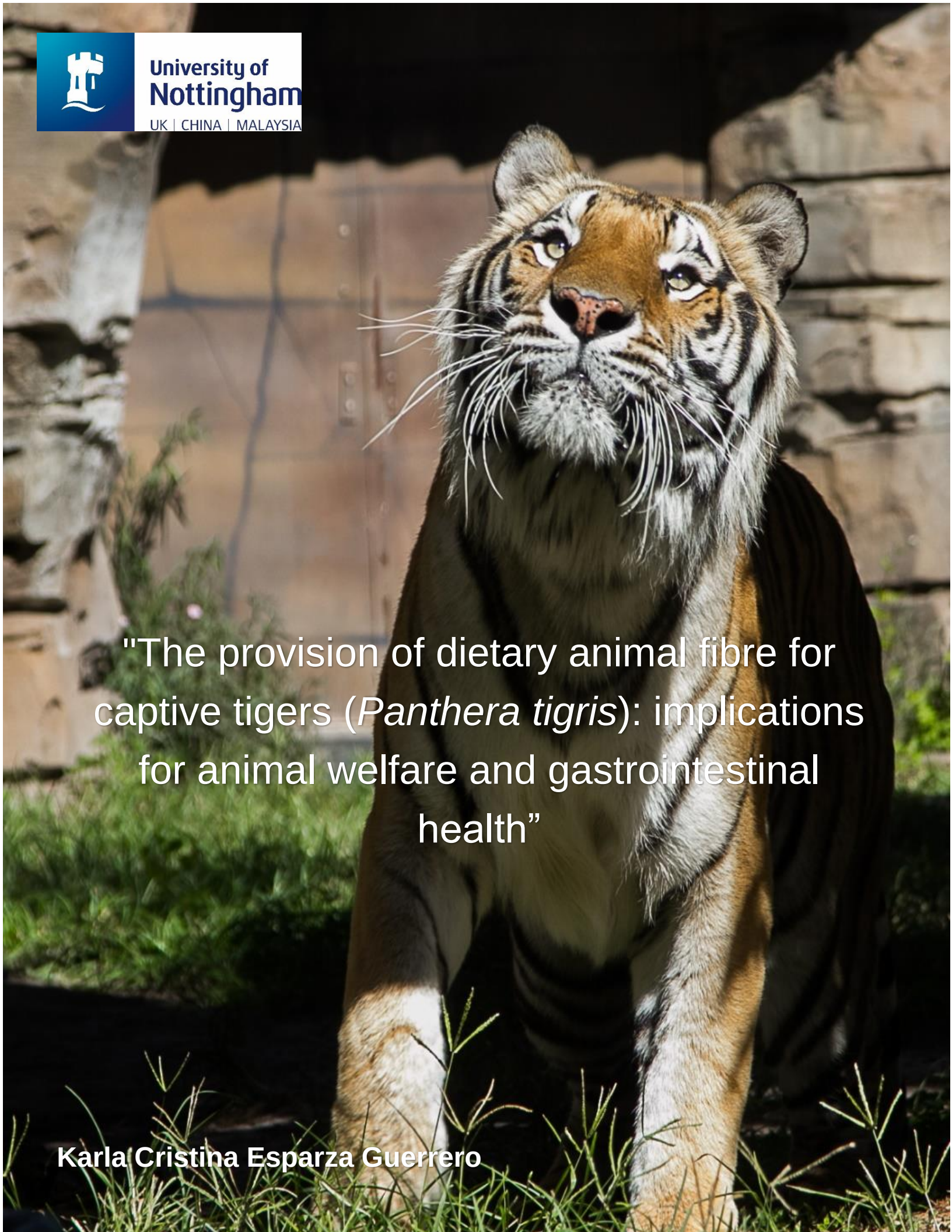

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"The provision of dietary animal fibre for captive tigers (*Panthera tigris*): implications for animal welfare and gastrointestinal health"

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Karla Cristina Esparza Guerrero, DVM, MSc

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

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16/December/2020

“Do not cut down the forest with its tigers and do not banish the tigers from the forest. The tiger perishes without the forest, and the forest perishes without its tigers. Therefore the tigers should stand guard over the forest and the forest should protect all its tigers.”

Mahabharata, circa 400 B.C.

“Iba y venía, delicado y fatal, cargado de infinita energía, del otro lado de los firmes barrotes y todos lo mirábamos. Era el tigre de esa mañana, en Palermo, y el tigre del Oriente y el tigre de Blake y de Hugo y Shere Khan, y los tigres que fueron y que serán y asimismo el tigre arquetipo, ya que el individuo, en su caso, es toda la especie. Pensamos que era sanguinario y hermoso. Norah, una niña, dijo: Está hecho para el amor”.

Jorge Luis Borges, El Tigre

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Chapter 3

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I would like to thank Drs Naomi Harvey and Giuliana Miguel for their help during the design phase of the behavioural data collection. Watching tiger videos might not sound like hard work but going over thousands of hours of video footage would not have been possible without the help of Natasha Clark, Leonie Dommett (both undergraduate students at the University of Nottingham) and Dr Ellen Williams (Nottingham Trent University, and who also explained the set-up for one of the recording systems used in this project).

Chapter 5

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List of abbreviations

A/P	acetate/propionate
ADF	acid detergent fibre
AZA	Association of Zoos and Aquariums
BCa	bias-corrected and bootstrapped
BCFA	branched-chain fatty acids
BD	baseline diet
BW	body weight
CD	control diet
CF	crude fibre
CI	confidence interval
CP	crude protein
DM	dry matter
ED	experimental diet
FAO	Food and Agriculture Organization
FCCM	faecal corticosterone metabolites
FCM	faecal cortisol metabolites
FGMs	faecal glucocorticoid metabolites
GC-MS	gas chromatography-mass spectrometry
GE	gross energy
GI	gastrointestinal
GIT	gastrointestinal tract
GLM	general linear model
HCD	histidine-containing dipeptide
IBD	inflammatory bowel disease
IOM	Institute of Medicine
IQR	interquartile range

IUCN	International Union for Conservation of Nature
Md	median
ME	metabolizable energy
NAG	Nutritional Advisory Group
NDF	neutral detergent fibre
NMH	N-methylhistamine
O/E	observed/expected
PFP	pentafluoropropionyl
QoL	quality of life
RO	reverse osmosis
SCFA	short-chain fatty acids
SI	small intestine
TAG	Taxon Advisory Group
TFA	time of first appearance

Abstract

Previous research has demonstrated that poorly digestible components of whole prey such as tendons, ligaments, fur, and skin (i.e. animal fibre) can positively influence the health and welfare of strict carnivores. Through non-invasive methods, the impact of dietary animal fibre was assessed in captive tigers using two common North American diets: (1) 100% commercial raw horsemeat, compared with (2) the same raw horsemeat (80%) with added whole prey (20%). A randomised crossover study was performed over 8-week periods with eight animals. Faecal consistency, pH, fermentation profiles (short-chain fatty acids and end-product concentrations), time of first appearance, and total tract apparent macronutrient digestibility were employed as gastrointestinal (GI) functional parameters. Two faecal inflammatory biomarkers, N-methylhistamine and S100A12, were measured as non-invasive GI health indicators. Finally, behavioural time budget and faecal glucocorticoid metabolites were evaluated as part of the welfare assessment.

An inclusion rate of 20% whole prey was insufficient to elicit any changes in the parameters measured. No significant differences in GI functional or health parameters or welfare indicators were detectable between dietary conditions. One exception was the faecal consistency score; tigers fed the diet with added whole prey exhibited significantly lower values. However, the mean scores for the tigers on each of the two diets were considered ideal for the species. Given the lack of impact seen in the suite of GI parameters, this suggests that the difference was of limited biological importance and highlights the need to use a panel of measures when evaluating dietary interventions.

As part of the holistic assessment approach of this study, behavioural and physiological welfare indicators were used to investigate dietary impacts beyond the GI tract. Aligning with the GI findings, both welfare indicators similarly supported a lack of dietary effect.

Diets incorporating ≤ 20% whole prey are unlikely to promote any of the previously reported benefits as seen in other felid species fed higher fibre concentrations. Future research should evaluate a wider range of inclusion rates, different types of whole prey and/or fibre sources. The results from this study clearly demonstrate the importance of using multiple integrated indicators rather than single isolated parameters to ensure a comprehensive evaluation of the dietary impact on animal health and welfare.

Chapter 1. Introduction

1.1 Tigers' biology

1.1.1 Taxonomy, distribution, and population status in-situ

Tigers (*Panthera tigris*) are the largest members of the Felidae family, which includes 37 extant species (Mazák 1981; Tilson and Nyhus 2010; Kitchener et al. 2017). Tigers are classified in the genus Panthera, along with lions (*Panthera leo*), jaguars (*Panthera onca*), leopards (*Panthera pardus*) and snow leopards (*Panthera uncia*) (Goodrich et al. 2015; Kitchener et al. 2017). Based on geographical isolation, morphological characteristics and population genetic structure, investigators have recognised the existence of six subspecies of tiger: Amur or Siberian tiger (*P. t. altaica*), Northern Indochinese tiger (*P. t. corbetti*), South Chinese tiger (*P. t. amoyensis*), Malayan tiger (*P. t. jacksoni*), Sumatran tiger (*P. t. sumatrae*) and Bengal tiger (*P. t. tigris*) (Luo et al. 2004; Goodrich et al. 2015).

Historical distribution of tigers extended from Turkey, across Asia up to Russia, and southward to the island of Bali in Indonesia (Dinerstein et al. 2007; Goodrich et al. 2015). At present, wild tigers occupy only 7% of their historical geographic range (see Figure 1.1) with wild breeding populations remaining only in Bangladesh, Bhutan, India, Indonesia, Malaysia, Nepal, Russia and Thailand (Goodrich *et al.,* 2015).

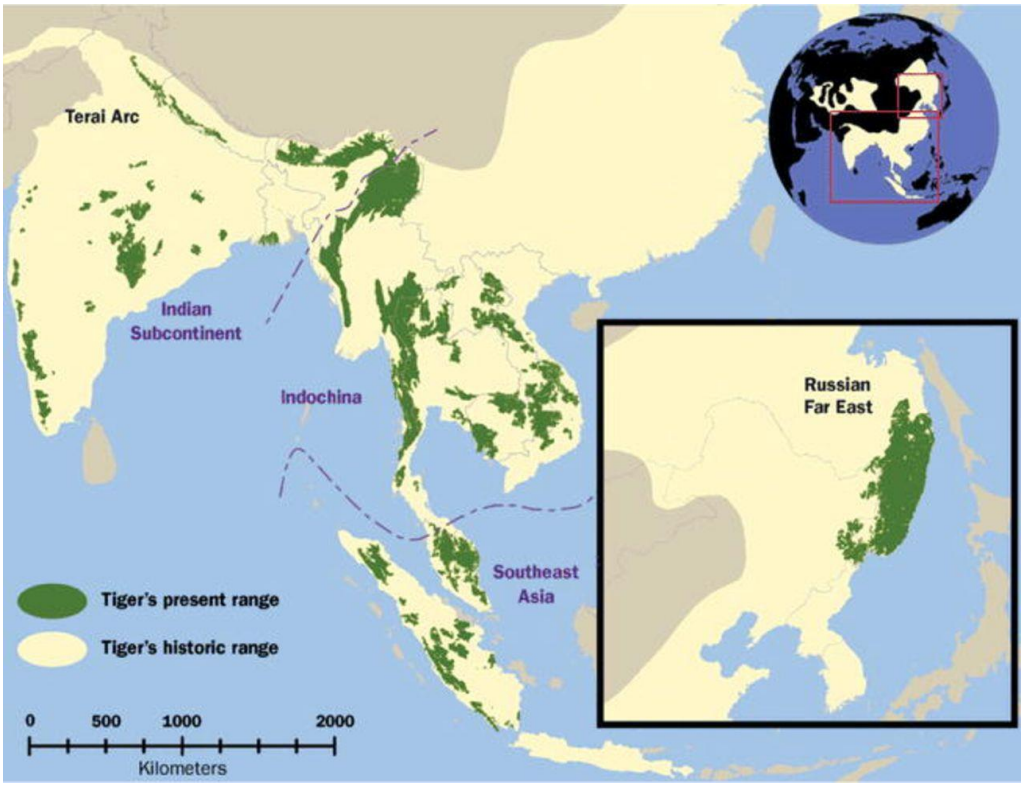

Figure 1.1 Present and historic (circa 1850) distribution of tigers (from Dinerstein *et al.*, 2007; p. 509).

The global tiger population in the early 1900s was estimated at over 100,000 individuals, decreasing to < 7,000 in 1998 (Luo *et al.,* 2004) and with a further drop to ~ 3,500 by 2014 (Goodrich *et al.,* 2015). Based on the International Union for Conservation of Nature (IUCN) criteria, which considers breeding range, total population and number of mature individuals *in situ*, tigers are classified as an endangered species (Goodrich *et al.,* 2015). Among the main causes of population's decline are prey depletion, habitat loss and the persistent trade of tiger parts for traditional medicines, clothing and decorative purposes (Dinerstein et al. 2007; Goodrich et al. 2015; Robinson et al. 2015). A summary of tigers' subspecies, distribution, and estimated population in the wild can be found in Table 1.1.

Table 1.1 Tiger subspecies, distribution and population *in situ.*

Scientific name	Common name	Geographic distribution	Estimated population (No. tigers)	References
Panthera tigris altaica	Amur or Siberian Tiger	Siberia, Northeast China	< 360	(Miquelle et al. 2011)
Panthera tigris corbetti	Northern Indochinese Tiger	Cambodia, Laos, Myanmar, Thailand, Vietnam, Southwest China	< 350	(Lynam and Nowell 2011)
Panthera tigris jacksoni	Malayan Tiger	Peninsular Malaysia	< 350	(Kawanishi 2015)
Panthera tigris sumatrae	Sumatran Tiger	Sumatra	< 500	(Linkie et al. 2008)
Panthera tigris tigris	Bengal Tiger	Bangladesh, Bhutan, India, Myanmar and Nepal	< 2,000	(Chundawat et al. 2011)
Panthera tigris amoyensis	South China Tiger	China	No official records since 1970, possibly extinct	(Nyhus 2008)

1.1.2 Feeding ecology

Tigers spend a considerable part of their active time travelling to defend their territory, locate prey or in search of potential reproductive mates (Kerley et al. 2003; Tilson and Nyhus 2010; Biolatti et al. 2016). In Siberia, tigers can roam 5-30 km/day on average (Szokalski et al. 2012; Breton and Barrot 2014), while in South India, where prey tend to be more abundant, tigers cover an average of 3 km/day (Tilson and Nyhus 2010). Like other felids, tigers are excellent hunters, adapting their technique to prey availability, skills and experience (Karanth and Sunquist 2000; Tilson and Nyhus 2010; Plantinga et al. 2011). Tigers tend to synchronise their activity

patterns with those of their prey and are classified as crepuscular and nocturnal hunters (Bashaw et al. 2003; Szokalski et al. 2012; Naha et al. 2016).

The main components of a tiger's diet depend on geographical location. Common prey species reported include wild boar (*Sus scofra*), chital (*Axis axis*), sambar (*Rusa unicolor*), sika deer (*Cervus nippon*), red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), muntjac (*Muntiacus* spp.), nilgai (*Boselaphus tragocamelus*) and common langur (*Presbytis entellus*) (Karanth and Sunquist 2000; Bagchi et al. 2003; Reddy et al. 2004; Hayward et al. 2012; Miller et al. 2013b, 2014; Fàbregas et al. 2017). Regardless of the geographical region, tigers seem to prefer medium to large prey size (i.e. body weight -BW- ranging between 60 and 250 kg) (Reddy et al. 2004; Tilson and Nyhus 2010; Miller et al. 2013b). However, if necessary, they can adapt to smaller prey such as hares (*Lepus nigricollis ruficaudatu*) (<5 kg BW) (Reddy et al. 2004; Hayward et al. 2012).

Tigers are known to consume most parts of a prey item, starting usually on the rump, after which the abdominal cavity is opened and the digestive system of the prey is removed before other internal organs are consumed (Tilson and Nyhus 2010; Miller et al. 2013b; Fàbregas et al. 2017). Small prey is generally eaten during a single meal, while larger prey could take up to a week before the carcass is abandoned (Mazák 1981; Tilson and Nyhus 2010). Anecdotal observations described tigers consuming up to 40 kg of food in a single day (Mazák 1981; Tilson and Nyhus 2010). However, considering prey availability and the interval between kills, it has been estimated that free-ranging Amur tigers can eat an average of 6 kg of prey/day (Miller et al. 2013b). Tilson and Nyhus (2010) suggested that, in order to survive, a single adult tiger in India would need to kill 40-50 medium to large prey every year and that a female rearing cubs needed between 60-70 kills per year. However, in Russia, the number of medium

to large prey per year was estimated by Miller *et al.* (2014) to be 25 items for adult male tigers, 18 for non-reproducing females, and 54 for females raising cubs.

1.1.3 Digestive system: anatomy and physiology

Although the anatomy and physiology of the gastrointestinal tract (GIT) of the domestic cat (*Felis catus*) have been thoroughly investigated, the equivalent characterisation of the GIT of non-domestic felids is limited. Studies of non-domestic species have focused mainly on the intestinal length and weight (Davis 1962; Stevens and Hume 1996; O'Regan and Kitchener 2005; Lamberski 2015; McGrosky et al. 2016).

The tigers' dental formula corresponds to 3/3 (incisors), 1/1 (canines), 3/2 (premolars) and 1/1 (molars), with a total of 30 teeth (Mazák 1981; Lamberski 2015; Kapoor et al. 2016). Large felids evolved to manipulate structurally complex diets and each tooth has a determined function (Hartstone-Rose et al. 2014; Kapoor et al. 2016). For example, small incisors nip the tissues from the carcass, while premolars slice meat and the long canines are used to capture and kill prey (Mazák 1981; Lamberski 2015; Kapoor et al. 2016). Relative to muscle mass, felids have the second most powerful bite among mammals, just below mustelids (Haberstroh et al. 1984; Hartstone-Rose et al. 2014; Lamberski 2015).

Another characteristic of felids is their inability to detect sweet-tasting compounds (Coradini et al. 2015; Furness et al. 2015), due to the absence of the taste bud receptor expressed by the gene Tas1r2 (Li et al. 2005). While felids do not appear to be attracted by sweet foods (Morris 2002; Zoran 2002; Plantinga et al. 2011), it is believed that they could distinguish between "sweet" and "bitter" amino acids as a possible adaptation to discriminate meat quality (Beauchamp et al. 1977; Bradshaw et al. 1996).

As seen in other members of the order Carnivora, tigers have a non-compartmentalized stomach, short small intestine (SI), rudimentary caecum and a non-sacculated short colon (see Figure 1.2) (Zhang et al. 2012; McGrosky et al. 2016; Tilson et al. 2016). The relatively large stomach of tigers facilitates gorging– a characteristic behaviour of big felids (Altman et al. 2005; Tilson et al. 2016). The colon of felids is smaller and shorter compared to that of omnivores and herbivores, which is characteristic of species that do not consume plant material as part of their diets (Stevens and Hume 1996; McGrosky et al. 2016; Weber et al. 2016). However, tiger intestines appear to be relatively short compared to the domestic cat (Stevens and Hume 1996; Furness et al. 2015). According to Davis (1962), intestinal length does not seem to have a consistent allometric relation to BW. Kitchener (1998) hypothesized that domestic cats have a longer GIT compared to other felid species as an adaptation to higher carbohydrate intake associated with manufactured diets (e.g. kibble or extruded commercial diets). Nevertheless, further research is needed to determine the link between GIT length and diet (O'Regan and Kitchener 2005).

Figure 1.2 Digestive system of a tiger (*Panthera tigris*) (photograph by McGrosky *et al*., 2016; p. 399).

Members of the Felidae family are considered obligate carnivores that rely primarily on animal tissues to meet their nutritional requirements (Mazák 1981; Karanth and Sunquist 2000; Morris 2002; Zoran 2002; Kim et al. 2016). Felids have evolved to cope with a strictly carnivorous diet through a series of physiological adaptations, such as the reduction of unnecessary enzymes, changes in enzymatic activity and a particular carbohydrate metabolism (a summary of which can be found in Figure 1.3). For example, the activity of salivary, pancreatic and intestinal amylase, as well as intestinal disaccharidase, is marginal in felids; therefore, breakdown of simple sugars is not as efficient as compared to other species (McGeachin and Akin 1979; Zoran 2002; Hewson-Hughes et al. 2011; McDonald et al. 2011; Furness et al. 2015; Kim et al. 2016). The high activities of hepatic enzymes, including fructose-1,6-biphosphatase, glucose-6-phosphatase and pyruvate carboxylase, suggest that glucose synthesis in the liver occurs via gluconeogenesis (Washizu et al. 1999; Verbrugghe et al. 2010;

Hewson-Hughes et al. 2011; Villaverde and Fascetti 2014). In addition, the lack of hepatic glucokinase activity and the decreased activity of glycogen synthetase have been associated with lower glycolytic capacity in comparison to domestic dogs (*Canis lupus familiaris*) or other more omnivorous species (Washizu et al. 1999; Zoran 2002). Reduced insulin secretion was observed in cats compared to dogs when both were fed a high carbohydrate diet (51% of daily metabolizable energy, ME), hence suggesting a reduced capacity to metabolise elevated carbohydrate loads (Hewson-Hughes et al. 2011; Coradini et al. 2015). The overall activity of enzymes involved in glycolysis and gluconeogenic pathways in cats are considered a metabolic adaptation to a strict carnivore diet by promoting the use of amino acids and fat rather than carbohydrates as primary sources of energy (Verbrugghe et al. 2010, 2012; Hewson-Hughes et al. 2011; Coradini et al. 2015). Further research is still needed to fully understand carbohydrate metabolism and elucidate if such adaptations are shared by all members of the Felidae family.

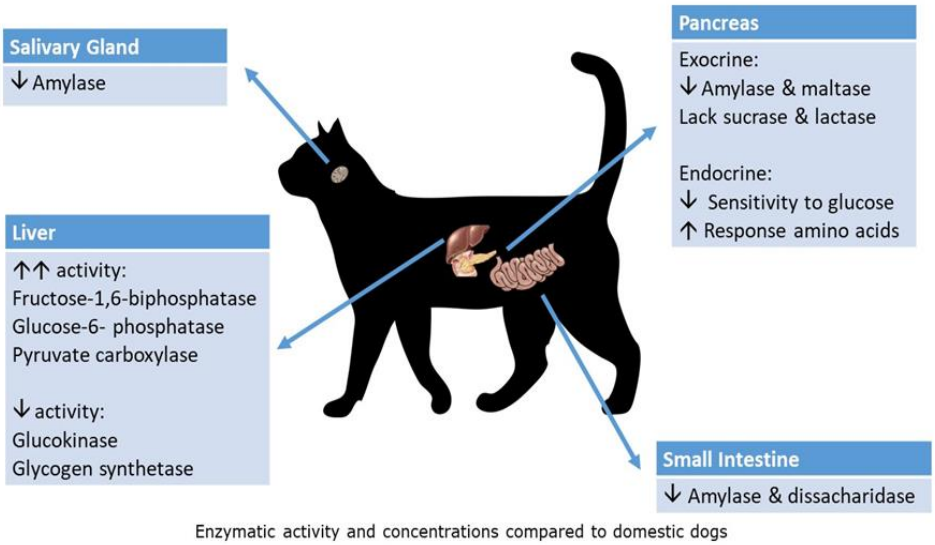

Figure 1.3 Physiological adaptations of domestic cats (adapted from Verbrugghe *et al.,* 2012).

1.2 Tigers in captivity

1.2.1 Ex-situ population

Display of non-domestic animals, including tigers, has taken place for centuries, evolving from ancient menageries to the modern zoological collections now found around the world (Tilson and Nyhus 2010; Kawata 2013; Lyles and Wharton 2013; Brown 2014). Over recent decades, zoos have focused on performing roles as education centres and promoters of conservation (Kohn 1994; Lyles and Wharton 2013; Zoological Society of London 2016). In an effort to fully address the well-being of the animals under their care, zoos have undertaken major changes to integrate different disciplines into their management practices, such as animal husbandry, nutrition and behaviour, to achieve the highest possible level of care (Kohn 1994; Robinson 1997; Lyles and Wharton 2013; Blackett et al. 2016).

Studies have estimated the total population of tigers under human care to be between 13,000 and 20,000 individuals worldwide, with China (>5,200) and the United States (~5,000) holding the largest number of individuals (Nowell and Xu 2007; Lovely 2009; Tilson and Nyhus 2010). However, data were obtained from unverifiable sources; therefore, information must be taken cautiously since figures might be overestimated or completely unrealistic. Using specialized zoological management software like Species360©, a *Panthera tigris* holding report obtained in May 2019 showed that a total of 1,856 individuals were kept in 466 zoological institutions worldwide (Species360© 2019). Europe had the largest population of tigers, with 698 individuals, followed by Asia with 588 and North America with 422, while Australia (67), South America (43) and Africa (38) reported the lowest populations. Yet, the data provided by Species360© represent an underestimation of the current captive tiger

population, since the information does not account for facilities that are not subscribed to the software. To date, the total population of captive tigers remains unknown since information on privately owned, non-accredited zoos and roadside shows worldwide is unavailable (Tilson and Nyhus, 2010).

Nevertheless, captive population of tigers might play a vital role in the future survival of the species. Through breeding programmes, public education and research, zoological collections have highlighted the role of captive populations in wildlife conservation (Lovely 2009; Sajjad et al. 2011; Blackett et al. 2016). Zoo-housed tigers could act as a population source for future reintroductions and already provide scientific information for field research (e.g. anaesthesia protocols) (Kohn 1994; Tilson et al. 1997). Additionally, captive tigers could support conservation of their wild conspecifics through public awareness, inspiration, and fundraising for *in situ* conservation programmes, with some evidence of this occurring at present (Tilson and Nyhus 2010; Mellor et al. 2015; Animal Welfare Committee 2019).

1.2.2 Dietary management

1.2.2.1 Nutritional requirements

The nutritional requirements for tigers have not yet been documented. Instead, the domestic cat is commonly used as the model species for estimating the nutritional requirements of non-domestic felids such as tigers (Vester et al. 2010b, a; Tilson et al. 2016). Nutritional idiosyncrasies of captive (pet) and free-ranging (feral) domestic cats that are likely to apply to all felids have been documented in several reviews (Bradshaw et al. 1996; Morris 2002; Zoran 2002; Hewson-Hughes et al. 2011; Plantinga et al. 2011). Studies assessing the macronutrient composition of diets

selected by laboratory and feral cats reported low carbohydrate content (2-12%), high protein levels (>52%) and variable fat content (36-46%) (Hewson-Hughes et al. 2011; Plantinga et al. 2011; Villaverde and Fascetti 2014). This feeding strategy aligns with the unique characteristics reported for domestic cats, including the need for high protein and fat diets, compared to other non-felid carnivores, along with the need for dietary provision of particular amino acids (e.g. taurine and arginine), arachidonic acid, active forms of vitamins A, D, and niacin (Dierenfeld et al. 1994; Morris 2002; Zoran 2002; Villaverde and Fascetti 2014; Tilson et al. 2016).

Cats' increased dietary protein requirements may be explained by their limited downregulation of amino acid catabolism (e.g. aminotransferases and urea cycle enzymes). This results in a constant amino acid break down and therefore a higher N loss rate compared to omnivorous species (Russell et al. 2000, 2002, 2003; Morris 2002; Plantinga et al. 2011; Villaverde and Fascetti 2014). In addition, a high protein requirement in cats could be the result of using amino acids for gluconeogenesis to supply energy to glucose-requiring tissues such as the brain (Eisert 2011; Rochus et al. 2013; Villaverde and Fascetti 2014), unlike other species in which carbohydrates are the main energy suppliers for such tissues. In contrast to the ten essential amino acids required by dogs, cats are known to require eleven: methionine, lysine, threonine, tryptophan, histidine, leucine, isoleucine, valine, arginine, phenylalanine, and taurine (National Research Council 2006; Villaverde and Fascetti 2014). The necessity for higher intake of arginine and taurine in felids, compared to other carnivores, could be explained by a marginal activity of the enzymes responsible for the synthesis of those amino acids and a high use as bile conjugate (Salter et al. 1999; Morris 2002; Zoran 2002).

Pre-formed or active forms of vitamins A and D are considered dietary essentials for cats since they have reduced capacity to synthesise retinol (vitamin A) and calcitriol (vitamin D) from their respective precursors

carotenoids and 7-dehydrocholesterol (How et al. 1994; Zoran 2002; Crissey et al. 2003). The insufficient endogenous synthesis of these two vitamins has been proposed as an adaptation to their natural diet, which would typically already contain high levels of these vitamins (Gershoff et al. 1957; Zoran 2002; O'Regan and Kitchener 2005). In a similar way, niacin is present in animal tissues, therefore cats eating whole prey or meat-based diets may not have the evolutionary pressures for the synthesis of this vitamin from dietary precursors. The supplementation of these vitamins has, therefore, been recommended for non-domestic felids in captivity that do not consume whole prey as part of their diet (Dierenfeld et al. 1994; Tilson et al. 2016). Similarly, arachidonic acid is considered an essential fatty acid in cats' diets due to the lower activity of enzymes responsible for the conversion of linoleic acid into arachidonic acid (δ-6 and δ-8 desaturase) compared to dogs (Salter et al. 1999; Morris 2002; National Research Council 2006; Villaverde and Fascetti 2014).

Despite domestic cats having been used as models to establish nutritional requirements for non-domestic felids (Vester et al. 2010a; Tilson et al. 2016), recent investigations have revealed physiological or functional differences across felid species. For example, Vester *et al*. (2010) found that apparent nutrient digestibility of CP, fat and energy was significantly lower for tigers and jaguars (*Panthera onca*) compared to domestic cats consuming the same raw meat diet. They also reported that relative daily food intake was higher in tigers (33.5 g/d Dry Matter–DM–/kg $BW^{0.75}$) compared to domestic cats (22.4 g/d DM/kg $BW^{0.75}$) (Vester *et al.* 2010). These results are similar to the findings of Kerr *et al.* (2013), who reported that tigers had a significantly higher food intake (33.2 g/d DM/kg $BW^{0.75}$) compared to domestic cats (15.9 g/d DM/kg $BW^{0.75}$), jaguars (26.3 g/d DM/kg $BW^{0.75}$) and African wildcats (*Felis silvestris lybica*) (22.1 g/d DM/kg $BW^{0.75}$) consuming a raw horsemeat diet. Likewise, the apparent digestibility of CP, fat and energy was significantly lower in tigers than in the other species. Both studies concluded that tigers would require either a

higher caloric intake or more digestible diets to maintain their BW compared to domestic cats.

Although both domestic cats and tigers are excellent hunters who prey mainly on mammals, differences in feeding ecology between species are evident. For example, domestic cats tend to kill prey which are small compared to a cat's BW (e.g. mice, rats, moles) (Bradshaw et al. 1996; Plantinga et al. 2011), while tigers are believed to prefer preys equivalent to their BW (e.g. sambar deer, red deer, wild boar) resulting in a prey:predator ratio of almost 1:1 (Bagchi et al. 2003; Reddy et al. 2004; Hayward et al. 2012). Another discrepancy between these species corresponds to hunting frequency. Researchers have estimated that, in order to survive, tigers need between 25-50 medium to large kills/year (Tilson and Nyhus, 2010; Miller *et al.*, 2014). The comparatively small prey size of domestic cats requires them to hunt and kill several preys each day to fulfil their energy requirements (Bradshaw et al. 1996; Hewson-Hughes et al. 2011). Finally, domestic cats ingest their preys entirely within a single meal, while tigers are known to discard the GIT of prey and, depending on prey size, it can take them several days to fully consume it (Mazák 1981; Bradshaw et al. 1996; Plantinga et al. 2011; Fàbregas et al. 2017). When formulating diets for captive tigers, such differences across species should be considered. To date, no evidence exists to suggest that tigers' metabolic adaptations are different from those described in domestic cats. As such, the latter continues to be the only available model for tigers' nutritional guidelines. However, tigers should not be considered as large domestic cats; further research is needed to elucidate if other dietary differences across felid species occur. (Salter et al. 1999; Vester et al. 2010a; Kerr et al. 2013a).

1.2.2.2 Feeding practices

Diets and feeding patterns of free-ranging felids are challenging to replicate in captivity (Altman et al. 2005). Furthermore, little information is available about wild prey nutrient composition or energy requirements of free-ranging tigers; therefore, nutrient guidelines for tigers continue to be extrapolated from nutritional requirements of domestic cats (Dierenfeld et al. 1994; Salter et al. 1999; National Research Council 2006; Tilson et al. 2016). However, captive diets must not focus exclusively on fulfilling the nutritional needs of animals; ideally, they should also ensure that natural feeding behaviours are elicited (Dierenfeld et al. 1994; Van Valkenburgh 1996; Altman et al. 2005; Gaengler and Clum 2015; Fàbregas et al. 2017).

An epidemiological survey on the captive tiger population revealed that North American facilities commonly fed commercially manufactured raw meat as the main component of the diet (Lefebvre et al. 2020). Similarly, previous studies with captive tigers reported that animals were fed with ground/chunk meat-based products (Salter et al. 1999; Crissey et al. 2003; Vester et al. 2010a; Kerr et al. 2013a; Iske et al. 2016). Carcass or small whole prey are used as part of enrichment programmes or during fasting days (Dierenfeld et al. 1994, 2002; Mcphee 2002; Crissey et al. 2003). The main protein sources include beef, horse and, more recently, pork (Salter et al. 1999; Crissey et al. 2003; Vester et al. 2010a; Iske et al. 2016). In China, Gu *et al.* (2016) indicated that the diet of captive tigers consisted of beef, chicken and pig meat; while He *et al.* (2018) reported duck as the only component of the diet in a different facility in China. By contrast, Mishra *et al.* (2013) and Mohapatra *et al.* (2014) reported buffalo meat as the main component of the diet in an Indian facility, where chicken was used only for enrichment. Hartstone-Rose *et al.* (2014) mentioned that some European collections practised carcass feeding, without specifying the prey species used. Regional differences in the type of diet provided to captive tigers may be explained by economic factors, item availability,

health-related concerns, cultural or historical use of ingredients, as well as local husbandry practices (Dierenfeld et al. 1994; Salter et al. 1999; Hartstone-Rose et al. 2014; Whitehouse-Tedd et al. 2015).

The non-nutritive properties of a diet, such as consistency, texture, palatability and temperature might be beneficial for the oral health and the behaviour of felids and should also be considered when formulating a diet (Haberstroh et al. 1984; Duckler and Binder 1997; Altman et al. 2005; Plantinga et al. 2011). A growing number of zoological collections are becoming more aware of the non-nutritive properties of food and of the potential benefits of feeding whole prey or carcass on behaviour, gastrointestinal (GI) health and welfare of carnivorous species (Dierenfeld et al. 2002; Depauw et al. 2011; Plantinga et al. 2011; Hartstone-Rose et al. 2014; Kerr et al. 2014b; Kapoor et al. 2016). To ensure the safe and effective employment of whole prey feeding, the Nutritional Advisory Group (NAG) of the Association of Zoos and Aquariums (AZA) has provided a series of guidelines to regulate such practice (NAG 2020). Fibre has not been considered an important nutrient for carnivores -including felids- and no official intake recommendations currently exist (National Research Council 2006; Tilson et al. 2016). However, evidence has suggested that fibre might play an important role in GIT function and overall health of non-domestic felids (Vester et al. 2010a; Depauw et al. 2011; Kerr et al. 2013a). The beneficial aspects of dietary fibre will be discussed in section 1.3.3.

Commercial nutritionally complete zoo diets are less time-consuming to prepare and are formulated to fulfil the nutritional requirements of domestic cats (Dierenfeld et al. 1994; Salter et al. 1999; National Research Council 2006). While they can be used to supply essential nutrients, these diets do not simulate the physical challenges of consuming whole prey, thus questioning their benefit for oral and behavioural health in the long term (Bond and Lindburg 1990; Dierenfeld et al. 1994; Salter et al. 1999;

Hartstone-Rose et al. 2014; Gaengler and Clum 2015; Kapoor et al. 2016). Unfortunately, for most North American zoological facilities, the provision of whole prey on a regular basis is still limited and the use of commercially available diets continues to be the basis of large felids diets in this region (Iske et al. 2016; Lefebvre et al. 2020).

In an attempt to promote natural feeding behaviours in captive tigers, zoological collections have employed dietary items as a source of enrichment. For example, it is common practice for some institutions to hide part (or the full extent) of the diet, to elicit foraging behaviours (Van Valkenburgh 1996; Szokalski et al. 2012) or to provide tigers with novel or complex food items, such as bones or whole prey, to increase the variety of feeding behaviours in carnivorous species (Bond and Lindburg 1990; Bashaw et al. 2003; Gaengler and Clum 2015). Food provision has also been employed as a mean of enrichment by mimicking the unpredictable access to prey faced by free-ranging tigers (Dierenfeld et al. *1994;* Szokalski et al. 2012). Another common practice with captive carnivores is the use of feeding-enrichment devices such as feeding poles or bungees-carcass (Ruskell et al. 2015; Law and Kitchener 2019). The feeding pole was developed in the mid-1990s to provide captive tigers with an opportunity to replicate the burst of energy employed when hunting prey (Law et al. 1997). Furthermore, zoological collections that have developed training programmes to facilitate medical and routine husbandry procedures (e.g., shifting on cue, weighing, voluntary blood sample collection) utilize the tigers' regular diet for such purposes (see Figure 1.4) (Tilson et al. 2016). Training is normally performed using positive reinforcement by providing animals with a highly valuable reward which is commonly food-based (Szokalski et al. 2012; Tilson et al. 2016). The benefits of using food as a type of enrichment in a wide range of captive species including tigers expand beyond increasing physical activity to promoting natural feeding behaviours, all of which could positively impact animal welfare (Hartstone-Rose et al. 2014; Law and Kitchener 2019).

Figure 1.4 Training sessions of captive tigers (*Panthera tigris*) where food was employed as a reward.

1.3 Dietary fibre and “Animal fibre”

1.3.1 Definition

Traditionally, only complex carbohydrates derived from plant sources (i.e. non-starch polysaccharides and lignin) that cannot be hydrolysed into monomers by mammalian digestive enzymes were classified as fibres (FAO 2001; Institute of Medicine 2001; Turner and Lupton 2011). In 2001, the Institute of Medicine (IOM) of the National Academies proposed a new definition of fibre in human diets, where animal-derived compounds were included for the first time (Institute of Medicine, 2001). Similarly, the dietary fibre definition in the Codex Alimentarius of the Food and Agriculture Organization (FAO) of the United Nations corresponds to “edible plant and animal material not hydrolysed by the endogenous enzymes of the human digestive tract” (FAO, 2001). The rationale underlying the inclusion of animal products in FAO and IOM definitions was to recognize the large diversity of non-digestible plant and animal compounds present in human diets. On the contrary, the diet of free-ranging felids like tigers contains negligible amounts of plant materials and is composed of all edible parts of a carcass, including bone, cartilage, tendons, ligaments, fur or feathers in addition to muscle meat (Fàbregas *et al.,* 2017; Mazák, 1981; Tilson and Nyhus, 2010). These poorly digestible components, rich in protein and/or glycoproteins, have been categorised as ‘animal fibre’ (Banta et al. 1979; Burrows et al. 1982; Depauw et al. 2011). Researchers have suggested that ‘animal fibre’ might play a similar physiological role in strict carnivores as that of plant-based fibres in herbivores acting as substrates and modulators of microbial metabolism in the GIT (Cummings and Macfarlane 1991; Depauw et al. 2011, 2012).

1.3.2 Classification and characteristics

Historically, plant fibres have been classified according to their food sources or chemical structure (see Figure 1.5) (Eastwood 1992; Guillon and Champ 2000; Stephen et al. 2017; Makki et al. 2018). Unfortunately, for animal-derived components, a classification system similar to that of plant fibres is not currently available. A broad categorization of fermentable substrates was proposed by Cummings and Macfarlane (1991), which consisted of starch, non-starch polysaccharides (i.e. cellulose and non-cellulosic polysaccharides), other sugars (e.g. lactose, raffinose, stachyose, lactulose, alcohols and fructooligosaccharides), mucin (e.g. components of GI mucus, glycoproteins and chondroitin-sulphate) and proteins (e.g. connective tissue, elastin, collagen, serum albumin, plant and microbial proteins, and digestive enzymes). Based on the Cummings and Macfarlane system, most animal-derived components will correspond either to the mucin or to the protein categories.

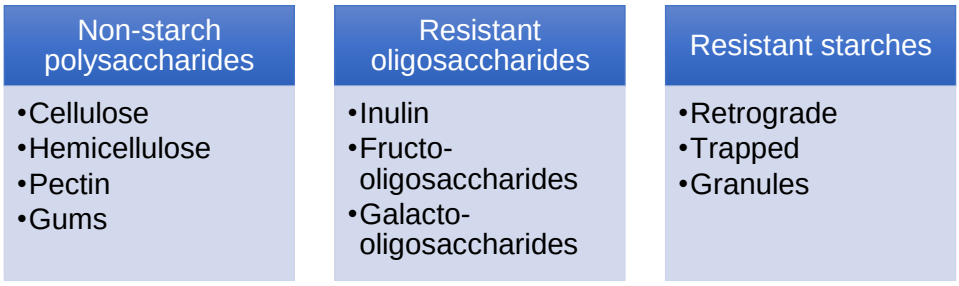

Figure 1.5 Classification of plant-based fibres according to their chemical structure (adapted from Stephen *et al.*, 2017 and Makki *et al.,* 2018).

Physical and chemical properties of plant fibres have been linked with changes in biological parameters such as total transit time, satiety, nutrient digestibility, fermentation metabolites concentrations and faecal consistency in different animal species, including felids (Eastwood 1992;

Bueno et al. 2000a; Propst et al. 2003; Vester et al. 2008; Bosch et al. 2009; Verbrugghe et al. 2010; Prola et al. 2010; Kanakupt et al. 2011; Rist et al. 2013; Rochus et al. 2013; Koppel et al. 2015; Hours et al. 2016; Loureiro et al. 2016; Deb-Choudhury et al. 2018). In an attempt to predict the physiological properties of dietary fibres, researchers have suggested that characteristics such as solubility, viscosity and fermentability should be considered when classifying fibres (Guillon and Champ 2000; Brownlee et al. 2006; Dikeman and Fahey 2006; Stephen et al. 2017). However, such information is still limited mostly to plant fibre sources, with little research done on animal fibre components.

Soluble fibres are those capable of being dissolved in water (e.g. pectin or oligofructose) (Patil et al. 2014; Purslow 2014). Soluble fibre sources have been associated with an increased passage rate in cats (Sunvold *et al.,* 1995), while the opposite effect has been reported in humans, cats and dogs fed insoluble fibres (e.g. cellulose, hemicellulose, lignin) (Burrows et al. 1982; Sunvold et al. 1995b, a; Loureiro et al. 2016; Makki et al. 2018). Higher faecal bulking was reported in cats fed diets containing insoluble fibres (i.e. cellulose or sugar cane fibre) compared to control diets (Verbrugghe *et al.,* 2010; Loureiro *et al.,* 2016). Similarly, in dogs, faecal weight increased when animals were offered a diet with added cellulose (Burrows *et al.,* 1982). In rats, the addition of soluble fibres (fructooligosaccharides, beta-glucans or pectin) was linked with decreased food intake and weight gain compared to those fed an insoluble fibre source (cellulose) (Adam et al. 2014).

Viscosity refers to the ability of fibres to thicken and form gels when mixed with fluids; these gels can entangle other molecules, which in turn affects gastric emptying and digestibility (Dikeman and Fahey 2006). In domestic cats, the use of highly viscous fibres (i.e. guar gum) resulted in increased defaecation frequency (Bueno *et al.*, 2000), lower protein digestibility and higher indole and p-cresol concentrations–indicative of protein

fermentation– (Rochus *et al.,* 2013). In humans, viscous fibre sources have been reported to increase satiety (Harrold et al. 2014; Yong et al. 2016; Stephen et al. 2017).

Fibre may be used by the intestinal microbiota– a wide range of microorganisms including bacteria, archea, fungus and virus inhabiting the GIT– as fermentation substrates, even in carnivorous species such as felids (Burrows et al. 1982; Sunvold et al. 1995a; Verbrugghe et al. 2010; Turner and Lupton 2011; Depauw et al. 2012; Rochus et al. 2013; Kerr et al. 2014a; Pradhan et al. 2016). Fermentation involves the hydrolysis of β-linkages, found in complex polysaccharides, to their basic units by bacterial hydrolases (McDonald et al. 2011). Additionally, microbial enzymes can hydrolyse other complex molecules (e.g. non-starch polysaccharides, synthetic sugars, mucin and proteins) (Brosey et al. 2000). The outcomes of microbial fermentation depend upon the substrate fermented but are commonly products such as short-chain fatty acids (SCFA), branched-chain fatty acids, organic acids, metabolic end-products (e.g. phenol, indole, sulphates, amines and ammonia) and gas (Cummings and Macfarlane 1991; Topping and Clifton 2001).

Fermentable fibre sources can be efficiently broken down by intestinal bacteria, a process characterized by gas and SCFA production; the degree of fermentability yields differing outcomes (Flickinger et al. 2003; Bosch et al. 2008; Barry et al. 2011; Den Besten et al. 2013). Increased faecal bulking was reported in cats and humans fed diets with low-fermentable fibre sources (Topping and Clifton 2001; Fekete et al. 2004; Prola et al. 2010). In cats, low fermentable fibre sources, like cellulose and lignin, have been associated with decreased protein digestibility (Fekete *et al.,* 2004) and lower concentrations of acetate and butyrate (Bueno et al. 2000a). Responses to dietary fibre in cats and dogs do not appear consistent either within or between species. In dogs, the use of a diet including cellulose resulted in higher digestibility of fat and protein and higher production of

SCFA compared to a diet including sugarbeet pulp (a highly fermentable fibre) (Propst *et al.,* 2003; Bosch *et al.,* 2009). However, in the study by Flickinger *et al.,* (2003), the use of a fermentable fibre (fructooligosaccharides) decreased macronutrient digestibilities, increased SCFA concentrations but had no effect on faecal characteristics of dogs (i.e. score and daily output). On the contrary, in cats, highly fermentable fibres (i.e. citrus pectin, bean gum, and guar gum) were linked with poor stool consistency (Fekete *et al.,* 2004) and decreased macronutrient digestibility (Sunvold *et al.,* 1995).

To date, only two published studies have evaluated the fermentability characteristics of animal-based fibre components. The first study assessed microbial degradability of collagen, glucosamine, chicken cartilage, rabbit bone, rabbit hair and rabbit skin using cheetah faecal inoculum (Depauw *et al.,* 2012). Results of the *in vitro* fermentation in this study revealed that animal components yielded different gas volumes and concentrations of SCFA, with glucosamine producing similar gas concentration as fructooligosaccharides (a highly fermentable fibre source), while rabbit by-products resulted in the lowest gas production of all components comparable to cellulose (a low fermentable fibre). Similarly, SCFA varied across animal substrates, with higher concentrations observed for collagen and glucosamine, while rabbit hair produced negligible concentrations similar to those observed with cellulose. The second study evaluated *in vitro* fermentation characteristics of three type of insects (black soldier fly (*Hermetia illucens*), housefly (*Musca domestica*) and yellow mealworm (*Tenebrio molitor*)) using dog inoculum (Bosch et al. 2016). The main results indicated that SCFA profiles and concentrations were similar across insects but, compared with the positive control fructooligosaccharides, lower concentrations of SCFA were observed. Overall, results from both studies suggest that the microorganisms present in the large intestine of carnivorous and omnivorous species can use animal-derived components as fermentation substrates. The extent to which different animal fibre

components are fermented, and if such characteristics vary across prey species, requires further study. Variables including substrate type and quantities offered also must be further examined.

Finally, it is worth noticing that physiological effects triggered by fibre appear to be species as well as substrate-dependent. For example, Vester and colleagues (2008) found that bobcats, jaguars, cheetahs and tigers fed a beef-based diet containing beet pulp showed significant differences in fat and energy digestibility, faecal score and indole concentrations across species. When tigers were offered a diet containing beet pulp, protein digestibility decreased, faecal scores increased (i.e. scats were more liquid), ammonia and SCFA concentrations increased compared to when fed a diet with added cellulose (Vester *et al.,* 2010). Based on their results, Vester *et al.* (2008, 2010) suggested that tigers are better suited to consume a diet with low-fermentable fibre than other medium to large felid species. However, since beet pulp was included in a beef-based diet, and the cellulose diet was horse-based, the authors could not confirm if the differences observed could be associated exclusively with the fibre source, the macronutrient composition of the diets or a combination of both. Kerr *et al.* (2013), later confirmed Vester's hypothesis in a study where tigers fed a horse-based diet including cellulose showed better faecal characteristics and macronutrient digestibility compared to the same horse-based diet with beet pulp. Therefore, it seems possible that the ideal fibre source and inclusion rates might differ across species.

1.3.3 Influence on health

Although dietary fibre is not considered a macronutrient and no recommended inclusion levels for felids exist, effects within and upon the GIT have long been known. In recent years, a broader array of beneficial health effects in a wide range of species have been recognised (a

summary of which is presented in Figure 1.6). As mentioned previously, changes in faecal consistency, modulation of passage rate and macronutrient digestibility have been linked to the use of plant fibres in the diets of a wide variety of species.

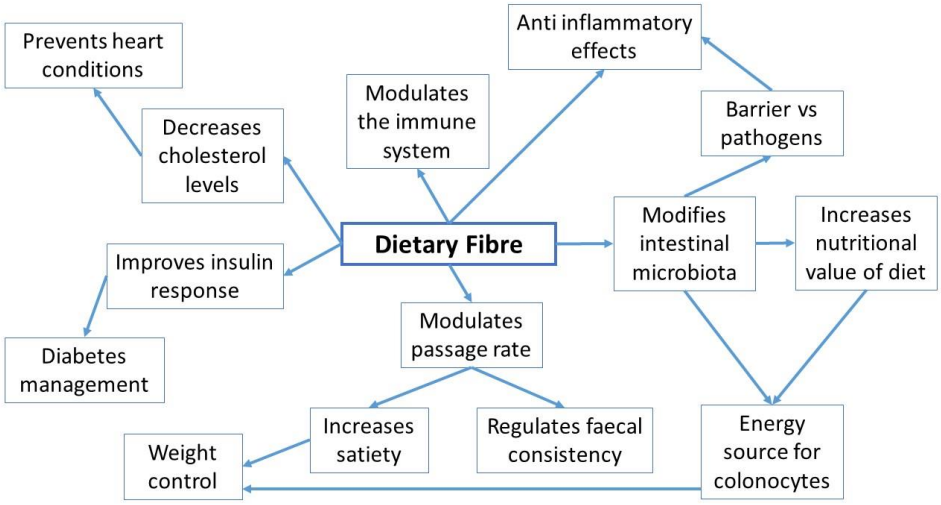

Figure 1.6 Summary of beneficial health effects of fibre.

Fibre is commonly employed in weight management programmes by adding volume yet negligible calories to the diet (Owens et al. 2014). Energy restriction is considered essential for body weight loss, hence commercial dog and cat diets designed to treat obesity are commonly high in fibre content (Laflamme 2012; Hamper 2016; Hours et al. 2016). However, fibre might help control weight gain through other means, for example by influencing satiety (Bosch et al. 2009; Backus and Wara 2016). In humans, the addition of highly viscous fibres to food items significantly increased satiety and resulted in lower food intake compared to placebo food items (Harrold et al. 2014; Yong et al. 2016). Similarly, in dogs and cats, reduced voluntary food intake– linked to satiety– was observed with high-fibre diets (Hours *et al.,* 2016). The authors hypothesised that the

fibre present in the commercial diets could have increased the viscosity of digesta and delayed gastric emptying, hence improving satiety. However, since viscosity was not measured in this study, such an explanation remains speculative.

Other comorbidities associated with obesity include chronic inflammatory conditions, diabetes and cardiovascular disease (Murphy 2016; Tarkosova et al. 2016). A considerable number of studies have investigated the ability of dietary fibre to modulate blood glucose and insulin (as reviewed by Fardet 2010 and Stephen *et al.,* 2017). Meta-analyses from such studies support the claim that increased consumption of soluble and insoluble fibres can decrease the risk of type 2 diabetes in humans (Silva et al. 2013; Threapleton et al. 2013; Yao et al. 2014). A possible mechanism by which this is achieved is the reduction of postprandial glycaemia (Fardet 2010). Similarly, a higher level of dietary fibre induced lower blood pressure, fasting glucose and cholesterol concentrations in a high-risk cohort, therefore reducing cardiovascular risk factors (Estruch et al. 2009). Finally, in dogs and cats, diets with high fibre content resulted in lower postprandial glycaemia, corroborating their clinical use for the management of diabetes in these species (Blaxter et al. 1990; Nelson et al. 2000; Mazzaferro et al. 2003; Bennett et al. 2006).

Evidence suggests that some of the key roles of dietary fibre in improving the host's health are linked to its ability to modulate intestinal microbiota (as reviewed by Den Besten *et al.*, 2013; Hagen-Plantinga and Hendriks, 2015; Makki *et al.*, 2018). Gut microbiota is believed to influence the host's physiology and health by creating a physical, chemical or competitive barrier(s) against pathogens, modulating the immune system, and enhancing the nutritional value of the diet by providing energy sources for enterocytes (Cummings and Macfarlane 1991; Lubbs et al. 2009; Guilloteau et al. 2010; Meijer et al. 2010; Wing et al. 2015; Blake and Suchodolski 2016; Pinna et al. 2016).

Bacteria can ferment complex carbohydrates and glycoproteins and produce, in return, molecules such as short-chain fatty acids (e.g. butyrate, acetate and propionate), along with some B vitamins that can be beneficial to the host (Brosey et al. 2000; Topping and Clifton 2001; Brouns et al. 2002; Bosch et al. 2009; Verbrugghe et al. 2010; Wing et al. 2015; Young et al. 2016). Although SCFA probably provide little contribution to metabolic energy in carnivores, other numerous health benefits have been proposed (Brosey et al. 2000; Fardet 2010; Rinttilä and Apajalahti 2013). For example, colonocytes use butyrate as their main energy source (Brouns et al. 2002), while proliferation and differentiation of colonocytes have been linked to high colonic butyrate concentrations (Guilloteau *et al.,* 2010). By preventing chemotaxis and cell adhesion, SCFA might inhibit infiltration of immune cells in peripheral tissues, therefore providing a protective effect against systemic inflammation (Meijer *et al.* 2010). SCFA are believed to regulate the immune system by promoting T-cell differentiation and proliferation while, at the same time, reducing the expression of pro-inflammatory cytokines (Wing *et al.* 2015). Numerous mechanisms are believed to contribute to these beneficial effects of SCFA; however, to date, most of them remain unclear.

Unfortunately, only a few studies have focused attention on the description of the impact of animal-based fibres in the diets of carnivores (Depauw et al. 2011, 2014; Zhang et al. 2014). Results of the study by Depauw *et al.* (2011) suggested that feeding captive cheetahs (*Acinonyx jubatus*) with whole rabbit instead of chunk beef meat did not alter the total faecal concentration of SCFA; however, concentrations of harmful compounds such as branched-chain fatty acids (BCFA), indole and phenol decreased when animals were fed whole rabbit. In addition, greater concentrations of faecal inflammatory biomarkers (calprotectin and S100A12) were detected in cheetahs fed the beef diet compared with whole rabbit diet. Overall, findings with cheetahs suggested that feeding whole prey may reduce intestinal inflammation in this species (Depauw *et al.,* 2014). In captive

arctic foxes (*Alopex lagopus*), the addition of disinfected poultry feathers to a commercial diet increased the diversity of faecal bacterial species compared to the control diet lacking feathers (Zhang *et al.,* 2014). Exposure to a wide variety of bacterial phyla has been associated with the improved immune capacity of the host, possibly due to a higher antigen exposure (Suchodolski 2011; Wing et al. 2015). Results from these two studies highlight the need to better understand the role of animal fibre components in the GI physiology and overall health of carnivorous species.

Although fibre is generally regarded as beneficial, some adverse effects have been reported. Texture and palatability of food can be negatively affected by the type and amount of fibre used (Harrold et al. 2014; Koppel et al. 2015). Fibre can bind minerals and other macronutrients, thus reducing their bioavailability for the host, which could then lead to deficiencies (Prola et al. 2010; Baye et al. 2017). Other undesirable effects of fibre include bloating, flatulence, diarrhoea and constipation (Turner and Lupton 2011; Stephen et al. 2017; Makki et al. 2018). In certain diseases, fibre appears to be counterproductive; for example, the addition of inulin (a fermentable fibre) exacerbated disease severity in mice with acute colitis (Miles et al. 2017). Similarly, butyrate– originating from microbial fermentation– was linked with hyperproliferation of colonocytes, which in turn promoted colorectal cancer in mice (Belcheva et al. 2014).

Other potentially harmful compounds produced by microbial fermentation include BCFA, indole, phenol, amines and ammonia (Cummings and Macfarlane, 1991; Davila *et al.,* 2013). These undesirable end-products are the result of the fermentation of proteins and amino acids that escaped enzymatic digestion from the host (Macfarlane and Macfarlane 1997; Davila et al. 2013; Rinttilä and Apajalahti 2013). Such end-products have been linked to chronic kidney disease, GI and cardiovascular diseases in humans and rats (Macfarlane and Macfarlane 1997; Ramezani and Raj 2014; Hagen-Plantinga and Hendriks 2015; Wing et al. 2015; Castaño-

Rodríguez et al. 2018). However, as reviewed by Wernimont *et al.*, (2020) some of these putrefactive compounds seemed to positively impact health. For example, indole (an end-product of tryptophan and tyrosine fermentation) is commonly regarded as a co-carcinogen in rodents (Sims and Renwick 1983; Lawrie et al. 1985; Chung and Gadupudi 2011); however, in humans, indole increased epithelial-cell junction resistance– an essential component of a healthy gut barrier– and decreased indicators of inflammation (Bansal et al. 2010). Although most data available suggest that fibre and by-products of fermentation such as SCFA can be beneficial, caution is needed before generalizing recommendations because, as already mentioned, compounds from microbial fermentation might act differently depending on the species or on their concentration. For this reason, further studies are needed to determine the ideal fermentation profile for each species.

1.4 Diet-related gastrointestinal health disorders

Captive felids can be susceptible to GI diseases that could have a major impact on their welfare and survival (Longley 2011; Lamberski 2015; Whitehouse-Tedd et al. 2015). Unfortunately, to date, information regarding the morbidity of GI disorders in captive tigers is limited. In a study performed by Srivastav and Chakrabarty (2002) in zoo-housed tigers in India, the principal causes of death corresponded to GI, respiratory and behavioural disorders (i.e. cannibalism and maternal rejection), accounting for 15.9% each. Gastrointestinal disorders included enteritis, gastritis, gastric ulcers, gastroenteritis and hepatitis (Srivastav and Chakrabarty 2002). While necropsy reports from German zoos reported renal lesions (94%), GI inflammation (50%), neoplasia (39%) and pneumonia (33%) as the most frequent findings in tigers (Junginger et al. 2015). Among tigers

presenting GI conditions, enteritis (89%), gastritis (22%) and exocrine pancreatic insufficiency (EPI) (22%) were the main pathological changes observed by Junginger et al. (2015). The only published epidemiological survey on captive tigers presented information from 32 facilities, of which 25 were located in the United States; however, the authors considered information from only one tiger per facility for those zoos housing multiple individuals (Lefebvre *et al.,* 2020). The prevalence of GI disease in surveyed tigers corresponded to 9%; however, Lefebvre *et al.,* (2020) estimated that the number of tigers represented by their survey covered only 1.7% of the population registered in the specialized software Species360© (see section 1.2.1 Ex-situ population).

1.4.1 Tiger Disease

Historically, GI conditions of unknown aetiology in captive tigers were classified as “Tiger Disease”, a condition first described in a German zoo in 1963, and which by 1978 was reported worldwide (Seidel and Wisser 1987). Clinical signs included regurgitation of undigested food and defaecation of foul-smelling soft faeces containing undigested food particles (Klos and Lang 1976; Siefert and Muller 1987). Blood tests showed decreased pancreatic enzyme concentrations in some individuals, hence suggesting a possible involvement of the pancreas in the development of the disease (Seidel and Wisser, 1987). In addition, necropsy findings included gastric and SI ulcers and chronic interstitial nephritis (Siefert and Muller, 1987). Bacteriological examination of vomit, gastric content and faeces of affected tigers showed increased presence of coliforms, bacilli and endospore-producing bacteria such as *Clostridium perfringens* (Siefert and Muller, 1987).

Originally, it was hypothesized that the disease was a disturbance in macronutrient metabolism leading to secondary gastroenteritis, with a

concomitant intestinal dysbiosis (an alteration in the composition and/or richness of the intestinal microorganisms) (Klos and Lang, 1976). Later on, Siefert and Muller (1987) proposed that “Tiger Disease” was the result of an enterotoxaemia due to a mixed infection with *Escherichia coli* and *C perfringens.* It was believed that the release of bacterial toxins in the GIT of affected tigers would alter the digestion of food, favouring further dysbiosis. The proposed treatment consisted of antibiotics, glucocorticoids, pancreatic enzymes, and dietary management– providing whole prey in small portions several times a day (Seidel and Wisser 1987; Siefert and Muller 1987). Diet was considered an essential component of such a condition; however, at present, “Tiger Disease” points towards a different aetiology, more compatible with inflammatory bowel disease (IBD) (Travis and Carpenter 2011).

1.4.2 Inflammatory bowel disease

Inflammatory bowel disease (IBD) has been defined as an idiopathic, immune-mediated inflammation of the SI and/or colon, with a predominant lymphocytic or plasmacytic infiltrate, without underlying causes (Willard 1999; Zoran 2002). This condition was first described in humans and, only in recent decades, in other species (Raithel et al. 2001; German et al. 2003; Travis and Carpenter 2011). Although it has been recognised for decades, controversy over aetiology, diagnosis and treatment persists (Willard 1999). A combination of altered intestinal microbial composition, genetic susceptibility of the host, dietary and/or environmental factors are suspected to be the main contributing factors in the pathogenesis of IBD (Willard 1999; Minamoto et al. 2012; Honneffer et al. 2014).

The most common signs of IBD in dogs and cats include weight loss, vomiting, diarrhoea and haematochezia (Willard 1999; Suchodolski 2011). Clinical signs are not always related to the severity of the inflammatory

infiltrate; however, a chronic presentation (>4 weeks) is frequently observed (Hall et al. 2005; Willard and Mansell 2011). Since clinical signs of IBD are not pathognomonic, diagnosis becomes challenging. Diseases that can mimic IBD clinical signs include dietary intolerance, microbial infections, pancreatic insufficiency and lymphosarcoma; for this reason, a diagnosis of exclusion is commonly performed (Willard 1999; Zoran 2002; Hall et al. 2005; Steiner 2012; Xenoulis et al. 2016). For a conclusive diagnosis, ideally, intestinal inflammation should be confirmed through histopathology– a method requiring the obtention of biopsy samples (Willard 1999). Recently, non-invasive methods such as the quantification of faecal inflammatory biomarkers, e.g. N-methylhistamine and S100A12, have been evaluated for the diagnosis and monitoring of chronic enteropathies like IBD (Collins 2013; Heilmann and Steiner 2018).

1.4.3 Non-invasive assessment of GI health: use of faecal inflammatory biomarkers.

Highly invasive diagnostic tools such as endoscopy, x-rays, biopsy collection and blood samples are commonly needed for diagnosing and monitoring the progress of chronic enteropathies such as IBD (Kaiser et al. 2007; Foell et al. 2009; Heilmann and Steiner 2018; Parambeth et al. 2019). These tools require intense manipulation of individuals and the use of anaesthesia, which pose an additional risk for the animal, especially if its health status is already compromised (Hall et al. 2005; Bechert 2012; Steiner 2012). Medical procedures can be a source of stress and perceived as negative experiences even in captive wildlife trained for such purposes (e.g. voluntary blood draws) (Blackett et al. 2016; Justice et al. 2017). In addition, handling dangerous species such as tigers involves a potential hazard for the staff taking part in the procedure (Lovely 2009). Concerns over possible adverse impacts associated with the use of these invasive diagnostic tools have generated interest in identifying and

validating less intrusive methods that could be applied even in a zoo setting. For example, a range of faecal inflammatory biomarkers has been studied as potential non-invasive indicators to diagnose and assess chronic enteropathies like IBD in humans (Bischoff et al. 1997; Winterkamp et al. 2002; De Jong et al. 2006; Kaiser et al. 2007), domestic dogs (Heilmann and Suchodolski 2008; Anfinsen et al. 2014; Berghoff et al. 2014), cheetahs (Depauw et al. 2014) and, more recently, common marmosets (Parambeth et al. 2019) and domestic cats (Bridges et al. 2019).

Indicators of mast cell degranulation such as N-methylhistamine (NMH) and neutrophil infiltration (e.g. S100A12) are among the most promising and commonly employed markers of inflammation in humans and dogs (Steiner 2014). Histamine, a biogenic amine, is a nitrogenous compound of low molecular weight, formed by decarboxylation of free histidine (Bodmer et al. 1999). Histamine detected in the GIT originates from either exogenous or endogenous sources. Exogenous sources include the ingestion of histamine contained in food (e.g. red meat and fish), while endogenous histamine is released by mast cells during inflammatory processes and is believed to contribute to some of the clinical signs seen in patients with enteropathies (Rolfe et al. 2002; Berghoff and Steiner 2011; Steiner 2014). In contrast, the role of mast cells in the pathophysiology of chronic GI disease is still not completely understood, but some of the effects linked to the release of this inflammatory mediator include tissue damage, motility disruption and possible alteration of intestinal transport and permeability (Raithel et al. 1995; Rolfe et al. 2002; Heilmann and Steiner 2018). After release, plasma histamine has a short half-life– approximately six minutes in humans– (Laroche et al. 1991; Pollock et al. 1991) and catabolic pathways include oxidation by amine oxidases (e.g. monoamine oxidase and diamine oxidase), methylation by methyl-transferase, or acetylation (Schayer 1966; Halász et al. 1994; Spano et al. 2010). The rapid metabolism of this biogenic amine makes its

quantification impractical (Collins 2013; Berghoff et al. 2014). However, N-methylhistamine, a stable metabolite of histamine, has been successfully measured in serum, urine and faeces using gas chromatography (Ruaux et al. 2009; Heilmann and Steiner 2018). This assay has been developed for use in dogs; however, since the methodology is not species-specific, researchers have proposed its use in other domestic and non-domestic species, including felids (Ruaux *et al.,* 2009; Berghoff *et al.,* 2011).

Previous research suggested that NMH could be a useful clinical biomarker of inflammatory bowel disorders. Winterkamp et al. (2002) found that urinary levels of NMH increased significantly in human patients with IBD compared to healthy controls. Results from domestic dogs showed a wide variation in faecal concentrations of NMH in healthy individuals (Ruaux et al. 2009) and dogs with chronic enteropathies (Anfinsen et al. 2014). Despite this variation, Berghoff *et al.* (2014) reported a significantly higher NMH concentration in dogs with GI disease compared to healthy groups. In addition, faecal NMH was also increased in common marmosets diagnosed with chronic lymphocytic enteritis compared to healthy controls (Parambeth et al. 2019). Overall, these studies seemed to suggest that increased faecal NMH concentrations could serve as a potential marker of chronic inflammatory enteropathies in a wide range of species. However, to establish reference values to help in the diagnosis and monitor the efficacy of treatment of these GI conditions, further research across different species is needed.

Other inflammatory mediators released primarily by neutrophils during infection or inflammation include proteins of the S100 family (Kaiser et al. 2007; Heilmann et al. 2016b). This family comprises more than 20 calcium-binding proteins, of which three have been of interest as markers of GI inflammation: S100A8, S100A9 and S100A12 (Heizmann 2007; Foell et al. 2009). Concentrations of faecal S100A12 have been quantified in humans (De Jong et al. 2006; Kaiser et al. 2007), dogs (Heilmann et al. 2016a,

2018a), cheetahs (Depauw et al. 2014) and, more recently, in domestic cats (Bridges *et al.,* 2019). Each protein has characteristic tissue-specific expression patterns (Heizmann 2007); however, in recent studies, S100A12 has shown a higher correlation with intestinal inflammation than S100A8/A9 in humans and dogs (Kaiser et al. 2007; Foell et al. 2009; Heilmann et al. 2014; Heilmann and Steiner 2018). S100A12 can be released by a variety of cells including neutrophils, activated granulocytes and monocytes, during inflammatory processes (Hofmann et al. 1999; Day and Jones 2000; De Jong et al. 2006; Kaiser et al. 2007).

Previous findings showed higher S100A12 concentrations in the duodenal and colonic mucosa of dogs with chronic enteropathies compared to healthy individuals (Hanifeh et al. 2018). Furthermore, a strong correlation between faecal concentrations and the number of cells with detectable concentrations of S100A12 along the GIT in dogs with chronic inflammatory enteropathies has been reported (Heilmann et al. 2019). Hence, faecal concentrations of S100A12 have been proposed as an indirect measure of GI inflammation in patients with chronic enteropathies (De Jong et al. 2006; Heilmann and Steiner 2018).

Although IBD is a common pathology in domestic cats, studies on S100 proteins in this species have to date only described the analytical validation of the assay, with no reports on differences in concentrations between healthy and sick individuals (Jergens 2012; Bridges et al. 2019). In a different felid species, the cheetah, effect of diet as a modulator of intestinal inflammation was assessed using faecal S100A12 concentrations (Depauw *et al.,* 2014). In this case, significantly higher concentrations were observed in cheetahs fed a supplemented beef diet compared to those fed a whole rabbit diet. In addition, other parameters associated with compromised GI health such as putrefactive compounds, softer faecal consistency and incidence of diarrhoea were also increased in cheetahs fed beef compared to those fed whole rabbit (Depauw *et al.,* 2014). Results

from the cheetah study supported the assumption that low animal fibre intake in meat-based diets could compromise GI health in the species compared with whole prey diets (Depauw *et al.,* 2011).

Finally, similar to NMH, large individual variation in daily faecal concentrations of S100A12 has been reported (Heilmann et al. 2011; Depauw et al. 2014; Bridges et al. 2019). Therefore, the recommended sampling strategy involves the evaluation of faecal samples from three consecutive days (Heilmann *et al.,* 2011; Depauw *et al.,* 2014). N-methylhistamine and S100A12 have shown promising results as non-invasive tools to detect GI diseases characterised by chronic inflammation; however, further research is needed to corroborate the clinical utility of such markers in the diagnosis and monitoring of chronic enteropathies.

While the role of nutrition in the prevention and treatment of digestive pathologies in dogs and cats has been well recognised, the role of diet in the development and management of GI conditions in captive non-domestic felids is an area that requires further investigation (Whitehouse-Tedd *et al.,* 2015; Lefebvre *et al.,* 2020).

1.5 Animal welfare

1.5.1 Animal welfare definition

The concept of animal suffering has been present since ancient Greece; growing concern over the past century or more led to the development of the “Five Freedoms” which are considered the starting point for modern animal welfare (see Table 1.2) (Kohn 1994; Farm Animal Welfare Council 2009; Broom 2011; Szűcs et al. 2012). The notion of animal welfare has evolved over the years: some definitions focused on the animals’ feelings,

others considered a state of harmony with the environment, while the more recent ones emphasised the fulfilment of animals' needs (Broom 2011; Green and Mellor 2011; Whitham and Wielebnowski 2013). The first welfare definition– inspired by the Five Freedoms– considered the physical status of animals, i.e. that individuals were free from hunger, pain or disease (Dawkins 2004; Farm Animal Welfare Council 2009; Broom 2011). Yet, it became evident that such definition represented an idealized goal rather than an achievable condition (Mellor et al. 2015; Blackett et al. 2016; Webster 2016). For example, it is biologically impossible for any animal to be completely free from hunger at all times. To feel motivated to seek food and eat, animals must initially feel hungry; hence, it could be argued that some of these "negative" states are essential to trigger behaviours needed to secure animals' survival (Dawkins 1998; Mellor 2016a).

Table 1.2 Five freedoms and their respective provision to promote animal welfare (adapted from Farm Animal Welfare Council, 2009).

Freedom	Provision
1. From thirst, hunger, and malnutrition	Access to fresh water and an adequate diet to maintain full health and vigour
2. From discomfort and exposure	Appropriate environment including shelter and resting areas
3. From pain, injury and disease	Prevention or opportune diagnosis and treatment
4. From fear and distress	Ensuring an environment and handling which avoids mental suffering
5. To express normal behaviours	Appropriate space, suitable facilities, and social grouping when adequate

Moving on from the Five Freedoms, researchers acknowledged that animals can also have positive experiences, examples of which are related to behavioural expressions that generate interest, comfort, and pleasure, as environment-focused exploration, food acquisition and non-aggressive social interactions (Mellor, 2016a, 2016b). Therefore the mental comfort of

animals, with a focus on the presence of positive experiences, is still considered by some researchers as a key aspect of welfare (Dawkins 2004; Wemelsfelder 2007; Mellor 2016a,b; Stilwell 2016).

From the acknowledgement that welfare should emphasize positive experiences, the concept of “Quality of Life” (QoL) emerged (Taylor and Mills 2007; Webster 2016). Taylor & Mills (2007) defined it as “the perceived state of an animal which can be predicted by the fulfilment of species-specific and individual needs and is reflected by its health and behaviour at any time point”. The concept of QoL led to another definition of animal welfare, which focused more on the reduction of abnormal behaviours, alongside an increase in species-specific behaviours (Bashaw et al. 2003; Gartner et al. 2016). Although animal welfare and QoL definitions might seem similar, some authors have argued that QoL is intended as a holistic approach to assessing an animal’s positive welfare based on the individual’s whole life rather than a replacement or synonym of animal welfare, which in turn encompasses consideration of both positive and negative welfare (Broom 2007; Yeates 2011).

Despite the broad range of definitions, animal welfare remains an area of core interest for modern zoological collections, and meeting established minimum welfare standards is enforced by both local authorities and zoo associations (DEFRA 2012; Draper and Harris 2012; Mellor et al. 2015; Blackett et al. 2016; Tilson et al. 2016). For the current project, animal welfare refers to “an animal’s collective physical, mental and emotional states over a period of time” as proposed by the Animal Welfare Committee of AZA (Animal Welfare Committee 2019).

1.5.2 Welfare assessment

To assess animal welfare, indicators of success or failure to cope with the environment may be used (Broom 2008; Hill and Broom 2009; Rose et al. 2017). Such indicators can include physiological, behavioural or environmental parameters (examples of each category can be found in Table 1.3), and may evaluate either a population or an individual by using invasive or non-invasive techniques (Hill and Broom 2009; Draper and Harris 2012; Hockenhull and Whay 2014).

Classically, the predominant approach to evaluate welfare in zoo animals has consisted of environmentally-based indicators, i.e. what is provided to the animals, for instance, access to food, water, shelter, and veterinary services (Kagan et al. 2015). Although important components of animal welfare, such indicators do not guarantee a good welfare status (Wemelsfelder 2007; Pastorino et al. 2017; Phillips et al. 2017): for this reason, researchers have emphasized the importance of including animal-based indicators during welfare evaluation (Kagan et al. 2015; Gartner et al. 2016; Webster 2016). A good understanding of the species' biology plays a key role in accurately assessing welfare, by enabling the identification of the most appropriate indicators (Broom, 2008; Hill and Broom, 2009; Rose *et al.,* 2017). Moreover, individual differences should be accounted for, because even when exposed to the same environment, animals can respond to it in different ways, depending on their particular life history, age, health and reproductive status (Mohapatra et al. 2014; Fureix and Meagher 2015).

Among the challenges faced when assessing the welfare of zoo housed animals is, firstly, the possible lack of reference values for measures for the majority of species (Mesa et al. 2014; Lamberski 2015; Parnell et al. 2015). Secondly, invasive methods that require animals handling (e.g. blood sample collection) can affect the welfare of the individuals assessed; hence, the use of non-invasive methods are preferred (e.g. collection of

faecal samples or hair from enclosures) (Veasey et al. 1996; Bechert 2012; Hockenhull and Whay 2014). Finally, resources needed for the assessment, i.e. the time involved, and financial cost can be a constraint for smaller zoological collections with limited staff (Kohn 1994; Justice et al. 2017). Ideally, welfare indicators should be non-invasive, affordable to analyse while at the same time providing important information on the welfare status of the animals assessed (Blackett *et al.,* 2016; Broom, 2008; Hockenhull and Whay, 2014; Veasey *et al.,* 1996). In addition, welfare indicators should be validated, reliable and feasible under different scenarios, while avoiding possible human bias (Hockenhull and Whay, 2014; Blackett *et al.,* 2016; Stilwell, 2016). For these reasons, physiological and behavioural indicators are the two most common animal-based parameters employed to assess welfare (Broom, 2008; Hockenhull and Whay, 2014).

Table 1.3 Selected examples of welfare indicators.

Indicator	Examples	References
	Environmental	
Enclosure	Size Presence of shelter/refuge areas Complexity Temperature, ventilation and humidity	(Mazák 1981; Morgan and Tromborg 2007; Breton and Barrot 2014; Blackett et al. 2016)
	Physiological	
Heart rate	Increased heart rate	(Broom 2007; Morgan and Tromborg 2007; Bechert 2012; Hockenhull and Whay 2014; Blackett et al. 2016).
Health status	Presence of disease, injuries or pain Mortality rates Lifespan	(Bechert 2012; Hockenhull and Whay 2014; Blackett et al. 2016)
Glucocorticoid levels	Concentration levels (blood, urine, saliva and/or faeces).	(Palme et al. 2005; Sajjad et al. 2011; Narayan et al. 2013; Ralph and Tilbrook 2016)
	Behavioural	
Behavioural diversity	Scope of behaviours expressed by an individual.	(Rabin 2003; Miller et al. 2016; Cronin and Ross 2019)
Time budget	Frequency of behaviour occurrences in a given period of time.	(Martin and Bateson 2007; Breton and Barrot 2014; Ruskell et al. 2015; Biolatti et al. 2016; Munita et al. 2016)
Stereotypies	Repeated patterns of movement that do not vary in form and do not have any apparent function.	(De Rouck et al. 2005; Breton and Barrot 2014; Mohapatra et al. 2014; Biolatti et al. 2016; Munita et al. 2016).

1.5.2.1 Behavioural indicators of welfare

Behaviour reflects one of the first attempts of an individual to cope with its environment, hence behavioural observations are widely used as an early indicator of the presence of factors adversely affecting welfare (Hockenhull and Whay 2014; Biolatti et al. 2016). Time budget and presence/absence of specific behaviours are among the most common methods to evaluate welfare through the observation of behaviour (Bashaw et al. 2003; Morgan and Tromborg 2007; Mohapatra et al. 2014; Ward et al. 2018).

Animals spend variable amounts of time performing different activities throughout the day, therefore time budgets are used to record the frequency of occurrence of different behaviours in a given period of time (Mohapatra et al. 2014; Veasey 2017). Abnormal levels of expression of some behaviours have been linked to decreased welfare. For example, either excessive inactivity–like freezing and hiding– (Ishiwata et al. 2008; Fureix and Meagher 2015) or the performance of abnormal behaviours– such as stereotypies, repeated patterns of movement that do not vary in form and do not have any apparent function (Mason 1991; Mason and Rushen 2006; Mishra et al. 2013; Vaz et al. 2017)– are considered indicators of diminished welfare (Gunn and Morton 1995; Rabin 2003; Sajjad et al. 2011; Mohapatra et al. 2014; Rose et al. 2017; Cronin and Ross 2019).

A common practice in zoo animal welfare evaluation is the comparison of time budgets of captive species against their free-living conspecifics (Spiezio et al. 2018; Browning 2020). However, since the range of behaviours expressed can be a reflection of the resources present (De Rouck et al. 2005; Yu et al. 2009; Macri and Patterson-Kane 2011), some authors have argued that the lack of “natural” behaviours performed in captivity, compared to free-ranging, might not necessarily implicate diminished welfare in captive individuals (Veasey et al. 1996; Wolfensohn et al. 2018; Browning 2020). The disparities between time budgets for free-living compared with captive wild animals may simply reflect the considerable difference in environmental resources available for these two different populations (Mohapatra et al. 2014; Veasey 2020). Therefore, it has been suggested that, if the time budget of captive species is to be compared to that of free-living conspecifics, additional indicators should be used to corroborate assessments of their welfare status (Veasey, Waran and Young, 1996; Browning, 2020).

Historically, the use of negative welfare indicators– such as the presence of stereotypic behaviours– have been employed to monitor animal welfare (Terlouw et al. 1991; Mason and Latham 2004; Miller et al. 2016; Spiezio et al. 2018; Martin et al. 2020). Stereotypies vary widely across species. In felids, common stereotypical behaviours include pacing and over-grooming (Mason and Rushen 2006; Mohapatra et al. 2014; Stanton et al. 2015). According to Mohapatra et al. (2014) and Szokalski et al. (2012), captive tigers spent up to 23 % of their day engaged in stereotypical locomotion. In captive tigers, increased pacing has been correlated with a wide range of features, such as small enclosures (size <190 m^2) (Breton and Barrot 2014), lack of water pools (Biolatti *et al.,* 2016), visual contact with neighbouring tigers (De Rouck et al. 2005; Miller et al. 2013a) and anticipation of feeding times (Mohapatra *et al.,* 2014).

Researchers have proposed three mechanisms by which stereotypies develop: as an attempt to cope with unfavourable conditions, as behavioural frustration, or as impaired cognitive development (Mason 1991; Mason and Latham 2004; Bacon 2018). Regardless of their origin, stereotypies have been associated with the presence of past or present stressors and are commonly regarded as indicators of poor welfare (Sajjad et al. 2011; Mohapatra et al. 2014; Rose et al. 2017). In addition, stereotypical behaviours can affect animal welfare either by impacting health– e.g. excessive footpad wear when pacing– or preventing individuals from interacting with their surroundings– e.g. decreased social interactions (Benhajali et al. 2014; Hu et al. 2015; Martin et al. 2020). Overall, stereotypies might pose more questions than answers, yet they might reflect differences in coping mechanisms across individuals. A better understanding of their origin and motivation seems necessary to improve husbandry practices and work towards positive welfare for animals under human care.

1.5.2.2 Physiological indicators of welfare

When animals are confronted with what they perceive as stressful situations– environmental, physiological or psychological–, activation of the hypothalamic-pituitary-adrenal axis occurs; the result is the release of glucocorticoids (i.e. cortisol and corticosterone) from the cortex of the adrenal glands (Palme et al. 2005; Lane 2006; Narayan et al. 2013; Ralph and Tilbrook 2016). Most mammals, including tigers, secrete mainly cortisol (Graham and Brown 1996; Schatz and Palme 2001); yet, corticosterone is also detected in cortisol-dominant species, such as tigers (Koren et al. 2012). The main difference between these glucocorticoids is the presence of an additional hydroxy group on the 17th carbon in cortisol, making it more hydrophilic than corticosterone (Palme 2019).

Once secreted, glucocorticoids trigger a variety of physiological responses, (including increased heart rate, blood pressure, respiration rate) aimed at regulating energy and preparing the organism to confront the stressor (Sapolsky et al. 2000; Busch and Hayward 2009). After circulating in the plasma, glucocorticoids are metabolized principally by the liver and the resulting metabolites are mainly excreted in the bile (to end up in the intestines) or in the urine, via the kidneys (82% and 18% respectively in domestic cats) (Schatz and Palme 2001; Goymann 2012; Palme 2019). In the intestines, glucocorticoid metabolites can be reabsorbed back into the blood or further degraded by bacteria and enzymes and end up in the faeces (Goymann 2012). Faecal glucocorticoid metabolites (FGMs) can be detected in scats after a time delay corresponding to the GI passage rate of the species (Graham and Brown 1996; Schatz and Palme 2001). For example, in captive tigers and domestic cats, a delay of up to two days between glucocorticoid release in the blood and faecal appearance has been reported (Palme et al. 2005; Narayan et al. 2013).

Glucocorticoids metabolites have been successfully measured in urine and faeces of a wide range of species using immunoassays (Washburn and

Millspaugh 2002; Palme et al. 2005; Narayan et al. 2013). In the immunoassays, the analyte of interest (cortisol or corticosterone) competes against a labelled analyte for limited binding places of the analyte-antibody (see Brown *et al.*, 2004 for an in-depth explanation). Cross-reactivity between antibody and analyte is essential for assay accuracy (Brown et al. 2004; Dias et al. 2008). Since the cortisol and corticosterone measured in faeces correspond primarily to their metabolites and not the intact hormones, special care should be taken while running the analytical assays (Palme *et al.,* 2005; Lane, 2006; Palme, 2019). Ideally, cross-reactivity between metabolites and antibody should be known and the assay should be validated for the species to ensure that glucocorticoid metabolites are being accurately measured (Dias, Nichi and Guimarães, 2008; Goymann, 2012; Palme, 2019). Validation can be performed through stimulation of the adrenal gland, to demonstrate significant elevations in glucocorticoid metabolites, using adrenocorticotropic hormone (Carlstead et al. 1992; Graham and Brown 1996; Schatz and Palme 2001) or by exposing individuals to stressful situations (e.g. physical restraint, relocation or transportation) (Naidenko et al. 2011; Watson et al. 2013). In addition, extrinsic factors such as diet, environmental conditions and storage of the samples can influence FGMs concentrations; therefore, careful selection and application of analytical methods, alongside cautious interpretation of results are recommended (Washburn and Millspaugh 2002; Palme et al. 2005; Touma and Palme 2005; Metrione and Harder 2011).

Although the release of glucocorticoids is essential for the survival of any individual, sustained periods of high glucocorticoids concentrations are considered detrimental to health and fitness (Sapolsky et al. 2000; Busch and Hayward 2009). Long-term elevation of glucocorticoids can affect the reproductive success (Moreira et al. 2007; Huang et al. 2020) and cause immune system suppression (Omidi et al. 2017). The disruptive effect of glucocorticoids on reproductive physiology is related to a reduced release

in females of the gonadotropin-releasing and the luteinizing hormone by the hypothalamus and pituitary respectively; while in males, responsiveness to the luteinizing hormone is diminished in the gonads (Smith and French 1997; Sapolsky et al. 2000; Busch and Hayward 2009). Glucocorticoid concentrations appear to suppress the immune system through three main mechanisms: firstly, by inhibiting the synthesis and efficacy of mediators of the immune and inflammatory reactions (e.g. cytokines); secondly by decreasing the number of circulating white blood cells (i.e. lymphocytes, eosinophils, basophils and macrophages) and finally by reducing the agglomeration of phagocytic cells at inflammation sites (as reviewed by Munck, Guyre and Holbrook, 1984; Sapolsky, Romero and Munck, 2000). Despite these apparently negative effects, some authors have proposed that, through the suppressive physiological action previously described, glucocorticoids contribute to recovering from the stress response rather than enhancing it (Munck, Guyre and Holbrook, 1984). Munch and colleagues believed that glucocorticoids downregulate the defence reactions induced by stress to prevent an overload of the system which in turn could compromise homeostasis, hence protecting an organism not against the source of stress itself, but from the physiological reactions triggered by it.

One of the most widespread methods to assess animals welfare is the measurement of glucocorticoids, which are used as physiological indicators of stress (Graham and Brown 1996; Schwarzenberger 2007; Sajjad et al. 2011; Ruskell et al. 2015). However, several authors have highlighted the limitations and confounding factors associated with the use of glucocorticoids as welfare indicators (see reviews by Goymann, 2012; Dickens and Romero, 2013; Palme, 2019). Secretion of cortisol and corticosterone can occur in non-stressful circumstances that generate pleasure, excitement, or arousal (Ralph and Tilbrook, 2016; Hockenhull and Whay, 2014). For example, significantly higher levels of cortisol have been observed in humans, rats and dogs undergoing intensive exercise

(Jacks et al., 2002; Jahr et al., 2019; Gashi et al., 2020; Tsuda et al., 2020); while reproductive status and social hierarchy position can also affect glucocorticoids concentrations (Smith and French, 1997; Kondo et al., 2003; Behie, Pavelka and Chapman, 2010; Huzzey et al., 2015). In chronically stressed animals, the direction of changes in glucocorticoids concentrations (i.e. increase or decrease) compared to a baseline appears to be affected by the type of stressor, potential habituation to that stressor, and the taxon of the animal (see review by Dickens and Romero, 2013) For this reason, it has been suggested that glucocorticoid concentrations should be evaluated alongside other welfare indicators to more accurately evaluate the welfare status of an individual (Blackett et al., 2016; Broom, 2007; Hockenhull and Whay, 2014).

1.6 Scientific aims

The aim of this project was to describe changes in non-invasive indicators of gastrointestinal health and function in captive tigers fed two of the most common diets used by North-American zoo collections: a diet comprising exclusively a commercially supplemented ground horsemeat- muscle-based diet or the same diet with 20% added whole rabbit (as a source of animal fibre). To provide a holistic approach to health evaluation, the potential influence of the dietary intervention on behavioural and physiological welfare parameters was also evaluated. The project is divided into two key areas: the first involves the evaluation of physiological aspects of the gastrointestinal tract, while the second focuses on animal welfare evaluation. Specific objectives for each area included:

1) Health and function of the gastrointestinal tract

- Investigate diet-related changes on functional GIT parameters such as GIT transit time, total tract apparent nutrient digestibility, faecal concentration of fermentation products (i.e. SCFA, indole, phenol and p-cresol), faecal consistency and faecal pH (Chapter 2).

- Assess the impact of added animal fibre on two faecal inflammatory biomarkers: S100A12 and N-methylhistamine (Chapter 3).

2) Welfare assessment

- Describe changes in behavioural indicators of animal welfare (i.e. time budgets and frequency of stereotypical behaviours) in captive tigers, associated with consumption of two alternative diets (Chapter 4).

– Evaluate a physiological indicator of animal welfare (i.e. FGMs) of tigers undergoing a dietary intervention (Chapter 5).

This project contributes to the existing literature on captive tiger gastrointestinal health by evaluating the application of some novel, non-invasive parameters such as inflammatory biomarkers. In addition, faecal and behavioural indicators provide comparative values that can be used in future studies on this species. Finally, I expect that the outcomes of this research project will provide evidence-based recommendations for managed feeding programmes that support and/or improve GI health and welfare of tigers under human care.

Chapter 2. The influence of dietary animal fibre on digestive function and fermentation profiles in captive tigers.

2.1 Introduction

Although research has been carried out over the past decades, specific nutrient requirements for tigers are still unknown and continue to be extrapolated from domestic cat (*Felis catus*) data (Dierenfeld et al. 1994; Salter et al. 1999; Vester et al. 2008). Commercial raw meat diets for carnivores are common among North American zoos (Kerr et al. 2013a; Kapoor et al. 2016), with the use of horse-based (*Equus caballus*) diets particularly prevalent for felids (Kerr et al. 2013b; Lefebvre et al. 2020). Commercial horsemeat diets are highly digestible and tend to be nutritionally balanced (Kerr, Beloshapka, *et al.*, 2013; Vester *et al.*, 2010). However, raw-meat diets have been linked to gastrointestinal (GI) disturbances in captive felids around the world, including tigers (*Panthera tigri*s) and cheetahs (*Acinonyx jubatus*) (see section 1.4.1 Diet-related gastrointestinal health disorders) (Seidel and Wisser 1987; Siefert and Muller 1987; Whitehouse-Tedd et al. 2015).

Over the years, zoological collections have acknowledged the importance of nutrition to promote optimal health of the species under their care (Haberstroh et al. 1984; Duckler and Binder 1997; Bechert et al. 2002; Mellor et al. 2015). For example, a previous study with captive cheetahs suggested that the use of a whole rabbit diet (*Oryctolagus cuniculus*), compared to raw-beef meat (*Bos taurus*) only diet, can prove beneficial for the GI function and overall health of this species (Depauw et al. 2011). Among the reported benefits were improved faecal consistency, lower concentrations of faecal putrefactive compounds and lower concentrations of faecal inflammatory markers (Depauw et al. 2011, 2014).

Strict carnivores, such as tigers, have evolved to cope with a high protein diet (Zoran 2002; Clauss et al. 2010; Kim et al. 2016). However, in captivity, concern over the detrimental impact of protein fermentation on the gut health of carnivorous species has been raised (Macfarlane and Macfarlane 1997; Vester et al. 2010a; Depauw et al. 2011). End-products of protein fermentation (e.g. phenol, indole, ammonia) have been linked with gastrointestinal inflammation, chronic kidney disease and cardiovascular conditions (as reviewed by Ramezani and Raj, 2014 and Wing *et al.*, 2015). Since renal and GI inflammatory conditions are commonly reported in captive tigers (Siedel and Wisser 1987; Srivastav and Chakrabarty 2002; Junginger et al. 2015), diets that promote lower concentrations of these detrimental putrefactive compounds are of interest.

Researchers have tried to elucidate the role of dietary fibre in carnivores' intestinal function and its possible health benefits. Several studies in domestic cats and other felid species including ocelots (*Leopardus pardalis*), cheetahs, jaguars (*Panthera onca*), leopards (*Panthera pardus*), lions (*Panthera leo*), pumas (*Puma concolor*) and tigers have demonstrated that the addition of plant-derived fibres can improve faecal consistency, enhance nutrient absorption and digestibility (Sunvold et al. 1995a; Bueno et al. 2000b; Fekete et al. 2004; Hernot et al. 2009; Bennett et al. 2010; Vester et al. 2010a; Prola et al. 2010; Verbrugghe et al. 2010; Kerr et al. 2012; Rochus et al. 2013; Iske et al. 2016). In addition, fibre promoted the production of beneficial compounds such as short-chain fatty acids (SCFA) that can provide overall energy as well as a primary metabolic substrate for enterocytes, while decreasing harmful fermentation end-products like indole, phenol, and p-cresol (Bueno et al. 2000a; Vester et al. 2010a; Rochus et al. 2013).

Since the natural diet of strict carnivores contains negligible plant-fibre components (sans prey GI tract contents), particular interest has been given to understanding the functional role of 'animal fibre', the low or non-

digestible glycoproteins found in the skin, fur, feathers, bone and cartilage of whole prey (Banta et al. 1979; Burrows et al. 1982; Depauw et al. 2011). Some authors have proposed that animal fibre could play a similar role in carnivore digestive physiology as that of plant-based fibres in herbivores (Depauw et al. 2012; Kerr et al. 2013b). Therefore, it could be hypothesized that a diet containing whole prey will positively modify GI parameters by minimizing the formation of putrefactive compounds and promoting the production of beneficial metabolites such as SCFA.

The objective of the current study was to describe the differences in apparent total tract macronutrient and energy digestibility, faecal characteristics, SCFA and fermentation end-products (e.g. indole, phenol and p-cresol) concentrations in captive tigers fed two of the most common dietary regimens in North-American zoos (Lefebvre *et al.,* 2020): a raw-horsemeat only diet and a diet with 20% added whole prey.

2.2 Material and Methods

2.2.1 Experimental design and diets

This study was performed in a zoological institution located in Tampa, Florida (USA) from October 2017 to January 2018. The project was approved by the Ethical Panel of the School of Veterinary Medicine & Science, University of Nottingham (UK), authorization number 1681-160215. Eight adult captive tigers were part of a randomised crossover feeding trial. Detailed information on tigers' demographics, housing conditions and feeding routine can be found in Chapter 4, section 4.2.1 Animals, enclosures, and management. Tigers were assigned to one of two groups (A or B); each group was offered either the Control Diet (CD) or the Experimental Diet (ED) for 8 weeks after which tigers were offered the

opposite diet (see Figure 2.1). However, one tiger refused to consume the ED and was fed the CD throughout the 16 weeks of feeding trials. The CD consisted exclusively of a commercial supplemented raw horsemeat-based diet, the Toronto Feline Diet, (Milliken Meat Products Ltd, Ontario Canada), while the ED contained the commercial supplemented raw horsemeat-based diet (80% of total daily intake as offered), with the addition of whole rabbit carcasses (20% of total daily intake as offered). Rabbits were farmed and locally sourced; the rabbits' stomach and intestines and their contents were removed before being offered to the tigers. To ensure that each tiger consumed the allocated amount of whole rabbit required, rabbits were categorized into small (<0.544 kg), medium (0.544-1.2 kg) or large (>1.2 kg) based on body weight. Large rabbits tended to exceed the inclusion rate needed for most tigers, hence a mixture of small and medium rabbits was used to supply the 20% inclusion rate needed for the ED for each tiger (see Figure 2.2).

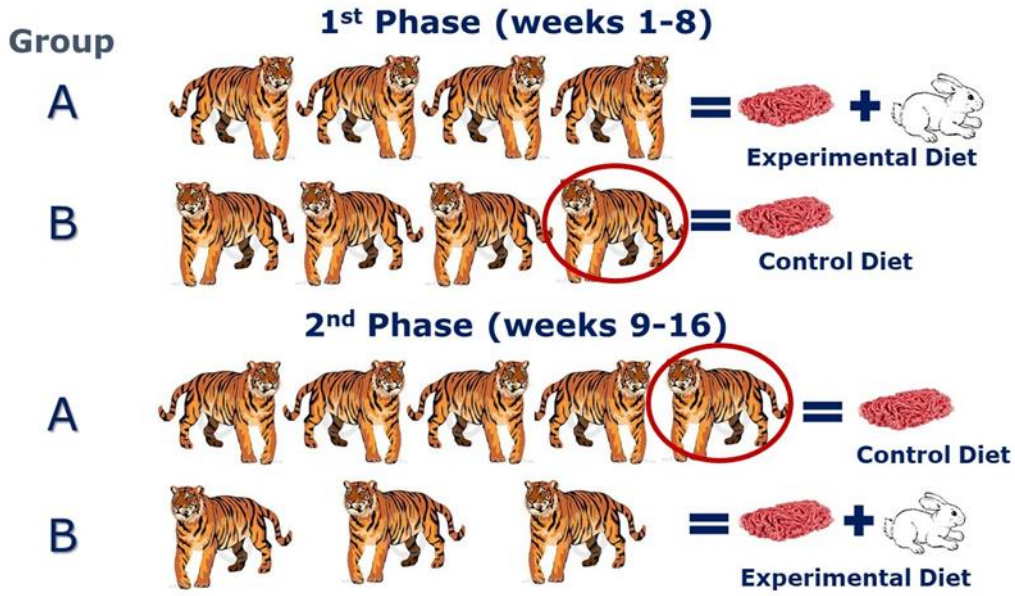

Figure 2.1 Diagram of the experimental design (the tiger circled in red refused to consume the Experimental Diet and was fed the Control Diet throughout the 16 weeks of the experiment).

Figure 2.2 Classification of whole rabbits according to body weight.

2.2.2 Faecal consistency score, faecal pH

Scats were collected during the morning cleaning routine hence time after voiding was estimated as maximum 24 h. Faeces were identified, faecal consistency scored, and scats were photographed prior to collection. Consistency scores were based on the 5-point scale recommended by the Felid Taxon Advisory Group (TAG) as follows: 1 = hard, dry pellets easy to crumble; 2 = very firm with minimal moisture and more than one pellet; 3 = pliable and formed surface, faecal units retain shape; 4 = moist stool, occurs in piles or spots, unformed and 5 corresponded to watery liquid

stools with a minimal texture that can be poured (Felid TAG 2014). Some scats had both a firmer and a looser consistency in different sections of the same scat (see

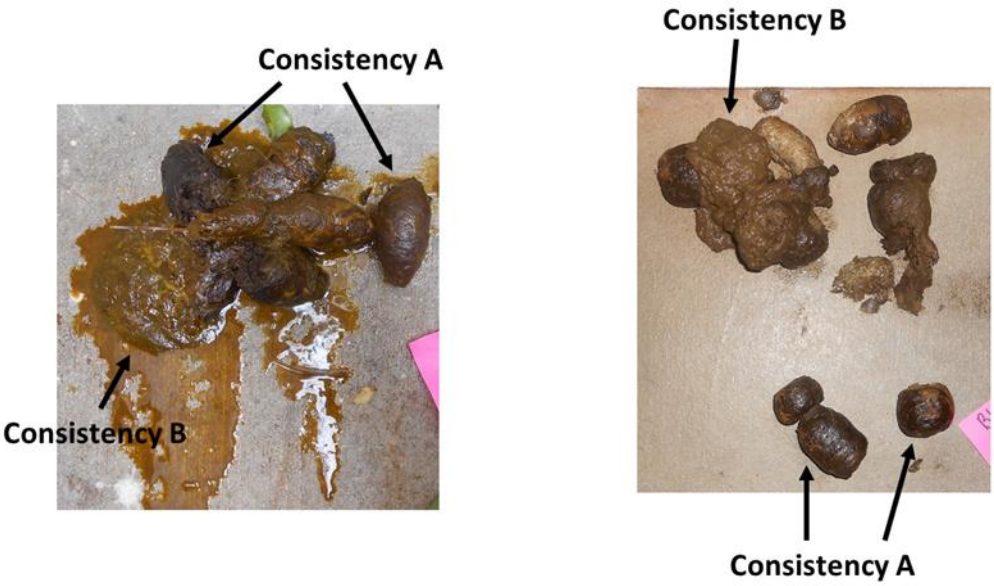

Figure 2.3); for which each consistency was scored independently (i.e. consistency A and consistency B). These dual consistency scats were not considered for the analyses described in this chapter. Faecal pH was determined from the core of the scat using a portable pH-meter (model ST20, Ohaus Corporation, Parsippany, NJ, USA).

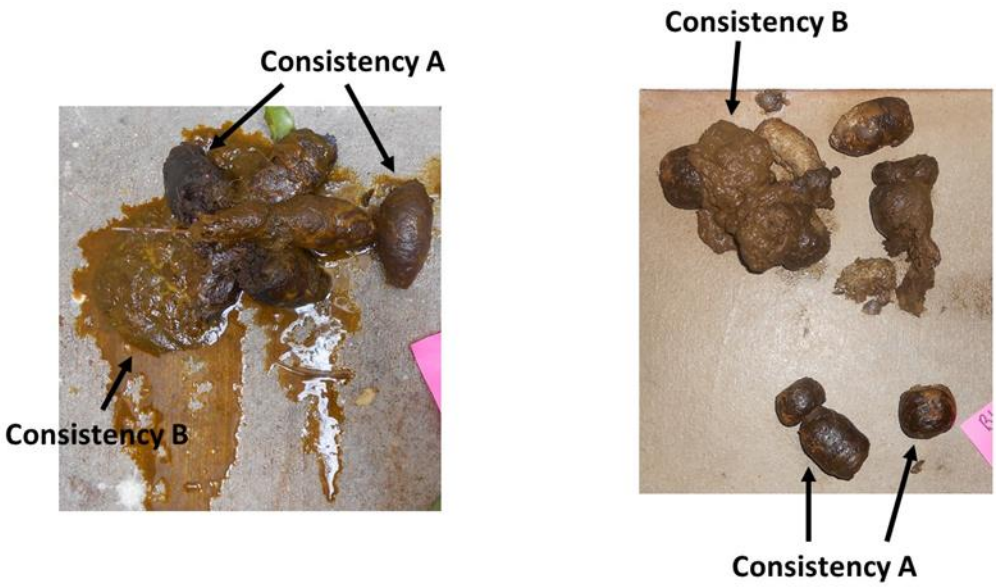

Figure 2.3 Dual faecal consistency scoring system for captive tigers (*Panthera tigris*).

2.2.3 Proximate nutrient composition

Samples of rabbits and commercial horsemeat-based diet were collected prior to the dietary intervention to establish dietary provision and throughout the 16 weeks of the experiment to confirm nutrient intake. In total, 5 samples of the commercial horsemeat and 12 samples of whole rabbit (of which 5 corresponded to small rabbits and 7 to medium rabbits) were analysed for nutrient composition. The proximate nutrient composition of the ED was calculated mathematically by considering the results of both ingredients (i.e., the commercial horsemeat and the whole rabbit) and based on the inclusion rates of each ingredient. Rabbits were thawed at room temperature for 3 hours, after which stomach, intestines and their contents were manually removed, before being ground using a meat grinder model 4346 (Hobart Corp., Troy, OH, USA). Once particle size was smaller than 1 cm, the minced rabbits were thoroughly mixed to homogenise the sample before collecting an aliquot of 200 g in a polyethene plastic bag. To obtain a sample of the commercial diet, the contents of the package unit (i.e. 2 kg bag) were manually mixed, and a

200 g sample was collected in a polyethene plastic bag. Rabbit and commercial diet samples were then stored at -4°C until shipped for analysis (by overnight courier in a cooler with cold packs). Food and faecal samples were analysed for proximate nutrient composition in a commercial laboratory (Dairy One, Inc.; Ithaca, NY, USA) using the methods below as described by the Association of Official Analytical Chemists (AOAC 1990).

Ash was determined by combustion at 600 °C (method 942.05), dry matter was determined by drying to a constant weight at 135°C (method 930.15). Crude protein (CP) was determined by combustion using a Carbon/Nitrogen Determinator model CN628 (Leco Corporation, St Joseph, MI, USA) (method 992.15). Crude fat (CF) was analysed following the crude ether extraction method (method 2003.05). The acid/alkaline titration method (method 973.18) was used to determine acid detergent fibre (ADF). Neutral detergent fibre (NDF) was determined using the AOAC method (AOAC 1990; Van Soest et al. 1991). Finally, gross energy (GE) was determined using a bomb calorimeter model C2000 (IKA Works, Inc., Wilmington, NC, USA) (Henken et al. 1986).

2.2.4 Digestibility

Tigers were fed separately; food offered was weighed daily throughout the 16 weeks of trial. Uneaten food was rarely detected and assumed to have equivalent composition to ingested portions; hence it was not analysed for nutrient composition. Nutrient intake was therefore calculated based on the amount of food offered and considering the average nutrient composition of the ingredients of the diets (see section Proximate nutrient composition for details). Since some tigers were group-housed and/or external enclosures were shared by all the individuals, non-toxic plastic glitter (Colorations®, Discount School Supply, Carol Stream, IL, USA) was used as an indigestible marker for identification of faecal sample origin (Fuller et

al. 2011; Hogan et al. 2011). Each tiger was assigned a different colour to enable identification of individual tigers' stools; 1 g of plastic glitter was mixed with the horsemeat and fed daily throughout the experiment (see Figure 2.4).

After allowing 21 days of adaptation, a modified protocol for determining apparent total tract digestibility was implemented. Over three consecutive days, total intake and total faecal outputs were weighed and recorded for each tiger. Due to the complexity of the outdoor enclosures, tigers had to be kept in the indoor enclosures to ensure the total collection of all scats produced. For welfare reasons, the use of a standard 5-day total collection was therefore not possible, hence the modification to a 3-day collection. Whole scats were collected using plastic polyethene bags, avoiding external contamination with sand, dust or vegetation. Samples were stored at -20 °C until the digestibility trial was over, after which all faecal samples were freeze-dried to a constant weight (Model 2000, Freeze Dry Company, Inc., Nisswa, MI, USA). Once faeces were dried, all samples for each tiger were pooled and homogenised to create one single sample per tiger per diet/trial (Iske *et al.*, 2016; Kerr *et al.*, 2012; Vester *et al.*, 2008). Finally, a 200 g aliquot from the pooled sample was collected for each tiger and stored at −20 °C until transported to an external laboratory for nutrient composition analysis (Dairy One, Inc.; Ithaca, NY, USA). Apparent total tract macronutrient digestibility was calculated using the following equation (all parameters on a DM basis) (Vester et al., 2010):

$$\text{Apparent total tract digestibility} = \frac{\text{nutrient intake - nutrient output}}{\text{nutrient intake}} \times 100$$

Figure 2.4 Plastic glitter colours used to identify scats from tigers housed in groups or sharing outdoor enclosures. Each tiger was assigned a specific colour. One g of glitter was mixed daily with the commercial supplemented raw meat diet throughout 16 weeks.

2.2.5 Time of first appearance

Corn kernels and plastic glitter, with different colours from those used to differentiate between individual tigers, were used as non-digestible, non-absorbable markers to determine the time of first appearance (TFA): TFA-marker (see Figure 2.5). The TFA was measured over three consecutive days, concurrent with the modified total tract apparent digestibility trial, following a variation of the methods described by Peachey, Dawson and Harper, (2000) and Loureiro *et al.* (2016). Individual tigers were offered a bolus comprising 1 g of TFA-marker mixed in 30 g of commercial supplemented feline diet, immediately followed by the remainder of their daily portion. The time of the TFA-marker administration was recorded, and tigers were monitored continuously for three consecutive days (i.e. 72 h)

using video equipment (with infra-red light for overnight observation) to determine the exact times of defaecation. All faeces voided after TFA-marker administration were collected for each individual. The aforementioned stools were thoroughly examined to ascertain the presence of the TFA-marker colour combinations (see Figure 2.5). The calculated TFA corresponded to the time interval between the ingestion of the marked food and the time of defaecation of any stools containing the TFA-marker assigned to the tiger for this trial (Peachey, Dawson and Harper, 2000). TFA was measured in each tiger over three consecutive days during each dietary treatment to account for individual variability.

TFA group	Tiger	Colour ID	Day 1 Food ingredient: Horsemeat	Day 1 Food ingredient: Rabbit	Day 2 Food ingredient: Horsemeat	Day 2 Food ingredient: Rabbit	Day 3 Food ingredient: Horsemeat	Day 3 Food ingredient: Rabbit
1	5	Green	Turquoise	Orange	Corn kernel	Gold	Violet	Green
	8	Red						
	9	Silver						
	4	No colour						
2	1	Violet	Gold	Orange	Corn kernel	Silver	Green	Turquoise
	2	Blue						
	6	No colour						
	7	Red						

Figure 2.5 Schematic representation of markers used for time of first appearance (TFA) trial. Individual tigers were offered a bolus comprising 1 g of this special plastic glitter or corn kernel mixed in 30 g of the commercial supplemented feline diet. Tigers 4 and 7 were housed in a different location within the zoo while the rest of the tigers shared outdoor enclosures.

2.2.6 Short-Chain Fatty Acids

Faecal SCFA were measured in fresh faecal samples collected within one hour after defaecation. Faeces were homogenised before collecting three 5g subsamples, the first for determination of DM, the second for SCFA and end-products concentrations and the last as a backup. All samples were stored at -20°C before overnight shipment on dry ice, then maintained at –20°C until analysed. Determination of faecal SCFA concentrations was performed at the Laboratory for Animal Nutrition and Animal Product Quality, Ghent University (Ghent, BE). Samples were thawed at room temperature for 15 min prior to analysis. One g of faecal material was then transferred to a plastic centrifuge tube containing 5 ml of 10% formic acid and internal standard (10 mg 2-ethyl butyric acid/ml formic acid) (Chaney and Marbach 1962). Plastic tubes were shaken for 5 min at maximum speed in a Unimax 2010 shaker (Heidolph Instruments GmbH & CO. KG; Schwabach, GER), after which samples were centrifuged for 15 min at 15,000 rpm at 4°C (Avanti J-E High-Performance Centrifuge No. 369003, Beckman Coulter, Indianapolis, USA) and supernatants were filtered using glass wool. Gas chromatography analysis followed the method described by Castro-Montoya *et al.* (2012) using a gas chromatographer Agilent 7890A with autosampler and a 30 m x 0.25 mm (internal diameter) and 0.25 µm column (Agilent Technologies, Inc., Wilmington, USA).

2.2.7 End-products

Determination of indole, phenol and p-cresol was performed at the Department of Veterinary Public Health and Food Safety, Ghent University (Merelbeke, Belgium). Modifications to the method reported by Depauw *et al.* (2011) are described below. End-products were extracted from 0.25 g thawed faecal samples by adding 40 µL of internal standard (100 ng µL-1

5-methyl indole) and 1960 µL of methanol. Extracts were vortexed for 30 sec, ultrasonically vibrated for 10 min, rotated for 10 min, and finally centrifuged for 10 min at 9000 rpm. Supernatants were collected and reduced to a volume of 200 µL by evaporation under nitrogen at a temperature of 40 °C. A sample of 60 µL of the concentrated supernatant was collected and diluted in 140 µL of water and centrifuged for 10 min at 9000 rpm. Finally, 10 µL of this final dilution was injected on a liquid chromatography (LC) system consisting of an Accela UHPLC pump coupled to an Accela autosampler and PDA detector (Thermo Fisher Scientific, San Jose, CA, USA). Chromatographic separation was achieved on a column model HSS-C18 kept at 40 °C (Waters, Milford, MA, USA). The mobile phase consisted of 50 mM of ammonium acetate and acetonitrile (70/30) pumped at a flow rate of 0.3 ml/min for 15 min; detection was performed at 270 nm.

2.2.8 Statistical analyses

Data were analysed using the SPSS version 24 (IBM SPSS Statistics, IBM Inc, Armonk, NY, USA). A linear mixed model with diet as fixed effect and tiger selected as random effect was used to analyse differences in faecal consistency score, faecal pH, intake (food and calories), output (faeces and TFA), nutrient digestibility (DM, crude protein, ADF, NDF, crude fat and GE), SCFA and end-products concentrations between dietary treatments (Goldstein et al. 2002; Kerr et al. 2013a; Iske et al. 2016). Results are reported as mean ± standard deviation (SD), statistical significance was set at $p < 0.05$.

2.3 Results

2.3.1 Nutrient composition, intake and output

Both dietary treatments were formulated isocalorically to maintain body weight, based on the previously calculated caloric intake for each tiger. Proximate nutrient composition and list of ingredients for ED and CD are shown in Table 2.1. Food intake (g DM/d), caloric intake (kcal/d) and faecal output (g DM/d) were not significantly different between diets ($p > 0.05$) (see Table 2.2).

Table 2.1 Nutrient composition and ingredients of the Control Diet (CD) and Experimental diet (ED) fed to eight captive tigers (*Panthera tigris*). Values reported as mean ± SD on dry matter basis (DM).

	Small rabbit	**Medium rabbit**	**Control Diet**	**Experimental Diet**
Dry matter %	27.6 ± 3.9	26.9 ± 3	31.9 ± 1.6	31.2 ± 3.2
Crude protein %	68.7 ± 5.2	66.7 ± 6.3	63.2 ± 4.3	64.2 ± 5.7
ADF %	12.9 ± 8.5	9.5 ± 4.6	8.2 ± 6	8.1 ± 6.4
NDF %	25.7 ± 9.6	25.6 ± 5.2	22.6 ± 6.7	22.7 ± 6.9
Crude fat %	14.2 ± 6.4	16.4 ± 5.9	21.5 ± 5.3	20.5 ± 5.9
Ash %	14.6 ± 5	13.3 ± 3.1	8.4 ± 0.9	9.4 ± 3.9
GE (Kcal/kg DM)	5,228 ± 358	5,351 ± 299	5,981 ± 255	5,882 ± 315
n	5	7	5	Mathematical calculation based on 5 CD and 12 rabbits
Composition (as fed)	whole rabbit <0.544 kg (stomach and intestines removed)	whole rabbit 0.544-1.2 kg (stomach and intestines removed)	100% commercially supplemented ground muscle horsemeat	80% commercially supplemented ground muscle horsemeat 20% whole rabbit (stomach and intestines removed)
Ingredient composition of the control diet: Horsemeat, cellulose, tri-calcium phosphate, Toronto Zoo Vitamin-Mineral Carnivore Premix, vitamin E, fatty acid supplement and taurine.				
ADF – acid detergent fibre; NDF – neutral detergent fibre; GE – gross energy; DM – dry matter				

2.3.2 Faecal characteristics

Faecal consistency scores were significantly lower (i.e. stools were firmer) when tigers were fed the ED (3.08 ± 0.69) compared to the CD (3.38 ± 0.77) ($p = 0.002$). Faecal pH differed significantly ($p < 0.001$) between treatments, with a more acidic pH during CD (6.58 ± 0.63) compared to ED (6.80 ± 0.68) (see Table 2.2).

Table 2.2 Food intake, faecal output, and apparent total tract nutrient digestibility of eight captive tigers (*Panthera tigris*) fed either a commercially supplemented ground muscle horsemeat diet (Control Diet) or a diet with 20% added whole rabbit (Experimental Diet). Values reported as mean ± SD on dry matter basis.

	Control Diet	**Experimental Diet**	***p***
		Intake	
Food g/d	1,187.2 ± 195.8	1,063.8 ± 99.2	0.231
Caloric intake kcal/d	7,100.8 ± 1,171.1	6,259.9 ± 583.9	0.758
		Faecal output	
Faeces g/d	288.6 ± 78.8	252.5 ± 80.4	0.056
Time of first appearance (h:min)	22:58 ± 9:26	28:12 ± 14:11	0.040
		Faecal characteristics	
Faecal score	3.38 ± 0.77	3.08 ± 0.69	0.002
Faecal pH	6.58 ± 0.63	6.80 ± 0.68	0.000
		Digestibility	
DM %	75 ± 7	76 ± 8	0.400
Crude protein %	92 ± 2	90 ± 4	0.345
ADF %	51 ± 18	40 ± 24	0.502
NDF %	68 ± 13	66 ± 13	0.224
Crude fat %	96 ± 4	97 ± 2	0.130
GE %	86 ± 4	86 ± 5	0.059

DM – dry matter; ADF – acid detergent fibre; NDF – neutral detergent fibre; GE – gross energy

2.3.3 Digestibility and time of first appearance

Apparent total tract digestibility of DM, CP, ADF, NDF, crude fat and GE did not differ significantly between diets ($p > 0.05$) (see Table 2.2). The time of first appearance was significantly different ($p = 0.04$) between diets, with a shorter period observed during the CD (22 h 58 m) compared to the ED (28 h 12 m) (see Table 2.2).

2.3.4 Short Chain Fatty Acids

The total concentration of SCFA was 505.9 ± 101.1 µmol/g of DM for the CD and 497.7 ± 109.5 µmol/g of DM during the ED. No significant difference in faecal SCFA concentration was detected between dietary treatments for acetate, butyrate, isovalerate, isobutyrate and total SCFA ($p > 0.05$) (see Table 2.3). Faecal concentrations of propionate were significantly higher ($p = 0.03$) during CD (94.9 ± 24 µmol/g of DM) compared to ED (72.9 ± 31.4 µmol/g of DM). Molar ratios of SCFA (acetate:propionate:butyrate) were similar between diets: 65:20:15 for CD and 68:15:17 for ED. Acetate to propionate ratio (A/P) corresponded to 3.3 for CD and 4.4 for ED.

2.3.5 End products

Concentrations of faecal indole and phenol showed no significant differences ($p >0.05$) when tigers consumed the ED (107.5 ± 33 µg/g of DM, 89.1 ± 82.4 µg/g of DM respectively) compared to when fed the CD (92.1 ± 28.9 µg/g of DM, 272.2 ± 213.1 µg/g of DM respectively). Faecal concentrations of p-cresol were numerically lower during ED (44.6 ± 12.7

µg/g of DM) compared to CD (67.6 ± 49.7 µg/g of DM) but failed to reach significance (p = 0.052) (see Table 2.3).

Table 2.3 Faecal short-chain fatty acid (µmol/g of DM) and fermentation end-products concentrations (µg/g of DM) of eight captive tigers (*Panthera tigris*) fed a commercially supplemented ground horsemeat only diet (Control Diet) or a diet with 20% added whole rabbit (Experimental Diet). Values reported as mean ± SD on dry matter basis.

	Control Diet	**Experimental Diet**	***p***
	SCFA (µmol/g of DM)		
Acetate	313.3 ± 65.7	319.9 ± 81.8	0.608
Propionate	94.9 ± 24	72.9 ± 31.4	0.03
Butyrate	72.1 ± 21.3	77.7 ± 23.4	0.428
Isovalerate	16 ± 3.5	17.6 ± 4.2	0.112
Isobutyrate	9.4 ± 2.3	9.6 ± 2.2	0.822
Total SCFA	505.9 ± 101.1	497.7 ± 109.5	0.259
	End-products (µg/g of DM)		
Indole	92.1 ± 28.9	107.5 ± 33	0.129
Phenol	272.2 ± 213.1	89.1 ± 82.4	0.104
p-cresol	67.6 ± 49.7	44.6 ± 12.7	0.052

Total SCFA = acetate + propionate + butyrate + isovalerate + isobutyrate + valerate + caproate
SCFA- short-chain fatty acids; DM- dry matter

2.4 Discussion

The present study evaluates differences in total tract apparent digestibility of macronutrients and energy, faecal fermentation profiles, stool characteristics and time of first appearance in a group of captive tigers fed either a raw horsemeat diet or a diet containing added whole prey. Both dietary treatments in this study resulted in similar apparent digestibility of DM, CP, ADF, NDF, crude fat and GE; values obtained were in agreement with previous studies conducted in dogs, domestic cats, and other captive felid species for these nutrients (Bueno et al. 2000b; Li et al. 2006; Zhihong

et al. 2007; Vester et al. 2008, 2010a; Prola et al. 2010; Depauw et al. 2011; Hooda et al. 2012; Kerr et al. 2013b; Iske et al. 2016).

Most digestibility studies conducted with captive non-domestic felids and domestic cats focused on evaluating changes associated with protein sources (i.e. beef vs horse) or protein:carbohydrate ratios. Only a few authors have investigated the effects of the type or concentration of plant-based fibres in the diet of felids (Vester et al. 2010a; Kerr et al. 2013b). To date, metabolic changes and possible health benefits associated with the use of animal fibre in captive carnivores diets have only been studied in cheetahs (Depauw *et al.*, 2011, 2014), Arctic fox (*Alopex lagopus*) (Zhang et al. 2014), American mink (*Neovison vison*) and silver fox (*Vulpes vulpes*) (Gugołek et al. 2015).

2.4.1 Crude protein

Protein quantity and/or quality, the presence of fibre and even storage conditions have been listed as factors influencing protein digestibility (Fekete et al. 2004; Becker and Yu 2013; Hamper et al. 2015). Despite differences in ingredients, CP levels were similar between diets fed in this study, and apparent CP digestibility of both diets (~90%) was within ranges (91 to 95.1%) previously reported from other studies with Malayan (*Panthera tigris jacksoni*), Amur (*Panthera tigris altaica*) (Kerr *et al.*, 2013; Vester *et al.*, 2008; Vester *et al.*, 2010) and Indochinese (*Panthera tigris corbetti)* tiger subspecies (Vester *et al.*, 2008). The inclusion of 20% whole rabbit to the diet of captive tigers did not affect apparent digestibility of CP.

High inclusion rates of whole prey (i.e. 100%) have been associated with lower apparent CP digestibility in domestic cats and ocelots compared to raw meat diets (Kerr *et al.,* 2014; Bennett, Booth-Binczik and Steele,

2010). In these studies, reduced digestibility was attributed to the higher amount of animal fibre in whole prey items compared to raw muscle diets. It is well documented that bones, tendons, ligaments, cartilages, skin and hair– i.e. components known as 'animal fibre'– are poorly digested compared to muscle meat (Bowland and Bowland 1991; Depauw et al. 2012; Zhang et al. 2014). These animal fibre components can correspond to approximately 25% of a rabbit's weight (Pla 2008; Zeferino et al. 2013; Khan et al. 2016; Moumen and Melizi 2017). Previous research has confirmed that even within whole prey, differences in digestibility can occur. For example, small prey tends to be less digestible (45-55%) than larger prey (>60%) (Jethva and Jhala 2004; Bennett et al. 2010). Researchers believe that small prey has higher surface-area-to-mass-ratio (i.e. higher proportion of animal fibre components) compared to larger prey, which may explain the difference in digestibility observed between prey sizes (Jethva and Jhala, 2004). The present results suggest that whole prey added at a common inclusion rate for North American zoos (i.e. 20% as fed) did not decrease apparent CP digestibility.

Low protein digestibility is among the concerns of feeding captive carnivores because of its possible detrimental effect on health (Clauss et al. 2010; Depauw et al. 2011). When proteins are not digested and absorbed in the small intestine, they become available for fermentation in the large intestine (Macfarlane and Macfarlane 1997; Hall et al. 2013). Protein fermentation generates a wide range of metabolites, some of which can be harmful and have been linked with increased GI inflammation, colorectal cancer, and metabolic diseases (as reviewed by Davila *et al.*, 2013; Diether and Willing, 2019). Despite the modest numerical difference, results from the current study suggest that the addition of 20% whole rabbit had no detrimental effect on CP digestibility. Hence, it is likely that the

amount of protein available for fermentation by intestinal microorganisms was similar between diets.

Bacterial fermentation activity may also contribute to apparent CP digestibility values. Evidence from previous studies suggests that dietary components, such as fibre, can modify microbial abundance in the large intestine of carnivores (Zhang et al. 2014; Deb-Choudhury et al. 2018; Butowski et al. 2019). If a higher number of bacterial protein are abraded from the GIT, underestimation of protein digestibility is likely to occur (Verbrugghe et al. 2010; McDonald et al. 2011). However, faecal bacterial abundance did not differ significantly between dietary treatments (data not shown); therefore, the fermentative activity could have been comparable between dietary treatments and contributed to CP digestibility at similar rates.

Finally, considerable amounts of tiger hair found in the faecal samples are another factor that could influence CP apparent digestibility. Tiger hair ingested while grooming may have differed between dietary treatments, contributing to the minor numerical difference in apparent protein digestibility observed between dietary treatments. Yet, behavioural observations found similar time spent grooming between ED and CD (see results section Chapter 4). Although the amount of hair was not quantified, possible tiger hair contribution did not seem to be sufficient to elicit detectable differences between diets or to underestimate CP digestibility.

2.4.2 Crude fat

No significant difference in apparent digestibility of crude fat was observed between diets. The fat digestibility coefficients of this study were higher than those previously reported in Malayan and Amur tigers which ranged

from 84% to 94.1% when fed a commercial raw beef-based or horse-based diet respectively (Kerr *et al.*, 2013; Kerr, Morris *et al.*, 2013; Vester *et al.*, 2010) but similar to those reported in Indochinese and Amur tigers fed a commercial raw beef-based diet (96.1% and 96.2% respectively) (Vester *et al.*, 2008).

Longer retention times are associated with increased fat digestibility (Edwards and Ullrey 1999; Peachey et al. 2000; Van Weyenberg et al. 2006). In my study, TFA corresponded to an average of 22 h during the CD and 28 h during the ED, hence dietary fats could have been exposed to enzymatic breakdown for a longer period than in Kerr and Vester's studies (Van Weyenberg, Sales and Janssens, 2006). These previous studies did not measure passage rate; hence I cannot confirm if retention times could explain the numerically higher crude fat digestibility observed in the present study.

Another possible explanation for the results obtained could be differences in fibre sources across studies. The two types of plant fibres used in previous studies were beet pulp (commonly added in beef-based diets) and cellulose (commonly used in horse-based diets). Kerr *et al.* (2013) reported a significantly lower fat digestibility in captive felids fed beef diets with beet pulp compared to beef diets with cellulose. On the contrary, Vester *et al.* (2010) found no difference in fat digestibility in tigers fed a beef diet containing beet pulp compared to a horse diet containing cellulose. In Vester *et al.*'s (2008) study with captive tigers fed a beef diet containing beet pulp, fat digestibility was even higher than that reported by Kerr *et al.* (2013) in captive felids fed the same beef diet with beet pulp. Since protein sources and nutrient composition also varied across studies, it is hard to determine whether the type of fibre used was the only factor possibly affecting fat digestibility. Their results could be attributed to dietary factors including protein source, macronutrient composition, or fibre

source, among others or associated physiological aspects (overall gut health, passage considerations, microbiome).

The high digestibility of fat observed in the present study could be due to an adaptation of tigers to efficiently use dietary fat. Vester et al. (2008) found that tigers had significantly higher fat digestibility coefficients than other felid species such as jaguars or cheetahs. Similarly, domestic cats and ocelots appear to be highly efficient in fat digestion (digestibility coefficients >91%) (Bennett et al. 2010; Prola et al. 2010; Hall et al. 2013; Hamper et al. 2015). Felids are considered strict carnivores, therefore protein and fat are key components of their caloric intake (Morris 2002; Zoran 2002). It is believed that the dietary fat needs of felids are related to their gluconeogenic activity (where energy is provided from fat and amino acids rather than carbohydrates), which could explain the high digestibility coefficients reported in these species (Zoran 2002; Rochus et al. 2013; Verbrugghe and Hesta 2017). The fat digestibility observed in these tigers (which was not affected by the addition of 20% whole rabbit) should be taken into account during diet formulation, to avoid an excessive provision of calories that could lead to obesity. Further research is needed in captive felids to determine the role of different fibre sources on fat digestibility and to corroborate possible species differences in intake efficiency of this nutrient category.

2.4.3 ADF and NDF

A range of analytical techniques is available to measure dietary fibre fractions, all of which were developed for plant-based ingredients (Jeraci and Van Soest 1990). For the current study, I had access to the methods developed by Van Soest and collaborators (1991). It was hypothesized that by adding whole prey, fibre quantities would differ between diets, yet ADF

and NDF levels of ED and CD were quite similar. Higher concentrations of ADF for the ED were expected because components of rabbit carcass, such as hair, can reach concentrations of up to 71% of DM (Depauw *et al.,* 2012). The low overall inclusion rate of rabbit could have been responsible for the similarity in fibre content, since mean ADF values for whole rabbit corresponded to 10.9%. In addition, the CD contained added cellulose, a fibre source with reported ADF values of 56% of DM (Depauw *et al.,* 2012). To obtain a difference in fibre concentrations, a higher rabbit inclusion rate would have been needed; nevertheless, the aim of this project was to assess the possible benefits of the most common whole prey inclusion rate in North American zoos which corresponds to ~20% (Lefebvre *et al.*, 2020). ADF and NDF assays were originally developed to quantify cellulose, lignin and hemicellulose content of forages (Jeraci and Van Soest 1990; Prosky et al. 1994); hence, they might not accurately represent other non-digestible components such as (glycol)proteins found in animal fibre (Depauw *et al.,* 2011, 2012). To date, no validated assay to quantify fibre from animal-based ingredients exists, therefore determination of these components is still reliant on methods developed for plant material (Cools et al. 2014). However, it is believed that ADF content of animal-based products could correspond to the insoluble fractions of plant-based ingredients (i.e. cellulose and lignin) and could predict the fermentability of animal substrates (Depauw *et al.,* 2011, 2012).

In my study, regardless of the diet, the digestibility of ADF was lower than that of NDF. Similar results have been described in sows fed a corn-soybean commercial diet, with lower digestibility of ADF compared to NDF (Niu et al. 2019). A possible explanation for such results could be the presence of tiger hair found in the faeces. As already mentioned, hair is expected to have high ADF levels; with higher concentrations reported for dog hair (91% ADF) than rabbit hair (71% ADF) (de-Oliveira et al. 2012;

Depauw et al. 2012). If considerable amounts of tiger hair were ingested during grooming, they could have been detected in the faeces, therefore, underestimating the digestibility of both fibre fractions. Similarly, de-Oliveira *et al.* (2012) reported low ADF and NDF digestibility in dogs fed commercial extruded diets. They attributed their results to increased ADF and NDF in faeces– originating from hair and microbial constituents detected by the fibre analyses– which resulted in a lower apparent digestibility of both fibre fractions. Previous studies with domestic cats or captive felids that have quantified fibre used different methodologies reporting total dietary fibre, soluble/insoluble fibre fractions or crude fibre (Sunvold et al. 1995a; Bueno et al. 2000a; Fekete et al. 2004; Kerr et al. 2013a). The use of different methods to the ones presented in the current study limit the ability to properly compare fibre digestibility provided across studies. Since tiger hair was not quantified in the current study, its possible impact on the digestibility coefficients observed cannot be confirmed.

Another possible explanation for the low ADF and NDF digestibility in tigers could be that, following gastrointestinal digestion and post-gastric fermentation, fibre fractions are more easily detected in faeces than in the raw diets. In domestic cats, differences in fibre digestibility have been demonstrated with lignin + cellulose being less digestible than cellulose alone (Fekete *et al.,* 2004). In vitro fermentation using cheetah faecal inoculum showed differences in fermentation patterns across substrates with rabbit skin producing more gas (an indicator of fermentability) than rabbit hair and cellulose (Depauw *et al.,* 2011). Hence, either digestibility of both fibre fractions is low or the digestive and fermentation processes in the GIT make these compounds more prone to be detected by the detergent extraction methods, overestimating their faecal values.

Finally, grass ingested by tigers could have influenced the fibre fraction digestibility results. Although not quantified, considerable amounts of grass

were observed in tigers' faeces. Grass was only observed in 10 of the 28 scats collected during the modified apparent total tract digestibility protocol. It is possible that grass ingested contributed to an overestimation of faecal ADF and NDF, which in turn could have decreased fibre fraction digestibilities. The mean faecal ADF content when tigers were fed the ED was 20.3% versus 16.6% during the CD feeding period, while mean faecal NDF corresponded to 32.6% for ED and 29.1% for CD treatments. Since the exact amount of grass present in the faeces was not assessed, it is not possible to determine their contribution to the digestibility results observed during the present study. Previous studies evaluating digestibility of large felids have not reported the presence of ingested plant/foliage materials (Zhihong et al. 2007; Vester et al. 2008; Kerr et al. 2013b). It will be interesting to know if such behaviour is common among other species or was a peculiarity of this study's population. Future research should consider determining quantities and analysing the proximate nutrient composition of extraneous materials found in faeces to better understand their influence on apparent total tract nutrient digestibility coefficients.

2.4.4 Energy

Dietary treatments were calculated to be isocaloric. Apparent digestibility of energy was not affected by the addition of whole prey in the diet and was within the range previously reported in tigers fed meat-based diets (84-97%) (Li et al. 2006; Kerr et al. 2013b). Results found in the present study were similar to that of Malayan and Amur tigers fed with raw beef diet (Kerr, Morris *et al.*, 2013; Kerr *et al.*, 2013) but lower than those reported on Malayan, and Amur tigers fed a commercial horsemeat diet (Vester *et al.*, 2010; Kerr *et al.*, 2013) or Indochinese tigers fed a beef diet (Vester *et al.*, 2008). Such variations in energy digestibility could be due to

differences in GE content or macronutrient composition across diets used, individual animal behaviours/energy outputs or could reflect actual subspecies differences in metabolism or energy utilisation.

These findings have implications for the weight management of captive tigers. Obesity is a major health concern in zoo and domestic animals (Altman et al. 2005; Lamberski 2015; Tilson et al. 2016). Along with reduced physical activity, dietary fat and energy density have been associated with weight gain leading to obesity (Deng et al. 2014; Backus and Wara 2016; Tarkosova et al. 2016). A negative correlation between energy and total dietary fibre has been reported (Owens et al. 2014; Hours et al. 2016). Furthermore, Prola *et al.*, (2010) found a significant decrease in energy digestibility of domestic cats fed a commercial diet supplemented with 4% cellulose compared to the control diet without cellulose. Diets with high fibre content are considered lower in energy density because fibre increases volume yet adds negligible calories (Owens *et al.*, 2014). For this reason, high fibre diets are commonly employed for the treatment and prevention of obesity in domestic dogs and cats (Laflamme 2012; Hamper 2016). Current results showed that, at a 20% inclusion rate, the GE apparent digestibility was not affected by the whole rabbit portion of the diet, hence suggesting that, if animal fibre is to be included in the diet for weight management purposes, a higher inclusion might be needed. However, it is always possible that animal fibre can contribute to preventing obesity in captive felids through other means, such as by influencing satiety (Altman et al. 2005; Bosch et al. 2009; Backus and Wara 2016); to document this assumption, behavioural observations were undertaken; Results are presented in Chapter 4.

2.4.5 Fermentation profiles

Short-chain fatty acids are considered beneficial products of bacterial fermentation, particularly butyrate, which has been linked with colonocyte proliferation and anti-inflammatory properties (Brosey et al. 2000; Bueno et al. 2000b; Den Besten et al. 2013). Previous studies reported similar SCFA concentrations in Malayan and Amur tigers fed a commercial horsemeat diet; while tigers fed beef-based diets had higher concentrations of faecal SCFA compared to this study's results (Vester *et al.* 2008; Vester *et al.* 2010). The difference in total SCFA concentrations between beef and horsemeat diets could be due to the type of fibre present. In commercial diets for captive felids, beet pulp is commonly added to beef-based diets, while microcrystalline cellulose is the fibre of choice for horse-based products. Beet pulp is considered a moderately fermentable source of fibre, producing higher in vitro SCFA concentrations compared to cellulose, a non-soluble, low-fermentable fibre (Sunvold et al. 1995b, a; Bosch et al. 2008; Barry et al. 2011). However, since CP content also differed between the horse and beef diet in Vester et al.'s (2010), the results could not be attributed exclusively to the fibre sources.

Besides fibre, protein intake and digestibility are believed to influence SCFA production (Rochus et al. 2014; Gugołek et al. 2015), yet a lack of consensus in the results obtained by previous studies can be found. For example, significantly lower concentrations of SCFA were observed in tigers fed a horse-based diet (50% CP) than those fed a beef-based diet (58% CP) (Vester *et al.,* 2010). However, the total SCFA of domestic cats did not differ across raw meat sources with different CP content (52-77% CP) (Kerr *et al.,* 2013). Similarly, in cheetahs fed whole rabbit (61% CP) or a beef-based diet (86% CP), SCFA concentrations remained similar between treatments (Depauw *et al.,* 2011). Therefore, other factors such as the interaction between macronutrients and fibre, rather than just CP

content and digestibility, must be considered. For example, Rochus *et al.,* (2013) suggested that the gelling properties of guar gum (a source of fibre) were responsible for the lower protein digestibility observed in their study with domestic cats. As a result, higher protein fermentation was observed in cats fed a diet with guar gum compared to the diet with added cellulose; their hypothesis was supported by higher concentrations of fermentation by-products in the guar gum group. Results from this study highlight the complex mechanism by which fibre acts and remind us of the interactions among nutrients and fibre– even in carnivorous species– most of which are yet to be fully understood.

In the current study, no significant difference in total SCFA concentrations between diets was observed. This finding could be a result of the similar macronutrient composition of the dietary treatments. It is possible that the inclusion of 20% whole rabbit was insufficient to trigger changes in protein fermentation and SCFA production. This study's results could also be explained by the presence of cellulose in the commercial horsemeat product used for both dietary treatments. Although considered low-fermentable, in vitro studies with cheetah faecal inoculum showed that cellulose yielded small amounts of SCFA, thus demonstrating that it can be fermented by intestinal microbiota of a strict carnivore (Depauw *et al.*, 2012). In this same study, components of rabbit carcass such as hair, skin and bones produced higher concentrations of total SCFA compared to cellulose. The low inclusion rate of rabbit in the ED could have been insufficient to promote higher total SCFA yields compared to the CD. However, in cheetahs fed a whole rabbit diet, no significant difference in total SCFA concentrations was observed compared to animals fed a raw beef diet (Depauw *et al.*, 2011). Likewise, the use of wool hydrolysate, cellulose or inulin as fibre sources in cats fed a commercial extruded diet resulted in similar concentrations of SCFA (Deb-Choudhury *et al.*, 2018). In

contrast, tigers fed a commercial horsemeat diet with cellulose (~3%) yielded significantly lower SCFA compared to those fed a commercial beef-based diet with added beet pulp (~2.5%) (Vester *et al.,* 2010). Hence, a wider range of factors (such as fibre fermentability rate, fibre solubility, protein intake/digestibility, microbiota composition and abundance, among others) rather than just fibre inclusion rate is likely to be involved in felids' fermentation profiles and must be considered (Wernimont et al. 2020).

Despite differences in total SCFA concentrations reported across previous studies involving tigers, molar ratios remained relatively constant (e.g. Amur tigers fed horsemeat: 65:22:13; Amur tigers fed beef: 65:27:8 and 67:25:8) suggesting that fermentation patterns are highly conserved within the species (Vester *et al.,* 2008; Vester *et al.,* 2010). Other species of carnivores such as cheetahs fed a beef diet and foxes fed a mixture of poultry and beef by-products also demonstrated similar molar ratios to those reported in tigers (Depauw et al. 2011; Gugołek et al. 2015). These ratios are in accordance with those obtained during the CD treatment (65:20:15) in the current study. In parallel, molar ratios from the ED treatment seemed more similar to those of cheetahs fed whole rabbit or domestic cats fed horse meat (Depauw *et al.,* 2011; Kerr *et al.,* 2013). Molecular ratios can be used to understand the fermentation patterns and microbial activity occurring in the hindgut (Depauw *et al.,* 2011). For example, acetate and butyrate are considered the main SCFA produced after protein fermentation (Macfarlane and Allison, 1986; Davila *et al.,* 2013). In the present study, acetate and butyrate concentrations were not influenced by diet ($p > 0.05$), therefore it could be hypothesised that protein fermentation occurred at similar rates between treatments.

Only propionate showed significantly lower concentrations during ED compared to CD; this change resulted in a higher A/P during ED (4.4) than during CD (3.3). A possible explanation for such a result could be

attributed to increased cellulose content in the CD. In domestic cats, significantly higher production of propionate was observed when animals were fed a diet containing cellulose compared to fructo-oligosaccharides or pectin (Barry *et al.,* 2011). The effect of animal fibre in propionate production should also be considered; protein fermentation is believed to yield lower propionate concentrations than carbohydrate fermentation (Cummings and Macfarlene 1991; Macfarlene and Macfarlene 1997). In vitro experiments with cheetah faecal inoculum showed lower propionate production of animal-based substrates (Depauw et al., 2012). Similarly, in cheetahs fed whole rabbit significantly lower propionate concentrations were observed compared to a beef diet (Depauw al., 2011). Hence the lower propionate concentrations observed during ED could be due to a decrease in cellulose and the presence of animal fibre. High A/P have been linked with lower fermentation rates in domestic cats and cheetahs; possibly because of the presence of insoluble fibres such as cellulose and carcass components (Sunvold et al., 1995; Depauw et al., 2011). To date, faecal fermentation profiles of free-ranging tigers are still unknown, hence comparisons are limited to data obtained from captive individuals or other carnivorous species. Despite the lack of statistical significance, results from the current study could reflect a possible shift towards slower CP fermentation during ED– observed by an increase in A/P– compared to CD. Whether a higher inclusion rate of whole prey in the diet of tigers could have significantly influenced the production of other SCFA remains to be investigated. Besides, the “ideal” fermentation profile for the species is yet to be established.

On the other hand, phenol, indole and p-cresol are known catabolites of protein fermentation (Macfarlane and Allison 1986; Cummings and Macfarlane 1991; Macfarlane and Macfarlane 1997). These end-products have been linked with negative health outcomes such as diarrhoea (Hang

et al. 2013), co-carcinogenic compounds (Cummings and Macfarlane, 1991), cardiovascular and renal disease (Wing et al. 2015).

In the current study, concentrations of indole and phenol were not affected by diet, whereas P-cresol concentrations tended to decrease ($p = 0.052$) during ED compared to CD. Indole concentrations found in the current study are lower than those reported in Malayan and Amur tigers fed a horsemeat diet or a beef-based diet (Vester *et al.,* 2010). This study's results resemble those obtained in cheetahs fed a whole rabbit diet and were lower than those reported for cheetahs fed a raw beef diet (Depauw *et al.,* 2011). Contrastingly, phenol concentration found in tigers fed the CD were higher than those reported in these previous studies. During ED, phenol levels were numerically lower and similar to those of cheetahs fed a whole rabbit diet (Depauw *et al.,* 2011). P-cresol concentrations in the current population of tigers were higher than those of cheetahs fed raw beef (Depauw *et al.,* 2011).

Higher concentrations of indole, phenol and p-cresol have been linked with protein fermentation (Macfarlane and Allison 1986; Lubbs et al. 2009; Davila et al. 2013; De Cuyper et al. 2017). In the study by Vester *et al.* (2010), increased concentrations of phenol and indole were reported in tigers fed a beef-based diet compared to a horsemeat diet. They attributed the changes partially to the higher CP and collagen content of the beef diet– which resulted in lower CP digestibility– compared to the horsemeat diet and concluded that tigers could benefit from a more digestible diet or a diet with lower concentrations of collagen. Likewise, in cheetahs fed a raw beef diet, a 3-fold increase in end-products concentrations was observed compared to a whole rabbit diet (Depauw *et al.,* 2011). The authors hypothesized that such difference was due to the higher CP content of beef, which was not completely digested and resulted in more substrates available for microbial fermentation in the cheetah's hindgut. Lubbs *et al.*

(2009) previously confirmed that protein quantity can influence protein fermentation, resulting in a shift of intestinal bacteria to more proteolytic species in domestic cats fed a high-protein extruded diet (53% CP) compared to a moderate-protein extruded diet (34% CP). Although the diets of the current study did not differ to the same extent as those used in Depauw *et al.*'s trial, end-products concentrations obtained when tigers were offered the ED are similar to those reported in cheetahs fed whole prey. Despite similar CP content and apparent digestibility between dietary treatments, phenol and p-cresol concentrations were numerically lower during the ED compared to the CD, suggesting a possible difference in hindgut fermentation activity. As previously proposed by Depauw *et al.* (2011), animal fibre components could act as modulators of the fermentation patterns in the large intestine of strict carnivores, yielding lower putrefactive compounds while promoting the production of beneficial SCFA. The inclusion rate used in the ED may have not been sufficient to limit protein fermentation, hence decreasing end-products concentrations (at least numerically), yet, not enough to modify the production of beneficial SCFA.

2.4.6 Faecal characteristics

Faecal consistency scoring is a commonly used method to indirectly assess GI health in many species (Murdoch 1986; Bechert 2012; Lamberski 2015; Vandeputte et al. 2016). However, some authors have suggested that it should not be used as a stand-alone parameter to evaluate GI health due to the lack of specificity, the scoring subjectivity and the wide variation across species and individuals (Eastwood, 1992; Bechert, 2012; Depauw *et al.,* 2012) hence the use of it in combination with several other parameters. In the current study, tigers fed the CD had

significantly looser stools and a wider variation in stool consistency (3.38 ± 0.77) compared to those fed the ED (3.08 ± 0.69). However, despite the outcome of statistical comparisons, the magnitude of the differences in faecal consistency score is unlikely to be of biological importance; both ED and CD mean scores were within what is considered “ideal” for the species, and dual consistencies were seen on both diets. This lack of biologically relevant difference is largely in accord with the other parameters measured here, therefore providing some support for the use of faecal consistency scoring in assessing GIT function or health. Results from the present study could suggest that the addition of at least 20% whole prey in the diet of captive tigers is unlikely to improve their faecal consistency, although there is an indication that some changes may be occurring such that higher inclusion rates may yield differences of biological significance to animal health.

From a statistical perspective rather than a biological one, the differences in faecal consistency between diets align with previous studies which have reported changes in faecal consistency associated with diet. For example, tigers had a firmer faecal consistency when fed a horsemeat diet (2.8) than a beef-based diet (3.7) (Vester *et al.,* 2010), although in this case, the differences were larger than those seen in the present study. Likewise, cheetahs fed whole rabbit had firmer faeces compared to a beef diet (2.1 vs 3.1, respectively) (Depauw *et al.,* 2011). One possible explanation for the minor changes observed in the current study is that the animal fibre could have acted as bulking material, making faeces slightly firmer (Depauw *et al.,* 2011). Nevertheless, faecal output during ED remained lower than during CD, thus these results indicate that the differences in faecal consistency cannot be attributed to the increased bulk of animal fibre components alone. If this had been the case, the bulking properties of

animal fibre components would have increased faecal output during ED compared to CD.

Another possible explanation for the difference in faecal consistency could be linked to GI transit time. Since TFA was significantly longer during the ED compared to the CD feeding periods, it is possible that a longer colonic transit time may have influenced faecal consistency by increasing the time available for fluid and electrolyte absorption (Berrnier 1997; Weber et al. 2016). However, since transit time through different zones of the GIT could not be measured in this study, this interpretation warrants further detailed investigation.

2.4.7 Limitations & future research

A limitation of this study was the reduced number of sampling days for the digestibility trial; due to husbandry circumstances, only 3 days of total sample collection could be obtained compared to 5-7 days set considered best practice (Salter et al. 1999; Sales and Janssens 2003; Hamper et al. 2015). The shorter sampling time could have increased the effect of any outlier value in the samples and reduced the representativeness of the dietary performance. Nevertheless, to counter this limitation, the adaptation period consisted of 21 days compared to the normal 7-14 days implemented by previous studies (Prola et al. 2010; Hamper et al. 2015; Iske et al. 2016). In addition, keeping tigers indoors for 72 h– to guarantee the total collection of faecal samples– could have affected this study's TFA data since some individuals preferred to defaecate outdoors. However, this was the only way to ensure adequate sample collection for the digestibility trial. Similarly, it was not possible to determine faecal defaecation frequency throughout the duration of the trial due to the complexity of the outdoor enclosures. Even if care was taken during the morning cleaning

routine to ensure that all faecal samples were collected, some older scats without glitter colours were found, making it impossible to identify the producer of the scat. In addition, sampling collection was not performed daily and depended on the parameter to be analysed. For these reasons, defaecation frequency data could not be presented.

Another consideration lies in the diversity of analytical methods for macronutrient determination used across studies. For example, as explained by Iske and colleagues (2016), acid hydrolysis fat analysis yields greater fat recovery compared to the Soxhlet methodology; hence direct comparison of macronutrient digestibility with previous studies should be undertaken with caution.

This study was limited to the use of non-invasive methods, relying exclusively on faecal samples. Some authors have argued that faecal SCFA concentrations have limited reliability to explain SCFA production (Cummings and Macfarlane 1991; Van Nuenen et al. 2004; Den Besten et al. 2013). According to Cummings and Macfarlane (1991), up to 95% of SCFA generated in the large intestine are absorbed in the gut or degraded by intestinal microbiota, hence faecal concentrations are not a good indicator of SCFA production. The use of plasma indicators as a more accurate evaluation of SCFA production has been suggested (Verbrugghe et al. 2010); however, this methodology could be hard to implement in a zoo setting. Obtaining blood samples from endangered (and dangerous) species such as tigers involves a series of additional risk (e.g. hazards associated with anaesthetic procedure) and ethical implications (e.g. welfare concerns when performing invasive practices), which can limit the use of plasma parameters, especially if samples must be collected on a time-sensitive basis. For this reason, I attempted to collect faecal samples used for SCFA and end-products determination within 1 h of voiding to limit any change due to environmental and microbial activity.

Another important limitation of the study is the lack of validated animal dietary fibre determination methods, forcing us to rely on existing plant assays which could have under-or over-estimated the actual content of the different fibre portions of the diets, hence not differentiating between fibre types. Several questions on this topic remain to be answered. A natural progression of this work would be the development of an animal-ingredients fibre assay that accounts for various poorly and/or non-digestible protein-containing compounds found in whole prey. In addition, continuing with Depauw *et al.'s* (2011) work and analysing carcass components from other species in addition to rabbit could help to better characterize nutrient composition to understand the role of carcass consumption on gut health and function in carnivores.

2.5 Conclusion

This project was undertaken to describe the differences in apparent total tract macronutrient digestibility, faecal characteristics, gut passage time, and faecal concentrations of SCFA and end-products in tigers fed either a raw horsemeat diet alone (CD) or a diet with added whole prey (ED). The addition of 20% whole rabbit made no significant difference to macronutrient digestibility or SCFA concentrations, yet potentially beneficial effects such as slightly firmer faecal consistency and a trend towards lower p-cresol concentrations were observed. The results of this investigation partially support previous evidence that animal fibre can act as a modulator of fermentation in strict carnivores. It was determined here, for the first time, that an animal fibre inclusion rate considered typical of North American dietary provision, is insufficient to elicit changes of the extent previously reported using higher experimentally-derived inclusion rates. Results from this study can help to better identify changes in the

digestive process associated with the addition of animal fibre in the diet of a strict carnivore. The findings of this research provide evidence of the importance of continuing to investigate the benefits of animal fibre on gut function and health at practical and industry-relevant inclusion levels. A further study with more focus on characterising carcass components is therefore suggested to facilitate our understanding of the characteristics needed for adequate/optimal fibre sources for tigers. Despite its limitations, the study adds to our understanding of the importance of fibre during diet formulation on the gut function of captive carnivores.

Chapter 3. Faecal inflammatory biomarker response to dietary animal fibre in captive tigers.

3.1 Introduction

For decades, animal care staff worldwide have reported a high incidence of gastrointestinal (GI) conditions in captive tigers (*Panthera tigris*) (Klos and Lang 1976; Seidel and Wisser 1987); among which inflammatory bowel disease (IBD) might be more common than originally thought (Travis and Carpenter 2011; Tilson et al. 2016). Inflammatory bowel disease is defined as an idiopathic, immune-mediated inflammation of the small intestine (SI) and/or colon with a predominant lymphocytic or plasmacytic infiltrate of unknown cause (Willard 1999; Zoran 2002). Diagnosis has proven challenging due to the wide range of non-pathognomonic clinical signs present in IBD such as diarrhoea, weight loss, vomiting and anorexia (Zoran 2002; Hall et al. 2005; De Jong et al. 2006; Heilmann and Steiner 2018). Common procedures to diagnose IBD and other chronic enteropathies are highly invasive for routine use in humans and other animals (e.g. endoscopy, biopsy collection, blood samples) (Kaiser et al. 2007; Foell et al. 2009; Heilmann and Steiner 2018; Parambeth et al. 2019). Concerns over the complications associated with invasive diagnostic tools in animals, including tigers, have led researchers to identify and validate less intrusive methods for obtaining diagnostic samples. One such method is the assessment of biological markers of inflammation through the opportunistic collection of faecal samples.

Among the most promising and commonly investigated markers of inflammation are indicators of mast cell degranulation (e.g. N-methylhistamine– NMH) and neutrophil infiltration (e.g. S100A12) (Steiner 2014). Faecal concentrations of inflammatory biomarkers have been

studied as potential non-invasive indicators to diagnose chronic enteropathies in humans (Bischoff et al. 1997; Winterkamp et al. 2002; De Jong et al. 2006; Kaiser et al. 2007), domestic dogs (*Canis lupus familiaris*) (Heilmann and Suchodolski 2008; Anfinsen et al. 2014; Berghoff et al. 2014), cheetahs (*Acinonyx jubatus*) (Depauw et al. 2014), common marmosets (*Callithrix jacchus*) (Parambeth et al. 2019) and domestic cats (*Felis catus*) (Bridges et al. 2019).

A wide variation was shown in faecal concentrations of NMH in healthy domestic dogs (Ruaux et al. 2009) as well as those with chronic enteropathies (Anfinsen et al. 2014). Despite this variation, Berghoff *et al.* (2014) reported a significantly higher NMH concentration in dogs with GI disease compared to healthy groups. Similarly, faecal NMH increased in common marmosets diagnosed with chronic lymphocytic enteritis compared to healthy controls (Parambeth et al. 2019).

In humans, higher faecal concentrations of another biomarker, S100A12, were found in patients with IBD compared with healthy controls (De Jong et al. 2006; Kaiser et al. 2007); while in domestic dogs, histopathological and endoscopic lesions of the GIT have been associated with increased faecal S100A12 concentrations (Heilmann et al. 2014). Concentrations of faecal S100A12 have also been quantified in cheetahs (Depauw et al. 2014) and, more recently, in domestic cats (Bridges, et al., 2019). As such, these faecal nitrogenous compounds appear to have promising functionality as biomarkers of inflammation.

Diet has been proposed as a triggering factor of chronic GI conditions such as IBD; specifically, a link between fibre and gut health in domestic cats and captive felids has been suggested (Bueno et al. 2000b; Vester et al. 2010a; Kerr et al. 2013b; Deb-Choudhury et al. 2018). In captive cheetahs, research has highlighted that the non-digestible components of whole prey

(e.g. bone, fur and connective tissue), known as ‘animal fibre’, can prove beneficial for their GI health (Depauw et al. 2011). Depauw and collaborators (2014) demonstrated that concentrations of faecal S100A12 increased significantly when cheetahs were fed a diet consisting of supplemented beef meat (lower in animal fibre) compared to a whole rabbit (*Oryctolagus cuniculus*) diet (higher in animal fibre). Results from Depauw’s group highlighted the importance of diet, specifically animal fibre, as a possible modulator of the inflammatory process in the GIT of captive felids. Free-ranging tigers are known to consume most edible parts of their prey including animal fibre components (Tilson and Nyhus, 2010; Miller *et al.,* 2013; Fàbregas *et al.,* 2017). However, in North American collections, the most common diet consists of commercial raw meat blends that lack such components (Iske, Morris and Kappen, 2016; Lefebvre *et al.,* 2020). This raises the concern of whether such dietary management can negatively affect tigers’ GI health.

Non-invasive diagnostic tools to assess inflammatory GI conditions in captive felids, including tigers, are currently limited and tend to be extrapolated from domestic species or humans (Lamberski 2015). However, results from cheetahs suggest the potential utility of these markers as non-invasive methods to monitor GI inflammation in captive felids (Depauw *et al*., 2014). The aim of the current study was to quantify, for the first time in captive tigers, faecal concentrations of N-methylhistamine and S100A12 in animals fed either a diet comprising a commercially supplemented ground muscle horsemeat or a diet with 20% added whole prey. Based on previous research in captive cheetahs, a decrease in faecal concentrations of NMH and S100A12 for captive tigers fed a diet including whole prey compared with a diet based on ground muscle meat was hypothesised.

3.2 Materials and methods

The experimental design and dietary treatments are described in section 2.2. Material and Methods of Chapter 2. This project was approved by the Ethical Panel of the School of Veterinary Medicine & Science, University of Nottingham (UK). Detailed information on tigers' demographics, housing conditions and feeding routine can be found in Chapter 4, section 4.2.1 Animals, enclosures, and management. Briefly, a randomized crossover study with eight zoo-housed tigers was performed. The Baseline Diet (BD) (which corresponded to the historic diet these tigers consumed prior to the beginning of the feeding trial) consisted of a mixture of featherless whole chicken (*Gallus gallus domesticus*) (8% as fed), degutted whole rabbit (*Oryctolagus cuniculus*) (3% as fed), horse shanks (corresponding to the biceps femoralis and semitendinosus muscles) or horse necks (both cuts with skin and bones) (7% as fed) and a commercially supplemented ground muscle horsemeat diet (82% as fed). The Experimental Diet (ED) comprised degutted whole rabbit (20% as fed) and the commercial horsemeat diet (80% as fed), while the Control Diet (CD) contained exclusively the commercial horsemeat diet. Both dietary treatments were fed for 8 consecutive weeks without washout period between diets. One tiger refused to consume the ED and was fed the CD throughout the 16 weeks of feeding trials.

3.2.1 Faecal collection, sample processing and extraction

Prior to sample collection, faecal consistency was scored using the AZA Felid Taxon Advisory Group scale system described in Chapter 2 (Felid TAG 2014). For each tiger one faecal sample over three consecutive days was collected at three different time points: week 0, week 8 and week 16. Approximately 50 g of faeces (avoiding sections contaminated with soil or water) was gathered in a plastic bag. After collection, samples were

transported to the nutrition laboratory at Busch Gardens Tampa Bay for processing. Samples were homogenised inside their plastic bag using a sterile wooden tongue depressor, after which an aliquot of 1 g was placed into a pre-weighed polypropylene tube. Aliquots were freeze-dried until constant weight was obtained using freeze-drying equipment (Model 2000, Freeze Dry Company, Inc., Nisswa, MI, USA). Aliquots were stored at -40 °C until shipped on dry ice to the Gastrointestinal Laboratory at Texas A&M University (College Station, TX, USA) for the determination of faecal NMH and S100A12 concentrations.

Faecal samples were thawed and extracted following the procedure described by Heilmann and Suchodolski (2008): samples were thawed, weighed and diluted in a 1:5 solution using faecal extraction buffer (20mM $(CH_3)CO_2Na$ and 3mM $CaCl_2$ [pH, 7.6]) containing a proteinase inhibitor cocktail (complete EDTA-free proteinase inhibitor cocktail tablets, Roche Diagnostics GmbH, Mannheim, Germany,1 tablet/25 mL). Diluted samples were homogenised by vigorous shaking for 30 min at room temperature (23°C). The resulting suspensions were centrifuged at 5°C for 20 minutes at 2,100 X g. Supernatants from the previous step were filtered and centrifuged again for 30 minutes at 10,600 X g at 23°C. The final extracts were stored at -80 °C until assayed.

3.2.2 S100A12 assay

Faecal concentrations of S100A12 were measured using an enzymatic immune assay (EIA) (Heilmann et al. 2016a). Briefly, assay plates were incubated with 200 ng/well of polyclonal anti-S100A12 antibody in 200 mM carbonate-bicarbonate (pH 9.4). Faecal extracts were diluted 1:100 in assay buffer (25 mM Tris/HCl, 150 mM NaCl, 0.05% (v/v) polyoxyethylene-20 sorbitan monolaurate, 0.5% (w/v) bovine serum albumin, pH 8.0). The

standard curve was prepared by diluting the polyclonal anti-S100A12 antibody in assay buffer to obtain the following concentrations: 5, 2, 1, 0.5, 0.2, 0.1 and 0.02 ng/mL. Samples diluted in assay buffer were added to the plate in duplicate (100 µL/well), while standard curve and quality controls (four different S100A12 concentrations) were loaded only once. Blank wells contained exclusively assay buffer. Plates were then incubated for 1 h at room temperature and washed three times using wash buffer (25 mM Tris/HCl, 150 mM NaCl, 0.05% (v/v) polyoxyethylene-20 sorbitan monolaurate, at pH 8.0). After washing, S100A12-horseradish peroxidase (HRP) was added (concentration 15 ng/well) to each well and plates were incubated for another hour at room temperature before being washed three times again. The plates were developed with a stabilized tetramethylbenzidine (TMB) substrate (1-Step Ultra TMB-ELISA, Thermo Scientific) and incubated at room temperature for 5 min. Fifty µL of stop solution (4 M CH_3COOH and 0.5 M H_2SO_4) was added and absorbance was measured in each well at 450 nm using an automated plate reader (Synergy 2 Alpha Microplate Reader; BioTek, Winooski, VT, USA).

Assay validation indicators consisted of dilutional parallelism (linearity), spiking recovery (accuracy), intra and inter-assay variability (precision and reproducibility) (Brown et al. 2004; Heilmann et al. 2011). Dilutional parallelism was calculated using three different samples diluted two-fold from 1:1000 to 1:32000. The same three samples were used to assess accuracy by spiking them against each other. The observed/expected ratio for linearity and accuracy was calculated with the following formula: *% Recovery = (measured value / expected value) x 100.* To determine assay precision and reproducibility, coefficients of variation were used *(% CV = SD/mean x 100*). Intra-assay variability was obtained by assaying all samples in duplicates within the same assay. Inter-assay variability was determined by analysing three samples in six consecutive assay runs.

3.2.3 NMH assay

Determination of faecal NMH was performed following the gas chromatography-mass spectrometry (GC-MS) method described by Ruaux *et al.* (2009). A gas chromatograph (Thermo Finnigan Voyager, Thermo Corp, Waltham, MA, USA) was coupled to a mass spectrometer (J&W DB-1ms column, Agilent Technologies, Inc., Wilmington, USA), with a 30 m x 0.25 mm (internal diameter) and 0.25 µm thick column. The flow rate was 2.0 mL/min, with a splitless mode for 30 seconds at injection, and the remainder of the separation was performed at 15:1 split-flow ratio. Helium was selected as the carrier gas. Chromatographic conditions were similar to those described by Tredget *et al.*, 1997, with a transfer line temperature of 250 °C and a carrier gas flow rate of 2.0 mL/min. The mass spectrometer specifications included electron impact ionization mode (70 eV) with selected ion monitoring at masses specific for the bis-pentafluoropropionyl (PFP) derivative of NMH (*m/z* 417) and the trideuterated bis-PFP derivative of trideuterated NMH (*m/z* 420) (Tredget et al. 1997; Ruaux et al. 2009). Dwell times were 100 ms with 5 cycles/s. Peak area integration was calculated with the default settings of the software for the GC-MS system. Retention times were confirmed daily by GC-MS evaluation of a specimen of pure, nondeuterated NMH that was evaporated to dryness and derivatized directly.

3.2.4 Statistical analysis

Mean concentrations for the three consecutive-days sample collections were used for statistical analysis, as suggested by Heilmann & Suchodolski, (2008). Mean faecal consistency scores for each dietary

treatment were calculated and used for correlation analysis. Data were analysed using the IBM plugin Essentials for R for Statistics (IBM Inc, Armonk, NY, USA) to access the WRS2 software package (Mair and Wilcox 2018) from the SPSS version 24 (IBM SPSS Statistics, IBM Inc, Armonk, NY, USA). Normality of the data was tested using the Shapiro-Wilk test. Since data were not normally distributed ($p < 0.001$) and despite attempts for log-transformation data remained without a normal distribution, statistical analyses were performed using 'Robust Methods' for comparison across dietary treatments and non-parametric test for correlations. Robust Methods in statistics offer an alternative to non-parametric tests to analyse data that violates normality assumptions in complex experimental designs (Field 2018; Mair and Wilcox 2018). These methods are characterized by bootstrap, trimmed means and the use of M-estimators among other concepts (for further details see (Wilcox 2017)). Differences in faecal concentrations of NMH and S100A12 across dietary treatments were evaluated using Robust Repeated Measures General Linear Model (GLM). Spearman's correlations and linear regressions were calculated for the analytical validation of the S100A12 assay.

Effect sizes for the GLM tests were calculated using omega squared (ω^2) (Field, 2018). *Post hoc* pairwise comparisons with adjusted *p*-values using the Bonferroni correction factor were performed. Size effects of the pairwise comparisons were calculated using Pearson's correlation coefficient *r* (Nakagawa and Cuthill 2007; Field 2018). Effect size is considered an objective and standardized measure of the magnitude of the observed outcome (Nakagawa and Cuthill 2007; Ialongo 2016). The following benchmark values have been proposed for effect sizes: small ($r = 0.1$) medium ($r = 0.3$) and large ($r = 0.5$) (Cohen 1988). Kendall's tau test was used to evaluate the association between faecal concentrations of

NMH and S100A12 and between each biomarker and faecal consistency score.

Results are reported as median (Md), range in nanograms per gram (ng/g) on a faecal dry matter (DM) basis. Bias-corrected and accelerated bootstrap 95% confidence intervals (CI) are reported in square brackets. To obtain values comparable with previously published studies, faecal concentrations of both biomarkers were calculated for the wet weight of the sample and were analysed following the same procedure as for the DM values.

3.3 Results

3.3.1 S100A12 assay validation results

Following dilutional parallelism testing, the linear regression's line equation was y = 0.004 + 1.198x (p <0.001) and dilutions were closely correlated (r_s = .98; p <0.001; Table 3.1). The linear regression equation obtained from spiking recovery tests was y =0.26 + 0.901x. Spiked samples did not correlate significantly (r_s = .87; p = 0.33; Table 3.2). Intra- and inter-assay variability corresponded to 6.93% and 8.29% respectively.

Table 3.1 Results of dilution parallelism study of S100A12 enzymatic immune assay for three tigers (*Panthera tigris*) faecal extracts.

Faecal extract	Dilution	Observed (ng/g)	Expected (ng/g)	O/E (%)
1	1:1000	4.07	NA	NA
	1:2000	2.54	2.04	124.5
	1:4000	1.26	1.02	123.8
	1:8000	0.74	0.51	146.2
	1:16000	0.43	0.26	167.8
	1:32000	0.22	0.13	169.7
2	1:1000	1.601	NA	NA
	1:2000	0.853	0.8005	106.6
	1:4000	0.458	0.4265	107.4
	1:8000	0.292	0.229	127.5
	1:16000	0.168	0.146	115.1
	1:32000	0.092	0.084	109.5
3	1:1000	1.792	NA	NA
	1:2000	0.854	0.896	95.3
	1:4000	0.481	0.427	112.6
	1:8000	0.269	0.2405	111.9
	1:16000	0.159	0.1345	118.2
	1:32000	0.121	0.0795	152.2

NA- not applicable; O/E- observed-to-expected ratio

Table 3.2 Results of spiking recovery test of S100A12 enzymatic immune assay for three tigers (*Panthera tigris*) faecal extracts.

Faecal extract	Observed (ng/g)	Expected (ng/g)	O/E (%)
1+2	0.94	1.02	92.2
1+3	0.94	1.01	93.1
2+3	0.44	0.46	95.7
Mean			93.6
SD			1.8

O/E- observed-to-expected ratio; SD standard deviation

3.3.2 Dry Matter basis results

Figure 3.1presents tigers' faecal concentrations of NMH and S100A12 on a DM basis. For NMH, the Greenhouse-Geisser estimate of sphericity showed a substantial deviation (ε = 0.523). A trend towards significance was observed for the variation of faecal NMH concentrations across dietary treatments (F (1.25, 4.98) = 4.53, p = 0.084, ω^2 = 0.14). However, pairwise comparisons with corrected p-values showed no significant difference between diets. Effect size was large between BD and ED (r = 0.58), between BD and CD (r = 0.65) and between ED and CD (r = 0.69) according to Cohen's (1988) coefficient benchmarks.

Similarly, a departure from sphericity was confirmed for S100A12 concentrations (ε = 0.839). No significant difference was detected in S100A12 concentrations across diets (F (1.1, 4.44) = 1.386, p = 0.306, ω^2 = -0.04). Effect sizes for the pairwise comparisons between BD-ED, and ED-CD were weak (r = 0.19 and r = 0.20 respectively), while BD-CD comparison resulted in a medium effect size (r = 0.47) according to Cohen's (1988) coefficient benchmark values.

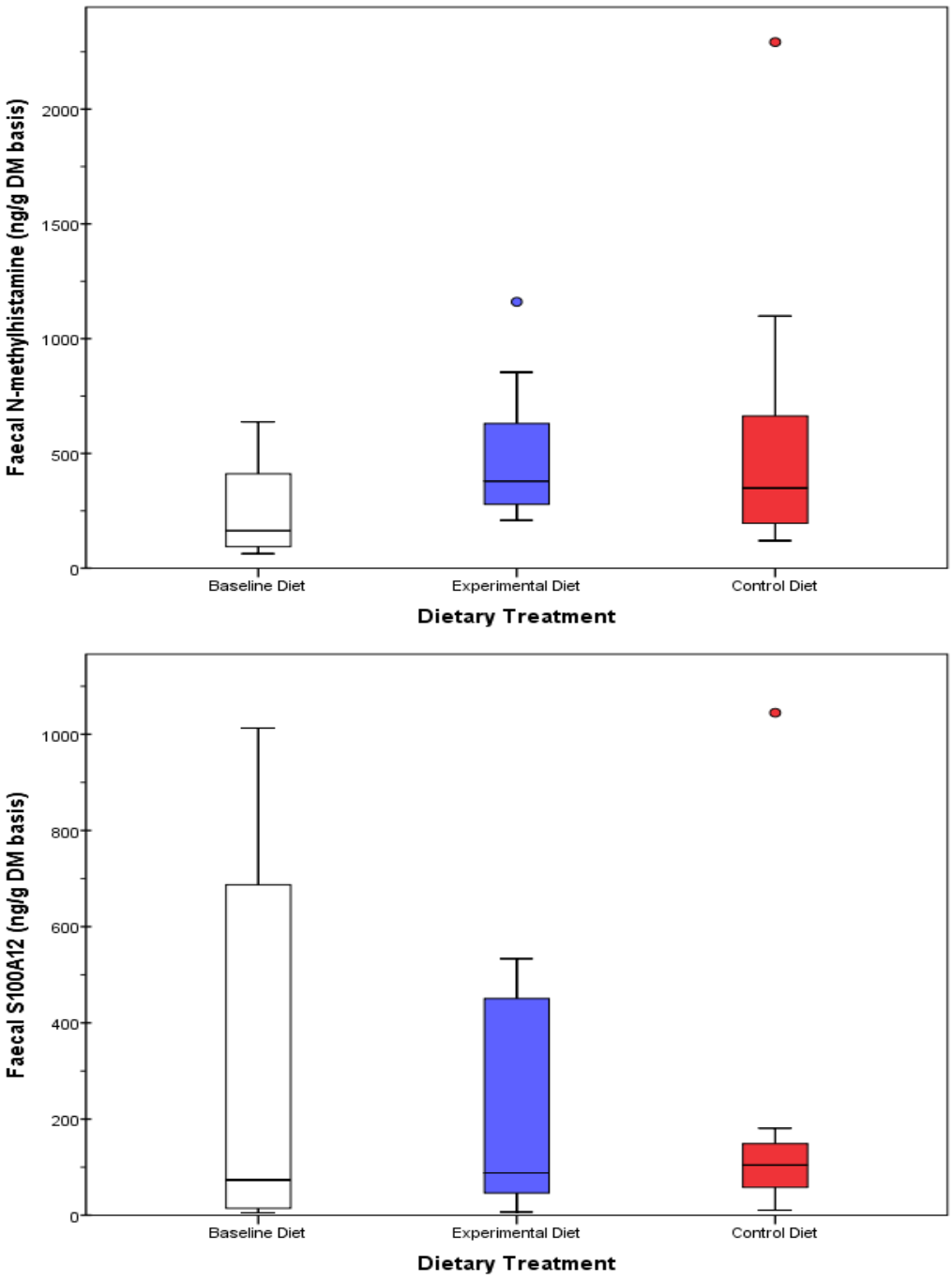

Figure 3.1 Faecal concentrations of N-methylhistamine and S100A12 from eight captive tigers (Panthera tigris) fed: Baseline Diet (mixture of commercially supplemented ground muscle horsemeat, chicken, rabbit, and bones), Experimental Diet (20% whole rabbit + 80% commercial horsemeat) and Control Diet (100% commercial horsemeat). All values expressed on dry matter basis; circles represent outliers; n = 24 samples.

Faecal concentrations of NMH and S100A12 did not correlate significantly (τ = -0.188, [-0.495, 0.114], p = 0.197). No significant correlation was found between mean faecal consistency scores and NMH concentrations (τ = -0.234, [-0.464, 0.027], p = 0.112). In contrast, a trend towards significance was observed between S100A12 concentrations and mean faecal consistency scores (τ = 0.255, [-0.083, 0.551], p = 0.082); however, since the 95% CI contains the value zero, such correlation is not likely to be meaningful (see Figure 3.2).

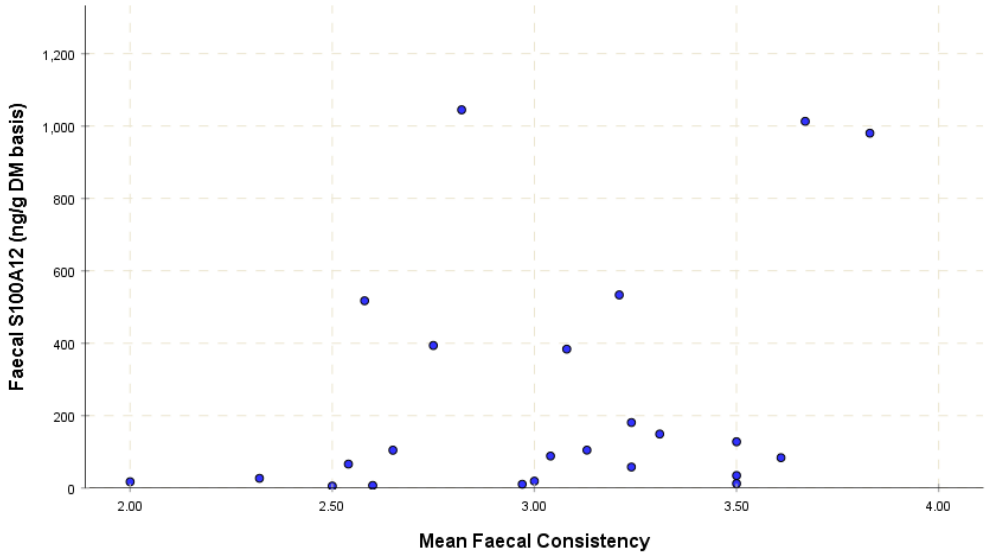

Figure 3.2 Faecal S100A12 concentrations (values expressed on DM (dry matter) basis) in relation to mean faecal consistency score in eight captive tigers (*Panthera tigris*) (n = 24 samples) regardless of dietary treatment.

3.3.3 Wet basis results

Figure 3.3 shows wet weight concentrations of faecal NMH and S100A12 from captive tigers. For NMH, a substantial deviation from sphericity was demonstrated by the Greenhouse-Geisser estimate (ε = 0.544). A significant difference was observed in faecal NMH concentrations among dietary treatments (F_t (1.84, 7.36) = 5.41, p = 0.038, ω^2 = 0.28). Nevertheless, none of the pairwise comparisons with corrected p-values was significant. The effect size was large between BD and CD (r = 0.81), of a medium magnitude between BD and ED (r = 0.47) and weak between ED and CD (r = 0.02) based on Cohen's (1988) coefficient benchmarks.

Likewise, a departure from sphericity was confirmed for faecal concentrations of S100A12 (ε = 0.773). S100A12 concentrations across diets were not significantly different (F (1.19, 4.78) = 1.48, p = 0.292, ω^2 = -0.06). Effect sizes for the pairwise comparisons between BD and ED (r = 0.10) and between BD and CD (r = 0.24) were weak, while the effect size between ED and CD (r = 0.48) was of medium magnitude according to Cohen's (1988) coefficient benchmarks.

No significant correlation was found between faecal concentrations of NMH and S100A12 (τ = -0.188, [-0.500, 0.149], p = 0.197). Relation between mean faecal consistency scores and NMH concentrations showed a trend towards significance (τ = -0.255, [-0.460, -0.011], p = 0.082; Figure 3.4). The correlation between S100A12 concentrations and mean faecal consistency scores also showed a trend towards significance (τ = 0.248, [-0.113, 0.556], p = 0.091); however, since the 95% CI contains the value zero, the correlation is not likely to be meaningful (Figure 3.5).

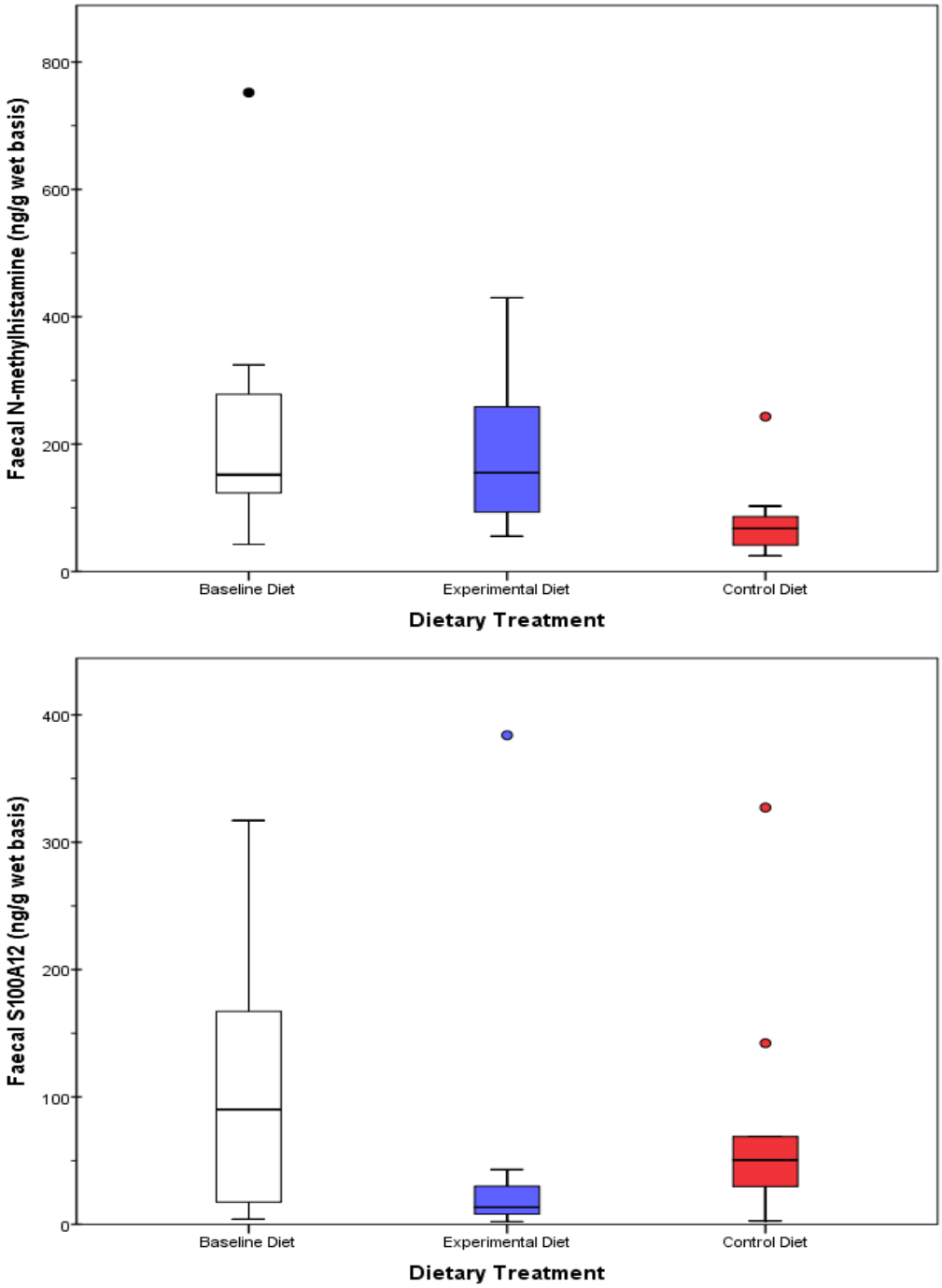

Figure 3.3 Faecal concentrations of N-methylhistamine and S100A12 from eight captive tigers (*Panthera tigris*) fed: Baseline Diet (mixture of commercially supplemented ground muscle horsemeat, chicken, rabbit, and bones), Experimental Diet (20% whole rabbit + 80% commercial horsemeat) and Control Diet (100% commercial horsemeat). All values expressed on wet basis; circles represent outliers; n = 24 samples.

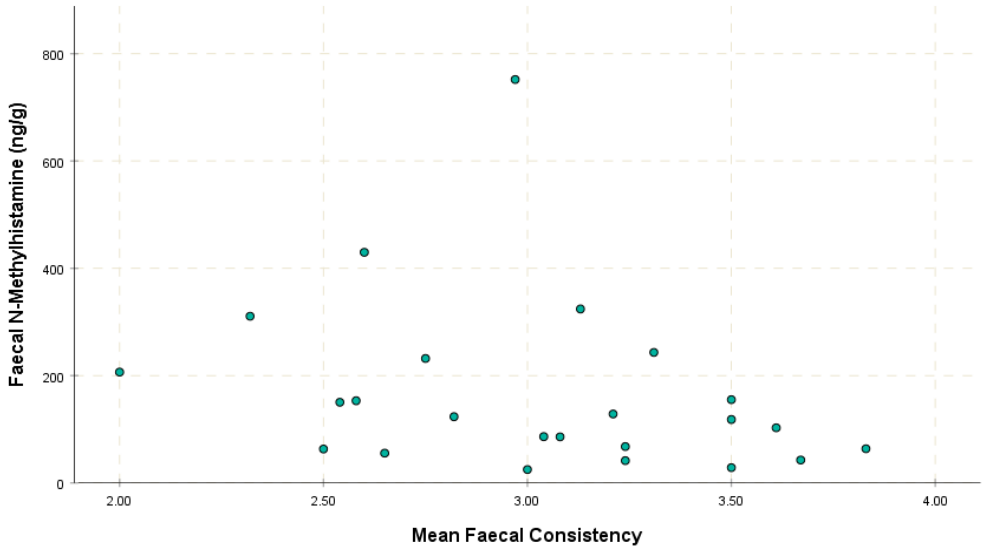

Figure 3.4 Faecal N-methylhistamine concentrations (values expressed on wet basis) in relation to mean faecal consistency score in eight captive tigers (*Panthera tigris*) (n = 24 samples, 3 samples/tiger) regardless of dietary treatment.

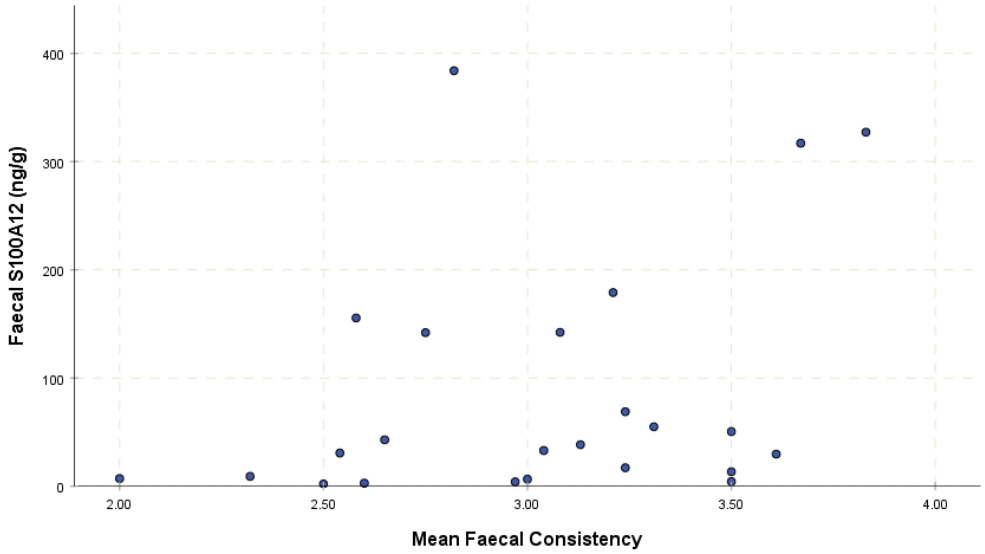

Figure 3.5 Faecal S100A12 concentrations (values expressed on wet basis) in relation to mean faecal consistency score in eight captive tigers (*Panthera tigris*) (n = 24 samples, 3 samples/tiger) regardless of dietary treatment.

3.4 Discussion

Researchers have investigated faecal NMH and S100A12 as potential markers of GI inflammation in a wide range of mammals including humans, dogs, marmosets, cats, and cheetahs. This study population comprised of eight apparently healthy zoo-housed tigers with no clinical signs of GI conditions before or during the trial. The aim of the current study was to describe changes in faecal concentrations of NMH and S100A12 in tigers undergoing a dietary intervention. Contrary to my hypothesis, no significant differences were observed in faecal concentration of either marker when tigers were fed a diet comprising a commercially supplemented ground muscle horsemeat or a diet with 20% added whole rabbit.

3.4.1 N-Methylhistamine

The lack of detectable difference in NMH in tigers fed diets of divergent fibre concentrations may be explained by three possible scenarios. Firstly, besides inflammatory processes, diet can affect NMH concentrations (Winterkamp et al. 2002; Raithel et al. 2015; Heilmann and Steiner 2018). A possible mechanism by which prey items can influence NMH concentrations includes histidine-containing dipeptide (HCD), amines found in mammalian muscle (i.e. carnosine, anserine and ophidine) (Crush 1970; Dragsted 2010; Boldyrev et al. 2013; Kwiatkowski et al. 2018). HCD should be degraded into histamine by similar enzymes (Tamaki et al. 1980; Flancbaum et al. 1990; Peters et al. 2015). However, in humans and rats (*Rattus norvegicus domestica*) methylhistidine, a component of anserine, is excreted in urine without being reutilized for protein synthesis nor metabolized (Long et al. 1975; Sjölin et al. 1987). Moreover, only carnosine has been reported as a precursor of histamine during trauma, inflammatory processes or in response to stress (Fitzpatrick and Fisher

1982; Flancbaum et al. 1990). Hence, carnosine and anserine may have independent metabolism yielding different concentrations of histamine that are later detected as NMH (Tamaki *et al.,*1980).

Differences in concentrations and proportions of HCD across prey items have been described previously. For example, chicken meat has higher HCD concentrations than rabbit and horse meat (Crush, 1970; Dragsted, 2010; Boldyrev, Aldini and Derave, 2013). Horse meat contains high levels of carnosine and low amounts of anserine, while the inverse trend is observed in chicken and rabbit meat, where anserine is more abundant (Crush 1970; Peiretti et al. 2011). Because of the mixture of prey items, BD could be considered a diet high in HCD with similar proportions of carnosine and anserine. In contrast, the ED may have contained lower concentrations of HCD but similar carnosine and anserine proportions, while CD was low in HCD but with higher carnosine than anserine. If carnosine metabolism differed from that of anserine– yielding more histamine– higher NMH concentrations should have been observed during the CD treatment. However, although non-significant, tigers fed the CD had a numerically lower NMH range compared to the ED and BD. Unfortunately, to date, no published study has compared the conversion rate of the different HCDs into histamine. Based on this study's results and the lack of supportive evidence for differences in the catabolism of carnosine and anserine, NMH concentrations obtained in the current study could have been influenced by prey histamine or HCD concentrations rather than differences in prey HCD composition.

Secondly, upon intake of substantial amounts of exogenous histamine– or the presence of GI disease–, the catabolic system is unable to eliminate it at an adequate rate. This leads to higher histamine concentrations in the GI lumen (Halász et al., 1994; Bodmer, Imark and Kneubühl, 1999). Products containing high histamine levels include tuna (*Thunnus*

albacares), mackerel (*Scomber scombrus*), meat and dairy products (Halász et al. 1994; Winterkamp et al. 2002; Spano et al. 2010). These elevated histamine levels could then increase concentrations of NMH. For example, in healthy humans, and those with food allergies, urinary NMH levels increased when subjects were fed an omnivorous diet containing meat compared to a diet comprising only potatoes and rice (Raithel *et al*., 2015). Hence, regardless of health status, ingested histamine seems to affect its metabolite concentrations. Previous studies in other species did not consider diet in their experimental designs and are limited to describe differences in NMH concentrations between individuals with chronic enteropathies and healthy controls. Although non-significantly different, the highest faecal NMH range was found during the BD, when animals consumed a wide variety of prey items, while the lowest range belonged to CD– a monotypic diet comprising only horsemeat. Since diet can act as an exogenous source of histamine, the NMH pattern (i.e. BD > ED > CD) obtained in the present study could be a reflection of dietary histamine rather than GI inflammation.

Thirdly, differences in mast cell infiltration in the GIT could explain the wide variation across NMH concentrations reported in humans, marmosets and dogs with chronic enteropathies (see Table 3.3). In dogs and humans, mast cells have been implicated in the pathophysiology of GI disorders such as IBD (Raithel et al. 1995, 2001; Vaden et al. 2000; Winterkamp et al. 2002; German et al. 2003; Boeckxstaens 2015). In dogs with chronic GI disease, the presence of mast cells in the small intestine was detected but did not correlate with faecal or urinary NMH concentrations (Anfinsen et al. 2014; Berghoff et al. 2014). These authors questioned the utility of NMH as an inflammatory marker for the species. However, Berghoff *et al.* (2014) noticed that for a subset of dogs increased NMH and mast cell counts were correlated; hence NMH can be used as an indicator of GI disorders where

mast cells are major contributors to the inflammatory process. In domestic cats, evidence to support the involvement of mast cells in IBD has been provided (Kleinschmidt et al. 2010). The authors reported a significant increase in mast cells counts in cats with IBD compared to healthy controls. To date, mast cell infiltration has not been described in tigers' GIT and information about the pathophysiology of intestinal disorders is still extrapolated from domestic cats. Tigers' mast cells may play a similar role to that of cats in some forms of GI inflammation making NMH an appropriate indicator for such conditions. Nevertheless, further research in tigers is needed to corroborate mast cell involvement in chronic enteropathies and the utility of NMH as an inflammation marker.

Table 3.3 Faecal concentrations of N-methylhistamine in different mammal species; reported values are expressed as median for 3-day collection (range) unless otherwise stated.

Species	Study group	Reported values	Stability	Reference
Human	Control	300 ng/g[a]	21 days (-20°C) 1 day (RT)	Bischoff *et al.,* (1997)
	Chron's disease	500 ng/g[a]		
	Food allergy	350 ng/g[a]		
Dog	Chronic gastroenteropathies (Norwegian Lundehunds)	285 ng/g (67-9319)		Berghoff, Suchodolski and Steiner (2008)
	Healthy	46 ng/g (9-197)		
	Healthy	(nd-1959 ng/g) [a]		Ruaux *et al.* (2009)
	Chronic enteropathies (mast cell activation)	48 ng/g (0-1451) [a]		Anfinsen *et al.,* (2014)
	Chronic enteropathies	126 ng/g (14-4515)		Berghoff *et al.,* (2014)
	Healthy	53 ng/g (9-252)		
Marmoset	Healthy	≤122 ng/g (nd-216)		Parambeth *et al.,* (2019)
	Chronic lymphocytic enteritis	≥118 ng/g (107-995) [a]		
Tiger	Commercial ground horsemeat	68 ng/g (25-243)		Current study
	Commercial ground horsemeat + 20% whole rabbit	155 ng/g (55-430)		
	Mixture of meat sources, bones, and whole prey	152 ng/g (43-752)		

[a] 1-day collection; RT- room temperature; nd- non detectable

Finally, faecal concentrations of NMH detected in captive tigers ranged from 25 to 752 ng/g, these results are consistent with previous data obtained in other species (see Table 3.3) (Bischoff et al. 1997; Ruaux et al. 2009; Anfinsen et al. 2014; Berghoff et al. 2014; Parambeth et al. 2019). Range values obtained during the CD were similar to those reported for healthy dogs and marmosets, while BD values compared to concentrations of individuals with chronic enteropathies (Berghoff et al. 2008, 2014; Parambeth et al. 2019). Although the median concentration of NMH obtained during the ED was similar to that of subjects with chronic GI conditions, the highest ED concentration was below the highest concentration reported in dogs and marmosets with chronic enteropathies (Berghoff *et al*., 2014; Parambeth *et al*., 2019). Results from Chapter 2 suggest that functional GI parameters remained unaltered despite dietary treatment. Since tigers participating in the trial were apparently healthy and no significant difference in NMH concentration across diets was detected, such values could correspond to a normal degree of biological variability in this marker for tigers (Ruaux *et al.*, 2009; Berghoff *et al.,* 2014; Parambeth *et al.,* 2019). Yet, to establish reference values for the species and corroborate if these values corresponded to the normal variation expected within the species, a wider sample size is needed.

3.4.2 S100A12

Levels of faecal S100A12 found in captive tigers in the present study were within the range reported in other species (Kaiser et al. 2007; Depauw et al. 2014; Heilmann et al. 2016b; Bridges et al. 2019). In other species, an apparent overlap in S100A12 concentrations between healthy individuals and those with IBD, particularly in dogs, was noticeable (see Table 3.4). Despite the wide ranges in reported concentrations, this marker was able

to differentiate between healthy individuals and those suffering chronic GI conditions (De Jong, Leach and Day, 2009; Foell, Wittkowski and Roth, 2009). In addition, faecal levels of S100A12 have been found positively correlated with the severity of both intestinal endoscopic lesions and clinical signs in dogs with chronic GI conditions (Heilmann *et al*., 2014; Heilmann *et al*., 2018). Heilmann *et al.* (2016) proposed an S100A12 reference interval for healthy dogs of 2-484 ng/g, which overlaps with the range observed in tigers fed either the BD, CD or ED.

For healthy cats, the suggested reference interval is 0-20 ng/g (Bridges *et al.* 2019). Since the information for cats with chronic enteropathies is not available, I cannot assume that tigers' results suggest an inflammatory process based on healthy domestic cats' reference values. The use of population-based reference values in healthy cats was considered of limited utility due to the high biological and inter-individual variations observed in S100A12 faecal values (Petersen et al. 1999; Walton 2012; Bridges et al. 2019). Researchers proposed instead the use of individual reference intervals, or changes of ≥3.9 ng/g in serial faecal samples from the same individual to be considered clinically significant in cats (Bridges *et al.,* 2019). As such, S100A12 values may need to be interpreted within the study population rather than simply compared with previous reference values. The overlap in faecal S100A12 values between healthy and diseased individuals of other species, in addition to the biological variation and high intra-individual variation, supports the suggestion that inflammatory biomarkers need to be evaluated alongside other GI parameters for a better interpretation of results.

Table 3.4 Faecal concentrations of S100A12 in different mammal species; reported values are expressed as median 3-day collection (range) unless otherwise stated.

Species	Study group	Reported values	Stability	Reference
Human	IBD (children) Healthy children	95400 ng/g (6190-49900) 690 ng/g (390-17730)	7 days (RT)	De Jong, Leach and Day (2009)
	IBD Healthy IBS	2450 ng/g 6 ng/g 50 ng/g		Kaiser *et al.,* (2007)
Dog	Healthy	<24 ng/g (<24-926)		Heilmann *et al.,* (2011)
	Healthy	2-484 ng/g	7 days (RT)	Heilmann, Cranford *et al.,* (2016)
	IBD Healthy	223 ng/g (IQR 21–3477)[a] 9 ng/g (IQR 5-31)[a]		Heilmann *et al.,* (2014)
	IRE/NRE versus FRE or ARE NRE versus CR/PR ARE FRE IBD	≥ 490 ng/g ≥ 2700 ng/g 16 ng/g (3-3,673) 27 ng/g (1-7040) 802 ng/g (2-34500)		Heilmann, Volkmann *et al.,* (2016)
	Healthy puppies	24 ng/g (<24-14363)		Heilmann, Grellet *et al.,* (2018)
	Chronic inflammatory enteropathies	65 ng/g (1-41660)		Heilmann *et al.,* (2018)
Cheetah	Whole rabbit diet Supplemented beef diet	301 ng/g (Q_1 223, Q_3 1,85) 1671 ng/g (Q_1 494, Q_3 4,15)		Depauw *et al.,* (2014)
Cat	Healthy	2 ng/g[a] (<2-56)		Bridges *et al.,* (2019)
Tiger	Commercial ground horsemeat Commercial ground horsemeat + 20% whole rabbit Mixture of meat sources, bones, and whole prey	51 ng/g (3-327) 13 ng/g (2-384) 90 ng/g (4-317)		Current study

[a] 1-day collection; CLE- chronic lymphocytic enteritis; IRE- immunosuppressive-responsive enteropathy; NRE- non-responsive enteropathy; FRE- food-responsive enteropathy; ARE- antibiotic-responsive enteropathy; CR- complete remission; PR- partial response; IBD- inflammatory bowel disease; RT- room temperature; Q_1- quartile; Q_3- quartile; nd- non detectable; IQR- inter quartile range

Previous studies have demonstrated the influence of diet on faecal calprotectin and S100A12 levels in cheetahs and dogs (Hang *et al.,* 2013; Depauw *et al*., 2014). In cheetahs, faecal concentrations of S100A12 decreased significantly when animals were fed exclusively with whole rabbit compared to a diet of supplemented beef (Depauw *et al*., 2014), while in dogs fed a high protein diet, faecal calprotectin was significantly elevated compared to when fed a dry commercial diet (Hang et al. 2013). In both studies, other parameters indicative of GI health such as putrefactive compounds and faecal consistency, worsened when animals were fed the supplemented beef or high protein diet. Results observed in both species used in these previous studies, when fed the beef or high protein diet, are commonly associated with poor gut health, hence indicating an influence of diet as a possible modulator of GI inflammation (Macfarlane and Macfarlane 1997; Davila et al. 2013). Median S100A12 concentration for ED, CD or BD in the current study were lower than those reported for captive cheetahs fed either whole rabbit or supplemented beef or dogs with IBD (see Table 4.4), which may indicate a lack of inflammation in this study population or that, for apparently healthy tigers, the diets used in the current study failed to modulate GI inflammation.

Another possible explanation for the S100A12 results obtained is that, in this population of tigers, neutrophil infiltration, or the contribution of neutrophils in the inflammatory process, was marginal; therefore, faecal S100A12 was not likely to differ, regardless of the diet. Evidence exists that S100A12 levels can change depending on the participation of neutrophils in the inflammatory process (Kaiser et al. 2007; Heilmann et al. 2018b). The most common type of cell infiltrates observed in cats and dogs with IBD corresponds to a lymphoplasmacytic infiltration (Marsilio et al. 2014). Neutrophilic enteropathies in cats have an unknown aetiology and are a less common variant of IBD (Jergens 2012; Maunder et al. 2016). In tigers, histopathological descriptions of GI inflammatory conditions are still scarce, hence extrapolation of information from domestic species is common but might not be adequate. Further research is essential to better understand the

pathophysiology of GI inflammatory processes in tigers to select the most suitable GI inflammation markers for the species.

3.4.3 Correlation between NMH, S100A12 and faecal consistency

Previous research highlighted significant correlations across inflammatory markers belonging to the same biochemical family. For example, a very strong correlation (r = 0.918, p <0.001) was found by Depauw *et al.* (2014) between faecal calprotectin and S100A12 in cheetahs undergoing a dietary intervention. Both compounds are part of the S100 family, produced by activated granulocytes (Heizmann 2007; Foell et al. 2009); thus, similar excretion patterns could be expected for calprotectin and S100A12. However, markers belonging to different biochemical families or expressed by different cellular lines, may not have the same relationship. In agreement with previous studies, no significant correlation was found between NMH and S100A12 values on either a wet or DM basis during the present study. Similarly, in dogs with chronic GI diseases no correlation was found between faecal NMH and faecal α_1-proteinase inhibitor, a clinical marker for gastrointestinal protein loss and epithelial damage (Hall et al. 2005; Berghoff et al. 2014; Heilmann and Steiner 2018). In humans with IBD, no correlation between NMH and eosinophil markers (e.g. eosinophil cationic protein and eosinophil protein X) was highlighted (Bischoff *et al*., 1997).

The lack of correlation between S100A12 and NMH was not surprising, because although they are derived from a common myeloid progenitor, neutrophils and mast cells are two well-differentiated cell types, which are found in different proportions across different tissues (Aughey and Frye 2001; Collins 2013; Heilmann and Steiner 2018). It is possible that since the markers in this study originate from different cell types (e.g. mast cells for NMH and neutrophils for S100A12) and are present at different stages of the inflammatory process, a significant correlation between them was unlikely to exist. However, as the cell types involved

in tigers' GI inflammatory conditions are still unknown, but evidence from domestic dogs and cats suggests the involvement of mast cells and neutrophils in IBD, it was necessary to evaluate both markers and a possible correlation between them.

Since inflammatory biomarkers are not exclusive to the GIT, it has been suggested that these markers should be used alongside other tools to assess gut health. Faecal consistency scoring is a common, non-invasive method to assess the health and function of the GIT in a wide range of species (Murdoch 1986; Bechert 2012; Vandeputte et al. 2016) but the relationship between faecal consistency score and faecal NMH concentrations has not previously been investigated. Regarding NMH, this study's results showed a trend towards significance for an inverse linear relationship between NMH and mean faecal consistency score ($p = 0.082$) when analysed on a wet basis. However, no significant correlation with faecal consistency score was detectable when NMH concentrations were analysed on a DM basis. Similarly, no correlation between S100A12 and faecal consistency score on either wet or DM basis was apparent.

With regard to S100A12, current results differ from Heilmann and colleagues', who reported that faecal consistency scores were inversely correlated with faecal S100A12 levels in juvenile dogs affected with a range of enteropathogens causing diarrhoea (Heilmann et al. 2018b). The lack of correlation between faecal consistency and biomarker concentrations in tigers may be explained by the fact that looser stools in the present study might not have been a consequence of an inflammatory condition, but rather a mechanical response to the diet, as opposed to Heilmann *et al.* (2018) where the causal agents of the diarrhoea were known to be enteropathogens.

In recent years, it has been proposed that in medical and biological studies, rather than using a null hypothesis testing approach, researchers should evaluate biological importance through the estimation of effect size (Nakagawa and Cuthill 2007; Cooper et al. 2009). Previous research on inflammatory biomarkers has not

evaluated effect size, hence forcing the comparison of effect size to the benchmarks established by Cohen (1988). The estimation of effect size in the current study represents the magnitude of the change in faecal concentrations of inflammatory biomarkers between dietary treatments. The high variability in faecal concentrations of S100A12 within the population could explain the weaker magnitude of the effect size observed for this biomarker. On the contrary, effect sizes for NMH were of a medium to high magnitude, which could support the hypothesis that dietary histamine can strongly influence faecal concentrations of this marker and hence act as a possible confounding factor. However, to corroborate if the changes observed in biomarker concentrations between diets were biologically important or just the expected variation within a healthy population, results need to be evaluated alongside other GI parameters for a more meaningful interpretation (see Chapter 6. General discussion).

There are, however, other possible explanations for the results obtained. Similar concentrations of macronutrients and fibre fractions were found in ED and CD (see Chapter 2). It is possible that, despite the provision of whole prey, the overall fibre content and the fermentation properties of the animal fibre fraction of the ED were insufficiently different from the CD to induce a significant change in GI inflammation status; this, therefore, precluded changes in inflammatory biomarker concentrations between dietary treatments that could then be detected. In captive cheetahs and dogs, evidence of GI inflammation was inferred after animals were exposed to extreme changes in diet format and/or nutrient composition (Depauw *et al*., 2011, Depauw *et al*., 2014; Hang *et al*., 2013). In the present study, changes between ED and CD were subtle since they both represented diets commonly fed in North American zoo collections (Lefebvre et al. 2020). Nevertheless, faecal consistency improved when tigers were fed the ED compared to CD (see Chapter 2), demonstrating an influence of diet on this less specific GI health parameter. However, the CD did not appear to trigger an inflammatory response in these healthy individuals, since no other GI parameter changed significantly between ED and CD. Whether this

population of captive tigers was clinically healthy and therefore had no signs of GI inflammation before the start of the study (in the first place) or the ED had no impact on GI inflammation remains to be elucidated through further detailed studies.

Another possible factor to consider for the interpretation of the results is the water content in the faeces. The water content of tiger faecal samples evaluated during this project fluctuated between 45 to 75%. Despite the similarities in the results when evaluating differences in NMH and S100A12 concentrations across dietary treatments using values on either a DM or wet basis, some important differences were found. When assessing the effect of diet on faecal NMH concentrations, the difference observed on a wet basis was significant ($p = 0.038$); however, when values were analysed on a DM basis, the difference decreased to only a trend towards significance ($p = 0.084$).

Substantial variation in faecal water content can be a critical factor when comparing faecal metabolite concentrations between individuals or species (Rolfe et al. 2002; Vester et al. 2008; Hang et al. 2013). To minimize variation in faecal water content due to differences across individuals or species, faecal samples could be homogenised for water content by drying prior to analysis; hence, results would be expressed on a DM basis. Reporting values on a DM basis has been extensively used in other fields such as toxicology (Scanlon 1982; Grandjean et al. 2005), endocrinology (Young et al. 2004; Parnell et al. 2015) and nutrition (Ardente and Hill 2015; Iske et al. 2016) to reduce parameter variation associated with the water content of the sample. However, for faecal biomarkers, authors have reported results exclusively on wet basis (Depauw et al. 2014; Heilmann et al. 2016b; Bridges et al. 2019; Parambeth et al. 2019). Therefore, to allow meaningful comparisons of faecal inflammatory biomarkers between animals and studies, it would be advisable to analyse and report values on a DM basis.

3.4.4 Limitations & future research

To the best of my knowledge, this is the first report of faecal NMH and S100A12 determination in captive tigers; thus, no previous reference values were available for the species. The small study population did not allow to establish reference intervals for faecal NMH and S100A12 concentrations in captive tigers. The study population was composed of individuals that seemed to be clinically healthy; hence, I could not determine if concentrations of either marker would change significantly in animals with clinical signs of GI inflammation. It will be too speculative to suggest the values observed in the present study correspond to healthy tigers or animals with GI inflammation due to the wide concentration variability of both markers, the small study population, the lack of reference value for the species and the absence of clinical signs associated with GI conditions. However, I provided comparative values and have identified possible pitfalls of relevance for future research. For example, researchers should consider evaluating dietary histamine and its correlation with faecal NMH concentrations; otherwise, prey histamine levels could act as a confounding factor for an accurate interpretation of NMH as a marker of intestinal inflammation in carnivorous species.

Another limitation of the present study was the use of similar ingredients across all dietary treatments (i.e. the commercial horsemeat). Similarities in nutritional composition between dietary treatments may have failed to modulate inflammatory response in the gastrointestinal tract of healthy tigers, as opposed to Depauw's results which were apparent following a radical change in diet (i.e. beef meat vs whole rabbit).

Based on previous findings and results from the current study, interpretation of faecal inflammatory biomarkers levels in tigers, and likely in other carnivores, should be assessed with caution and in parallel with other indicators of GI health. The use of a panel of diagnostic tools rather than a single indicator would cover more aspects of gut health and provide a better understanding of health status. To

elucidate the effect of diet on GI health, the association between putrefactive compounds, faecal fermentation metabolites and inflammatory biomarkers will be considered in Chapter 6 General Discussion. Future studies with a larger sample size, including healthy and clinically compromised tigers, need to be carried out to assess the biological variation and relevance of faecal NMH and S100A12 concentrations in this species. Such investigations could help to establish reference values to assist with the interpretation of biomarker results in tigers.

3.5 Conclusion

Clinical management of chronic enteropathies in mammals currently requires highly invasive diagnostic tools and lifelong monitoring. The use of non-invasive inflammatory markers to diagnose and track the progress of these conditions would be preferable, particularly in a zoo setting. This study quantified, for the first time, faecal NMH and S100A12 concentrations in captive tigers. Despite variations in NMH and S100A12 concentrations across dietary treatments, no significant differences were observed among diet groups. These results provide evidence in direct contrast with previous studies in which dietary influence over faecal concentrations of two inflammatory biomarkers has been reported. It remains to be determined whether this was due to tiger-, diet-, or marker-specific factors, or indeed whether the lack of difference reflects a lack of effect or poor detectability. A longitudinal study with a larger tiger population with different GI health status would be suitable to estimate reference values for the species. Moreover, confounding factors such as dietary histamine levels and the involvement of mast cells and neutrophils in tigers' GI inflammatory processes will require further research. If used as stand-alone indicators, interpretation of inflammatory biomarkers results can become challenging, especially when reference values are lacking for most species. For this reason, I will interpret these results alongside a wider set of GI parameters to better elucidate the influence of animal

fibre on intestinal inflammation in captive tigers (see Chapter 6 Integrated discussion).

Chapter 4. The impact of dietary animal fibre on the behaviour of captive tigers

4.1 Introduction

Providing adequate diets to ensure that the nutritional needs of animals in captivity are met is among the stated priorities of modern zoological collections (DEFRA 2012; Ward et al. 2018). For this reason, in the past 20 years, researchers have evaluated captive diets in an effort to understand how they may meet species' nutritional requirements (Vester et al. 2008, 2010a; Kerr et al. 2013b) and how they may affect digestive tract health (Bechert et al. 2002; Kapoor et al. 2016). More recently, zoological associations have acknowledged other important aspects of diets, such as encouraging natural feeding behaviours in carnivorous species like tigers (Blackett et al. 2016; Tilson et al. 2016). However, when dietary interventions that modify nutritional content are performed, the impact on animal welfare is normally not evaluated, and studies have focused exclusively on health (Bechert *et al.,* 2002; Depauw *et al.,* 2014), or physiological parameters such as digestibility, fermentation profiles or faecal consistency (e.g. Zhihong *et al.*, 2007; Vester *et al.*, 2008; Kerr, Beloshapka, *et al.*, 2013). On the other hand, when the diet is used as a mean of providing environmental enrichment, the nutrient composition is not evaluated, the impact on gastrointestinal parameters is overlooked, and studies focused on changes in welfare indicators such as behaviour or faecal glucocorticoid metabolites (e.g. Bashaw *et al.*, 2003; Mishra, Guru and Patnaik, 2013; Ruskell *et al.*, 2015). Nevertheless, it is likely that changes in nutrient content influence other aspects of an animal's biology– beyond gastrointestinal physiology– including behaviour.

The use of commercially processed diets for carnivores is common practice in many North American facilities (Salter et al. 1999; Bennett et al. 2010; Iske et al. 2016). It has been hypothesised that such diets could produce a reduced feeling of satiety compared to whole prey or

carcass feeding (Bosch et al. 2007; Veasey 2017). Researchers have proposed that hunger could stimulate foraging behaviours; when the expression of these behaviours is restricted in captivity, it has been suggested they could then evolve into stereotypies such as pacing (Clubb and Mason 2007; Mason 2010). Stereotypies are defined as repeated patterns of movement that do not vary in form and do not have any apparent function (Rose et al. 2017; Veasey 2017). In captive felids, stereotypical behaviours include pacing, head rolling, self-biting and tail sucking (Mason and Rushen 2006; Mohapatra et al. 2014; Stanton et al. 2015). Stereotypies are commonly used as indicators of sub-optimal welfare status, indicating exposure to acute or chronic stressors (Clubb and Mason 2003; Sajjad et al. 2011; Mohapatra et al. 2014; Vaz et al. 2017).

Modern zoological collections worldwide have recognised the importance of ensuring the best husbandry standards for the animals in their care (Mellor et al. 2015; Tilson et al. 2016). By promoting the good welfare of individuals in captivity, modern zoos can then contribute to the conservation of endangered species (Whitham and Wielebnowski 2013; Marchant-Forde 2015; Blackett et al. 2016; Tilson et al. 2016). In fact, improved welfare has been linked with increased lifespan and reproductive success (Moreira et al. 2007; Walker et al. 2012; Benhajali et al. 2014; Martin et al. 2020). Past research has also highlighted the possible beneficial effects of diet on the well-being of captive animals (D'Eath et al. 2009; Hothersall and Nicol 2009; Van Krimpen and De Jong 2014). For example, sows (*Sus scrofa domesticus*) fed high fibre diets spent less time in stereotypic behaviour and showed reduced aggressive behaviours compared to those fed a low-fibre diet (Stewart et al. 2010; Oelke et al. 2018). Researchers suggested that high fibre diets increased satiety, which in turn was responsible for the decrease in stereotypic behaviours observed (D'Eath *et al.,* 2009; Stewart *et al.,* 2010).

The natural diet of strict carnivores contains negligible plant-fibre components, yet it has been proposed that animal fibre–i.e. the low or

non-digestible glycoproteins found in the skin, fur, feathers, bone and cartilage of whole prey– play a similar role in carnivore digestive physiology as that of plant-based fibres in herbivores (Banta et al. 1979; Depauw et al. 2011, 2012; Kerr et al. 2013b). Therefore, the use of a diet containing whole prey could influence tigers' health beyond gastrointestinal benefits by increasing satiety and possibly reducing the occurrence of stereotypies.

To date, no study has taken a holistic approach to evaluates behavioural indicators of welfare in tigers undergoing a dietary intervention. The aim of the present study was to explore possible changes in behaviour-based welfare indicators (i.e. time budget and pacing frequency) in a group of captive tigers fed the two most common dietary regimes in North-American zoos: one consisting of exclusively commercial supplemented raw meat and another including 20% whole prey (Lefebvre et al. 2020). It was hypothesized that a diet containing whole prey would lead to higher satiety, compared to a diet of solely raw meat, resulting in a reduced expression of stereotypical pacing behaviour and hence a better welfare state.

4.2 Material and Methods

The experimental design and dietary treatments are described in section 2.2. Material and Methods of Chapter 2. Briefly, a randomized crossover study with eight zoo-housed tigers was performed. The Control Diet (CD), contained exclusively a commercially supplemented ground muscle horsemeat diet (100% as fed) and the Experimental Diet (ED), comprised degutted whole rabbit (20% as fed) and the commercial horsemeat diet (80% as fed). Both dietary treatments were fed for 8 consecutive weeks without washout period between diets. One tiger refused to consume the ED and was therefore fed with the CD for the whole duration of the trial.

4.2.1 Animals, enclosures, and management

Eight adult tigers were included in the current study; demographic information of the individuals can be found in Table 4.1. Tigers were housed in two different locations within the zoo and remained within their allocated location for the whole duration of the experiment (see Figure 4.1). The first location, Tiger House, consisted of two outdoor enclosures (Gorge and River habitats) and 13 indoor enclosures. This location was open for public viewing, six tigers were housed individually, in pairs, or groups of three individuals in the outdoor enclosures (see Table 4.1). The second location, Oasis, housed three tigers out of the sight of the public and consisted of two outdoor enclosures (North and South yards) and four indoor enclosures. Tigers at Oasis were always housed individually and had access to either the outdoor or indoor enclosures. Social housing was determined by animal managers based on social groups established upon first arrival of tigers at the facility, however, tigers were always kept individually in the indoor enclosures. All outdoor and indoor enclosures complied with spatial and design recommendations made by the American Zoo and Aquarium Association (AZA) for accredited institutions (Tilson *et al.,* 2016).

Table 4.1 Tigers' (Panthera tigris) demographics. Sex, subspecies, date of birth, zoo location and housing of subjects at the time of the study.

Tiger	Subspecies	Sex	Date of birth	Location	Housing group [a]
1	Bengal	F N	03/08/06	Tiger House	With tigers 2, 5, 6
2	Bengal	M N	03/08/06	Tiger House	With tigers 1, 6
3 [b]	Malayan	M	30/03/13	Oasis Habitat	Alone
4	Bengal	F N	04/04/01	Oasis Habitat	Alone
5	Bengal	M N	10/09/06	Tiger House	With tigers 1, 6
6	Bengal	F N	10/09/06	Tiger House	With tigers 1, 2, 5
7	Malayan	M	30/03/13	Oasis Habitat	Alone
8	Bengal	M N	23/03/06	Tiger House	Alone
9	Bengal	F N	23/03/06	Tiger House	Alone

F – Female; M – Male; N- neutered
[a] tigers could be housed alone or with any combination of tigers mentioned
[b] this tiger was housed at the same facilities but did not take part in the study

Tigers in both locations did not have a set feeding or training routine, or a typical routine for animals to move between enclosures, i.e. the time of the day for these activities was not consistent on a day to day basis and vary during the week. This management system was chosen to prevent boredom and promote tigers' welfare. Staff members were present at both locations all week from 0600 to 1800 h. Animals in the Tiger House were moved to indoor enclosures around 0630 h to facilitate the cleaning of the outdoor enclosures, after which tigers were randomly allocated to indoor or outdoor enclosures either in groups or individually. Throughout the day additional movement of tigers could occur to try and ensure all tigers had the opportunity to access the outdoor enclosures. Animals spent the night in indoor enclosures at the Tiger House only when the zoo had night events (e.g. during October and December due to special events that took place after the normal closure hours of the zoo).

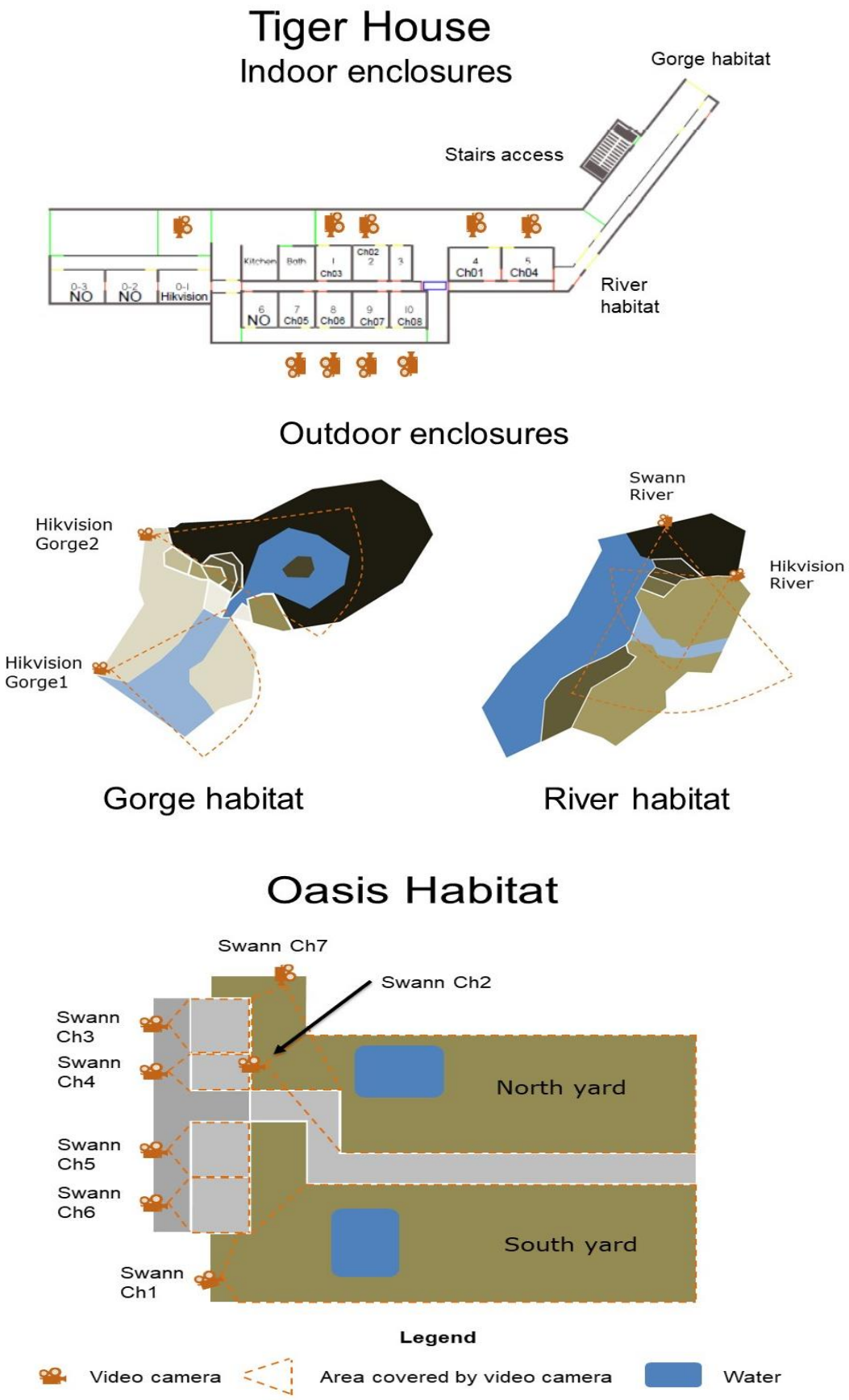

Figure 4.1 Schematic of enclosures and camera location.

Likewise, Tigers at Oasis were shifted indoors at around 0800 h to facilitate cleaning procedures. Animals remained in their allocated yard (i.e. north or south yard) during the whole day. Yard swaps occurred every other month and animals were allowed access to both yards for a few hours a day at least twice a month. Feeding and training sessions at both locations were planned around the enclosure movement schedule. The quantity of food provided to each tiger remained constant throughout the experimental trial, however, feeding timing and amounts offered at a given feeding varied during the week. All the management choices, including moving between enclosures, were planned the day before by a member of the staff.

4.2.2 Ethogram and time budget

To detect possible changes in the tigers' behavioural repertoire associated with the dietary intervention, the activity budget was determined using video footage obtained from two different recording systems: Hikvision cameras (model DS-2CD2632F-IS, Hikvision Digital Technology Co, City of Industry, CA, USA) and Swann cameras (model pro-series HD, Swann Communications U.S.A. Inc, Santa Fe Springs, CA, USA). A total of 20 cameras were installed to cover outdoor and indoor enclosures (see Figure 4.1, Figure 4.2 and Figure 4.3). Video footage was obtained during the first and last week of each dietary treatment (i.e. in weeks 1, 8, 9, and 16 of the study). Tigers were recorded 24 h per day during those weeks to obtain 168 h of observations per individual.

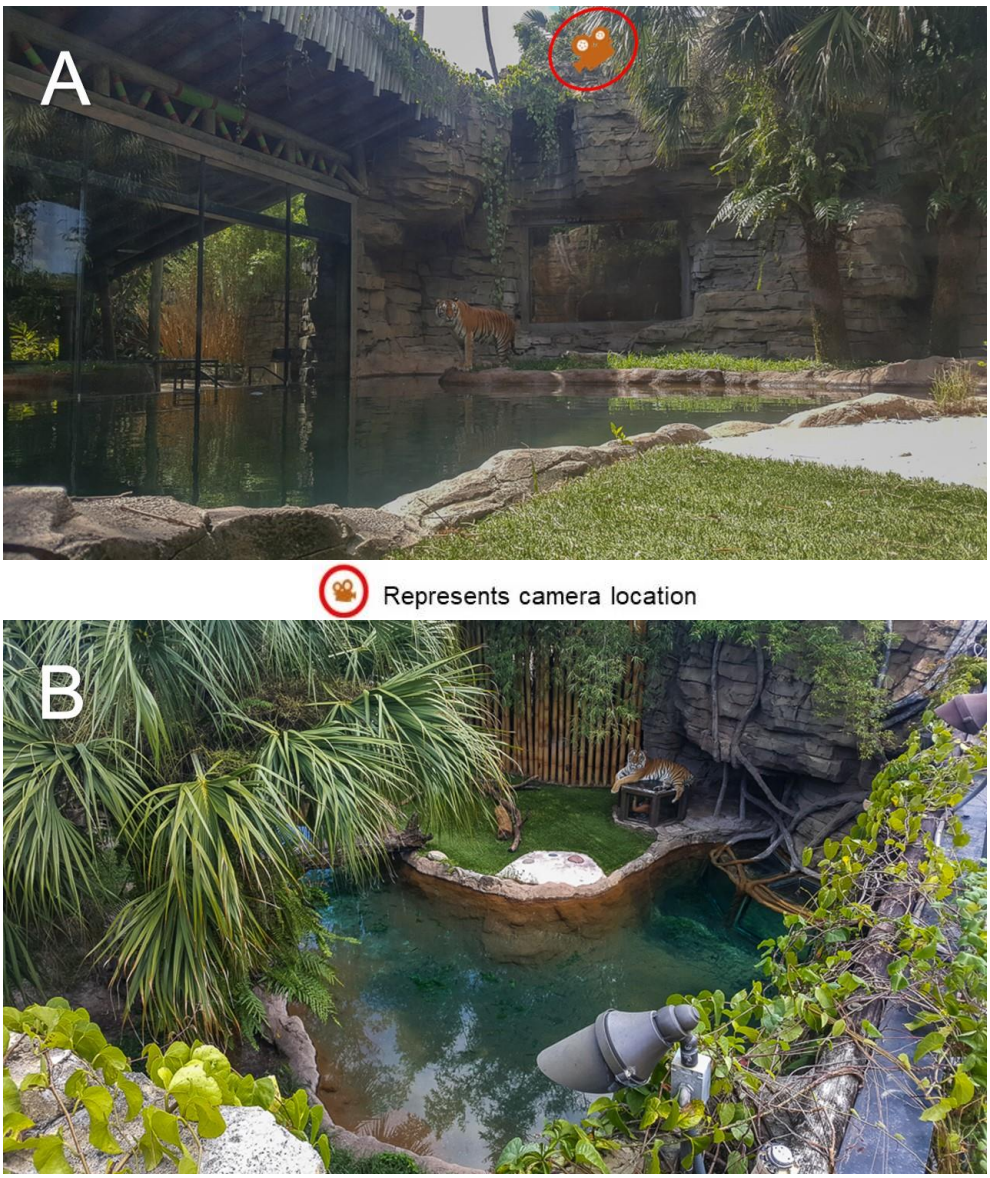

Figure 4.2 Gorge habitat from public view (A) and camera view (B)

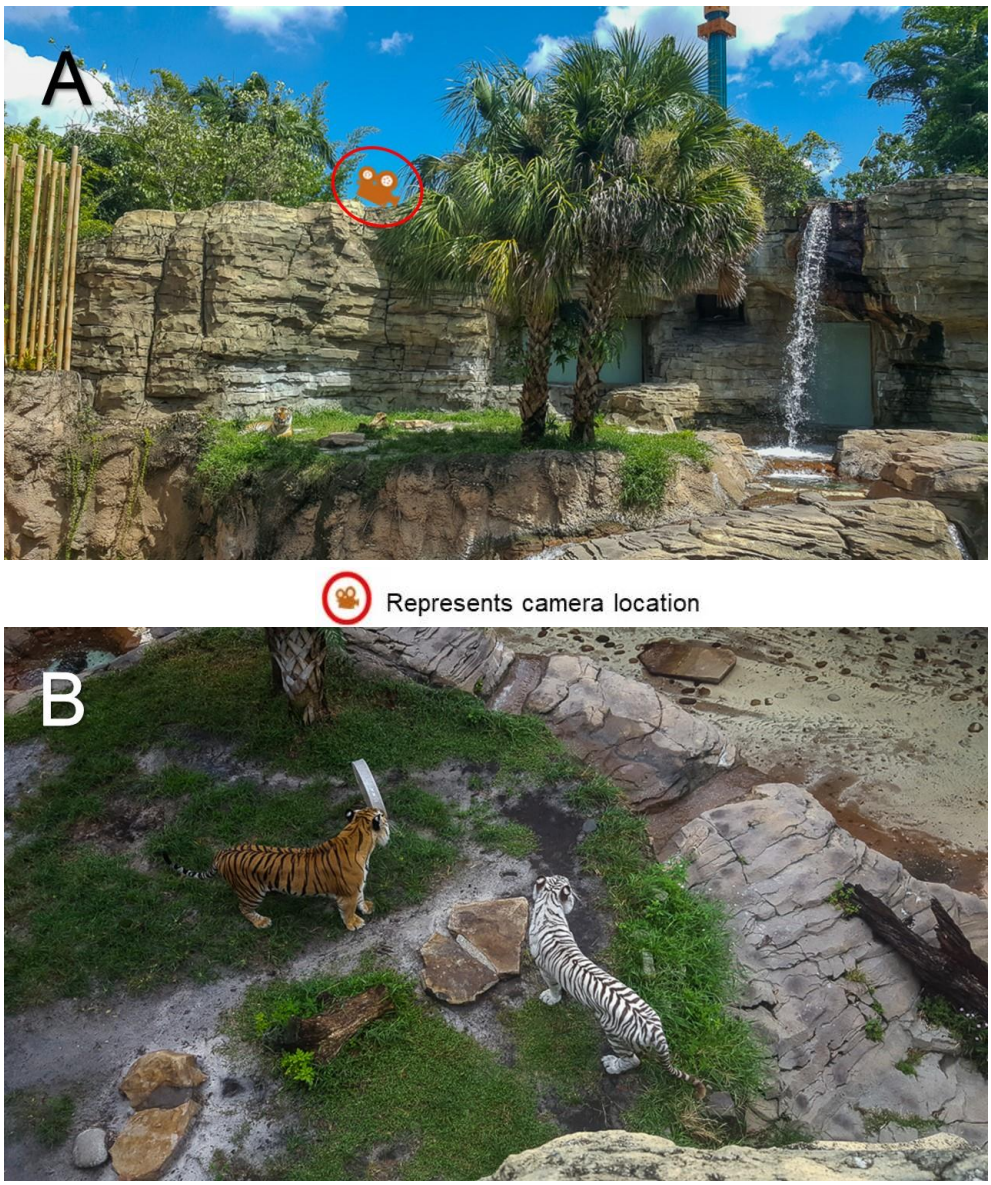

Figure 4.3 River Habitat from public view (A) and camera view (B).

Time budget was expressed as the frequencies of occurrence of the different behaviours recorded over a determined period (Munita et al. 2016). An initial ethogram was created based on the standardized ethogram for felids proposed by Stanton *et al*. (2015). To refine the ethogram, a week before the start of the feeding trial, an informal period of preliminary observations was conducted (Biolatti *et al*., 2016). The final list of behaviours included in the ethogram for the study can be found in Table 4.2. In order to distinguish between 'sleep' and 'rest' when the tiger's eyes were not visible, the animal was observed

continuously for 30 seconds before and after the observation time point. If the tiger did not move during those time periods, then this was scored as ‘sleep’; if movement such as tail flicking was perceived then the behaviour was scored as ‘rest’. Five observers were involved in the analysis of the video footage; to ensure the standardized classification of behaviours, all observers were asked to watch and score the same five video clips (Biolatti *et al.*, 2016). Inter-observer reliability was assessed by evaluating the degree of agreement in behavioural scoring between observers, which corresponded to 90% (Bashaw et al. 2003; Martin and Bateson 2007).

To improve efficiency without losing accuracy, an appropriate sampling interval was determined following the method suggested by Hämäläinen *et al.*, (2016). Briefly, every tiger was recorded for a week prior to the beginning of the dietary intervention. Total observation time equivalent to 50 h per tiger (10 hr/day) was then scored using focal sampling at 5 min intervals (Bashaw *et al.*, 2003; Mohapatra *et al*., 2014). A time budget was calculated for each tiger using the 5-min interval scans; then using every 2nd scan (representing a 10-min interval), every 3rd scan (15-min interval) and every 4th scan (20-min interval). Activity budgets were then calculated for each of these time intervals. Finally, the mean proportion of behaviours observed in these longer observation intervals was compared to the 5-min interval activity budget. The time interval that produced less than 10% difference (for the greatest number of behaviours) compared to the 5-min interval was the 10-min interval, hence this was the selected interval for the subsequent analysis of the videos (Hämäläinen *et al.*, 2016).

4.2.3 Statistical analysis

Due to technical difficulties with both the recording systems and some time periods when tigers were housed in indoor enclosures without cameras, behavioural data were not equivalent in time for all individuals (i.e. not all tigers had 1,008 observations or 168 h/tiger per week of

observation). To ensure that all tigers had equal representation in the dataset, data were standardized for the minimum amount of observation points obtained according to the procedure described by Farine and Whitehead (2015). The minimum number of observations for all tigers was 403 observation points (67 h), so the frequency of each behaviour for each tiger was adjusted using the following equation (Harvey et al. 2018)

$$Standardized\ behaviour = \left(\frac{Raw\ behaviour\ frequency}{Total\ observation\ points}\right) \times min\ observation\ points$$

After standardization, the behavioural frequencies were converted to percentages based on the total standardized observation time (Mishra, Guru and Patnaik, 2013). The percentage of 'out of view' was subtracted and not considered for further analysis as recommended by De Rouck *et al.* (2005) and Biolatti *et al.* (2016). Behaviours with <2% of occurrence were not considered for statistical analysis and included: body rub, climb, drink, eat, immersion, jump, rear, roll, run, sit, sniff, spray, stare, stretch, strike with paw, threaten, void, watch and yawn. 'Tail suck' was analysed with the rest of the behaviours since it is considered a stereotypy in felids despite occurring only in 1.1% of the observed time. One tiger's data had to be discarded from the statistical analysis since he refused to consume the ED and was fed with the CD during the whole duration of the trial, hence the behavioural data of only seven tigers were analysed.

All statistical analyses were performed using SPSS v. 24 (SPSS Inc., Chicago, Illinois, USA). Data were first analysed for normal distribution using the Shapiro Wilk test. The statistical significance threshold was set at $p = 0.05$. Behavioural data were not normally distributed, and arc-sine transformation did not result in a normal distribution; hence, non-parametric tests were performed for this dataset. All summary statistics are reported as medians and interquartile ranges (IQR). The 95% confidence intervals (CI) bias-corrected and bootstrapped (BCa) values are reported in square brackets. Assessment of correlation, using

Spearman's coefficient, was used as a form of test-retest reliability to determine if activity budget was consistent for each individual tiger's behaviours within each dietary treatment (Jacoby et al. 2014; Harvey et al. 2018). Finally, a Wilcoxon signed-rank test was used to compare the percentage of behaviours observed between the two dietary treatments, to determine if they differed.

4.3 Results

A total of 19,447 observation points (equivalent to 3,241 h) were collected during the study. Twenty-six behaviours were observed in this population (see Table 4.2). Behaviours with a low proportion of scans (i.e. <2%) can be found in Figure 4.4 The most frequently observed behaviours are shown in Figure 4.5, of which 'sleep' had the highest percentage of occurrence (ED = 57.7% and CD = 55.9%) followed by 'rest' (ED = 15.2% and CD = 14.8%), 'pace' (ED = 9.8% and CD = 11.2%), 'walk' (ED = 3.4% and CD = 4.4%), 'groom' (ED = 3.4% and CD = 4.4%) and 'stand' (ED = 2.2% and CD = 2.2%).

Table 4.2 Ethogram used for behavioural data collection of captive tigers (*Panthera tigris*) based on Hall and Bradshaw (1998), Stanton *et al.* (2015) and Biolatti *et al.* (2016).

Behaviour	Description
Body Rub	Tiger rubs any part or entire length of body against (modifier).
Climb	Tiger ascends and/or descends an object or structure.
Drink	Tiger ingests water (or other liquids) by lapping up with the tongue
Eat	Tiger ingests food (or other edible substances) by means of chewing with the teeth and swallowing
Groom	Tiger cleans itself by licking, scratching, biting or chewing the fur on its body. May also include the licking of a front paw and wiping it over one's head.
Immersion	Entering a water pool with any part of the body other than the mouth.
Jump	Tiger leaps from one point to another, either vertically or horizontally.
Out of sight	Tiger is not visible to the observer
Pace	Repetitive locomotion in a fixed pattern, such as back and forth along the same route. Can include walking, trotting and running. Must be performed at least two times in succession before qualifying as stereotypic.
Rear	Tiger stands up on its hind legs with forelegs toward or against (modifier). In the case of rear (object), forelegs may be reaching up to obtain something.
Rest	Tiger's body is on the ground in a horizontal position, including on its side, back, belly, or curled in a circular formation with eyes opened.
Roll	While lying on the ground, Tiger rotates body from one side to another. During the roll, the back is rubbed against ground, the belly is exposed, and all paws are in the air. Tiger may continue rolling repeatedly from side to side.
Run	Forward locomotion in a rapid gait, which is faster than walking or trotting
Sit	Tiger is in an upright position, with the hind legs flexed and resting on the ground, while front legs are extended and straight
Sleep	Tiger is lying with its head down and eyes closed, performing limited head or leg movement, and is not easily disturbed.
Sniff	Tiger smells (modifier) by inhaling air through the nose
Spray	While standing with tail raised vertically, Tiger releases a jet of urine backwards against a vertical surface or object. The tail may quiver as urine is discharged.

Stand	Tiger is in an upright position and immobile, with all four paws on the ground and legs extended, supporting the body
Stare	Tiger gazes fixedly at (modifier) and is not easily distracted. In the case of social stare, gaze may be directed at another Tiger's eyes
Stretch	Tiger extends its forelegs while curving its back inwards
Strike with paw	Tiger strikes at (modifier) with forepaw and contact is made. Claws are usually extended.
Tail suck	Tiger excessively grooms its tail. May result in the removal and visible loss of fur, as well as skin irritation
Threaten	Tiger directs aggressive behaviours toward (modifier) without making any physical contact with it. Can include baring the teeth, snarling, arching the back, piloerection, ground slapping, striking at with the paw, extending claws, and producing various vocalizations (spitting, hissing, growling, etc.).
Void	Tiger releases urine/faeces on the ground while in a squatting position
Walk	Forward locomotion at a slow gait.
Watch	Tiger observes a specific stimulus (or modifier). Behaviour is distinguished by tracking movements of the eyes and head.
Yawn	Tiger opens its mouth widely while inhaling, then closes mouth while exhaling deeply.

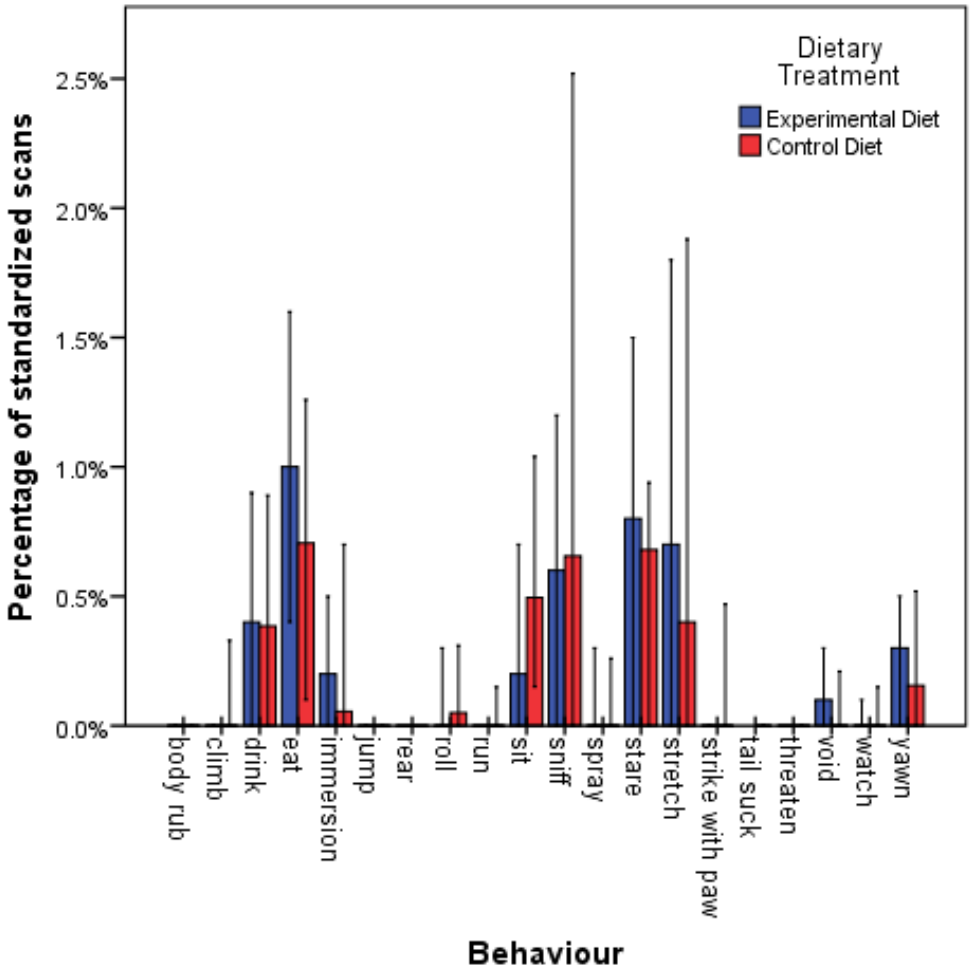

Figure 4.4 Percentage of rare behaviours observed in seven captive tigers (Panthera tigris) fed either an Experimental Diet containing 20% whole rabbit or a Control Diet comprising only a commercially supplemented ground muscle horsemeat. Values are expressed as the median for each category and error bars represent the 95% confidence interval. These behaviours were grouped in the category ‘others’ for further statistical analyses.

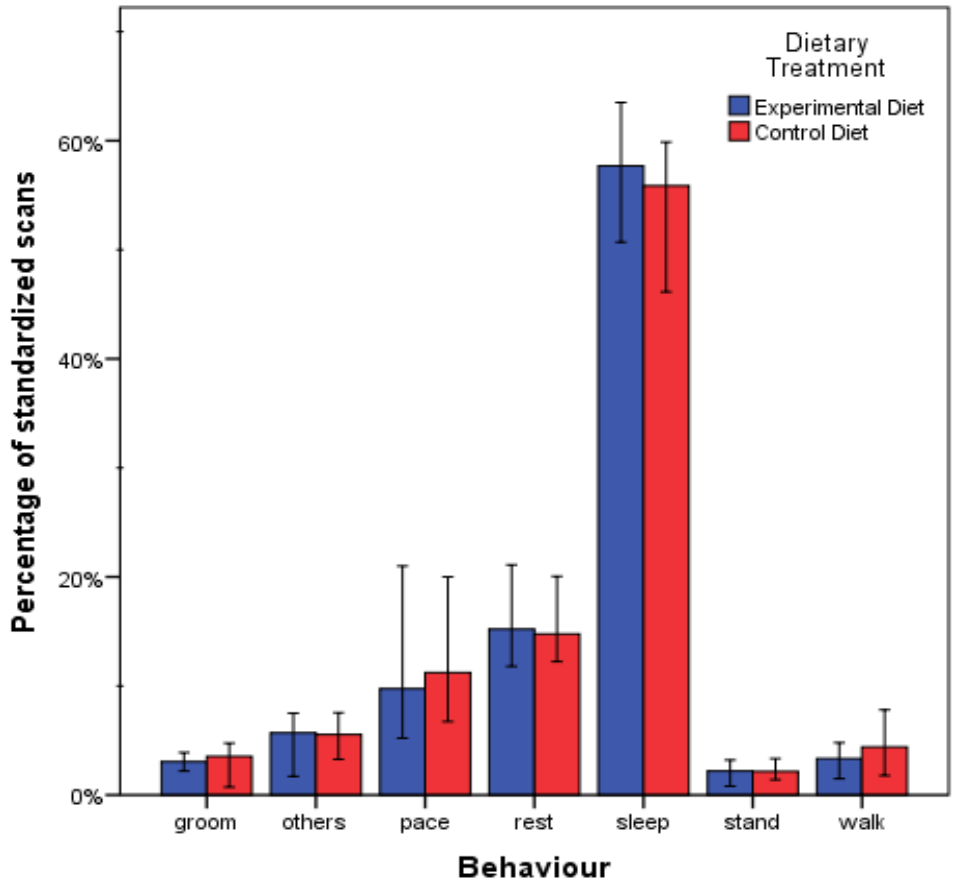

Figure 4.5 Principal behaviours observed for seven captive tigers (Panthera tigris) fed either an Experimental Diet containing 20% whole rabbit or a Control Diet comprising only a commercially supplemented ground muscle horsemeat. Values are expressed as the median for each category and error bars represent the 95% confidence interval.

Consistency of behaviours between the first and last week of the dietary intervention was statistically significant for six of seven tigers (r_S ≥0.79, p <0.05) for both dietary treatments.

With regard to the effect of dietary treatments, results of the Wilcoxon signed-rank test revealed that the percentage of occurrence of the most frequently observed behaviours (as well as 'tail suck') did not differ significantly between ED and CD (all p >0.05, see Table 4.3).

Table 4.3 Results of the Wilcoxon signed-rank test (T) for comparison of main behaviours observed in seven tigers (*Panthera tigris*) fed a commercially supplemented ground muscle horsemeat diet (Control Diet) or a diet with 20% added whole rabbit (Experimental Diet). Values represent the percentage of occurrence and are expressed as median (interquartile range).

Behaviour	Median (IQR)		*T*	*p*
	ED	CD		
Groom	3.1 (1.2)	3.5 (2.7)	40	0.433
Pace	9.8 (10.8)	11.2 (12.1)	34	0.245
Rest	15.2 (7.9)	14.8 (6.2)	51	0.925
Sleep	57.7 (11.4)	55.9 (9.5)	65	0.433
Stand	2.2 (1.8)	2.2 (1.5)	39	0.397
Tail suck	0 (0)	0 (0)	1	0.317
Walk	3.4 (2.7)	4.4 (5.3)	27	0.196

IQR- Interquartile Range; ED- Experimental Diet; CD- Control Diet

4.4 Discussion

This study assessed whether increased dietary fibre could modify behavioural indicators of welfare, in the form of time budget and pacing frequency, in captive tigers undergoing a dietary intervention. Results indicate that the activity budget of tigers fed a diet with 20% added whole prey (ED) did not differ from that of tigers fed a diet comprising exclusively a commercial supplemented raw horsemeat (CD). Contrary to my hypothesis, stereotypical behaviours were observed at similar frequencies during both dietary treatments.

Behaviour is often used as an early indicator of compromised welfare because it represents an individual's first attempt to cope with adverse situations (Dawkins, 1998; Hockenhull and Whay, 2014; Biolatti *et al*., 2016; Rose, Nash and Riley, 2017). The current investigation recorded several differences in activity budgets compared to previous tiger studies. For example, although inactive behaviours (i.e. 'sleep', 'rest', 'sit' and 'stand') are commonly reported as the predominant behavioural category in tigers (Biolatti *et al*., 2016), the study population seemed to be less active than other captive tiger populations. Tigers in the current

study spent 72.9-75.1% of the observation time performing inactive behaviours, while previous studies have reported values of 10.5% (Sajjad *et al*., 2011), 32.3-52.6% (Mishra, Guru and Patnaik, 2013) 59.9% (Mohapatra, Panda and Acharya, 2014), and 60.1% (Biolatti *et al*., 2016). A possible explanation for such a difference is that previous studies observed tigers only during zoos' opening hours (i.e. between 0900 and 1800 h); instead, in the present study observations occurred over 24 hours. Tigers were more prone to sleep while keepers were not present (i.e. between 1800 and 0500 h), which could explain the discrepancies in resting behaviours observed across studies (Miller et al. 2013a).

Stereotypic behaviours, such as pacing, are commonly used as indicators of diminished welfare in large felids (Clubb and Mason 2003; Sajjad et al. 2011; Mohapatra et al. 2014; Vaz et al. 2017). In the current study, pacing was observed between 9.8% (ED) and 11.2% (CD) of the time, a difference that was not significant. Previous studies in captive tigers have reported a wide range of pacing frequencies from 0.43% (Biolatti *et al.*, 2016) to 31% (Mishra, Guru and Patnaik, 2013). This study's results are similar to those of Vaz *et al.* (2017) who found that captive tigers in Indian zoos spent 12% of the time observed pacing. Authors have tried to elucidate the motivation underlying such behaviours but, to date, the meaning or significance of stereotypical behaviours is still controversial (Mason and Latham 2004; Bacon 2018). Nevertheless, stereotypies are still considered indicators of diminished welfare (for a comprehensive review see Mason, 2010; Rose, Nash and Riley, 2017).

In addition to pacing, another stereotypical behaviour observed during the current study was 'tail suck'. Although this stereotypy was only performed by two tigers at a low frequency (1.8-2.9% of observations), it was included in the statistical analyses as an indicator of reduced welfare (Stanton et al. 2015). Similar to pacing, there was no significant difference in the observed frequency of 'tail suck' between dietary treatments. Although 'tail suck' is among the stereotypies described for

felids (Stanton et al. 2015; Phillips et al. 2017) previous studies in captive tigers have not reported this behaviour (Bashaw et al. 2003; De Rouck et al. 2005; Sajjad et al. 2011; Miller et al. 2013a; Mohapatra et al. 2014; Ruskell et al. 2015; Phillips et al. 2017). The difficulty for inexperienced observers to distinguish between normal and excessive grooming behaviours could have affected the ability of previous studies to score this stereotypy. In addition, the use of longer sampling time intervals in those studies (e.g. 20 min) could have decreased the possibility of detecting low-frequency behaviours such as 'tail suck' (Martin and Bateson 2007). Due to the rare occurrence of 'tail suck' in this study population, it might have been challenging to detect a change associated with the diet even if such difference was larger in magnitude.

Different factors have been reported to influence stereotypic behaviours in tigers, such as social housing, husbandry routines, the anticipation of food delivery and environmental factors (e.g. enclosure size, number of visitors, the presence of water pool, trees, dens or visual barriers) (De Rouck *et al.*, 2005; Miller, Leighty and Bettinger, 2013; Vaz *et al.*, 2017). Hence, comparing pacing frequency among different institutions might be inappropriate given that all aforementioned variables could act as confounding factors (Vaz *et al.*, 2017). For this reason, a crossover design was selected: firstly, to allow comparisons within the study population when tigers were fed the CD or the ED and secondly, to minimise possible confounding factors (Bate and Clark 2014). For example, it is possible that the out-of-hours events happening in October and December, where tigers had to be kept indoors at night, might have influenced the frequency of stereotypical behaviours observed in the present study. However, tigers were habituated to be housed indoors during such events and these situations happened during both phases of the feeding trial, hence a similar effect on both dietary treatments was expected. In addition, consistency of behaviours was analysed to corroborate that within dietary treatments behaviours occurred at similar frequencies rates (Jacoby et al. 2014).

An association between diet and stereotypical behaviours has been previously reported in a wide range of species such as pigs, dogs and chickens (Robert et al. 1993; Hothersall and Nicol 2009; Van Krimpen and De Jong 2014). These studies proposed that signs of hunger, linked to a decreased satiety, promoted foraging-oriented behaviours which developed into stereotypies or accentuated aggression in group-housed species (Meunier-Salaün et al. 2001; D'Eath et al. 2009). To promote satiety without increasing nutrient allowance, fibre is commonly employed as a bulking agent in the diet (Owens et al. 2014; Hours et al. 2016). Possible mechanisms by which fibre can affect satiety range from physical effects (e.g. gastric distension) to more complex physiological interactions (e.g. release of satiety-related hormones like leptin and ghrelin) (Butterwick et al. 1994; Bosch et al. 2009; D'Eath et al. 2009; Backus and Wara 2016). High fibre diets have been linked with increased time spent eating, reduced time engaged in stereotypies and reduced aggression, in sows (Robert et al., 1993; Stewart et al., 2010), broilers (Van Krimpen and De Jong, 2014), horses (Bulmer et al. 2015) and giraffes (*Giraffa camelopardalis*) (Baxter and Plowman 2001). Similarly, in dogs, a reduction in stereotypical behaviours was reported when animals were fed a high-fibre diet containing soybean hulls compared to a non-fibre diet (Scheraiber et al. 2018).

Although in the wild, carnivore diets contain negligible amounts of plant fibre, it is believed that animal fibre plays a similar role to that of plant fibre in herbivores (Depauw *et al.,* 2011; Depauw *et al.,* 2012). Unfortunately, for North American facilities, whole prey diets can be challenging to source thus carnivores are fed with highly digestible raw meat or commercial ground-meat diets (Salter *et al.,* 1999; Iske, Morris and Kappen, 2016; Lefebvre *et al.,* 2020). Researchers have suggested that highly digestible diets fail to maintain the feeling of satiety, making carnivorous species prone to develop stereotypical behaviours such as pacing (Bosch et al. 2007; D'Eath et al. 2009; Veasey 2017; Scheraiber et al. 2018). Based on these previous studies, it was hypothesised that the animal fibre contained in the ED would have provided a longer feeling of satiety compared to the CD, which comprised raw muscle

tissue and cellulose. It was expected that enhanced satiety would have influenced behaviour and reduced pacing occurrence in tigers fed the ED compared to the CD; however, this study's results did not support such ideas.

There are two likely diet-related causes for the lack of difference in pacing frequency between dietary treatments. Firstly, the particular type of whole prey selected for the ED. In the wild, tigers spend the vast majority of their time engaged in foraging and prey consumption behaviours (see Chapter 1 section 1.1.2 Natural diet and feeding behaviours) (Mazák 1981; Szokalski et al. 2012; Mishra et al. 2013). While in captivity, commercial diets like the CD require little manipulation and are readily consumed, hence minimizing the time spent in feeding-related behaviours (Bond and Lindburg 1990; O'Regan and Kitchener 2005). During the ED, a numerical reduction in 'pace' was accompanied by a modest increase in 'sleep' and 'rest' compared to CD, though such differences were not statistically significant. The use of bones or large-size carcass has proven beneficial for carnivorous species including cheetahs (*Acinonyx jubatus*) (Bond and Lindburg, 1990), Andean condors (*Vultur gryphus*) (Gaengler and Clum 2015) and tigers (Ruskell *et al.,* 2015). In these studies, animals spent more time physically engaged with the consumption of food and less time involved in stereotypic behaviours (Bond and Lindburg, 1990; Gaengler and Clum, 2015; Ruskell et al., 2015); in addition, frequencies of other behaviours such as 'groom', 'rest' or 'walk' increased (Ruskell et al., 2015). Since rabbits are a small-size prey for tigers, the amount of time spent eating the ED was probably too similar to that required to consume the CD, and thus not likely to cause a significant change in any of the observed behaviours. It is possible that the use of larger prey size could have resulted in significant changes in activity budget between diets, similar to those reported by Ruskell *et al.* (2015). However, the diets used in the current experiment were selected to represent two typical dietary regimes of North American zoos, in which rabbit is the most common whole prey source (Lefebvre *et al.,* 2020).

Secondly, the low inclusion rate of whole prey in the ED (20% as fed) could explain the lack of difference in frequency of pacing between diets. As a result of the proportion of rabbit in the ED, the amount of animal fibre may have been insufficient to result in a significant change in satiety– due to the similarity in fibre composition between dietary treatments– which, in turn, could have influenced behaviour. Significant differences in behaviour (e.g. stereotypies frequency, presence of aggressive behaviours or handling reactivity) and improved satiety have been reported when animals are fed diets with significant differences in fibre content. For example, in sows, a reduction in stereotypical behaviour and increased satiety was observed when animals were fed diets with 10-20% crude fibre compared to a control diet with 2% crude fibre (Robert et al. 1993). While in dogs, no significant effect of diet on food intake– used as a proxy of satiety– was observed with treatments ranging from 0.2 to 1.2% of crude fibre (Butterwick, Markwell and Thorne, 1994). This suggests that to exert an observable effect on satiety, high fibre content might be needed. However, a study with domestic cats fed a 27.1% total dietary fibre (TDF) found no difference in satiety compared to a control diet with 10.4% TDF (Loureiro et al. 2016). However, not all prior studies support the theory that higher fibre intake improves satiety; dogs fed a diet with 12.4% TDF showed a tendency to increase voluntary food intake compared to a diet with 9.4% TDF (Bosch *et al.,* 2009). Results from Bosch *et al.* (2009) indicate that not only inclusion rate or fibre content but also fibre characteristics, such as fermentability, could influence satiety through higher production of short-chain fatty acids and their effect on the secretion of satiety-related hormones (Brownlee *et al.,* 2006; Hooda *et al.,* 2013; Loureiro *et al.,* 2016). The apparently conflicting results obtained from previous studies evaluating fibre in relation to satiety and behaviour highlight the possibility that complex mechanisms, that remain to be fully understood, are involved in the control of satiety and hunger. Future studies should assess if diets with more substantial differences in dietary fibre content (ideally 100% whole prey vs 100%

ground meat) can exert a more profound effect on satiety to also influence behaviour.

Finally, such results might be explained by the fact that tigers were managed under a randomised schedule. Previous research has highlighted the importance of providing captive felids with variability and novelty in their environment (Clubb and Mason 2007; Quirke and O'Riordan 2011; Szokalski et al. 2012). Quirke and O'Riordan (2011) hypothesised that, when captive cheetahs were managed through predictable schedules, their response to various stimuli (such as environmental enrichment) diminished due to a habituation factor, e.g. lower pacing and increased exploratory behaviours were observed when cheetahs were fed at different times throughout the day compared to a feeding schedule with consistent feeding times. As a consequence, they believed that predictable schedules had a negative impact on cheetah welfare. In the current study, the timing for moving animals between enclosures, training, and feeding varied from day to day, the facility managers indicated that the rationale for this system was to enhance tigers' welfare. Hence, this study population may have been accustomed to experiencing a wide variety of stimuli considering the unpredictable management schedule, hence, the small dietary variation provided by the dietary treatments was not sufficient to result in detectable changes in behavioural indicators. Previous studies of captive tigers have reported predictable schedules with set times for access to outdoor enclosures and feeding times (Bashaw *et al.,* 2003; Miller, Leighty and Bettinger, 2013; Phillips *et al.,* 2017). Therefore, reports of reductions (compared to baseline values) in time spent pacing after the use of environmental enrichment could simply be associated with the novelty introduced by the enrichment provided. In addition, increased pacing has been observed prior to feeding in cheetahs (Quirke and O'Riordan, 2011) and lions (*Panthera leo*) (Altman et al. 2005). In cheetahs, the lowest levels of pacing were reported when animals had an unpredictable feeding schedule (Quirke and O'Riordan, 2011). Since the study population had no fixed feeding timetable, pacing was observed mainly before other predictable events,

such as the arrival of staff members in the morning or before being moved to a different indoor/outdoor enclosure. Therefore, the unpredictable husbandry schedule could be a major confounding factor in the present study and a possible explanation for the lack of difference in activity budget between dietary treatments. To confirm if daily novelty could have surpassed any possible influence of animal fibre as a modulator of behaviour, future studies should investigate animals under a fixed and a random schedule.

4.4.1 Limitations & future research

Our findings may be limited by the small sample size and the fact that only one zoological institution was evaluated. Due to technical difficulties, behavioural data had to be standardized for all tigers to a third of what was originally planned (i.e. 405 of 1,008 observation points). In addition, due to the complexity of the enclosures and the location of the video cameras (see Figure 4.2 and Figure 4.3), some individuals were 'out of sight' for up to 83% of the observation time. All of these limitations could have introduced confounding factors that should be considered for the interpretation of this study's results.

Another limitation of the current study was the similarity between dietary treatments. The inclusion rates of the present study were based on two of the most common feeding regimes of captive North American tigers: a diet with 20% added whole prey (ED) and a diet comprising exclusively commercial supplemented raw meat (CD) (Lefebvre et al. 2020). The current study tested practical inclusion rates rather than experimental rates– designed to achieve a maximal effect– because I wanted to evaluate the benefits of adding animal fibre in the concentrations currently used by zoological collections. Future research using different prey sources could determine if larger prey size can influence behaviour in a significant way as opposed to small prey like rabbits. In addition, the use of more clearly differentiated diets, such as 100% whole prey vs exclusively muscle meat, could be used to

determine whether higher concentrations of animal fibre can influence satiety and thus modulate behaviour. However, it will be essential to determine first if changes in activity budget or behavioural repertoire reflect indeed an enhanced welfare status for captive tigers.

Although the fibre fractions of both the CD and ED seemed to be similar (see Results section Chapter 2), the analytical methods used to quantify fibre were developed for plant material products, and might not represent less-fermentable animal fibre components adequately (such as hair, sinew, skin and bones) (Depauw et al. 2012; Cools et al. 2014). Until a suitable assay for quantification of fibre content originating from animal products is validated, the only option available is the more traditional, but likely insufficient, descriptive-analytical methods. Further research is needed in this area to elucidate the possible behavioural benefits of a diet containing whole prey compared to a solely commercial raw meat-based diet.

Considerably more work is needed to determine whether the use of animal fibre in the diet of captive tigers can influence behavioural welfare indicators. Further studies could assess other animal-based indicators such as demeanour– using Qualitative Behavioural Assessment (Wemelsfelder et al. 2001). Qualitative Behavioural Assessment provides information on emotional state by assessing demeanour and body language, i.e. subtle changes in its expressions, posture, or movement (Andreasen et al. 2013; Minero et al. 2016). This methodology has been validated as a welfare indicator and correlates significantly with other behavioural and physiological markers (Wemelsfelder 1997; Walker et al. 2016; Hintze et al. 2017).

4.5 Conclusion

In summary, the current study showed that the addition of 20% animal fibre (as fed) made no significant difference in the activity budget of captive tigers compared to when the same tigers consumed a commercial raw meat diet. Stereotypical behaviours observed in these tigers included pacing and tail suck and were performed at similar frequencies during both dietary treatments; similarly, the rest of the behaviours observed did not vary between diets. These results failed to support the hypothesis that feeding whole prey at a 20% inclusion rate might prove beneficial at the behavioural level to improve tigers' welfare. However, higher inclusion rates need to be evaluated to determine if behaviour can be influenced by dietary fibre. Further research is needed to investigate the impact of predictable and unpredictable husbandry schedules on tigers' welfare. To extend such findings, the use of a larger sample size and diets with higher concentrations of animal fibre should be trialled.

When undertaking a welfare assessment, many authors have advocated the use of an array of indicators rather than relying exclusively on a single measure, such as the presence or frequency of stereotypies (Mason and Latham 2004; Broom 2007). Since animal welfare is a complex construct, no single welfare measure is adequate as a standalone (Veasey et al. 1996; Broom 2008; Hockenhull and Whay 2014; Marchant-Forde 2015; Blackett et al. 2016). The use of a panel of indicators (e.g. environmental, physiological and behavioural) could provide a more complete evaluation of an individual's welfare status (Dawkins 1998, 2004; Whitham and Wielebnowski 2013). When evaluating the diets of captive tigers, a holistic approach considering physiological and behavioural parameters is thus required; this could promote adequate management guidelines and improve the welfare standards of tigers under human care. The correlation between behavioural and physiological welfare indicators will be discussed in Chapter 6, Integrated Discussion.

Chapter 5. Adrenal response of captive tigers to dietary animal fibre

5.1 Introduction

Animal welfare has become a priority area for most modern zoological collections worldwide in an attempt to improve conditions for animals under their care (Whitham and Wielebnowski 2013; Marchant-Forde 2015; Blackett et al. 2016; Tilson et al. 2016). Different welfare assessment indicators have been developed over the years; of these, the analysis of glucocorticoids metabolites is probably the most widely used physiological indicator of stress in zoo animals (Palme et al. 2005; Veasey 2017). Although the release of cortisol and/or corticosterone from the cortex of the adrenal glands, in the short term, is aimed at increasing the chances of animals to cope with a stressor (e.g. by mobilizing stored energy), prolonged periods of high glucocorticoid concentrations are believed to be detrimental to the overall health of individuals, for example by suppressing the immune system reaction or decreasing reproductive success (Touma and Palme 2005; Metrione and Harder 2011; Bhattacharjee et al. 2015). Since a vast array of non-stressful stimuli can influence the secretion of glucocorticoids (see section 1.5.2.2 Physiological indicators of welfare, Chapter 1) researchers have advised interpreting results with caution; nevertheless, quantification of glucocorticoids remains a common animal-based indicator employed during welfare assessment (Lane, 2006; Dickens and Romero, 2013; Palme, 2019).

Diet is considered a key domain of animal welfare, that not only fulfils physiological needs– essential for an animal's survival– but can promote the performance of behaviours potentially impacting welfare (D'Eath et al. 2009; Kasanen et al. 2010; Veasey 2017). For example, researchers have proposed that commercially processed raw meat diets– commonly fed to carnivores in North American facilities– could result in reduced satiety feeling compared to whole prey or carcass

feeding (Bosch et al. 2007; Iske et al. 2016; Veasey 2017; Lefebvre et al. 2020). Although subjective, hunger is considered a negative experience that can affect welfare (D'Eath et al. 2009; Verbeek et al. 2011). It has been hypothesised that a persistent state of hunger in carnivores can lead to unfulfilled foraging behaviours that, in turn, can develop into stereotypies- repetitive movements without any apparent function (Veasey, 2017). Yet, indicators of diminished welfare such as increased aggressive behaviours, oral stereotypies and increased glucocorticoid concentrations have been reported in a wide range of species (including pigs (*Sus scrofa*), chickens (*Gallus gallus domesticus*), rabbits (*Oryctolagus cuniculus*), howler monkeys (*Alouatta pigra*) and humans facing dietary restrictions (Parrott and Misson 1989; Anderson et al. 2002; Behie et al. 2010; Menchetti et al. 2015). On the other hand, diets promoting a feeling of satiety over a longer period of time have been associated with improved welfare in those same species (Oelke et al. 2018; Manu et al. 2020; Tahamtani et al. 2020).

The aim of this study was to evaluate the two most common feeding regimes for captive tigers in North American zoos: a diet exclusively comprising a commercial supplemented raw horsemeat, and a diet with 20% added whole prey (Lefebvre *et al.,* 2020). It was hypothesized that a diet with added whole prey would lead to an increased sense of satiety, and hence positively affect welfare, reflected as a reduction in faecal glucocorticoid metabolites (FGMs) compared to levels measured when the animal was fed the commercially supplemented diet.

5.2 Material and Methods

The experimental design and dietary treatments are described in section 2.2 Material and Methods Chapter 2. Detailed information on tigers' demographics, housing conditions and feeding routine can be found in Chapter 4, section 4.2.1 Animals, enclosures, and management. Briefly, a randomized crossover study was performed with eight zoo-housed tigers. The Baseline Diet (BD), which

corresponded to the historic diet these tigers consumed prior to the beginning of the feeding trial, consisted of a mixture of featherless whole chicken (*Gallus gallus domesticus*) (8% as fed), degutted whole rabbit (*Oryctolagus cuniculus*) (3% as fed), horse shanks (corresponding to the biceps femoralis and semitendinosus muscles) or horse necks (both cuts with skin and bones) (7% as fed) and a commercially supplemented ground muscle horsemeat diet (82% as fed). The Experimental Diet (ED) comprised degutted whole rabbit (20% as fed) and the commercial horsemeat diet (80% as fed), while the Control Diet (CD) contained exclusively the commercial horsemeat diet. Both the CD and ED were fed for eight consecutive weeks without washout period between diets. One tiger refused to consume the ED and was therefore fed with the CD for the entire duration of the trial. Since tigers shared external enclosures, each tiger was fed a different colour of non-toxic plastic glitter (Colorations®, Discount School Supply, Carol Stream, IL, USA). To enable identification of each tiger's stool, one gram of plastic glitter was mixed with the horsemeat and fed daily throughout the experiment (Fuller et al. 2011; Hogan et al. 2011).

5.2.1 Faecal sample collection

Throughout the 16 weeks of the experiment, fresh faecal samples were collected opportunistically every third day from the tigers' enclosures during the morning cleaning routine (between 0600 to 0800 h). Prior to collection, the consistency of each scat was scored using the Felid Taxon Advisory Group (TAG) faecal scoring system (Felid TAG 2014), a photograph of the sample was taken prior to sample collection, and a 50 g subsample was then collected using a plastic bag (avoiding sections contaminated with substrate, urine or water). Samples were transported in a container with ice packs to the laboratory for preparation, where they were immediately homogenised inside the plastic bag with the help of a wooden tongue depressor, and then an aliquot of 10 g was removed and stored at -40 °C in a sterile Whirl-Pak® collection bag (Nasco; Fort Atkinson, WI, USA). Before analysis,

all samples were freeze-dried to constant weight (model 2000, Freeze Dry Company, Inc; Nisswa, MI, USA).

5.2.2 Faecal hormone extraction and enzyme-immunoassay

Determination of FGMs was performed at the laboratory of the South-East Zoo Alliance for Reproduction & Conservation (University of North Florida, Jacksonville, FL, USA). Hair, bone, grass and any other visible extraneous materials were removed from the sample, leaving only faecal matter prior to weighing. An amount of 0.2 g of freeze-dried faeces was weighed into 16 x 100 mm extraction plastic tubes using an analytical scale (model USS-DBS8, U.S. Solid; Cleveland, OH, USA). When more than one scat was collected on the same day from an individual tiger, samples were combined during weighing, such that only one sample per day per individual was created for extraction (i.e. if two samples were collected on the same day, 0.1 g of each freeze-dried scat was weighed and then mixed within the plastic tube in order to create a single sample for extraction). For extraction, 0.5 mL of reverse osmosis (RO) water followed by 4.5 mL of anhydrous ethanol were added to each tube. Tubes were then vortexed for 15 minutes at 90 rpm in a multi-pulse vortexer (model 099A-VB4, Glas-Col; Terre Haute, IN, USA) and centrifuged for 10 minutes at 3100 rpm (Labofuge 400; Heraeus Instruments; Hanau, Germany). The supernatant was decanted into two pairs of 12 x 55 mm plastic tubes. The first pair contained 2 mL of undiluted ethanol extract each and was used for enzyme-immunoassay (EIA), while the second set contained 0.5 mL of undiluted ethanol extract each and was dried in a fume hood for 3 days and archived as a backup. Both sets of tubes were stored at -20 °C until further analysis.

- *Cortisol*

Faecal cortisol metabolites (FCM) were quantified via EIA using a cortisol polyclonal antiserum and cortisol: horseradish peroxidase (HRP) (R4866; C. Munro, University of California-Davis, CA, USA). Antibody cross-reactivity for the R4866 anti-cortisol antiserum has been previously reported as 100% with cortisol and less than 10% with other steroids (Young et al. 2004; Narayan et al. 2013). This cortisol antibody has previously been validated for use in tigers (Narayan et al. 2013; Parnell et al. 2014; Bhattacharjee et al. 2015; Ruskell et al. 2015). Assay plates (Ultra Cruz ELISA plate 96 wells, Santa Cruz Biotechnology, Inc; Santa Cruz, CA, USA) were coated with 10 mg/mL of goat anti-rabbit IgG (Arbor Assays, Ann Arbor, MI, USA). Neat samples were diluted 1:10 in assay buffer (saline phosphate buffer, pH 7) and standard curves were prepared by serially diluting 200 µL of cortisol standard stock (1000 pg/well, catalogue ID Q3880-000; Steraloids, Newport, RI) with 200 µL of assay buffer. Cortisol-HRP (1:100) and cortisol antiserum stock (1:85) were also diluted in assay buffer. Each well was then loaded in duplicate with 50 µl of the standard curve, samples and internal controls followed by 50 µl of cortisol-HRP and 50 µl of cortisol antibody, except for two blank wells that did not contain antibody. Internal controls were made from synthetic cortisol standard stock and were binding at 24% (high control) and 41% (low control) of HRP binding on the standard curve. The plate was covered with a plate sealer and shaken continuously for 1 hour at room temperature (Lab Line Instruments, Inc; Melrose Park, IL, USA). After shaking, the plate was washed four times with 300 µl of wash buffer (400µl Tween 20 in 5L of RO water). Each well was then loaded with 100 µl of high kinetic tetramethylbenzidine (TMB) (Moss, Inc; Pasadena, MDN, USA) and incubated at room temperature for 10 min, after which 50 µl of stop solution (1M HCl) was added. Light absorbance was read at 450 nm using a microplate reader BioTek model ELx808 (BioTek Instruments, Inc; Winooski, VT; USA).

- *Corticosterone*

An EIA was also performed to measure faecal corticosterone metabolites (FCCM) concentration using a corticosterone-3-carboxymethyl oxime polyclonal antiserum and corticosterone: HRP (CJM006; C. Munro, University of California-Davis, CA, USA). Cross-reactions of this polyclonal antibody to parent compounds have been determined at 100% for corticosterone and 0.23% for cortisol (Metrione and Harder 2011; Mesa et al. 2014). The CJM006 corticosterone antibody has been previously validated in a wide range of species, including tigers, to measure excreted FGMs (Watson et al. 2013). Neat samples were diluted 1:20 in assay buffer, while standard curves were prepared by serial dilution of corticosterone standard stock (1000 pg/well, catalogue ID Q1550-020, Steraloids, Newport, RI) as described above. Corticosterone-HRP (1:100) and corticosterone antiserum stock (1:50) were diluted in assay buffer. Plates were loaded following the same procedure used for the cortisol assay, and controls bound at 36% (high) and 64% (low) of HRP binding on the standard curve were also added. After being covered with a plate sealer, plates were shaken continuously for 2 hours, washed, loaded with 100 µl of TMB, and incubated at room temperature for 12 min, before adding 50 µl of stop solution (1M HCl). Light absorbance was measured as described above.

5.2.3 Assay validation

Laboratory validation of both cortisol and corticosterone assays was done based on parallelism and recovery tests following the guidelines suggested by Brown *et al.* (2004) and Touma and Palme (2005). Cortisol and corticosterone EIAs using the polyclonal antibodies R4866 and CJM006 have been validated in different felid species including jaguars (*Panthera onca*) (Mesa, Brown and Kelly, 2014), cheetah (*Acinonyx jubatus*), clouded leopard (*Neofelis nebulosa*), domestic cat (*Felis catus*) (Young et al., 2004) and tigers (Narayan et al., 2013). To

confirm parallelism between serial dilutions of pooled tiger faecal samples and standard hormone preparations, a serial 2-fold dilution of the pooled sample was prepared in assay buffer using the same method described for the preparation of the standard curve. Plates were loaded and run as described previously. For the accuracy checks, pooled samples were diluted in assay buffer using a 1:10 dilution (cortisol) or a 1:20 dilution (corticosterone). Diluted pooled samples were then spiked by adding increasing concentrations of known standard stock (0.078 to 10.0 ng/ml). Standard curves were prepared following the same procedure used in the EIA. Spiked samples were loaded in triplicate; plates were run and read as described previously. The results were plotted to obtain a linear regression equation. To calculate the percentage of recovery for each dilution the following formula was used:

% Recovery = (Measured amount / Expected amount) x 100

The final recovery percentage for each assay was calculated as the mean of spiked cortisol or corticosterone dilution. Assay sensitivity was 0.078 ng/ml for both assays, determined as the value obtained at 90% binding of the cortisol-HRP and corticosterone-HRP conjugates. Recovery of known amounts of synthetic cortisol and corticosterone added to pools of diluted faecal extract was 90.3% (regression equation: 0.783x + 4.580, r^2 = 0.998) and 96.1% (regression equation: 0.749x + 4.87, r^2 = 0.999), respectively. The intra-assay coefficient of variation was < 10% and the inter-assay coefficient of variation was 2.39% for cortisol and 2.58 % for corticosterone.

5.2.4 Statistical analysis

All statistical analyses were performed using SPSS v. 24 (SPSS Inc., Chicago, Illinois, USA). Data were first analysed for normal distribution using the Shapiro Wilk test. Faecal concentrations of cortisol and corticosterone metabolites were not normally distributed ($p < 0.05$). A non-parametric Friedman's two-way ANOVA was used to determine if

significant differences in FGMs concentrations across treatments existed. Pairwise comparisons with adjusted *p*-values were made using the Wilcoxon signed-rank test. To examine if concentrations of cortisone and corticosterone were correlated with one another, Kendall's tau test was used. Statistical significance threshold was set at $p = 0.05$. All summary statistics are reported as medians (Md) and interquartile ranges (IQR). The 95% confidence intervals (CI) bias-corrected and bootstrapped (BCa) values are reported in square brackets. All concentrations are expressed on dry matter (DM) basis.

5.3 Results

Summary statistics for cortisol and corticosterone metabolites are reported in Table 5.1. No significant difference was detected in cortisol metabolite concentrations across dietary treatments: $X^2(2) = 0.143$, $p = 0.931$. Faecal concentrations of cortisol metabolites did not differ significantly between BD and CD ($T = 58$), between BD and ED ($T = 52$) nor between CD and ED ($T = 4{,}710$). Similarly, no significant differences were found across treatments for faecal concentrations of corticosterone metabolites ($X^2(2) = 1.857$, $p = 0.395$). Pairwise comparisons with adjusted *p*-values determined that differences in faecal concentration of corticosterone metabolites between BD and CD ($T = 65$), BD and ED, ($T = 38$), or between CD and ED ($T = 4{,}677$) were not significant.

Table 5.1 Concentration (ng/g dry matter) of faecal glucocorticoid metabolites (cortisol and corticosterone) of seven captive tigers (*Panthera tigris*) fed a mixture of commercially supplemented ground muscle horsemeat (82% as fed), featherless whole chicken (*Gallus gallus domesticus*) (8% as fed), degutted whole rabbit (*Oryctolagus cuniculus*) (3% as fed), horse shanks (corresponding to the biceps femoralis and semitendinosus muscles) or horse necks (both cuts with skin and bones) (7% as fed) (Baseline Diet, n=15); exclusively commercially supplemented ground muscle horsemeat (Control Diet, n=131) or a diet containing 20% whole rabbit and 80% commercial supplemented ground muscle horsemeat (Experimental Diet, n=132).

Treatment	Median	Range	IQR
Faecal Cortisol Metabolite (ng/g DM basis)			
Baseline Diet	59	20-419	56
Experimental Diet	89	7-730	79
Control Diet	95	19-578	80
Faecal Corticosterone Metabolite (ng/g DM basis)			
Baseline Diet	184	105-484	96
Experimental Diet	197	39-544	90
Control Diet	201	82-606	84

IQR – Interquartile range; DM – dry matter

Kendall's tau test was used to determine whether faecal cortisol and corticosterone metabolite concentrations were correlated with each other (see Figure 5.1). Correlation between faecal cortisol and corticosterone metabolites was significant, $\tau = 0.494$, BCa 95% CI [0.429, 0.558], $p < 0.001$. In addition, faecal corticosterone metabolite concentrations seemed to follow similar patterns to faecal cortisol metabolite concentrations for all tigers (see Figure 5.2).

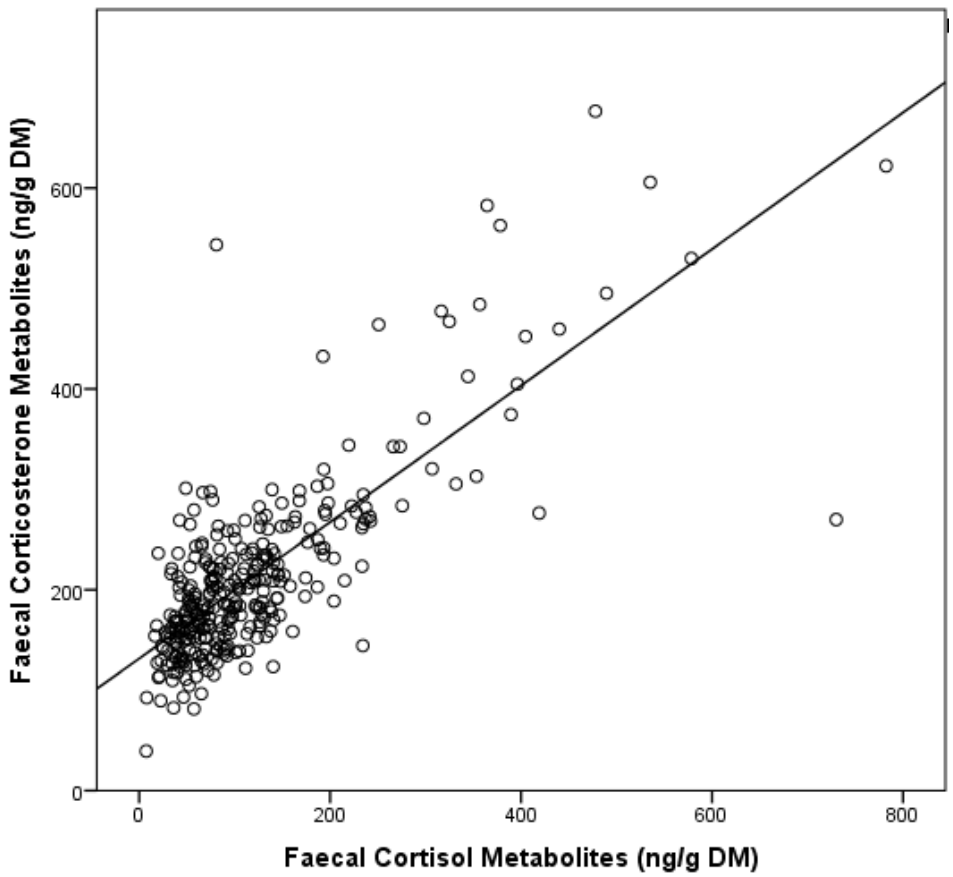

Figure 5.1 Scatterplot showing the correlation between faecal concentrations of cortisol and corticosterone metabolites of eight captive tigers (*Panthera tigris*). Metabolites expressed in ng/g dry matter basis (DM) (n = 317, τ = 0.494, BCa 95% CI [0.429, 0.558], $p < 0.001$).

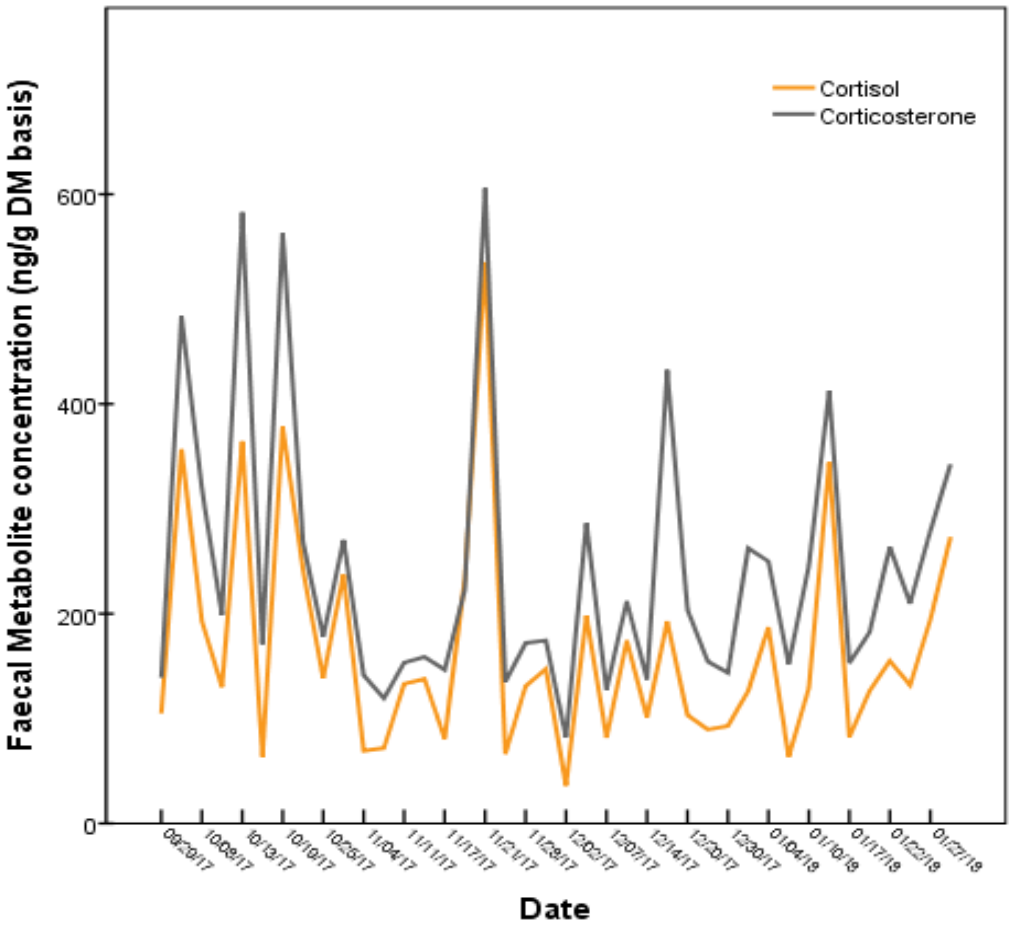

Figure 5.2 Faecal concentrations of cortisol and corticosterone metabolites of a neutered female captive tiger (Panthera tigris) fed a commercially supplemented ground muscle horsemeat diet: Control Diet (08/Oct/2017 to 02/Dec/2017) or a diet with 20% added whole rabbit: Experimental Diet (03/Dec/2017 to 24/Jan/2018).

5.4 Discussion

In this study, FGMs concentrations in tigers undergoing a dietary intervention are reported. Results indicated that faecal cortisol and corticosterone metabolites were not significantly different when tigers were fed a diet comprising a commercial supplemented ground muscle diet (CD), a diet with 20% added whole rabbit (ED) or a diet comprising a mixture of prey (BD).

Concentrations of FCM found in this experiment were within the ranges previously published for both wild and captive tigers. For example, Bhattacharjee *et al.* (2015) reported FCM concentrations between 47.90 and 115.68 ng/g dry faeces, in free-ranging Bengal tigers. In captive Bengal and Sumatran subspecies, mean FCM concentrations ranged from 63.49 to 153.4 ng/g dry faeces (Narayan *et al*., 2013).

Secretion and metabolism of glucocorticoids vary across species (Krass and Bazhan 1976; Palme et al. 2005). Although tigers are considered a cortisol-dominant species, synthesis and secretion of corticosterone may occur in response to stressful situations (Rozhnov et al. 2010; Naidenko et al. 2011; Koren et al. 2012). Evaluation of corticosterone concentrations is not as widely used as cortisol to assess stress in felids (Watson et al., 2013; Mesa, Brown and Kelly, 2014). Concentrations of FCCM obtained in this study were lower than those reported for captive cheetahs and clouded leopards (Young *et al*., 2004). However, FCCM concentrations were higher than those observed in captive Bengal tigers in Indian zoological collections (Vaz *et al.*, 2017). A possible explanation for such differences is the type of assay used. While Young *et al*. (2004) determined FCCM concentrations with a radioimmunoassay (RIA), Vaz *et al*. (2017) and the current study performed an EIA. Furthermore, the supplier for the polyclonal corticosterone antibody used by Vaz differed from the supplier used in the current study. It is well known that cross-reactivity with cortisol and corticosterone metabolites can vary significantly between RIAs and EIAs, and even between polyclonal cortisol and corticosterone antibodies used in different EIAs (Wasser et al. 2000; Dias et al. 2008); thus direct comparisons with previously published research must consider all of these potentially confounding factors. Hence, to compare FCCM values appropriately, studies should assess the same species, using the same assay and the same polyclonal antibodies.

Previous research reported the presence of both cortisol and corticosterone in different mammalian species; in addition, a strong

correlation between the concentrations of the two hormones was observed (Koren et al. 2012; Gong et al. 2015). In the current study, results from Kendall's tau test confirmed that a significant correlation between FCM and FCCM existed in tigers. Similarly, Young et al. (2004) reported a strong correlation between cortisol and corticosterone faecal metabolites in other felid species including domestic cats, clouded leopards, and cheetahs. However, along with the significant correlation, a numerical difference in median concentrations between cortisol and corticosterone analytes was observed, with the former being lower than the latter (see Table 5.1). Although median concentrations varied between glucocorticoids in the current study, corticosterone followed a similar fluctuation pattern to cortisol metabolites for all individuals (see Figure 5.2). Similarly, in other species like mice (*Mus muscuslus*) and sea otters (*Enhydra lutris*), excretion profiles of both cortisol and corticosterone showed similar patterns, i.e. an increase in concentrations, after animals were confronted with stressors (Gong *et al.,* 2015; Murray, Young and Santymire, 2020). The numerical changes between cortisol and corticosterone could be due to variations in cross-reactivity of the antibodies and metabolites between EIA (Schatz and Palme 2001). Palme *et al.* (2005) mentioned that special care should be taken with the selection of antibodies for faecal EIA since most cortisol or corticosterone found in faeces correspond to metabolites and not the intact hormones. The antibodies used in the present study have been previously validated for the species (Watson *et al.* 2013); hence, a high cross-reactivity with the relevant metabolites of the target hormones was expected. However, since antibodies can react with both glucocorticoid metabolites, the use of more specific analytical methods such as liquid chromatography coupled with mass spectrometry is recommended and should be considered for future work to more precisely identify glucocorticoid metabolites of target hormones (Koren *et al.,* 2012).

Another possible explication for the difference in median concentrations observed between the glucocorticoids could be due to some

peculiarities in the metabolism of these molecules. It is believed that the two glucocorticoids share similar physiological roles, yet little information on this topic is available. For example, in mice (a corticosterone dominant species), both corticosterone and cortisol levels increased when animals were exposed to stressors; however, cortisol was predominant during acute stress, while corticosterone was prevalent during chronic stress (Gong *et al.*, 2015). In sea otters and guanacos (*Lama guanicoe*) (species considered cortisol-dominant), cortisol and corticosterone concentrations were detected when animals faced an acute stressor; however, cortisol increased to a greater extent than corticosterone (Ovejero et al. 2013; Murray et al. 2020). Similarly, in domestic cats, Schatz and Palme (2001) reported that stimulation of the adrenal cortex– through an adrenocorticotropic hormone challenge– was not reflected appropriately by FCCM, hence the use of corticosterone to assess adrenal activity in cats was not recommended by the authors. Results from these studies might suggest physiological differences in regulation of cortisol and corticosterone, and the possibility of independent roles of these glucocorticoids within species. So even if cortisol is the dominant glucocorticoid for a species, the presence and possible effects of corticosterone should be considered when evaluating adrenal response. For this reason, validation– analytical, biological and/or physiological– is essential for a comprehensive evaluation of adrenal activity in felids (Touma and Palme, 2005). Although tigers are considered a cortisol-dominant species, whether corticosterone or cortisol better reflect the adrenal response to stressors or whether differences between acute or chronic stress are better reflected by either glucocorticoid still needs to be investigated.

5.4.1 Physiological stress & diet

No significant differences in FGMs concentrations were found in tigers consuming a diet with added whole prey (ED), compared to a diet solely consisting of commercial supplemented raw horsemeat (CD). The role

of diet in animals' welfare extends beyond providing nutrients to support life; for example, in sows, the use of high fibre diets decreased both stereotypical behaviours and plasma cortisol levels (Oelke et al. 2018; Huang et al. 2020). In captive cheetahs, improved appetite and longer time spent feeding– considered to reflect improved welfare– were observed when animals consumed whole carcasses instead of a commercial minced-meat diet (Bond and Lindburg 1990). Hunger has been associated with increased plasma cortisol levels in humans, accentuated aggression in pigs (Rushen et al. 1999; Al-Zubaidi et al. 2020; Huang et al. 2020) or the development of stereotypic behaviours in carnivores (Veasey, 2017). Researchers have proposed that, by modulating satiety, diet could improve animal welfare (Kasanen *et al.,* 2010; Verbeek *et al.,* 2011).

We predicted that the ED would promote a greater feeling of satiety (due to increased animal fibre content originating from the rabbit carcass) compared to the CD (containing only cellulose as a fibre source), and this enhanced satiety feeling might then result in lower glucocorticoid concentrations; however, FGMs levels remained similar between dietary treatments. One possible explanation for this study's results could be that dietary fibre content was not sufficiently different between treatments to trigger a difference in satiety. Although ED included added animal fibre compared to the CD (which contained exclusively a plant fibre source), the acid and neutral detergent fibre (ADF and NDF) fractions measured were similar between diets (see Table 2.1, Chapter 2). Previous studies in sows have reported a significant decrease in plasma cortisol concentrations in animals fed a diet with 0.6-5% added fibre; however, NDF concentrations were similar between dietary treatments used in such studies (between 19% and 19.5% NDF) (Sun et al. 2015; Huang et al. 2020). Since differences in NDF concentrations were negligible in such studies, but a reduction in cortisol levels was obtained, other properties of fibre beyond quantity must be involved in regulating satiety. For example, in humans, highly viscous fibres have been linked with a diminished feeling of hunger and increased satiety (Dikeman and Fahey 2006; Harrold et al. 2014; Yong

et al. 2016). Future research could evaluate other fibre characteristics, besides inclusion rate, to determine their possible role in the satiety of carnivorous species.

5.4.2 Confounding factors

Even if FGMs is considered the gold standard to evaluate stress in zoo animals, it is essential to consider some of the confounding factors associated with the metabolism, excretion and quantification of the metabolites of these hormones (Young et al. 2004; Parnell et al. 2014; Miller et al. 2016). For example, bioavailability and excretion of glucocorticoids ingested orally have been documented in multiple species (Cooper et al. 1996). Numerically higher median concentrations of FCM and FCCM were observed during the CD compared to BD. Ingested glucocorticoids can affect the measured faecal metabolites: for example, in Alaskan brown bears (*Ursus arctos horribilis*), FGMs peaks were observed during the months when their prey, salmon, had higher cortisol concentrations (Von Der Ohe et al. 2004). Similarly, McDonald *et al.* (2018) found that Arctic foxes (*Vulpes lagopus*) fed a supplemented amount of cortisol in their diet had significantly higher FGMs concentrations compared to controls. It is, therefore, possible that ingested glucocorticoids could have influenced tigers' FGMs.

Although the numerical differences in the present study were not statistically significant, it should be noticed that the higher FGMs concentrations were observed when the diet comprised horse muscle tissues (CD), while lower concentrations were obtained during the ED and BD when tigers were offered a wider variety of tissue sources like bones, skin, tendons. Pre-slaughter transport conditions and the health status of prey items can significantly impact cortisol and corticosterone levels in their plasma and muscle (Tharwat and Al-Sobayil 2014; Gehlen et al. 2018; Składanowska-Baryza et al. 2018; Trocino et al. 2018). If horse muscle contained higher glucocorticoid levels compared to other carcass tissues or prey items, this could explain the numerical increase in FGMs concentrations obtained during the CD. Since ED and BD contained similar inclusion rates of horsemeat (~80%), horse-

originating glucocorticoids could have been too similar to that of CD to produce a significant difference in FGMs concentrations across dietary treatments. Another possible explanation could be that, overall, dietary sources of glucocorticoids were negligible, and hence did not influence FGMs concentrations significantly. Unfortunately, cortisol and corticosterone concentrations of dietary items were not evaluated prior to feeding in the present study; therefore, the potential influence of prey glucocorticoid concentrations on tigers' FGMs cannot be ruled out.

Another confounding factor to consider when interpreting faecal glucocorticoid metabolites results is the influence of environmental conditions and bacterial degradation in the gastrointestinal tract or after defaecation (Morrow et al. 2002; Millspaugh and Washburn 2004; Touma and Palme 2005; Mesa et al. 2014; Palme 2019). Due to the nature of the exhibits and the husbandry practices of the zoological collection, it was not possible to collect samples within minutes of defaecation. Some faecal samples may have been voided up to 24 h hours before they were accessible for collection. Indoor and outdoor enclosures were cleaned thoroughly every morning, limiting the chance of collecting older scats; only faeces that looked less than one day old were considered for analysis. A previous study with tiger faeces demonstrated a significant increase in cortisol metabolite concentrations in samples exposed for more than 48 h to natural environmental conditions (Parnell et al. 2015). Researchers believed that the changes in FGM were due to faster steroid degradation under wet or room temperature conditions, associated with changes in the faecal microbiota that could transform cortisol and corticosterone metabolites (Washburn and Millspaugh, 2002; Goymann, 2012; Parnell *et al.,* 2015). To minimise enzymatic bacterial activity in faecal samples, it has been recommended to collect freshly voided samples and freeze them at -20°C within 1 h of collection (Morrow *et al*., 2002; Washburn and Millspaugh, 2002; Parnell *et al.*, 2015). However, although it was not possible to undertake very rapid collection and storage of samples, the impact of environmental conditions on glucocorticoid metabolites would have been similar across diets, since samples were collected

under the same conditions throughout the study (i.e. within 24 h of defaecation). However, bacterial degradation could still be a confounding factor if changes in faecal bacterial abundance or composition occurred in response to dietary changes. To date, no study has linked FGMs decay with specific bacteria genera; hence, knowledge on the effect of faecal microorganism in glucocorticoid metabolism remains limited.

Glucocorticoids have a wide range of metabolic functions that can be associated with both stress and non-stress related stimuli (see review by Lane 2006). It seems possible that results from studies reporting higher glucocorticoids levels in hungry individuals are due to the effect of cortisol on energy mobilization (Ralph and Tilbrook, 2016). For example, Naidenko *et al.*, (2011) found that free-ranging Amur tigers had higher FCM concentrations (1268 ng/g) than captive individuals (457 ng/g). They hypothesised that changes observed between populations were due to higher energy demand– due to physical activity and thermoregulation– in free-ranging animals compared to captive tigers. The use of FGM as stand-alone indicators of animal welfare remains highly questionable; however, when used in conjunction with other parameters, such as behaviour, FGMs can provide a more reliable picture of an individuals' welfare (Schwarzenberger 2007; Dickens and Romero 2013; Palme 2019). For instance, when assessing behaviour alongside FGMs concentrations, behavioural observations can help to determine whether changes in FGMs levels are due to increased activity or triggered by a particular stressor (Ralph and Tilbrook, 2016; Palme, 2019). For example, it could be argued that the out-of-hours events happening in October and December (see section 4.2.1 Animals, enclosures, and management), where tigers were housed indoors at night, might have been perceived as a stressful situation for the individuals, hence affecting FGMs results. However, no evidence of increased concentrations of either FCM or FCCM concentrations were observed during or after such events. Therefore, it is possible that tigers were habituated to be housed indoors during such

events and did not perceive these situations as a negative or aversive condition.

Another possible explanation for this study's results could be that the food items used for the dietary treatments were known by the tigers. In collared anteaters (*Tamandua tetradactyla*), a significant reduction in FCM was observed when animals were fed novel food items (i.e. fruit, vegetables or meat that the anteaters had not consumed before) compared to pre-and post-treatment values with their habitual diet (Eguizábal et al. 2013). Yet in the current study, even when compared to the BD (which offered a wider variety of food sources), no significant differences were found in FGMs values. Hence, results observed in anteaters could have been due to changes in feeding routine (i.e. frequency, time and presentation of food items) and not only the addition of novel items in their diet. Similarly, a study with captive cougars (*Felis concolor*) and tigers found no significant differences in FCM concentrations when animals were fed a deer carcass flank compared to pre-treatment concentrations where animals ate a commercial supplemented horsemeat diet (Ruskell *et al.,* 2015). These findings raise intriguing questions regarding the nature and extent of dietary components as a modulator of satiety and their impact on adrenocortical activity.

5.4.3 Limitations & future research

As already described in previous paragraphs, a wide range of confounding factors should be considered when interpreting FGMs results; in addition, our findings may be limited by the small sample size and the relative similarities in nutrient composition between the different diets used in the study.

To date, published information on the effect of prey glucocorticoid concentrations and their influence on predators' FGMs levels is still scarce. Further research is needed in this area to better understand the metabolism and excretion of glucocorticoids from prey origin in

carnivorous species. Until then, prey glucocorticoids should be considered as a potential additional confounding factor that could affect the evaluation of adrenal activity in carnivores through measurement of FGMs. Although prior studies in red squirrels (*Tamiasciurus hudsonicus*), collared anteaters, cougars and tigers acknowledged the influence of diet on glucocorticoid concentrations (e.g Dantzer *et al.,* 2011; Eguizábal *et al.,* 2013; Ruskell *et al.,* 2015), none of these studies analysed macronutrient composition of the diets offered; hence, the link between the nutritional profile and FGMs metabolism remains to be investigated. Instead, some evidence exists to support the role of fibre– through regulation of satiety– in improving the welfare of sows and chickens (Van Krimpen and De Jong 2014; Oelke et al. 2018; Manu et al. 2020; Tahamtani et al. 2020). However, more work is needed to determine whether animal fibre can have a similar effect and influence physiological welfare indicators in captive carnivores. Higher inclusion rates, such as 100% whole prey, could be used to elucidate more clearly if animal fibre can increase satiety compared to 100% muscle meat and hence prove beneficial to tigers' welfare.

5.5 Conclusion

This study contributes to the general knowledge of the effects of fibre on a highly threatened species: the tiger. Whilst the hypothesis that 20% added whole prey could decrease tigers' FGM was not supported by this study's results, the possible benefits of whole prey to improve animal welfare is worthy of further research. Although the influence of factors such as diet, environmental conditions, storage techniques and assay methods on the measurement of FGMs have been reported in the literature, they are yet to be fully understood. Determination of FGMs remains a frequently used non-invasive marker of adrenocortical activity to assess welfare in zoological collections. FGMs findings must be interpreted with caution since adrenocortical activity can be influenced by a vast array of potentially confounding factors; for this

reason, a panel of welfare indicators, rather than stand-alone markers, should be used when evaluating welfare in zoological collections.

Chapter 6. Integrated discussion

6.1 Summary of research findings

The effects of fibre on digestive physiology, including aspects such as passage rate, macronutrient digestibility and production of SCFA are well recognised in a wide range of species, including a number of carnivorous species (Edwards and Ullrey 1999; Moore-Colyer et al. 2003; Propst et al. 2003; Fekete et al. 2004; Vester et al. 2010a; Patra 2011). Over the past decade, the benefits of plant-based fibre for obligate carnivore species like the domestic cat have gained attention within the scientific community (Prola et al. 2010; Barry et al. 2011; Kanakupt et al. 2011; Deb-Choudhury et al. 2018). Although not considered a macronutrient, fibre is regarded as a valuable component of diet composition, with a broad array of health benefits that extend beyond the GIT such as increasing satiety, preventing the development of diabetes or modulating the immune system (as reviewed by Brownlee *et al.*, 2006; Threapleton *et al.*, 2013; Makki *et al.*, 2018). However, the effects of fibre seem to be highly variable across studies and depend upon the type of fibre used, the inclusion rate and the species studied. For example, when fed at the same inclusion level, tigers produced firmer stools on an added cellulose diet compared to a diet with beet-pulp; while the opposite effect was observed in domestic cats, who produced firmer stools when provided with the beet-pulp diet (Vester et al., 2008, 2010; Kerr et al., 2013). Thus, the ideal fibre source may differ among species.

However, the natural diet of non-domestic felids like tigers contains negligible amounts of plant-based material (Fàbregas *et al.,* 2017; Mazák, 1981; Tilson and Nyhus, 2010). Diets of free-ranging felids are challenging to replicate in captivity and, instead, commercial raw meat diets are commonly employed to feed non-domestic felids in North American collections (Kerr, Beloshapka, *et al.,* 2013; Kapoor *et al.,* 2016; Lefebvre *et al.*, 2020). Although nutritionally balanced and highly

digestible, these commercial diets have been associated with the presence of gastrointestinal conditions in captive cheetahs and tigers (Seidel and Wisser, 1987; Siefert and Muller, 1987; Whitehouse-Tedd, Lefebvre and Janssens, 2015). For this reason, previous studies have investigated whether the poorly digestible components of whole prey (the so-called 'animal fibre') consumed by obligate carnivores have similar functions to that of plant fibres in herbivores (Depauw *et al.,* 2011, 2012). Findings from the studies by Depauw *et al.* (2011, 2014) with captive cheetahs suggested that animal fibre was able to reduce both intestinal inflammation and the incidence of diarrhoea while promoting firmer stools.

Based on this previous evidence from cheetahs, it was hypothesised that the use of added animal fibre in an experimental diet (ED) compared to a muscle-meat diet used as a control (CD) would prove beneficial to tigers' GIT health and function by improving parameters such as faecal consistency and SCFA concentration, decreasing concentrations of fermentation end-products and inflammatory biomarkers, without detrimentally influencing macronutrient digestibility. In addition, it was expected that animal fibre would positively impact the welfare of tigers undergoing the dietary intervention by increasing satiety, reflected as reduced pacing frequency and lower FGMs concentrations, compared to the meat-only diet.

Examination of functional parameters of the GIT (Chapter 2) tested initial hypotheses. Findings confirmed that total tract apparent macronutrient digestibility coefficients were not affected by the presence of 20% added whole prey. Contrary to what was hypothesised, fermentation profiles (i.e. SCFA and end-products concentrations) showed no difference between dietary treatments. However, some evidence of beneficial effects (such as firmer faecal consistency and a trend towards lower p-cresol concentrations) were observed during the ED feeding period. To evaluate the influence of animal fibre on the GIT health of tigers, two inflammatory biomarkers – NMH and S100A12 (Chapter 3)– were quantified for the first time in this

species. I found no significant differences in faecal concentrations of these markers between dietary treatments nor when compared to baseline values collected prior to the start of the experiment (BD). In addition, no correlation between NMH and S100A12 concentrations was observed. Finally, welfare parameters were evaluated in relation to the dietary intervention. Firstly, behavioural observations of the tigers allowed me to determine the presence of two stereotypies in the study population: pacing and tail sucking (Chapter 4). Activity budgets of tigers fed the ED showed no significant differences compared to that of tigers consuming the CD; similarly, the frequency of time engaged in stereotypical behaviours did not vary significantly between diets. To complement the welfare assessment, FGMs were measured to determine adrenal activity and as a physiological indicator of welfare (Chapter 5). However, FGMs showed no significant differences between dietary treatments.

6.2 Animal fibre as a modulator of gastrointestinal function

This project aimed to investigate whether the addition of dietary animal fibre– at a common inclusion rate used for North American collections– can promote positive changes in captive tigers' welfare, health, and GIT function.

Previous research had demonstrated that functional aspects of the GIT can be modified by plant-based fibres, yet results varied depending on the characteristics of the fibres used. For example, Sunvold *et al.* (1995) reported that highly fermentable fibres resulted in poorer faecal consistency in domestic cats compared to diets without added fibre or with less-fermentable fibres like cellulose. Similarly, results presented by Vester *et al.* (2010) and Kerr *et al.* (2013) suggest that tigers are better adapted to low-fermentable fibre sources (e.g. cellulose) than fermentable sources (e.g. beet-pulp). In addition, differences at a species level might play a role in faecal consistency response to

different fibre sources. Significant differences in faecal scores were observed among felid species fed a beef-based diet with added beet-pulp (a fermentable fibre source): Indochinese tigers and cheetahs presented softer stools (3.9 and 3.6 respectively) compared to bobcats and jaguars (3.3 both) (Vester *et al.*, 2008).

Results reported in Chapter 2 documented that tigers fed the ED had significantly firmer stools (3.08) compared to the CD (3.38), however, although statistically significant, changes observed in the population, in response to added animal fibre, were subtle compared with previous studies and did not represent a shift in the actual score. In cheetahs, the use of a whole prey diet significantly improved faecal consistency (2.1) compared with a supplemented beef diet (3.1) (Depauw *et al.*, 2011). In domestic cats, faeces were significantly softer when animals consumed a commercial extruded diet compared to ground whole chicken (Kerr *et al.*, 2014). Possible mechanisms by which animal fibre can modulate faecal consistency include increasing passage rate, acting as bulking material or promoting the absorption of water from scats (Weber et al. 2002; Kim et al. 2016).

Since, in the current study, mean faecal consistency scores for both dietary treatments were within what is considered as “ideal” for the species and the magnitude of the change, as a result of added animal fibre, was small it could be questioned whether the current results are of biological importance. In this respect, none of the other parameters evaluated during the current study indicated that CD was detrimental or negatively impacted the health and function of tigers’ GIT. For example, no significant differences in faecal inflammatory biomarkers concentrations were observed between diets (Chapter 3) and macronutrient digestibility coefficients remained similar between dietary treatments (Chapter 2). It is also possible that the use of average faecal scores is less valuable to assess changes in faecal consistency due to the high disparity between individuals. Other analysis such as the coefficient of variation might help show the extent of such variability in relation to the population mean to better interpret results (Field 2018).

The coefficient of variation for ED was 22.4% and 22.8% for CD, hence further corroborating that the changes observed in faecal consistency between diets are likely to be of little biological importance.

Another key parameter associated with the use of fibre involves the production of SCFA, a group of compounds with numerous health benefits (Brosey, Hill and Scott, 2000; Fardet, 2010; Rinttilä and Apajalahti, 2013). Concentrations of total SCFA did not vary between dietary treatments (Chapter 2). Results from the current study are similar to those reported from previous research in domestic cats, cheetahs and tigers, where SCFA concentrations were not affected by dietary interventions (Vester *et al.,* 2010; Depauw *et al.*, 2011; Deb-Choudhury *et al.*, 2018). Although the ED dietary treatment included 20% added whole prey, ADF and NDF fibre fractions were similar to those of CD (Chapter 2), hence it remains possible that the amount of animal fibre present in the ED was not sufficient to increase the production of SCFA. However, even higher inclusion rates (i.e. 100% whole rabbit Vs. 100% ground meat diet) did not elicit a significant change in fermentation metabolite concentrations in cheetahs (Depauw *et al.*, 2011). Besides fibre, dietary protein can also influence SCFA production (Lubbs *et al.,* 2009; Rochus, Janssens and Hesta, 2014; Gugołek *et al*., 2015). Since diet composition and apparent protein digestibility were similar between dietary treatments, it is possible that the amount of protein available for fermentation by intestinal microbiota was comparable between dietary treatments. This assumption is supported by the lack of significant differences in end-product concentrations (Chapter 2). It is therefore likely that the production of SCFA is regulated by a wider range, or combination of factors such as fibre characteristics, fibre inclusion rate and macronutrient intake and digestibility.

End-products such as indole, phenol and p-cresol are commonly regarded as harmful compounds produced after fermentation of proteins and amino acids (Macfarlane and Macfarlane, 1997; Davila *et al.,* 2013; Rinttilä and Apajalahti, 2013). No significant differences in

end-product concentrations were observed between dietary treatments in the current study. However, although indole and phenol concentrations found during the ED resembled those reported in cheetahs fed a whole rabbit diet, p-cresol concentrations were up to 6 times higher than those described in cheetahs fed a supplemented beef diet (Depauw *et al.*, 2011). In addition, end-product concentrations were lower than those previously seen in tigers fed either beef or horsemeat diets (Vester *et al.,* 2010). With no significant correlation found between end-products and NMH or S100A12 concentrations (except for indole which was significantly correlated with S100A12 concentrations, see Table 6.1), this aligns with the lack of detectable inflammation concurrently observed in the study population during either ED or CD. However, as mentioned in Chapter 3, many confounding factors exist at the tiger and dietary levels for both inflammatory markers, hence further research will be needed to determine the clinical efficacy of the biomarkers used here to assess GI inflammation in this species. Since no signs of GI inflammation were detected during the current study, the difference in p-cresol concentrations observed between tigers and cheetahs could be due to variations in protein composition of the diets offered (p-cresol and phenol are products of tyrosine fermentation) or differences in microbiota activity (Davila et al. 2013; Wing et al. 2015; Diether and Willing 2019).

Table 6.1 Results of Pearson's correlation between faecal end-products concentrations and two faecal inflammatory biomarkers in captive tigers (*Panthera tigris*): N-Methylhistamine and S100A12 (n = 15).

Parameter	*r*	95% Confidence Interval	*p*
N-Methylhistamine			
Indole	-0.184	-0.696, 0.497	0.512
Phenol	0.390	-0.293, 0.799	0.151
p-cresol	-0.239	-0.470, 0.205	0.391
S100A12			
Indole	0.613	0.199, 0.845	0.015
Phenol	0.226	-0.417, 0.872	0.417
p-cresol	0.069	-0.342, 0.823	0.808

6.3 Animal fibre as an influencer of animal welfare

Changes in nutrient composition are likely to influence other aspects of an animals' biology beyond digestion. In the present study, whether dietary animal fibre could influence satiety in tigers and if such effect would manifest itself as being detectable were evaluated as two non-invasive welfare parameters: pacing frequency and FGMs concentrations (Chapter 4 and Chapter 5 respectively). To properly assess animal welfare, many authors have advocated the use of an array of indicators rather than relying exclusively on a single parameter (Mason and Latham 2004; Broom 2007). Stereotypical behaviours have been employed as indicators of acute or chronic stress since they are considered a coping mechanism to deal with adverse situations (Clubb and Mason, 2003; Sajjad *et al.*, 2011; Mohapatra, Panda and Acharya, 2014; Vaz *et al.*, 2017). To determine the possible relation between 'pace' and FGMs, a Kendall's tau test was performed. During the ED, a non-significant correlation between pacing and FCM was detected (τ = 0.095, 95% CI [-0.055, 0.231], p = 0.183); while although the

relationship between pacing and FCCM was statistically significant, the 95% CI contained the value zero, hence, such a correlation is not likely to be meaningful (τ = 0.142, 95% CI [-0.026, 0.293], p = 0.047). Similarly, the correlation between pacing and either glucocorticoid during the CD is not likely to be meaningful because the CI contained the value zero (FCM: τ = 0.120, 95% CI [-0.013, 0.254], p = 0.088 and FCCM: τ = 0.108, 95% CI [-0.017, 0.227], p = 0.124).

Previous studies evaluating welfare in captive tigers reported either cortisol concentrations (e.g. Naidenko *et al.*, 2011; Narayan *et al.*, 2013) or behavioural indicators (e.g. De Rouck *et al.*, 2005; Biolatti *et al.*, 2016), while few evaluated both parameters (Sajjad *et al.*, 2011; Ruskell *et al.*, 2015; Vaz *et al.*, 2017). Among these, only Vaz *et al.* (2017) examined the relation between FGMs and stereotypical behaviour. Findings from the present study are in agreement with Vaz *et al.* (2017) since a lack of correlation between stereotypic behaviour and FGMs levels in captive tigers was found. A possible explanation for the lack of correlation between indicators could be that physiological responses to adverse situations are highly variable across individuals and could depend on multiple factors, such as type, intensity, and frequency of the stressor (Dickens and Romero 2013; Mohapatra, Panda and Acharya, 2014; Fureix and Meagher, 2015).

Previous studies have shown an inconsistent association between glucocorticoid values and stereotypical behaviour frequencies. For example, some studies found no relationship between stereotypies and cortisol concentrations (Redbo 1993; Wilson et al. 2004; Liu et al. 2006; Fureix et al. 2013; Svendsen et al. 2013a; Vaz et al. 2017). Other studies reported a positive correlation, with animals performing higher rates of stereotypic behaviours exhibiting higher cortisol concentrations (Malmkvist et al. 2011; Eguizábal et al. 2019). Conversely, some researchers described a negative correlation, with animals engaged in stereotypies showing lower concentrations of glucocorticoids compared to non-stereotyping individuals (Verhoeven *et al.*, 1999; Svendsen, Palme and Malmkvist, 2013; Denham, Bradshaw and Rooney, 2014;

Briefer Freymond *et al.*, 2020). It seems possible that for some individuals, stereotypies develop as a coping mechanism that allows the animal to deal with stressors, consequently helping to maintain lower levels of circulating cortisol (Mason and Rushen 2006; Fureix et al. 2013; Briefer Freymond et al. 2020). On the contrary, for other individuals facing unfavourable conditions, the response to stressors is reflected by elevated glucocorticoid concentration despite the performance of stereotypies (Mason, 1991; Mason and Latham, 2004; Bacon, 2018). This individual stress response was confirmed by Dickens and Romero (2013), who evaluated more than 150 studies in a wide range of species and found that the direction of change in glucocorticoid concentrations (i.e. increase, decrease or no change) when animals were confronted with stressors was inconsistent between studies. The authors suggested that it is not possible to predict the endocrine response of stressed animals due to the highly variable response to stressors (Dickens and Romero, 2013).

Overall, these apparently contradictory results could indicate that physiological and behavioural responses to adverse situations are highly variable across individuals and could depend either on stressor factors (such as type, intensity, and frequency) or animal factors (such as age, sex and early life experience) (Dickens and Romero, 2013; Mohapatra, Panda and Acharya, 2014; Fureix and Meagher, 2015; Briefer Freymond *et al.,* 2020). No differences in behavioural (Chapter 4) or physiological (Chapter 5) indicators of welfare were observed between dietary treatments. The lack of correlation between these parameters could reflect the inability of a 20% added whole prey diet to impact the overall tigers' welfare or the high variability between individuals in stress response. Previous authors have suggested that changes in welfare indicators are essential to identify stressed individuals (Romero et al. 2009; Dickens and Romero 2013). The fact that neither welfare indicator varied between diets, and no correlation between parameters was found could indicate that the welfare status of the study population remained consistent throughout the trial and that minor changes in dietary nutrient composition level simply were not a

source of stress which sufficiently exceeded their baseline or everyday levels. In addition, the lack of detectable difference in the two welfare indicators suggests that the feeding regimes used in the current study are not detrimental to the welfare status of tigers and could be employed by other facilities. Further research is suggested to determine if higher inclusion rates of animal fibre can result in more detectable improvements in these two welfare indicators.

Over the years, scientists have conceived a romanticised interpretation of the animal welfare concept, in which negative experiences should be minimized to prevent captive animals from being stressed or suffering (Dawkins, 2004; Farm Animal Welfare Council, 2009; Broom, 2011). Although such a notion might seem attractive to justify keeping animals in captivity, the perception of comfort implied might seem rather anthropomorphic. If we are to maintain in captivity species such as tigers and provide them with a naturalistic environment and management, the hazards and rigours of the wild should also be considered. Free-ranging tigers are likely to encounter a variety of situations where social, mental and physical challenges are present, all of which might have a negative impact on their welfare (e.g. unsuccessful hunts might lead to hunger or physical injuries) (Rabin 2003; Law and Kitchener 2019). For ethical and practical reasons, zoos might not be able to expose tigers to the wide variety of situations they would naturally experience in the wild; however, simple changes such as incorporating fasting days or making food availability not immediate might provide a more realistic environment (Mazák 1981; Reddy et al. 2004; Kasanen et al. 2010). Therefore, it will be interesting for zoological collections to reflect and possibly modernize their approach towards animal welfare to avoid skewing the concept by creating a highly predictable environment, deprived of realistic conditions, where animals are no longer confronted with challenges similar to those experienced by their wild counterparts.

6.4 Holistic assessment of the gastrointestinal tract.

The GIT is a complex system that performs a wide range of functions beyond the processing of nutrients (Hall et al. 2005; Bischoff 2011; Zhang et al. 2012). Traditionally, evaluation of the GIT in zoo animals has been performed using non-invasive methods/studies such as faecal consistency scoring, digestibility, and passage rate (Li *et al.,* 2006; Zhihong *et al.,* 2007; Vester *et al.,* 2008, 2010; Depauw *et al.,* 2011; Kerr, Morris, *et al.,* 2013; Iske, Morris and Kappen, 2016). However, these techniques provide information encompassing only a few aspects of GI physiology. In the present study, changes associated with the addition of animal fibre in three main areas were evaluated: GIT function, GIT health and animal welfare. I considered that such an approach would provide a more comprehensive understanding of the impact of dietary animal fibre in captive tigers compared to previous studies that only assessed one of these areas.

The use of a panel of indicators allowed me to evaluate different facets of GIT health and corroborate the results obtained in the current study. For example, visual inspection of faeces is a common, non-invasive tool to assess GI health in a wide range of domestic and non-domestic species (Murdoch 1986; Bechert 2012; Lamberski 2015; Vandeputte *et al.,* 2016). When used in a zoo setting, faecal scoring provides a standardised tool for zoo staff to document faecal consistency as part of their daily records (Felid TAG 2014; Lamberski 2015). However, some authors consider the lack of sensitivity and possible subjectivity of this scoring method to render it questionable as a stand-alone parameter to evaluate GI health (Eastwood 1992; Depauw *et al.,* 2012; Bechert 2012). In addition, variation across species and even across individuals should be considered when performing faecal scoring to avoid introducing further bias (Depauw *et al.,* 2012; Felid TAG, 2014). Looser stools are considered a sign of poor GI health and commonly associated with the presence of pathogens (Hall, Simpson and Williams, 2005; Lamberski 2015). Yet, faecal consistency can be modified by parameters such as transit time, nutrient composition and

digestibility of the food ingested (Eastwood 1992; Murdoch 1986; Vester *et al.,* 2008; Fekete *et al.,* 2004; Kerr *et al.,* 2012; Kerr, Morris, *et al.,* 2013; Iske *et al.,* 2016). In the current study, a significant numerical difference between faecal scores was observed during CD compared to ED (Chapter 2). However, when evaluated alongside other parameters such as digestibility (Chapter 2) or faecal inflammatory biomarkers (Chapter 3)– where no significant differences between diets were observed– it might be assumed that such numerical difference is not likely to be due to a detrimental effect of the CD on the GI health of tigers. In addition, mean consistency scores for both dietary treatments fell within the range considered "ideal" for tigers (Felid TAG 2014; Lamberski 2015). A possible explanation for the significantly higher faecal scores observed during CD could be the significantly reduced transit time compared to the ED. A short passage rate may decrease faecal consistency by reducing the time available for fluid and electrolyte absorption (Berrnier 1997; Weber *et al.,* 2017). Evaluating faecal consistency in light of other parameters can help to better interpret results and confirm if changes observed could be considered of biological importance or are just part of the normal variation observed across individuals.

Another example of the importance of avoiding stand-alone parameters can be seen with FGMs. As mentioned in section 1.5.2.2 of Chapter 1 and as discussed in Chapter 5, the release of glucocorticoids can be influenced by a number of conditions such as exercise, reproductive and social status (Ralph and Tilbrook, 2016; Hockenhull and Whay, 2014; Huzzey *et al.,* 2015; Tsuda *et al*., 2020). In addition, many confounding factors such as GI transit time (Goldin et al. 1982; Dantzer et al. 2011), storage (Khan et al. 2002; Mesa et al. 2014) and antibody cross-reactivity in assays (Dias, Nichi and Guimarães, 2008; Goymann, 2012; Palme, 2019) are known to modify FGM concentrations pre and post-defaecation. To elucidate if changes in FGM concentrations were triggered by a stressor, behavioural observations can be useful (Ralph and Tilbrook 2016; Palme, 2019). No evidence of changes in the activity budget of tigers consuming either diet was observed in the current

study, and frequency of pacing remained constant and relatively low despite the diet change (Chapter 4). Similarly, FGM concentrations showed no significant differences between dietary treatments (Chapter 5). It is possible that the satiety produced by both diets was too similar to produce any detectable changes at the behavioural or physiological level. Taken together, these results suggest that tigers in the present study did not experience a decrease in welfare status when fed the CD compared to the ED since neither FGM nor activity budget changed significantly between dietary treatments. To provide a holistic assessment of animal welfare a behavioural and a physiological indicator were selected for the current project. The use of an array of indicators, rather than single measures allowed me to better interpret the results by verifying that FGMs and behavioural data indicated similar outcomes for the welfare status of the tigers undergoing the dietary intervention.

When working with non-domestic species, the lack of reference values is a common limitation (Lamberski, 2015). This study evaluated two inflammatory biomarkers for the first time in tigers, such that no reference values exist for the species. Besides obtaining baseline values for the study population, NMH and S100A12 results were evaluated alongside other parameters that could indicate a possible GI disturbance (e.g., faecal score, digestibility, or fermentation profiles). Since none of the other indicators was significantly affected by the dietary treatments, I can infer that the variation observed in the concentrations of both inflammatory markers could correspond to the normal biological variation reported in other species (Ruaux *et al.,* 2009; Anfisen *et al.,* 2014; Walton, 2012; Bridges *et al.,* 2019). This represents a first step towards determining the viability of these markers for use in tigers and as comparative values for future studies. However, evaluation of a larger population of tigers with and without GI disease is needed to corroborate if such markers could have a clinical application in diagnosing and monitoring GI inflammation in this species.

6.5 Limitations and future directions

6.5.1 Limitations

The current study provides a detailed investigation into the influence of animal fibre on the GI function and health of captive tigers using a suite of non-invasive parameters, yet some overall limitations must be considered.

Firstly, the sample size was limited to the number of tigers housed at the zoological facility. To address this situation, a randomized crossover study was utilised, so each tiger could act as its own control; however, with only eight individuals, extrapolation of the results might require caution. In addition, due to the small sample size, subtle effects occurring between diets could have been difficult to be detected by statistical analyses. Nevertheless, all the parameters evaluated during the current study indicated that no significant difference existed between dietary treatments.

Secondly, for those parameters that had not been previously evaluated in tigers (e.g. NMH and S100A12 concentrations), the lack of reference values for the species made it harder to determine if the values obtained corresponded to healthy individuals or a sign of compromised GI health. For this reason, results were evaluated and interpreted alongside other well-known indicators, to provide a more comprehensive interpretation of findings.

Thirdly, the current study relied on the non-invasive opportunistic collection of faecal samples in a zoo setting. Due to the complexity of the outdoor enclosures and the tigers' management schedule, it was not possible to collect samples immediately after defaecation, thus I aimed to obtain all samples within 24 h of being voided. Whenever a sample was judged to be older than 24 h (e.g. as indicated by the level of desiccation of the sample), it was discarded to avoid analysing inadequate material. In addition, I ensured that only analytes considered stable at room temperature for up to 24 h post defaecation

were used, to be sure that the collection method was not introducing variability to the data. For those less-stable parameters (e.g. SCFA and fermentation end-products), I decided to reduce the number of collection points and use exclusively “fresh” samples (i.e. scats defaecated no more than 30 min before collection) to ensure that the samples were adequate for analysis. Therefore, I am confident that despite the limitations of such a collection method, the effect of environmental conditions on the analytes measured was minimal and this study’s results were not subject to additional confounding factors.

Finally, as mentioned in each experimental chapter, other confounding factors should be considered, especially when dealing with a carnivorous species. For example, prey histamine or cortisol content can act as exogenous sources of these compounds, possibly influencing results. For this reason, results were interpreted alongside other indicators to obtain a more complete picture of the situation and a more rigorous evaluation of the GI health, function and welfare status of the tigers undergoing the dietary intervention.

6.5.2 Future directions

In the present study, a common whole prey inclusion rate for the ED was chosen, such that results could be representative of the current dietary management of many tigers in North American zoos. To determine at which concentration animal fibre proves beneficial for GI health and welfare of captive tigers, future studies could assess different and/or wider ranges of whole prey inclusion (i.e. from 30% to 100% inclusion rate). In addition, different whole prey types such as whole chicken, sheep, pigs or goats could be evaluated to determine if differences among prey nutrient composition can modulate GIT function and/or health.

Evaluation of intestinal microbiota could help determine if changes in microbial diversity, species richness and/or abundance occur when tigers are fed a diet with added animal fibre, compared to a sole muscle

meat diet. In recent years, researchers have recognised the importance of gut microbiota in an organism's physiology and GIT health, e.g. by creating a competitive barrier against pathogens, enhancing the nutritional value of the diet, providing energy sources for enterocytes, and regulating the hosts' immune system (Lubbs et al. 2009; Guilloteau et al. 2010; Meijer et al. 2010; Wing et al. 2015; Blake and Suchodolski 2016; Pinna et al. 2016). Research in non-domestic felids' gut microbiota in response to dietary interventions (and their possible health effects) is limited (Vester et al. 2010b; Eshar et al. 2019). Studies on exotic felids have described gut microbial diversity in captive and free-ranging individuals (Becker et al. 2014; Wasimuddin et al. 2017; He et al. 2018a,b; Eshar et al. 2019) or examined the stability of intestinal microbiota using longitudinal studies (Becker et al. 2015). Investigating changes in microbial diversity and its functional capacity could help elucidate how diet can shape the tigers' GI microbiota and if the adaptation to a dietary intervention is rather an acute or chronic process.

Establishing reference values for faecal NMH and S100A12 is necessary if these markers are to be included as a validated method for the assessment of tigers' GI health. Sampling a larger tiger population including animals of different ages and exposed to a variety of diets will be needed to determine reference values for the species.

A better understanding of the origin and motivations underlying stereotypies in captive non-domestic felids, and their relationship with glucocorticoid physiology, warrants further research. Similarly, further research is needed to understand the mechanisms by which dietary fibre may affect satiety, and how an increased feeling of satiety could, in turn, promote welfare by decreasing the frequency of time engaged in stereotypical behaviours (D'Eath *et al.,* 2009; Hothersall and Nicol, 2009; Van Krimpen and De Jong, 2014). Such information could help improve current management programmes, GI health and the welfare status of non-domestic felids in zoological collections.

Finally, animal fibre is a fairly recent concept, likely to be relevant to other carnivorous species. Future studies are needed to develop and validate a proper analytical method to assess animal-derived fibres; until then, methods developed to target plant fibre sources (i.e. ADF, NDF, TDF and CF) form the basis of diet composition analysis but may inaccurately represent the components present in animal fibre. Researchers interested in animal fibre could evaluate other characteristics such as solubility, viscosity and fermentability, to develop a classification of animal fibre components (such as the one proposed by Depauw *et al.,* 2012). Quantifying and elucidating these characteristics could help better understand the impact of animal fibre on the GIT of carnivorous species, to improve their management and welfare worldwide.

6.6 Conclusions and take-home message

Modern zoological collections recognise that appropriate nutrition is key to promoting good health and welfare in captive animals. However, mimicking the natural diet of strict carnivores in captivity represents a challenge for most facilities. Commercially manufactured diets, although nutritionally balanced and complete– based on domestic cats' requirements– very often lack the complexity of whole prey. Until now, detailed evidence of benefits from animal fibre (i.e. the poorly-digestible components of whole prey) – such as promoting firmer stools, reducing diarrhoea incidence, and decreasing the concentration(s) of inflammatory biomarkers– has only been reported in one felid species: the cheetah.

No significant differences were detectable in apparent digestibility, fermentation profiles, faecal pH, inflammatory biomarkers, or behavioural and physiological indicators of animal welfare in tigers fed diets containing 20% added whole prey compared to a diet consisting exclusively of a commercially supplemented horse-meat. A significantly lower faecal consistency and a trend for lower p-cresol levels were

however documented when tigers consumed the diet with added animal fibre. When taken together, results obtained in the present study suggest that an inclusion rate of 20% added whole prey (ED) as fed is insufficient to elicit, in captive tigers, changes of the same magnitude as those previously reported in cheetahs fed a diet comprising exclusively whole prey. The study population appeared to be clinically healthy before the feeding trial (BD) and while consuming the commercially supplemented horse-meat diet (CD); thus, the lack of animal fibre seemed to have no detrimental effects on the parameters measured during this study. However, dietary treatments were only used for 8-weeks each; hence, it cannot be determined if the beneficial effects of a diet with added animal fibre can be more apparent when fed on a long-term basis. The results of this project provide only minimal support for the suggested positive effects of animal fibre on gastrointestinal health and function when fed at a typical inclusion rate. This, therefore, highlights the importance of inclusion rate when considering dietary interventions. Previous beneficial impacts documented using experimentally high inclusion rates may be impractical to replicate in practice; zoological facilities may be unlikely to be able to incorporate such feeding regimes into general practice. Future research into defining the minimum threshold for achieving such beneficial health effects within the limitations of zoo practice is hence warranted. Nonetheless, other beneficial outcomes of added animal fibre –such as promoting oral health– should not be discounted (Haberstroh *et al.,* 1984; Duckler and Binder, 1997; Altman, Gross and Lowry, 2005; Plantinga, Bosch and Hendriks, 2011). For this reason, I support the recommendation of providing captive tigers with whole prey items as part of their routine diets.

Gastrointestinal health and animal welfare are complex constructs that require the use of a panel of indicators rather than single stand-alone measures to ensure an adequate evaluation. Thus, the use of a wide range of indicators and the implementation of novel methodologies are suggested in order to achieve a more holistic understanding of the influence of animal fibre in captive felids.

6.6.1 Take home message

The influence of nutrition and diet should be assessed holistically as impacts of a dietary intervention are likely to be seen in other aspects of an animal's biology beyond gastrointestinal physiology. The inclusion rate of animal fibre commonly used in North American zoos (20% whole prey as fed) appears insufficient to result in the beneficial effects reported in other species. Further research is needed to determine if a different inclusion rate is able to trigger positive changes in gastrointestinal function, health, and welfare indicators of captive tigers.

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