *in situ* and *ex situ* efforts, is necessary to save the species from extinction (Schwartz *et al.*, 2018). However, globally, captive cheetahs are known to suffer from multiple unusual diseases, limiting the sustainability of these populations (Terio *et al.*, 2018). Captivity-induced stress compounded by genetic and possibly dietary factors is thought to play an aetiological role in the various diseases prevalent in captive cheetahs (Bolton & Munson, 1999; Munson *et al.*, 2005). The conservation, captive management, and ultimate survival of cheetahs can benefit significantly from understanding the conditions of captivity inducing a stress response and why and the methodological advances used to quantify these reactions.

This thesis aimed to holistically assess cheetahs' resilience to the captive setting using emerging biologging technology and more traditional measures of stress-related behavioural and physiological responses. A major methodological challenge in animal welfare research is that, when quantifying activation of the physiological stress response, studies often compare absolute levels of arousal or stress with little consideration about the adaptive benefit or cost associated with the response (Selye, 1973). In some contexts, arousal and activation of the physiological stress response are adaptive and help an animal cope with stressors, while in others, they are maladaptive and have deleterious effects (Proudfoot & Habing, 2015). Measuring concurrent physiological parameters could help to differentiate between an adaptive and maladaptive stress response by providing a more comprehensive representation of an individual's physiological status (Brijs *et al.*, 2021; Laske *et al.*, 2021; Williams & Ponganis, 2021). Additionally, monitoring the behavioural responses of animals may offer context for

arousal and activation of the physiological stress response by indicating valence (Wascher, 2021).

A significant contribution of the research was findings supporting the influence of the more natural feeding pattern within the GI environment, which may have included microbial parameters and/or GI physiology or functional traits. As FCS is not a definitive measure of GI health, the results should be treated this way, and further investigation is needed. However, for management purposes, Chapter 2's findings are sufficient to encourage a more naturalistic reduced feeding days schedule to mediate the unnatural composition of horsemeat-based diets routinely fed to cheetahs in captivity.

The provision of EE encouraged the study cheetahs to engage actively with their environment. Consistent with the promoted stress-reducing effects of EE, its provision and a more naturalistic reduced feeding days schedule resulted in lower HR, demonstrating the latter's effectiveness as a food-based enrichment strategy for cheetahs. Higher active behaviour observed across treatments is an encouraging finding considering the propensity of carnivores in captivity to become obese from lack of exercise and experience physical injuries (Hope & Deem, 2006; Law *et al.*, 1997). Alternatively, that the study cheetahs were more active may infer behavioural agitation. Cheetahs and other carnivores in the wild spend a significant portion of their activity budget resting (Siegel, 2005). While reducing the risk of obesity, the motivation for increased activity levels, possibly hunger and human and predator avoidance, could have negative welfare implications. Regarding Chapter 2, the findings emphasise the importance of offering sufficiently large meals if additional fast days are introduced not to be contrary to animal welfare.

Of the simulated scenarios depicting conditions considered psychological stressors for captive cheetahs, participation in AVIs and close proximity to other large predators appeared

to have the most significant potential to elicit a maladaptive stress response. However, it is difficult to determine if the treatments per se were responsible for the results. During the treatment, the study cheetahs were housed in an on-exhibit enclosure smaller than the off-exhibit enclosure in which they were kept during the control (see section 1.1.6.1 for more information on the study site and animals). In the wild, cheetahs are solitary, have large home ranges, and avoid human contact (Caro, 1994), so it may not be surprising that a zoo exhibit environment is related to stress in this species. Housing in public exhibits has been associated with behavioural and physiological stress in cheetahs (Wells *et al.*, 2004) and other elusive cats (Mallapur & Chellam, 2002; Sellinger & Ha, 2005; Wielebnowski *et al.*, 2002a). Providing enclosures of sufficient size and additional opportunities for the study cheetahs on-exhibit to control their degree of exposure to visitors (e.g., hiding places, visual barriers, and indoor spaces) should be considered from a welfare perspective (Carlstead *et al.*, 1993; Wielebnowski *et al.*, 2002a).

Though it could not be determined whether participation in AVIs per se or some other factors (e.g., housing on-exhibit) were responsible for the results observed, evidence for the potentially enriching effects of AVIs is sufficient to support the continued participation of the study cheetahs in natural encounters. Hand-rearing may have increased the study cheetahs' overall tolerance for close contact with people (Carlstead, 2009; Bonato *et al.*, 2013), making them more suitable for interactive experiences (Acaralp-Rehnberg *et al.*, 2020; Narayan *et al.*, 2013; Szokalski *et al.*, 2013). For animals not intended for reintroduction into the wild or *ex situ* breeding programmes, positive early-life experiences with humans may down-regulate stress reactivity and reduce adult fearfulness (Bonato *et al.*, 2013; Feenders & Bateson, 2011; Hemsworth & Barnett, 1992), facilitating an adaptation to the captive environment. Indeed, a positive keeper-animal relationship has been proposed as a significant mediator in captive

animals' responses to visitors (Claxton, 2011; Hosey, 2008). This is a relatively unstudied research topic, but for which further scientific attention is justified.

A secondary aim of this thesis was to explore the potential of biologging technology for measuring stress in cheetahs. The multi-trait devices offered several benefits, including, but not limited to, that they remotely and continuously recorded fine-scale T_b, HR, and LA data, revealing how the various functions interact. Moreover, they could be deployed for extended periods, measuring short-term arousal levels and long-term physiological data without repeated capture and handling collection bias. However, a significant limitation of the research was the battery malfunction of the biologgers implanted in the study cheetahs CH-2207, -2271, CH-2276, and CH-2277, impeding the acquisition of complete T_b, HR, and LA data sets for those individuals. The reduced sample size could have affected the reliability and potentially, as complete data sets were only captured for the male study cheetahs and those three years old, the objectivity of the results. For the technology to be routinely utilised in animal welfare studies, there is still a need to develop more reliable devices capable of remote data transmission to avoid repeated capture and handling.

It must also be acknowledged that, across treatments, the effect on the physiological parameters measured, though statistically significant, was likely irrelevant to the study cheetahs' biology. The lack of biological relevance was possibly explained by the reliance of the research on only six study subjects, further compounded by the battery malfunction of four of six biologgers implanted in the study cheetahs. Like other large carnivores, cheetahs are generally kept in low densities at facilities such as the Cango Wildlife Ranch and Conservation Centre. As proposed by Swaisgood and Shepherdson (2005), future studies should focus on increasing sample size through multi-institutional studies to improve the quality of the findings

and the number of publications in peer-reviewed journals and to stop replications of anecdotic studies.

Furthermore, the research's experimental design was based on best accommodating the inverse relationship between the number of T_b, HR, and LA recordings made by the biologgers and the lifespan of their batteries. However, the 3-week study periods and 24-hour washout period between interventions may not have been of sufficient duration to elicit a clear response in the study cheetahs, further limiting the generalisations drawn from these results. For example, in mice, stress-related alterations in the composition and function of faecal microbiota have been described after eight days, while in rats, it appears after 10 days (Yoshikawa *et al.*, 2017; Zhang *et al.*, 2019). It is necessary to extend the study periods better to quantify the treatment effects and the washout periods to ensure the results are attributable to the correct intervention.

This thesis contributes to the growing evidence supporting a multi-method approach to measuring stress and animal welfare. The research relied on only hand-reared cheetahs and a relatively small sample size, limiting the inferences drawn from the results. However, the methodology for assessing cheetahs' responses to captivity should be helpful for future studies on this and other felids. The findings presented here will hopefully provide a basis for future research to build on appropriate management and husbandry protocols for cheetahs that better accommodate their requirements.

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APPENDIX

S.1 Phase Differences

From the continuous measurement of T_b by biotelemetry in its early application days (Asa & Sarrik, 1990), it was suggested that cheetahs and other large felids might not have a circadian rhythm of T_b or that of activity (Bircher & Noble, 1997). However, distinctive daily T_b, HR, and LA patterns were observed in the study cheetahs, whereby there was an increase in all parameters at around 0700 hours (shortly after sunrise) and again at about 1600 hours (shortly before sunset) for T_b (Fig. S.1.1). The median T_b varied cyclically throughout the day, following the established pattern of higher T_b in the afternoon and evening, ubiquitous in diverse species. In support of this finding, a similar circadian T_b rhythm was reported in the cheetah by the more recent deployment of temperature-sensitive biologgers (Hetem *et al.*, 2019; 2013).

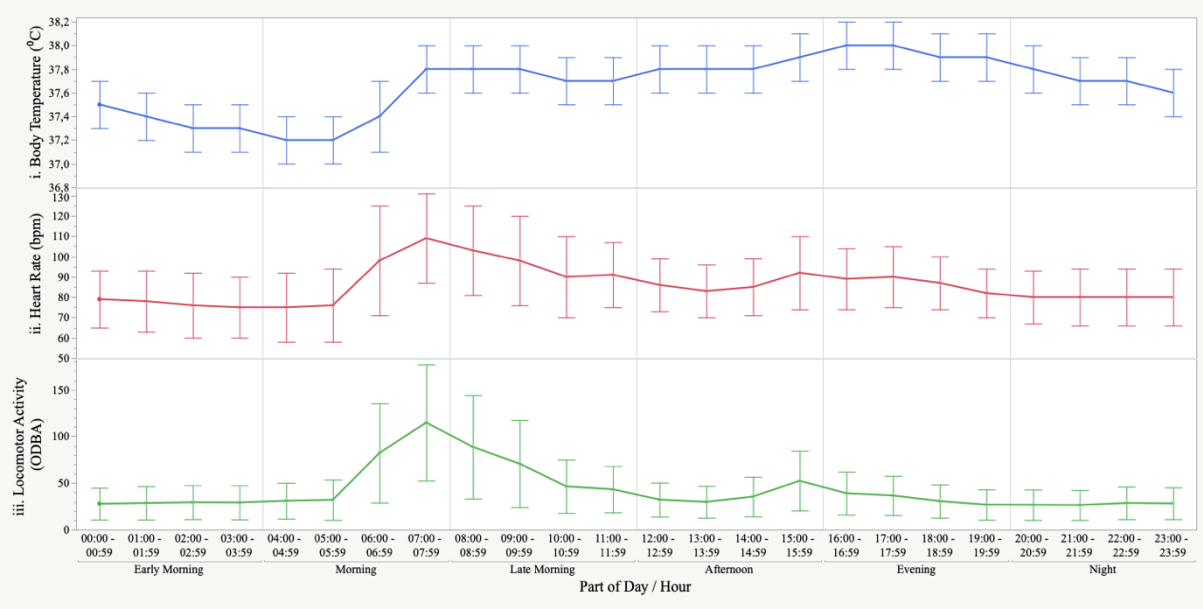

Figure S.1.1. Circadian rhythm of (i) body temperature (^{0}C), (ii) heart rate (beats per minute [bpm]), and (iii) locomotor activity (overall dynamic body acceleration [ODBA]) (median, median absolute deviation) recordings for the study cheetahs (CH-2012, -2014, -2205, -2206, -2207, -2271, -2276, and -2277).

The increase in LA, corresponding with increased T_b and HR around sunrise (Fig. S.1.2), is consistent with the diel activity pattern demonstrated in free-ranging cheetahs (Veldhuis *et al.*, 2020). Cheetahs are a diurnal species, an evolutionary adaptation to reduce the risk of encountering larger, more dominant predators, which are primarily nocturnal (Hayward & Slotow, 2009). In some regions, a preference for hunting at dawn has been observed in cheetahs (Cozzi *et al.*, 2012), with more than half of all hunts occurring between 0600 and 1000 hours (Schaller, 1968; Wilson *et al.*, 2013). Animals are acknowledged as having 'behavioural needs' (Brambell, 1965), generally conceptualised as behaviours they must perform regardless of environmental circumstances. That is, primarily internally motivated behaviours occurring even without appropriate external stimulation (Mench, 1998). The relationship between T_b, HR, and LA recordings and the timing of sunrise following a seasonal pattern may be attributable to the study cheetahs' intrinsic motivation to hunt.

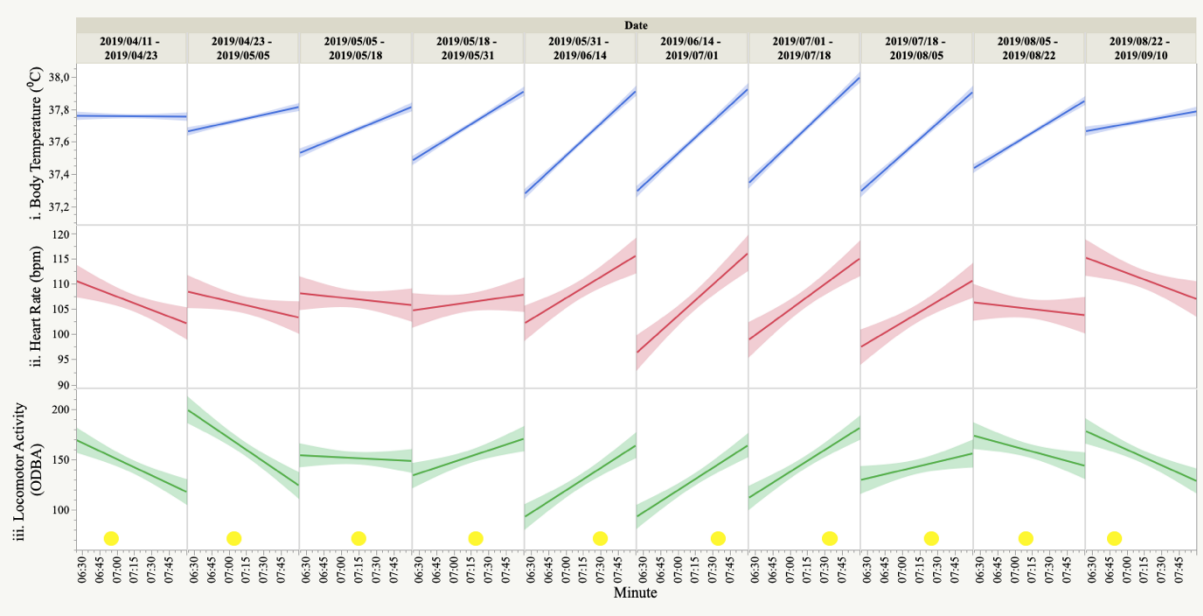

Figure S.1.2. Linear regression of (i) body temperature (^{0}C), (ii) heart rate (beats per minute [bpm]), and (iii) locomotor activity (overall dynamic body acceleration [ODBA]) (95% confidence interval) recordings for the study cheetahs (CH-2012, -2014, -2205, -2206, -2207, -2271, -2276, and -2277). The relationship between parameters and the timing of sunrise, as it follows a seasonal pattern, is indicated by the sun's position on the timeline.

S.2 Pilot Study: Responses to Surgical Procedures in Captive-Born Cheetahs (Acinonyx jubatus)

S.2.1 Introduction

Surgical intervention is integral to veterinary medicine, whether performed to treat a medical condition, lower disease incidence, or control the population. However, by design, surgery and related procedures are stressful (Desborough, 2000; Giannoudis *et al.*, 2006). Stress in the peri-operative period has four significant contributors: anxiety of unfamiliar surroundings associated with these procedures, discomfort and pain following incisions and tissue manipulation, the surgical stress response, and the potential neurotoxicity of anaesthetic agents (Borsook *et al.*, 2010). A hallmark of improving animal welfare is mitigating the pain

and stress experienced by an individual because of surgical intervention, accomplished using appropriate capture and restraint as well as peri- and postoperative care. However, the systemic reaction to surgical injury is less amenable, attended by the neuroendocrine-metabolic response and the haemato-immunological response (Cusack & Buggy, 2020; Desborough, 2000). The magnitude, invasiveness, and duration of the surgery are central to determining the degree of the stress response (Finnerty *et al.*, 2013; Giannoudis *et al.*, 2006; Horta *et al.*, 2015; Kehlet, 1997; Marana *et al.*, 2003).

This pilot study's overall aim was to investigate the response of captive-born (hand-reared) cheetahs to the surgical implantation of biologgers. Biologging technology has or will advance our understanding of diverse species today and in the future, but its application involves procedures that may have welfare implications for animal carriers (Hawkins, 2004). I hypothesised that minimally invasive surgery would induce a stress response followed by temporal recovery. As an established stress-related marker, fGCM concentration analysis (Terio *et al.*, 1999) was performed, and faecal consistency scoring as a preliminary indicator of the study cheetahs' GI health (Whitehouse-Tedd *et al.*, 2015). A secondary objective was to explore the potential of biologging technology for measuring stress in the cheetah by recording concurrent T_b, HR, and LA. I predicted higher fGCM concentration and lower FCS, consistent with a physiological stress response.

S.2.2 Materials and Methods

S.2.2.1 Study Site and Animals

The experimental trials occurred in April 2019 at the Cango Wildlife Ranch and Conservation Centre (33°33'S, 22°12'E) 4 km north of Oudtshoorn, in the Western Cape of South Africa (see section 1.1.6.1 for more information on the study site and animals). Four

male (CH-2012, -2205, -2206, and -2271) and four female (CH-2014, -2207, -2276, and -2277) resident adult cheetahs (Table S.2.1) were allocated to this pilot study.

Table S.2.1. Demographic information of the study cheetahs.

Identification Number	Origin	Housing	DOB (YY-MM-DD)	BM1 (kg)	BM2 (kg)	Sex
CH-2012	Captive-born (hand-reared)	Single	2013-08-18	45.45	43.4	M
CH-2014	Captive-born (hand-reared)	Single	2013-08-18	39.4	43.9	F
CH-2205	Captive-born (hand-reared)	Paired (with CH-2206)	2016-06-09	45.0	45.0	M
CH-2206	Captive-born (hand-reared)	Paired (with CH-2205)	2016-06-09	47.15	47.65	M
CH-2207	Captive-born (hand-reared)	Single	2016-06-09	36.6	37.1	F
CH-2271	Captive-born (hand-reared)	Single	2017-08-28	37.65	41.75	M
CH-2276	Captive-born (hand-reared)	Paired (with CH-2277)	2017-09-16	39.9	39.35	F
CH-2277	Captive-born (hand-reared)	Paired (with CH-2276)	2017-09-16	43.05	43.55	F

DOB: date of birth, BM1: body mass at the start of the experimental trials, BM2: body mass at the end of the experimental trials.

S.2.2.2 Body Temperature, Heart Rate, and Locomotor Activity Measurement Intervals

A single cardiac-, temperature- and movement-sensitive biologger (DST centi-HRT ACT, Star-Oddi, Gardabaer, Iceland) with the dimensions of 46 mm x 15 mm x 15 cm and weighing approximately 19 g was implanted in each of the study cheetahs. The biologgers were calibrated individually against a high-accuracy thermometer (Hart 1504, Fluke, Utah, US). Calibrated accuracy was better than 0.1 °C. Before surgical implantation, the biologgers were set to record tri-axial LA, i.e., heave, surge, sway, every minute for the ODBA and T_b (°C) and leadless single-channel ECG-derived HR (bpm) every 5 minutes at 200 Hz. Representative traces of raw ECG recordings were saved every 24 hours for validating the HR measurements' QI (where QI_0 was the highest quality and QI_1, QI_2, and QI_3 were of progressively reduced quality). Afterwards, the biologging devices were sterilised using ethylene oxide and implanted (see section 1.1.6.3 in the introduction for more information on the surgical procedure).

S.2.2.3 Experimental Design

The study cheetahs were monitored for five days pre-operatively regarding (i) fGCM concentration and (ii) FCS and 10 days post-operatively regarding (i) fGCM concentration and (ii) FCS, as well as (3) T_b, HR, and LA concurrently recorded by implanted biologgers.

S.2.2.4 Faecal Sample Collection and Consistency Scoring

To differentiate between individual faecal samples in the event of paired housing (Table S.2.1), 1 tbsp of uncooked rice was thoroughly mixed into the diet of study cheetahs CH-2206 and CH-2277 once per day. The caretakers monitored the study cheetahs during feeding times to ensure the sufficient consumption of rice and prevent meal sharing. Only faeces found to have uncooked rice were considered to have originated from those individuals fed rice. Due to the operating hours of the Cango Wildlife Ranch and Conservation Centre, the enclosures could not be entered between 1800–0800 hours. As such, once daily between 0800–1000 hours, faeces from the previous night were collected from each enclosure within 16 hours of defecation (4–10 °C T_a) (Ludwig *et al.*, 2013).

Following sample collection, the researcher (KLB) assigned FCS as per a five-point grading system, where two points (grade 4: firm and dry and grade 5: firm) were considered to be 'normal,' and three points (grades 1–3: liquid, soft without shape, and soft with shape) were considered to be 'suboptimal' (adapted from AZA Tiger Species Survival Plan®, 2016; Whitehouse-Tedd *et al.*, 2015). Afterwards, the samples were deposited into appropriately labelled (sample collection date and study cheetah and sample ID numbers) 50 mL polypropylene specimen containers and frozen at –20 °C.

S.2.2.5 Faecal Steroid Extraction and Quantification

Following completion of the experimental trials, faecal samples were transported frozen to the Endocrine Research Laboratory, University of Pretoria, South Africa. Faecal steroids were extracted and analysed for fGCM concentration as described in section 1.1.6.7.

S.2.2.6 Ethical Statement

All of the experimental procedures involving the study cheetahs were approved by the University of Pretoria's Animal (clearance number V075-18) and Research Ethics Committees (clearance number REC069-18).

S.2.2.7 Statistical Analysis

Statistical analysis was performed using Microsoft Excel (version 16.0) and JMP Pro software (version 16.0) for Windows, developed by SAS Institute Inc (North Carolina, US). Raw data was manipulated before detailed analyses (see section 1.1.6.9 for more information on the data preparation). The data were screened for univariate outliers greater than three interquartile ranges away from the 99.5th or 0.05th percentiles ($n = 0$), which were subsequently excluded from descriptive statistics and analyses. Normal distribution and homogeneity of variance were verified using Anderson-Darling and Levene's tests, respectively. Box-Cox transformations were used to more closely satisfy the assumption of normality and homogeneity in the case of departure. To maintain statistical integrity, the data were back-transformed for descriptive statistics and visual representation. An MMRM analysis was conducted to investigate the following:

- The random effect of the study cheetah, the independent fixed effect of the study period (i.e., pre- versus post-operative), and the interaction fixed effects of the study period and (i) sex and (ii) age group on fGCM concentration and FCS.

The surgery effect size was calculated using Cohen's d. Descriptive statistics were reported as median ± MAD and the significance level, α, was set at 0.05.

S.2.3 Results

This pilot study's overall aim was to investigate the response of captive-born (hand-reared) cheetahs to the surgical implantation of biologgers. It investigated whether demographic factors (sex and age group) and temporal recovery affected this response.

S.2.3.1 Faecal Glucocorticoid Metabolite Concentrations

Faecal glucocorticoid metabolite concentration data were Box-Cox transformed to more closely satisfy the assumption of normality and homogeneity. The MMRM analysis revealed that fGCM concentration was significantly higher during the post-operative period (1.18 ± 0.23 µg/g DW) than in the pre-operative period (0.95 ± 0.18 µg/g DW; $F_{1,99.07} = 23.68$, $p < .0001$) (Table S.2.2). Post hoc comparisons using Tukey's HSD test revealed that the fGCM concentration of the female study cheetahs was significantly higher during the post-operative period (1.21 ± 0.21 µg/g DW) than in the pre-operative period (0.84 ± 0.14 µg/g DW; $t_{98.0} = -4.28$, $p = .0002$) (Fig. S.2.1i). The fGCM concentration of the male study cheetahs was significantly higher during the post-operative period (1.13 ± 0.23 µg/g DW) than in the pre-operative period (0.97 ± 0.16 µg/g DW; $t_{98.0} = -2.64$, $p = .0473$). Post hoc comparisons using Tukey's HSD test revealed that the fGCM concentration of the two-year-old study cheetahs was significantly higher during the post-operative period (1.14 ± 0.12 µg/g DW) than in the pre-operative period (0.84 ± 0.16 µg/g DW; $t_{97.4} = -2.93$, $p = .0475$) (Fig. S.2.2i). The fGCM concentration of the three-year-old study cheetahs was significantly higher during the post-operative period (1.16 ± 0.34 µg/g DW) than in the pre-operative period (0.86 ± 0.18 µg/g DW; $t_{98.0} = -3.04$, $p = .0350$).

Table S.2.2. Median ± median absolute deviation (MAD) of faecal glucocorticoid metabolite (fGCM) concentration (µg/g dry weight [DW]), faecal consistency score, body temperature (^{0}C), heart rate (beats per minute [bpm]), and locomotor activity (overall dynamic body acceleration [ODBA]) for the study cheetahs (CH-2012, -2014, -2205, -2206, -2207, -2271, -2276, and -2277) during the pre- and post-operative period.

Study Period	fGCM Concentration (µg/g DW)	Faecal Consistency Score (grade)	Body Temperature (°C)	Heart Rate (bpm)	Locomotor Activity (ODBA)
Pre-operative period	**0.95 ± 0.18**	**4 ± 1**	-	-	-
Day 5	0.85 ± 0.10	3.5 ± 0.5	-	-	-
Day 4	0.76 ± 0.33	4 ± 1	-	-	-
Day 3	0.77 ± 0.13	4 ± 1	-	-	-
Day 2	0.97 ± 0.14	3.5 ± 1	-	-	-
Day 1	1.06 ± 0.29	4 ± 1	-	-	-
Post-operative period	**1,18 ± 0.23**	**4 ± 1**	**37.8 ± 0.2**	**86 ± 15**	**37.8 ± 23**
Day 1	1.12 ± 0.08	3 ± 0	37.8 ± 0.3	86 ± 13	41.2 ± 24.7
Day 2	1.23 ± 0.23	4 ± 1	37.7 ± 0.2	84 ± 14	39.8 ± 23
Day 3	1.25 ± 0.17	4 ± 1	37.8 ± 0.2	84.5 ± 13.5	36.9 ± 21.3
Day 4	1.28 ± 0.45	3 ± 0	37.7 ± 0.2	86 ± 17	41.6 ± 26.2
Day 5	1.33 ± 0.14	3 ± 0	37.8 ± 0.2	86 ± 16	34.6 ± 19.6
Day 6	1.31 ± 0.20	3.5 ± 0.5	37.8 ± 0.2	86 ± 13	38.3 ± 22.5
Day 7	1.15 ± 0.11	3 ± 0	37.8 ± 0.2	86.5 ± 13.5	38.3 ± 23.5
Day 8	1.09 ± 0.33	4.5 ± 0.5	37.8 ± 0.2	86 ± 15	35.2 ± 21.1
Day 9	1.08 ± 0.19	5 ± 0	37.7 ± 0.2	88 ± 15	35.2 ± 21.4
Day 10	1.08 ± 0.10	4 ± 1	37.7 ± 0.2	87 ± 16	38.2 ± 25.7

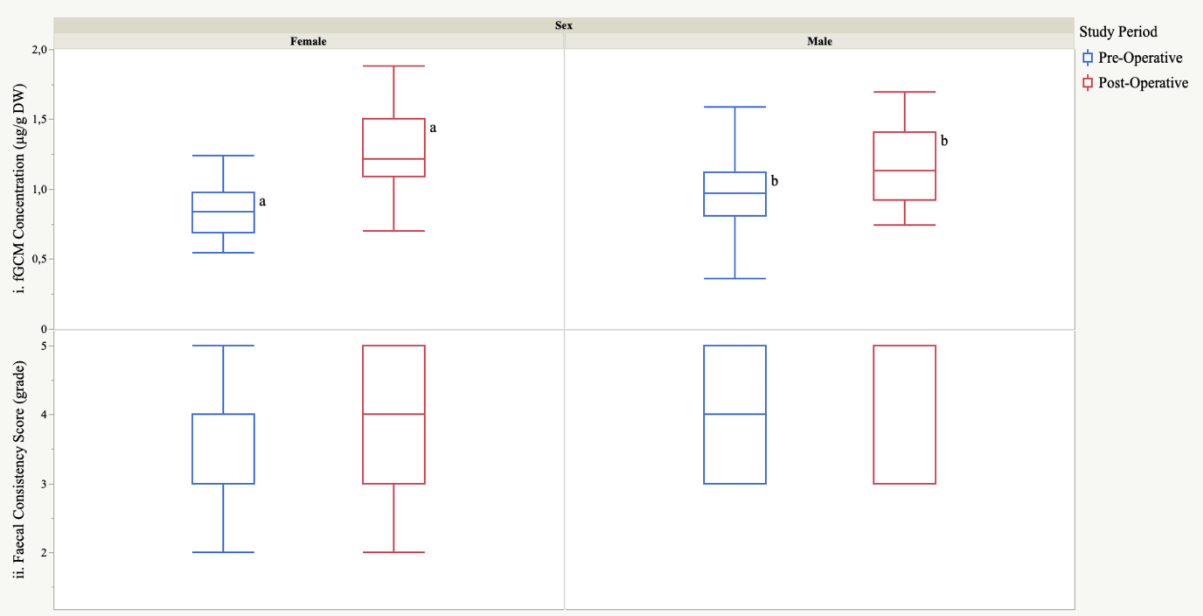

Figure S.2.1. Box and whisker plot of (i) faecal glucocorticoid metabolite (fGCM) concentration (µg/g dry weight [DW]) and (ii) faecal consistency score (grade) for the study cheetahs (CH-2012, -2014, -2205, -2206, -2207, -2271, -2276, and -2277). Effect of sex (female and male) during the pre- and post-operative period. Statistics were performed by a mixed model for repeated measures and Tukey's HSD post hoc test.

(i) a: $p = .0002$ and b: $p = .0473$.

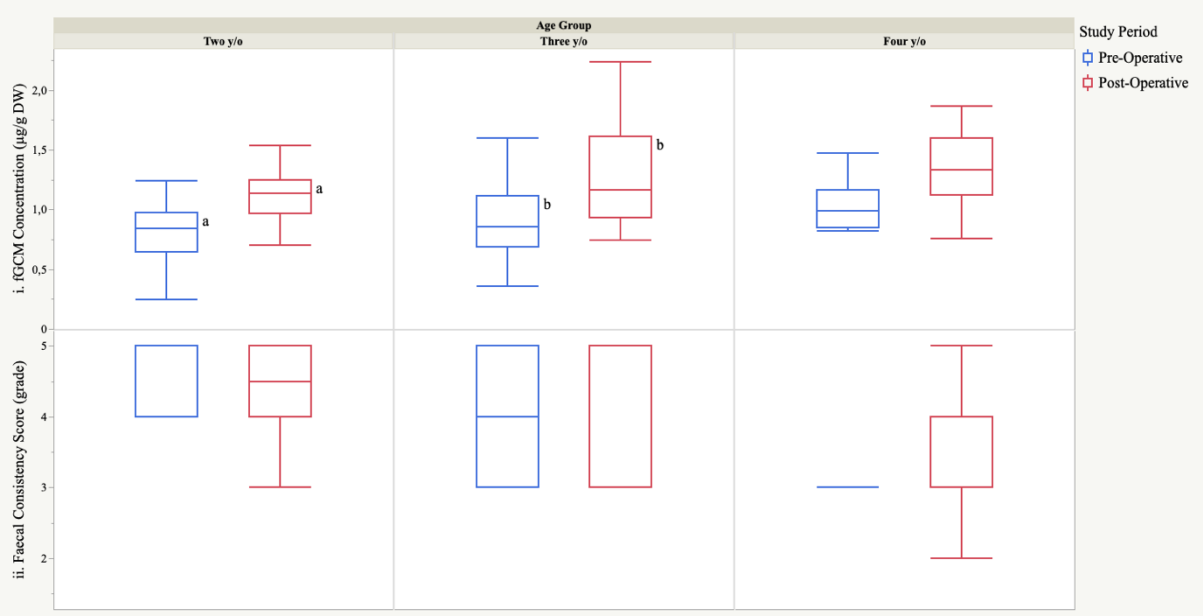

Figure S.2.2. Box and whisker plot of (i) faecal glucocorticoid metabolite (fGCM) concentration (µg/g dry weight [DW]) and (ii) faecal consistency score (grade) for the study cheetahs (CH-2012, -2014, -2205, -2206, -2207, -2271, -2276, and -2277). Effect of age group (two, three, and four years old [y/o]) during the pre- and post-operative period. Statistics were performed by a mixed model for repeated measures and Tukey's HSD post hoc test.

(i) a: $p = .0475$ and b: $p = .0350$.

The temporal profile of fGCM concentration pre- and post-operatively is shown in Figure S.2.3i.

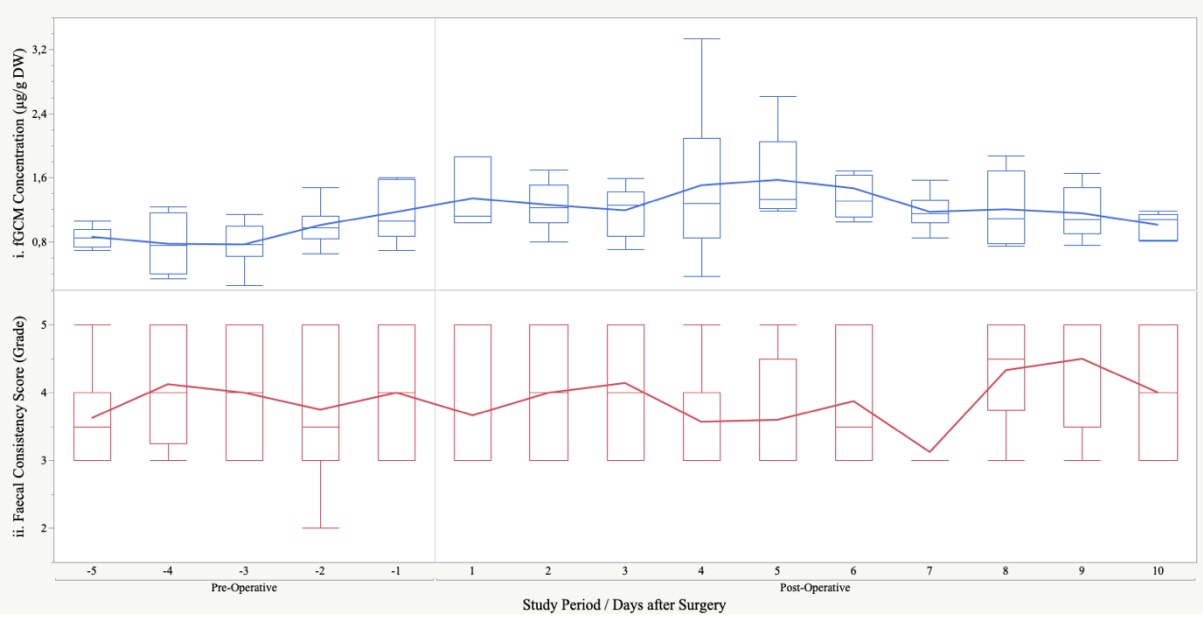

Figure S.2.3 Box and whisker plot with mean trendline of (i) faecal glucocorticoid metabolite (fGCM) concentration (µg/g dry weight [DW]) and (ii) faecal consistency score (grade) for the study cheetahs (CH-2012, -2014, -2205, -2206, -2207, -2271, -2276, and -2277). Temporal effect of surgery during the pre- and post-operative period.

Cohen's d revealed a large effect size (d = 0.94; t_{106} = 4.72, p < .0001) on fGCM concentration (Table S.2.3).

Table S.2.3. Surgery effect size on faecal glucocorticoid metabolite (fGCM) concentration (µg/g dry weight [DW]) and faecal consistency score for the study cheetahs (CH-2012, -2014, -2205, -2206, -2207, -2271, -2276, and -2277). Calculations were performed using Cohen's d.

	t	*p*	df	Cohen's d	95% CI for Cohen's d	
					Lower	Upper
fGCM Concentration (µg/g DW)	4.72	< .0001	106	0.94	0.53	1.35
Faecal Consistency Score (grade)	0.05	.9642	103	0.01	-0.39	0.40

df: degrees of freedom, CI: confidence interval.

S.2.3.2 Faecal Consistency Scores

Faecal consistency score data were Box-Cox transformed to more closely satisfy the assumption of normality and homogeneity. The soft with shape faecal grade was the most frequently recorded in the study cheetahs (for the pre-operative period: 35.0% and the post-

operative period: 42.6%) (Fig. S.2.2). Post-operatively, the 'normal' grade of firm was higher (~5.3%) and the 'normal' grade of firm and dry (~12.4%) and the 'suboptimal' grade of soft without shape (~1.0%) were lower than in the pre-operative period. There was no numerical difference in median FCS between the post-operative (4 ± 1) and pre-operative period (4 ± 1) (Table S.2.2).

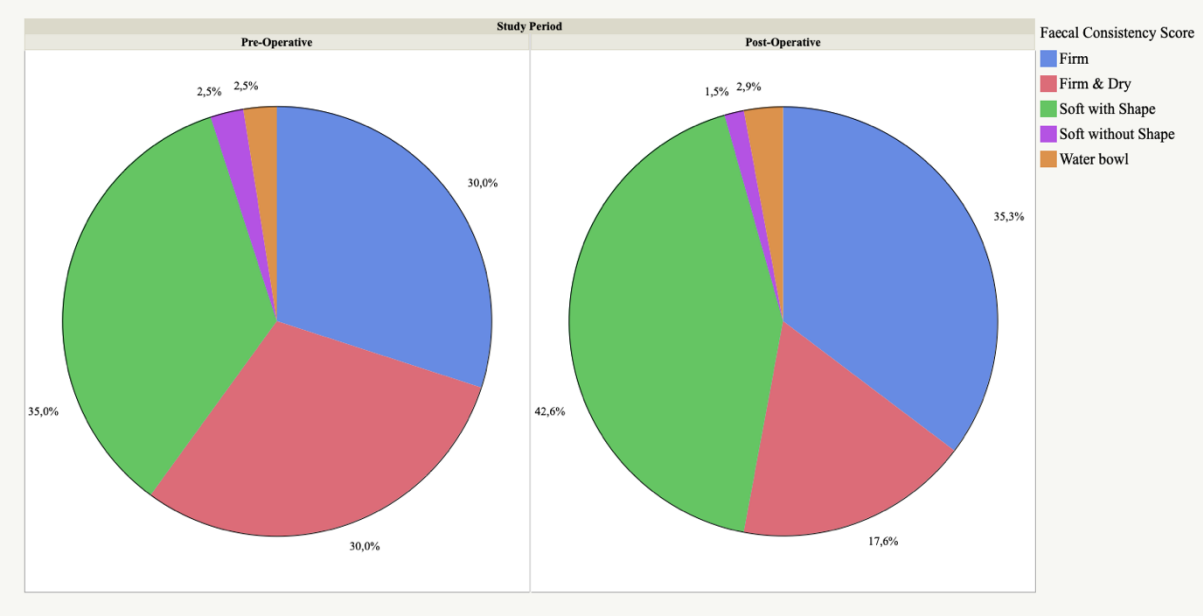

Figure S.2.4 Pie chart of faecal consistency score for the study cheetahs (CH-2012, -2014, -2205, -2206, -2207, -2271, -2276, and -2277) during the pre- and post-operative period, where firm and firm and dry were considered to be 'normal' and liquid, soft without shape, and soft with shape were considered to be 'suboptimal' (adapted from AZA Tiger Species Survival Plan®, 2016; Whitehouse-Tedd *et al.*, 2015). The number above the slice indicates the proportion of total faecal samples collected for each score.

The temporal profile of FCS pre- and post-operatively is shown in Figure S.2.3ii.

S.2.3.3 Body Temperature, Heart Rate, and Locomotor Activity Recordings

Complete T_b, HR, and LA data sets were acquired for all the study cheetahs (CH-2012, -2014, -2205, -2206, -2207, -2271, -2276, and -2277). The initial examination of the raw HR data revealed values ranging from 0 to 1005 bpm, the extremes of which were likely because

of incomplete, low-quality readings or implant movement within the pectoral muscle when the study cheetahs were active. To remove erroneous measurements, ensuring only plausible values were included in the analyses, upper and lower thresholds were created (see section 1.1.6.9.3 for more information on the data preparation of T_b, HR, and LA recordings). Once filtered, all HR recordings (n = 21174/23040, which represented over 90% of raw data initially obtained from the loggers) fell within the 30- and 200-bpm thresholds set.

The temporal profiles of T_b, HR, and LA pre- and post-operatively are shown in Figure S.2.5.

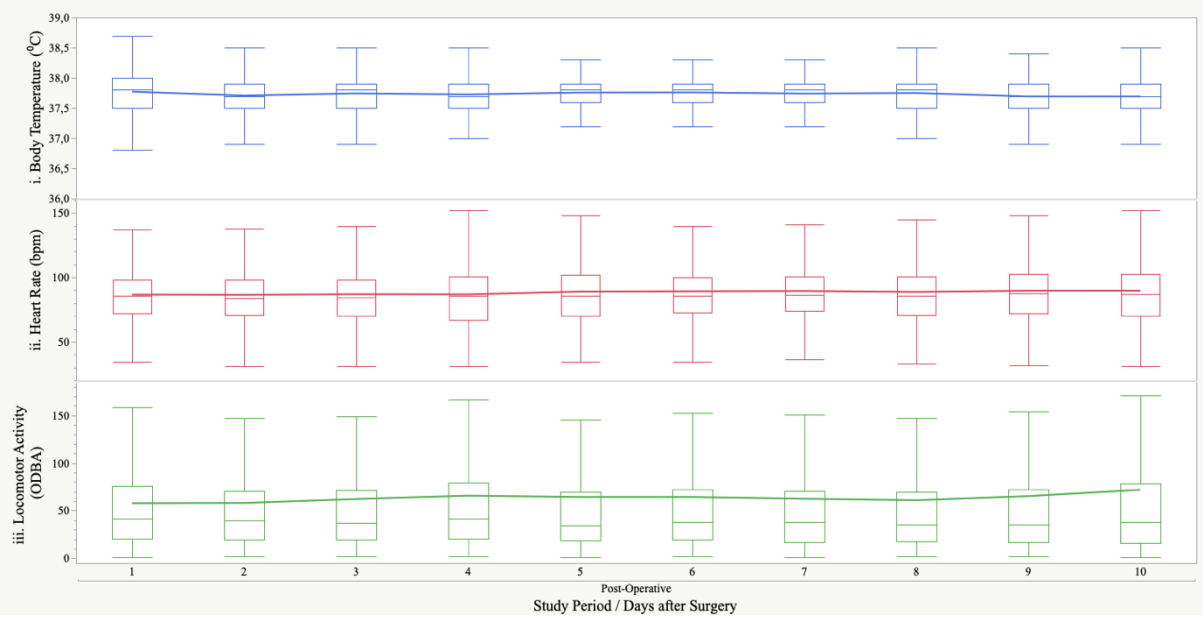

Figure S.2.5 Box and whisker plot with mean trendline of (i) body temperature (^{0}C), (ii) heart rate (beats per minute [bpm]), and (iii) locomotor activity (overall dynamic body acceleration [ODBA]) for the study cheetahs (CH-2012, -2014, -2205, -2206, -2207, -2271, -2276, and -2277). Temporal effect of surgery during the post-operative period.

S.2.4 Discussion

Following surgery, central stimulation is triggered by afferent nerve impulses arising at the injury site, activating the two branches of the neuroendocrine stress system, the SNS and HPA

axis (Kisani *et al.*, 2018; see section 1.1.2 for more information on the adaptive stress response). Both branches originate in the hypothalamus and converge on the adrenal gland, working together to maintain or re-establish homeostasis peri-operatively by orchestrating behavioural and physiological adaptations (Coffman, 2020). The SNS provides the immediate first wave of the stress response, mediating the rapid release of the catecholamine hormones E and NE from the adrenal medulla (Romero & Wingfield, 2015). The second wave is more gradual and involves the GCs, the end-products of the hormonal cascade along the HPA axis (Sapolsky *et al.*, 2000). Here, the surgical implantation of biologgers elicited in the study cheetahs significantly higher fGCM concentration. The results of this analysis support the researcher's hypothesis that minimally invasive surgery would induce a physiological stress response.

Immediately after surgery, circulating levels of ACTH and GC are reported to increase (Walker *et al.*, 2010). An apparent failure of negative feedback loops to downregulate further ACTH secretion intra- and post-operatively (Desborough, 2000; Finnerty *et al.*, 2013) results in prolonged elevation of GCs for up to seven days (Prete *et al.*, 2018). Surgery and anaesthesia have been found to increase plasma and serum cortisol concentrations in dogs (Church *et al.*, 1994; Fox *et al.*, 1994), cats (Smith *et al.*, 1999; 1996), horses (Taylor, 1989), sheep and goats (Fattah *et al.*, 2013), and rhesus monkeys (Puri *et al.*, 1981).

The demographic factors, sex and age group, did not affect the study cheetahs' responses to the surgical implantation of biologgers. Surgery likewise induced an adrenocortical response in all of the study cheetahs.

Anaesthesia can affect or modulate the stress response via afferent blockade, central modulation, and peripheral interaction with the endocrine system (Kahveci *et al.*, 2014). The parameters measured appeared to have a dampened initial response post-operatively, possibly

explained by anaesthetic intervention. Faecal glucocorticoid metabolite concentration and T_b and HR (salient features of the SNS-mediated reaction to stress) were numerically higher on post-operative days five, six, and seven, respectively. At the same time, FCS and LA were numerically lower on post-operative days seven and eight, respectively. Symbiotic gut microbes harboured in animals' GI tracts serve various essential functions (Wasimuddin *et al.*, 2017). However, disturbance-related deviation in the microbial diversity and abundance pattern beyond a natural range, i.e., gut dysbiosis, can advance pathophysiology and affect host health (Shreiner *et al.*, 2015). Certain circumstances and events during the peri-operative period can alter the gut microbiota, including stress (Accarie & Vanuytsel, 2020; Farzi *et al.*, 2018), anaesthesia (Serbanescu *et al.*, 2019), and surgery (Lederer *et al.*, 2017). Faecal consistency is purportedly linked to intestinal microbiota composition (Tigchelaar *et al.*, 2016; Vandeputte *et al.*, 2016) and could indicate GI health (Whitehouse-Tedd *et al.*, 2015).

After that, the parameters began stabilising with fGCM concentration approaching baseline and FCS returning to pre-operative values. The similar temporal profiles of HR and LA on post-operative days nine and 10 suggest that the increasing HR, as it corresponded with increasing LA, could have been caused by the demands of increased physical activity. During physical activity, requirements for oxygen and gluconeogenetic substrates in skeletal muscle are increased, as are the removal of metabolites and carbon dioxide (Burton *et al.*, 2004). The cardiac output is increased to meet the demand for blood flow by contracting muscles, attributed to sympathetically-mediated increases in HR and stroke volume.

Though to be considered preliminary and requiring further scientific attention, this pilot study's findings provide information for the refinement of procedures involved in applying biologging technology to cheetahs (e.g., anaesthesia-surgical protocols, implantation site, and other factors related to the device itself). That the parameters demonstrated, to some extent,

temporal recovery by post-operative day 10, I felt it appropriate to commence the experimental trials of the main study 12 to 13 days after surgery. Furthermore, I demonstrated that biologging technology could be used to record concurrent T_b, HR, and LA and, in addition to behavioural and physiological metrics, has tremendous potential as a tool to measure stress in the cheetah.

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