UNDERSTANDING THE IMPACTS OF ECOLOGICAL AND ANTHROPOGENIC FACTORS ON STRESS PHYSIOLOGY OF WILD TIGER (*Panthera tigris tigris*) AND LEOPARD (*Panthera pardus fusca*) IN THE TERAI-ARC LANDSCAPE, INDIA

Thesis submitted for the award of the degree of
Doctor of Philosophy

in

WILDLIFE SCIENCE

by

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to

Saurashtra University
Rajkot - 360005

Under the supervision of

Dr. Samrat Mondol, Scientist-E

भारतीय वन्यजीव संस्थान
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DECLARATION

I hereby declare that the work conducted under this thesis titled "**Understanding the impacts of ecological and anthropogenic factors on stress physiology of wild tiger (*Panthera tigris tigris*) and leopard (*Panthera pardus fusca*) in the Terai-Arc landscape, India**" is a record of original and independent research work done by me and subsequently submitted for the award of the degree of **Doctor of Philosophy in Wildlife Science** to the **Saurashtra University**, **Rajkot (Gujarat)**. This research work has been carried out under the guidance and supervision of Dr. Samrat Mondol, Scientist-E of Wildlife Institute of India, Dehradun. The work has not formed the basis for the award of any other degree, diploma or any other qualification. I also declare that the thesis embodies my own work, analysis, observation, understanding and the particulars given in it are true to the best of my knowledge.

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CERTIFICATE

This is to certify that the thesis by **Miss Shiv Kumari Patel** titled "**Understanding the impacts of ecological and anthropogenic factors on stress physiology of wild tiger (*Panthera tigris tigris*) and leopard (*Panthera pardus fusca*) in the Terai-Arc landscape, India**" is an original and independent research work submitted to the **Saurashtra University**, **Rajkot (Gujarat),** for the award of the degree of **Doctor of Philosophy in Wildlife Science.**

Miss Shiv Kumari Patel has put more than six semesters of research work embodied in this thesis under my guidance and supervision. The work presented in this thesis has not been submitted to any other University or Institute for the award of any degree, diploma or distinction.

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I certify that the research work was appreciated by all who were present, and the comments made by the faculty and researchers have been appropriately included in the thesis.

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CERTIFICATE OF PLAGIARISM CHECK

It is certified that the Ph.D. thesis titled "**Understanding the impacts of ecological and anthropogenic factors on stress physiology of wild tiger (*Panthera tigris tigris*) and leopard (*Panthera pardus fusca*) in the Terai-Arc landscape, India**" submitted by Miss Shiv Kumari Patel has been examined by us for plagiarism check as per UGC (Promotion of Academic Integrity and Prevention of Plagiarism in Higher Educational Institutions) Regulations. The following inferences are drawn from this check:

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List of publications:

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Introduction

1.1 Background

In the age of Anthropocene, major challenge for continued survival of free ranging terrestrial animals is rapid loss of their natural habitat and its fragmentation (Murphy and Romanuk, 2014; Wolf et al., 2021; Magioli 2021). Loss of global forest habitat has followed decline in forest dependent species (Andrén 1994; Tracewski et al., 2016; Betts et al., 2017; Wolf et al., 2021). Impact of loss of habitat and its intactness is different for different species based on their requirements and sensitivity (Andrén 1994; Devictor et al., 2008; Matthews et al., 2014). Apex predators with their requirement of large space and prey base have experienced largest range reduction (Ripple et al., 2014; Wolf and Ripple 2017). Tigers are one such large carnivore who have lost 95% of their historical range (Wolf and Ripple 2017). Primary requirement for tiger to survive is an undisturbed habitat with sufficient prey biomass and forest cover, but expanding human population and its pressure on forests and grasslands have resulted in continued shrinking of their natural habitats. Out of nine tiger species named four are extinct (South China functionally extinct from wild) and remaining five are categorized as either critically endangered or endangered. Existing tiger populations are designated as tiger conservation units (TCUs), majority of which are small and isolated (61%) and are considered not to be viable in long term (Dinerstein et al., 1997) unless managed at metapopulation level (Seidensticker et al., 1999).

Within India decades of conservation efforts have led to increase in tiger numbers but available natural habitat continues to decline (Jhala et al., 2020). Large number of studies on tigers focus on change in their number or distribution status as condition of their natural habitat improves or deteriorates. Natural change in population dynamics of a population depends on reproductive success of breeding females and tigers are known to respond reproductively to changes in environmental conditions. Number of females with young ones, litter size, dispersal age and consequently interbirth interval differs between habitats as available prey biomass changes (Schaller 1967; McDougal 1977). Given that reproduction along with growth, body condition and parental provisioning are mediated by physiological processes (Macleod et al., 2018; Clinchy et al., 2013) it would be important to quantify physiological responses of individuals of a population to understand future change in population parameters. Across taxa monitoring physiological changes of individuals or population have been advantageous in obtaining information that could guide management plans in identifying critical environmental stressors (Wasser et al., 2011; Ayres et al., 2012; Santicchia et al., 2018; Pokharel et al., 2019; Hunninck et al., 2020b) and populations at higher risk (Thiel et al. 2011; Sheriff et al., 2015; Bryan et al., 2015; Malviya et al., 2018). In this thesis I have utilized the ecological habitat gradient (in terms of habitat quality and disturbance) present in the Terai-Arc landscape, one of the major tiger landscapes of India, to understand how tigers physiologically respond to environmental disturbances in a human dominated landscape.

TAL forest habitat is also shared by another large carnivore from the same guild, the leopards (*Panthera pardus*). Among large cats, leopards have the widest distribution and as social subordinates are sympatric with dominant congeners like lions (Schaller 1972) and tigers (Seidensticker 1976) over much of Africa and Asia, respectively. As is for other large felids, habitat loss and fragmentation and resulting prey loss is the primary cause of leopard population decline globally (Fahrig, 2003; Nowell & Jackson, 1996). Out of currently recognized nine subspecies of leopards (Kitchener et al., 2017), seven are either endangered or critically endangered (Stein et al., 2016, Rostro-Garcı´a.,2019), two *P.p pardus* (African leopard) and *P.p fusca* (Indian leopard) are near threatened (Henschel et al., 2008). Globally leopard has lost 63-75% of their historical range, for Asian subspecies range loss is even more severe at 83-87% (Jacobson et al., 2016). Across India, Bhatt et al (2020) reported a decline of 75-90% (90 and 88% for Siwalik and Terai regions, respectively) of population due to human induced factors.

Efforts to restore natural habitats in India has largely been focused towards conserving tigers. Decades of conservation efforts have resulted in doubling of tiger population (Jhala et al., 2015; 2020) and TAL retains 22% of this current population (Jhala et al., 2020). Understanding of cascading impact of this increase in tiger population on leopard population dynamics is essential and should be an important consideration for overall conservation of large predator guild. Leopards are known to shift their habitat niche as the intensity of competition from dominant predators increases (Harihar et al., 2011; Mondal et al., 2012). Whether this shift affords leopard to co-exist with tigers or results in their exclusion from optimal habitats will depend and vary based on regional ecological settings. Hence It is important to study how leopard population get impacted at local scale, for example if leopards shift their activity from core to buffer areas or more suboptimal habitats with fewer natural prey, they may subsist their diet with domestic livestock that would increase their conflict with humans and consequently may result in retaliation from humans. Additionally, although leopards' adaptability may afford them to be able to survive in suboptimal habitats (Athreya et al., 2013; 2016), this persistence may come with certain level of compromised physiological health. As physiological health is important for long term survival of any population, this study seeks to understand how changing environmental factors impacts the physiological parameters of a leopard population. For this I have used leopard population in Rajaji tiger reserve (RTR) as model population. In eastern sector of RTR tiger population has increased three folds approaching its carrying capacity (Harihar et al., 2020) since creation of inviolate space in 2003 (Harihar et al., 2009; 2014). As opposed to eastern sector, tiger population in functionally disconnected western sector has dwindled over the years. Western and eastern sectors of RTR provides an excellent natural experimental set up to understand physiological dynamics of leopard in absence and presence of competition from dominant predator.

Environmental stressors and non-invasive physiological biomarkers

With the advancement of non-invasive (using faecal samples) approaches, measuring physiological responses have become possible for free ranging animals (Sheriff et al., 2016; Cook, 2012; Madliger et al., 2016; 2018). Usage of non-invasive faecal sampling are in fact considered a better option due to the long-term cumulative information obtained (Brown et al., 1996; Graham and Brown, 1996), as against blood sampling that itself induces stress (Möstl and Palme 2002; Palme et al., 2005a). The egested faeces of animal is known to contain metabolites of various circulating hormones (Palme et al., 1996;

Hodges et al., 2010; Palme et al., 2013), measure of which have been validated in numerous species to represent circulating levels, thereby providing an estimate of physiological state experienced by the animal (Wasser et al., 2000; Palme et al. 2005b; Sheriff et al., 2010). From a single faecal sample, one can obtain information on multiple physiological aspects like stress status (by quantifying glucocorticoids and mineralocorticoids), nutritional or metabolic state (by quantifying triiodothyronine) and reproductive state (by quantifying progesterone, estradiol, testosterone etc.) (Vynne et al., 2014; Joly et al., 2015; Corkeron et al., 2017; Valenzuela-Molina et al., 2018; Srivastava et al., 2021). Multiple hormones measures can be employed simultaneously to obtain a health profile (or physiological state) of animal or populations (Corkeron et al., 2017; Lemos et al., 2020; Mondol et al., 2020).

Glucocorticoids (GCs) are most extensively employed endocrine markers for studying free-ranging animals (Palme 2019). GCs are secreted in response to activation of hypothalamic-pituitary-adrenal (HPA) axis stimulated during stress full conditions (Sapolsky et al., 2000; Wingfield and Romero, 2010). Elevated GCs mobilizes energy resources to cope with the present challenge and returns to baseline, once the challenge is dealt (GC as anti-stress hormones see Wingfield and Kitaysky 2002; Palme 2019). However, when a challenge persists for a longer indefinite duration, elevated GCs and resultingly continued mobilization of energy resources becomes exhausting for animal resulting in suppression of vital life processes like growth and reproduction (Dallman and Bhatnagar, 2001; Wingfield and Romero, 2010). High levels of GCs are generally considered an indicator of chronic stress that compromises overall health. GCs as a sole parameter of animal fitness have been successfully associated with multiple environmental predictors (like disturbance, diet, predation risk, social dominance, poaching history, temperature etc.) in terrestrial (Gobush et al., 2008; Wasser et al., 2011; Stetz et al., 2013; Creel et al., 2013; Pokharel et al., 2019; Klich et al., 2021; Muller et al., 2021) as well as marine mammals (Burgess et al., 2013; Ayres et al., 2012; Hammerschlag et al., 2022), birds (Hayward et al., 2011; Thiel et al., 2011;), amphibians (Narayan et al., 2013; Santymire et al., 2021) and reptiles (MacLeod et al., 2021a; 2021b). A recent review on studies using GC as stress markers by Dickens & Romero 2013 revealed that GC responses to challenges can be bidirectional (or no change at all). It implies, in response to chronic stress GC measures can elevate as well as fall. Now, the elevation is known to be caused by the failure of negative feedback, whereas fall is reasoned to be caused by failure of HPA axis to respond (loss of coping response, Dickens & Romero 2013). Although a very useful marker, variation in GCs has to be carefully linked and interpreted based on ecological context.

For more concrete inferences, it is useful to have multiple-measure panels that would help in separating the effects of different stressors (Madliger et al., 2016; Behringer and Deschner, 2017). For example, a study by Ayres et al (2012) on killer whales employed fecal triiodothyronine (T3) measures along with GC to tease apart effect of psychological stress (vessel disturbance) from nutritional stress (long term prey deficit). Similar approach has been taken for many mammalian vertebrates (Wasser et al., 2011; 2017; Vynne et al., 2014; Joly et al., 2015; Dias et al., 2017). Thyroid hormone (TH) is secreted by thyroid glands in response to stimulation of hypothalamus-pituitary-thyroid (HPT) axis

(Eales, 1988; Flier et al., 2000) and is also regulated by cross-talk between axes (HPA-HPT) (Castañeda Cortés et al. 2014). In event of a stressful situation, activation of HPA axis and release of glucocorticoids downregulates TH secretion as well as peripheral conversion of T4 in to T3 (biologically active TH), but this effect is transient and HPT axis adapts to long lasting challenges (Behringer et al., 2018). Long term energy deficits have been consistently associated with reduced T3 levels, that helps in conserving energy by lowering overall metabolic rate (Eales, 1988; Behringer et al., 2018). As T3 have been reliably measured in faecal samples of numerous herbivore (Wasser et al., 2011; Joly et al., 2015; Hunninck et al., 2020a; Szott et al., 2020), carnivore (Ayres et al., 2012; Vynne et al., 2014; Wasser et al., 2017), as well as omnivore mammals (Dias et al., 2017; Gesquiere et al., 2018), together with GC it forms a powerful multiple-marker panel to assess overall physiological status (psychological as well as nutritional) in free ranging animals.

Despite immense potential of non-invasive endocrinology approaches, reliable measurements of hormone metabolites from faecal samples can get challenging. Apart from intrinsic species and sex specific differences (Brown et al., 1994; Goymann, 2012; Young et al., 2004), extrinsic factors such as sample condition and storage (Lafferty et al., 2019; Millspaugh et al., 2003; Wilkening et al., 2016), hormone extraction and assay protocols (Davidian et al., 2015; Gholib et al., 2018;; Nugraha et al., 2017) and hormone inert materials in faeces introduced by dietary differences (Goymann, 2012; Von der Ohe and Servheen, 2002) are known to impact reliable measures of hormone metabolites. These differences need to be controlled for obtaining accurate measurement and making biologically meaningful inferences. In this thesis, I first standardized the glucocorticoid and triiodothyronine metabolites measurement from wild tiger and leopard faecal samples. I specifically explored if faecal inorganic matter (IOM) like soil and sand that has been reported to be found in faeces of large wild felids (Khan, 2004; Schaller, 1967) can become a confounding factor in reliable hormone metabolite measurements.

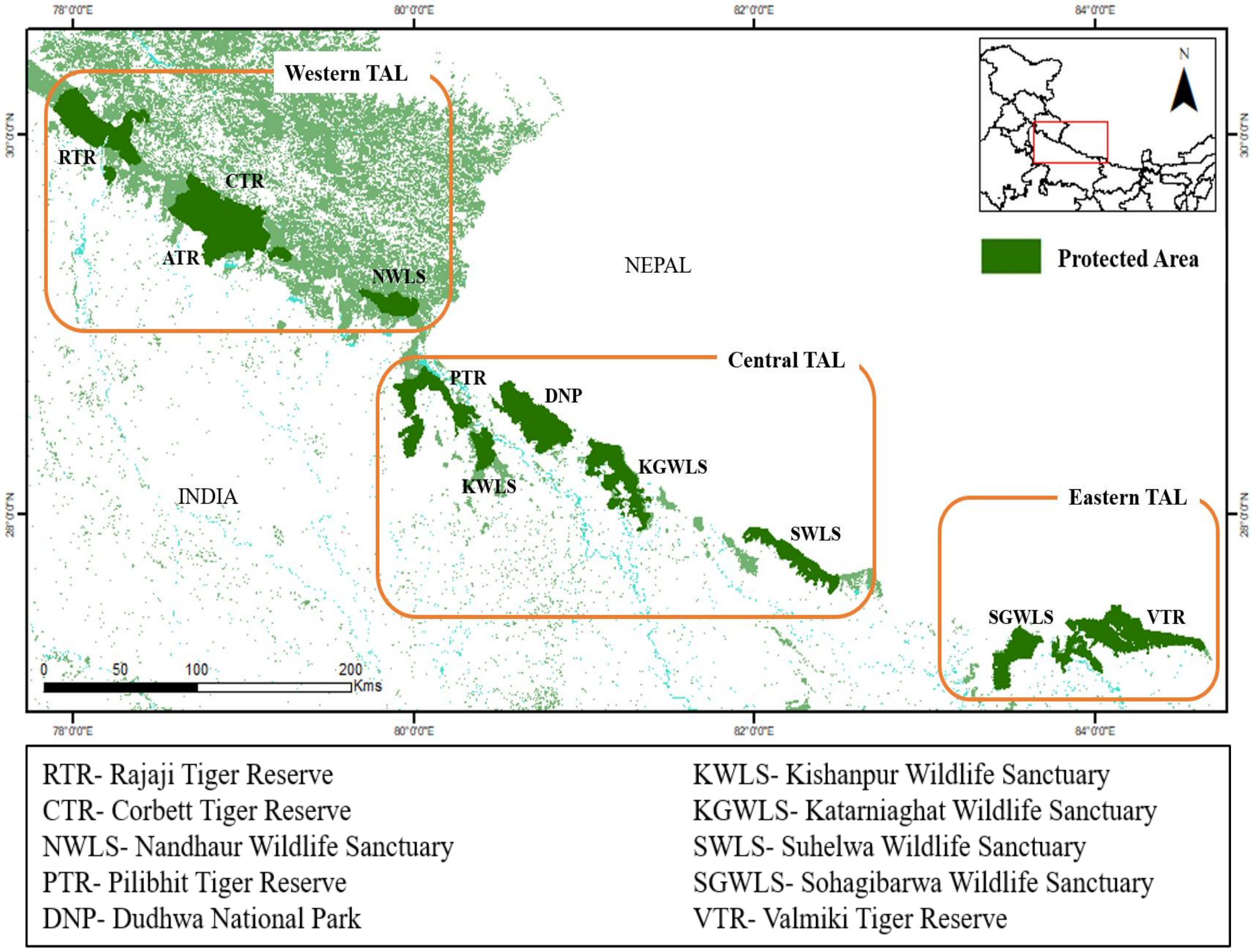
Study Area

Terai-Arc landscape-India

Some of the highest tiger densities in Indian subcontinent bioregion today occurs in tiger habitat of outer foothills of the Himalayas along the Siwalik range (Dinerstein et al., 1997), also known as Terai-Arc landscape (TAL). Once continuous belt of lowland forests (Gee 1964), TAL contains rich alluvial soil and receives ample rainfall that supports mosaic of rich habitat characterized largely by tropical moist deciduous, riverine and upland forest, interspersed with tall grasses (Stainton 1972; Tamang 1982). Productive mixture of alluvial grasslands and riverine forests are prime tiger habitats containing higher densities of tiger and its prey compared to upland Sal forests (Smith et al., 1998). In India, this linear landscape contains a total of 20800 km^2 of potential tiger habitat, covering the states of Uttarakhand, Uttar Pradesh and Bihar (Qureshi et al., 2006). Identified as ‘Global priority’ tiger conservation landscape (Sanderson et al., 2010), TAL retains about 22% of the India’s wild tiger population (Jhala et al., 2020). India and Nepal together share three level one and two level two tiger conservation units (Dinerstein et al., 1997). This study was conducted on Indian part of TAL, which can be further categorized as western, central and eastern TAL (Harihar et al., 2009; Chanchani et al., 2014). Western TAL consists of Rajaji-Corbett TCU (level I) with Rajaji tiger reserve (RTR) at the westernmost boundary and Corbett tiger reserve (CTR) towards east as major source population connected and surrounded by reserve forest areas. Rajaji-Corbett TCU with multiple core protected areas connected by forest lands is a macro reserve harboring the largest tiger population in this landscape (Jhala et al., 2020), its effective management can ensure long term persistence of tigers in this habitat. Central TAL is more fragmented and could be more aptly seen as specks of forests in sea of human settlement. It consists of Indian portion of Sukla-Phanta-Kishanpur (level II), Dudhwa-Kalali (level II) and Bardia- Banke (level I) TCUs. All major tiger habitats are protected areas in central TAL, from west to east, Pilibhit tiger reserve (PTR) is closer to Kishanpur wildlife sanctuary (KWS) lying toward its southeast (part of Sukla-Phanta-Kishanpur TCU), Dudhwa national park (DNP) is the smallest and most isolated unit in central TAL and Katerniaghat wildlife sanctuary (KGWS) is connected to Bardia national park in Nepal. Valmiki tiger reserve in eastern TAL is connected to Chitwan tiger population in Nepal (Chitwan-Parsa-Valmiki level I TCU). In this landscape in terms of habitat integrity we see a gradient, at one extreme we have well connected Rajaji-Corbett unit and at other extreme we have highly fragmented central TAL, with completely isolated DNP being most vulnerable tiger population (Chanchani et al., 2014). This study seeks to understand the underlying physiological responses of tiger population to ecological and anthropogenic stressors that vary between these extreme habitats within this landscape.

Rajaji Tiger Reserve

Within western TAL, Rajaji tiger reserve (RTR) was granted the status of tiger reserve in 2015. RTR is connected in east via corridor (Lansdown Forest division) to Corbett tiger reserve (CTR) which is a major tiger source population of the landscape. It holds one of the highest density of leopards (16.90 ±1.44/100km2) in the landscape (Jhala et al., 2021). Located at base of Himalayan foothills and starting of Indo-Gangetic plains, the reserve has an undulating topography and forest is mosaic of wood forest and grasslands drained by rivers and streams running north to south. Forest type is broadly classified as northern Indian moist deciduous forest and northern tropical dry deciduous Forest (Champion & Seth 2005). Reserve is separated by river Ganges in eastern (579 km2) and western (571km2) sectors structurally connected by the narrow Chilla-Motichur corridor that has lost its functionality for tigers due to heavy human activity on river banks. Eastern sector consists of a core zone that is surrounded by forest lands (buffer zones) in north, south and east directions. Towards west across Ganges is the western sector which is roughly rectangular and is divided into northeastern and southwestern half by Shiwalik ridge (with maximum elevation of 894m) that runs across its length. Towards the northern boundary flood plains of Song and Suswa rivers put some distance between park boundary and villages, whereas in south, villages and crop fields come right up to the park boundary (Berkmuller 1987). Inviolate western sector along with core zone of eastern sector supports higher wild-prey densities compared to buffer zones of east which are still under extensive pressure from Gujjars and their live stocks residing within and along park boundaries (Harihar et al., 2020).



RTR- Rajaji Tiger Reserve
CTR- Corbett Tiger Reserve
NWLS- Nandhaur Wildlife Sanctuary
PTR- Pilibhit Tiger Reserve
DNP- Dudhwa National Park
KWLS- Kishanpur Wildlife Sanctuary
KGWLS- Katarniaghat Wildlife Sanctuary
SWLS- Suhelwa Wildlife Sanctuary
SGWLS- Sohagibarwa Wildlife Sanctuary
VTR- Valmiki Tiger Reserve

Figure 1.1 *Protected areas of Terai-Arc landscape, India.*

1.3 Research questions:

Large carnivore conservation studies within India have largely evaded physiological context, mainly because of logistical difficulties in obtaining such information. With advancement in non-invasive endocrinology tools, it has become possible to assess animals and population health in context of the environment factors. Studies worldwide have established the potential of this approach in identifying the factors and the populations that need immediate conservation intervention. Considering the gap in understanding of free ranging large carnivore physiology at broad as well as fine scale I asked following questions:

1. If hormone metabolites can reliably be measured from tiger and leopard faecal samples, are there any confounding factors, if yes then how to overcome them?
2. What anthropogenic and ecological factors significantly impact tiger physiological health at landscape scale?
3. How competition from dominant predators in addition to anthropogenic disturbance impact physiological health of leopards?

1.4 Objectives:

To answer these questions, I conducted my study with following objectives:

Objective 1: Assessing effects of faecal inorganic content variability on quantifying glucocorticoid and thyroid hormone metabolites in large felines.

Objective 2: Evaluating the impacts of ecological and anthropogenic factors on tiger stress physiology within Terai-Arc landscape, India.

Objective 3: Spatio-temporal analyses of leopard physiological responses to varying tiger density in Rajaji Tiger Reserve, Uttarakhand, India.

1.5 Thesis structure:

The introduction of this thesis provides a background on suitability of physiological markers for conservation-oriented studies. I discussed the current distribution of tiger and leopard in study area and established the ecological context of the study.

In first chapter, I discussed the need for species specific standardization and validation of hormone extraction and quantification procedure. I looked at a potential factor that can confound hormone metabolite measures from faecal samples and provided solutions to control that factor in free ranging large carnivores.

In second chapter, based on extensive literature I quantified various fine and broad scale ecological and anthropogenic factors that can potentially impact physiological health measures of tigers at landscape level. I evaluated impact of each factor and discussed the factors, areas and population in immediate need of conservation attention.

In third chapter, taking advantage of a natural setup and using a spatio-temporal framework, I quantified physiological responses of leopard to changing competition intensity from dominant predators (tigers). I assessed and discussed the reasons of past and present physiological status of leopard in light of diet quality, competition intensity and habitat productivity.

The conclusion contains a summary of key findings of this thesis. I believe that this thesis with its methodological improvement, landscape level approach and an attempt to provide physiological evidence of cost of interspecies competition (first for tiger-leopard dynamics) is an important contribution in the field of conservation physiology. This work should encourage future studies on large carnivores to include physiological aspects in the list of factors tested, to provide insight into underlying physiological causes of change in population dynamics.

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CHAPTER 1

Assessing effects of faecal inorganic content variability on quantifying glucocorticoid and thyroid hormone metabolites in large felines

2.1 Introduction

The ever-increasing impacts of humans on wildlife through habitat destruction and other environmental alterations call for various reliable indices that can monitor temporal and spatial variations in physiological responses of free ranging animals. Faecal hormone analysis has become a powerful tool in this regard due to the variety of stress, nutrition and reproductive measures that can be used to evaluate the magnitude and direction of physiological responses of disturbances (Creel et al., 2002; Gobush et al., 2008; Keay et al., 2006; Wasser et al., 1997, 2005, 2011, 2017). Such non-invasive approach allows easy access of samples at spatial and temporal scales and provide a cumulative and feedback-free measure of circulating hormone concentrations over a longer time period (Brown et al., 1996; Graham and Brown, 1996; Palme et al., 1996, 2005) when compared with blood (Sheriff et al., 2010). The interactive effects of these stress, nutrition and reproductive hormones also allow relating wildlife health measures and adaptations to environmental changes in response to various disturbances (Ayres et al., 2012; Corkeron et al., 2017; Gobush et al., 2008; Hayward et al., 2011; Hunt et al., 2006; Mondol et al., 2020). For example, glucocorticoid (GC) concentrations rapidly increase in response to wide range of short or long-term psychophysiological stressors (Dallman and Bhatnagar, 2010; Wingfield and Romero, 2010), whereas the bioactive thyroid hormone triiodothyronine (T3) acts more slowly, altering metabolism in response to acute or chronic nutritional stress (Eales et al., 1988; Flier et al., 2000). When studied together, psychological and nutritional hormone metabolites provide complementary information on an animal's coping mechanism under different ecological or environmental conditions at different scales. Despite such potential of the non-invasive endocrinology approaches in wildlife conservation, reliable measurements of faecal hormone metabolites are often challenging. A myriad of extrinsic and intrinsic factors are known to affect measures of various faecal hormone metabolites including sampling and storage methods (Hunt and Wasser, 2003; Khan et al., 2002; Lafferty et al., 2019; Millspaugh et al., 2003b; Wilkening et al., 2016), species and sex-specific hormone metabolite compositions (Brown et al., 1994; Goymann et al., 2012; Young et al., 2004), and appropriate laboratory protocols for hormone extraction and assays (Davidian et al., 2015; Gholib et al., 2018; Millspaugh et al., 2003a; Nugraha et al., 2017; Pappano et al., 2010; Watson et al., 2013). The influences of these major factors in physiological assessments and their technical solutions have been the major focus of research over years (Millspaugh and Washburn, 2004; Palme et al., 2013, 2019; Sopinka et al., 2015; Touma and Palme, 2005). However, alterations introduced by dietary differences in free ranging animals that changes faecal composition and output have received less attention (Goymann et al., 2012; Von der Ohe and Servheen, 2002). To this end, undigested parts of the diet (e.g., dietary fiber) and other inorganic matters (e.g., urates, soil) that do not contribute hormone metabolites have been recognized to influence estimates of multiple steroid hormones in various species (Ganswindt et al., 2012; Wasser and Hunt, 2005). Studies suggest that these hormone-inert parts of faecal matter decreases hormone metabolite concentrations during extraction (Wasser and Hunt, 2005) and introduce errors in measuring physiological biomarkers for stress (for example, see Ganswindt et al., 2012 for aardwolves; Goymann, 2005 for stone chats; Wasser and Hunt, 2005, Hayward et al., 2010 for owls etc.) and reproductive status (for example, see Goymann, 2005 for stone chats; Wasser et al., 1993 for baboons; Wasser and Hunt, 2005 for owls etc.). Such effects seem to be more pronounced in free-ranging animals, possibly due to large variations in species-specific food quantity and quality in the wild compared to captivity (Dierenfeld et al., 1994; Tilson et al., 2016). If unaccounted, these variations can result in inaccurate assessment of stress-(GC) and nutrition-related (T3) hormones, resulting in misguided conservation efforts.

In this chapter, key methodological issues are addressed to examine the impacts of hormone-inert inorganic matters (henceforth IOM) on measures of stress (GC) and nutritional (T3) hormones in wild tigers in the Terai-Arc landscape, north India. The tiger (*Panthera tigris*) typifies global international conservation efforts across their range countries (Sanderson et al., 2010). Decades of intense conservation efforts have resulted in doubling their population in India (Jhala et al., 2015, 2020), and their future persistence will depend on managing these increasing population within the existing habitat (Gubbi et al., 2016, 2017). It is expected that the increasing tiger density will have distinct physiological impacts, particularly perceived stress (from intra-specific competition and anthropogenic disturbances), nutritional challenges (wild prey depletion) and possibly reproductive effects. Tigers occupy a wide variety of land cover types (Jhala et al., 2020) with varying dietary regimes (Basak et al., 2016; Harihar et al., 2011; Kumar et al., 2008) and their faeces is known to contain significant amount of soil and other inorganic matter (Khan, 2004; Schaller, 1967), thereby making this an informative system to study impacts of these materials on hormone quantification. While other studies have focused on stress status measurements in wild tigers in India (Bhattacharjee et al., 2015; Malviya et al., 2018; Naidenko et al., 2019; Tyagi et al., 2019), they have not looked at the impacts of inorganic matters on measurement of faecal glucocorticoid (fGCMs) and T3 metabolites (fT3Ms). In addition, we used another large feline carnivore species, the Asiatic lions (*Panthera leo persica*), with similar digestibility as tigers (Wynne 1989), housed under captive environment where differences in dietary regimes are generally negligible and hence variations in inorganic matter content and its resulting influence on hormone measurements are expected to be low. Main objectives in this study were: (1) to estimate the amount of IOM present in faecal samples from wild tigers and captive lions and evaluate their variations; (2) assess the impacts of IOM in measures of fGCMs and fT3Ms and explore approaches to minimize the effects; and (3) evaluate how errors introduced by IOM variability, if any, can affect the ecological interpretations of fGCM and fT3M measures in both species. I believe that results of this study have wider relevance for non-invasive endocrinology studies of wild animals, particularly carnivores with variable inorganic matter contents in their faeces.

2.2 Materials and Methods

2.2.1 Study area

Wild tiger faeces have been collected from five major protected areas in the Terai-Arc landscape (TAL) in India. This linear landscape of TAL contains a total of 20800 km^2 of potential tiger habitat, covering the states of Uttarakhand, Uttar Pradesh and Bihar

(Qureshi et al., 2006). Situated at the foothills of the Himalayas, this region supports tropical moist deciduous forests dominated by Sal (*Shorea robusta*), tall Terai swamp grasslands and permanently moist reed swamps (Champion and Seth, 2005). This landscape is identified as ‘Global priority’ tiger conservation landscape (Sanderson et al., 2006) and retains about 22% of the India’s wild tiger population (Jhala et al., 2020). Sampling was mostly concentrated inside five main tiger reserves in this landscape: Rajaji and Corbett Tiger Reserves in Uttarakhand (western TAL), Pilibhit and Dudhwa Tiger Reserves in Uttar Pradesh (central TAL) and Valmiki Tiger Reserve in Bihar (eastern TAL).

The captive Asiatic lion faecal sampling was conducted at the largest Asiatic lion conservation-breeding center at Sakkarbaug Zoological Garden (SZG), Gujarat. SZG hosts the largest captive population (N= 60) with the highest reported number of wild founders (Srivastav et al., 2018). Faecal samples were collected from 35 individuals housed at the off-display conservation-breeding center of SZG. The same samples were used in an earlier study to evaluate the effects of enrichment interventions on behaviour and stress measures (see details in Goswami et al., 2021). During the enrichment intervention, the animals were randomly assigned to control (n=16) and test (n=19) group to ascertain the physiological stress status during pre- and post-enrichment periods.

2.2.2 Faecal sample collection, species identification and hormone extraction

A team of experienced field personnel surveyed forest trails of aforementioned protected areas (see Bhatt et al., 2020) and collected tiger faecal samples that were visibly fresh, intact with minimal insect activities and contained strong odour (Vynne et al., 2014), during winter seasons of 2016-2018. Faecal samples were stored in -20^{0}C freezer till DNA extraction (Biswas et al. 2019). Tiger-specific mitochondrial DNA markers (Mukherjee et al., 2007) were used to identify tiger faeces and only confirmed tiger faecal samples were used for downstream hormone and inorganic matter analyses. A total of 193 tiger faecal samples were used in this study: Rajaji Tiger Reserve (RTR, n=57), Corbett Tiger Reserve (CTR, n=50), Dudhwa-Pilibhit region (DTR-PTR, n=52) and Valmiki Tiger Reserve (VTR, n=34). For lions, a total of 120 fresh faecal samples were collected from the SZG conservation breeding center over a period of three months. Two fresh faecal samples per week from each individual were collected during the enrichment experiment duration (covering both pre- and post-enrichment periods). The samples were stored in -20^{0}C freezer till further processing.

For hormone extraction from tiger (n=193) and lion (n=120), a modified hormone extraction protocol described in Wasser et al. (2010) was followed. Each frozen sample was broken down to smaller pieces and dried up to 72 hours in an oven (#Unilab-112HO, Haryana, India) at 60^{o}C prior to hormone extraction to control for moisture (Wasser et al., 1993). Dried samples were then pulverized and sieved through 0.5 mm steel mesh strainers to remove prey remains/other hard parts and obtain faecal powder. The dried faecal powder was thoroughly mixed and subsequently hormone extraction was performed by pulse-vortexing 0.1 grams of powder in 15 ml of 70% ethanol, followed by centrifugation at 2200 rpm for 20 min (Mondol et al., 2020; Wasser et al., 2010). The hormone extracts were collected in 2 ml cryochill vials (1:15 dilution) and stored in -20^{o}C freezer till further analyses. A total of 193 and 187 samples were used for tiger fGCM and fT3M analyses, respectively. For captive Asiatic lions, a total of 120 (from 35 individuals) and 46 samples (from 12 individuals) were used in fGCM and fT3M analyses, respectively.

2.2.3 Estimation of inorganic/organic content

To estimate inorganic/organic content in each faeces (both tiger as well as Asiatic lion), a slightly modified protocol described in Ganswindt et al. (2012) was used. About 0.1 grams (same weight as used for extraction) of dried faecal powder was measured in a crucible and ashed the powder in a muffle furnace (#NSW-101, NSW, New-Delhi, India) at 550^{0}C for 2 hours. Post-combustion, the residual IOM was weighed and amount of organic matter (OM) combusted in each sample was calculated using the formula: Organic matter = (0.1- weight of inorganic matter) grams.

2.2.4 fGCM and fT3M assays

Corticosterone (#K014, Arbor Assays, MI, USA) and Triiodothyronine (T3) (#K056, Arbor Assays, MI, USA) EIA kits were used to measure fGCMs and fT3Ms from tiger and lion faecal extracts. Physiological validation for the corticosterone EIA kit was conducted by quantifying fGCMs in sample extracts of tiger faeces collected before and after ACTH challenge study conducted in Mondol et al. (2020). Assays showed 2.5-fold increase in fGCM titers post ACTH challenge followed by gradual decrease in subsequent samples (Figure 2.1). For lions, biological validation of fGCM was earlier conducted by Goswami et al. (2021) where the levels of fGCM were measured during pre- and post-enrichment interventions under captive conditions. Although the T3 EIA kit has not been physiologically/biologically validated for tiger and lion, it has been earlier successfully used in many terrestrial and marine mammals (Ajó et al., 2020; Kozlowski et al., 2019; Szott et al., 2020). During assays, sample extracts were air-dried inside an incubator (#ATI-117, Obromax, Delhi, India) and re-suspended in assay buffer

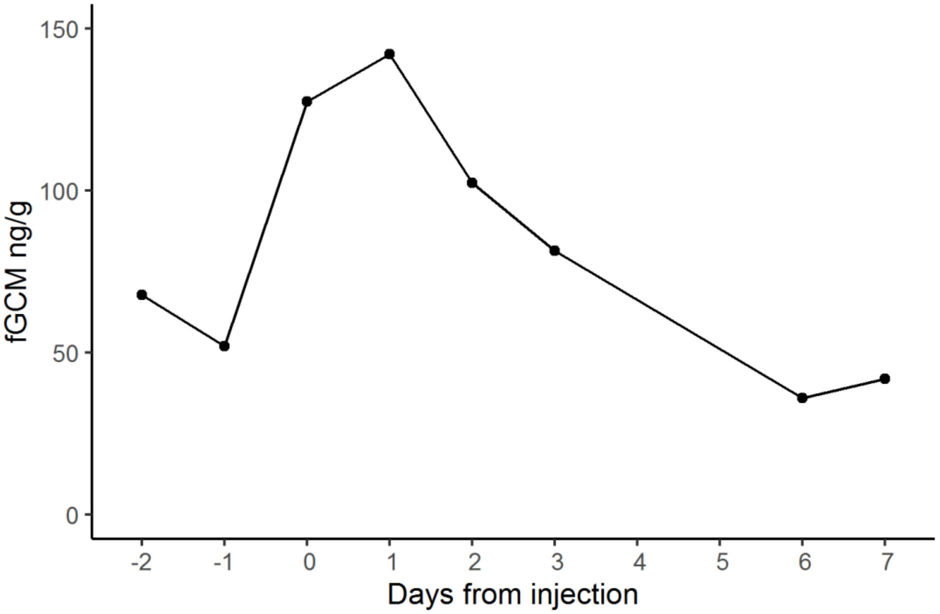

Figure 2.1: *Physiological validation of tiger GC EIA assay, where tiger fGCMs were measured on a single tiger individual following ACTH challenge (samples from Mondol et al., 2020). In X-axis 0 represent the day of ACTH challenge.*

as per required dilutions. Each sample was assayed in duplicate using respective kit protocols and the optical density was measured at 450 nm using GMB-580 automatic microplate ELISA plate reader (#GMB-580, Genetix Biotech Asia, New Delhi, India). Hormone metabolite concentration is interpolated using four parametric logistic (4PL) regression function of GraphPad prism version 5 (GraphPad Software, California, USA). Sensitivity of GC and T3 EIA assay was 20 pg/ml and 37.4 pg/ml, respectively. Cross-reactivities of respective antibodies are listed in table 2.1.

Table 2.1: *Details of the faecal hormone assays conducted for tiger and Asiatic lion in this study.*

Hormone	**Assay method**	**Dilution**	**Slope (R^2)**	**Inter-assay CV**	**Intra-assay CV**	**Cross-reactivity**
Corticosterone (Lion pool)	EIA	1:60	0.92 (0.99)	9.0	9.8	100% with corticosterone, 12.30% with Desoxycorticosterone, 2.30% with Tetrahydrocorticosterone and <1% with Aldosterone, Cortisol, Progesterone, Dexamethasone, Corticosterone-21-Hemisuccinate, Cortisone and Estradiol
Corticosterone (Tiger pool)	EIA	1:100	1.18 (0.99)	9.0	3.6	
Triiodothyronine (Lion Pool)	EIA	1:30	0.97 (0.99)	9.7	6.2	100% with T3, 0.88% with thyroxine and less than 0.1% with reverse T3 (3,3’,5’-Triiodo-L-thyronine)
Triiodothyronine (Tiger Pool)	EIA	1:15	0.98 (0.99)	6.2	8.8	

2.2.5 Parallelism and Accuracy

Parallelism and accuracy tests were used for laboratory validation of GC and T3 hormone antibodies for both species. Parallelism tests for fGCM and fT3M were conducted using dilutions of pooled tiger and lion faecal extracts from multiple random samples (n=20) to assess reliable quantification of respective hormone metabolites at different concentrations and to find optimal dilutions for final assays (at 50% binding). For both hormones, species-wise sigmoid curves were generated as relative dose vs. percent bound hormones for the pools and the standards, where parallel slopes indicate better antibody binding at different concentrations. Subsequently, accuracy tests were performed for both assays to determine any interference in faecal extracts during antibody interactions. Independently both hormone standards were spiked with equal volumes of diluted faecal extract of known concentration (dilution level close to 50% bound from parallelism test) and assayed with standards. Results were plotted as regression lines using observed and expected concentrations from accuracy tests to show that faecal components were not interfering with assay accuracy at the tested dilution. Inter- and intra- assay coefficients of variation were determined using repeated measures of same-pooled extract. Parallelism results were examined using an F ratio test for differences in slopes. Accuracy results were evaluated using analysis of linear regression. All analysis for parallelism and accuracy were performed in GraphPad prism version 5 (GraphPad Software, California, USA).

Parallelism and accuracy tests for tiger fGCM and fT3M indicated reliable measures across different concentration ranges. Serial dilutions of faecal extracts paralleled the standard curves (Figure 2.2). No differences between slopes of standard and pooled extract curves for fGCM ($F_{(1,12)} = 2.01$, $P = 0.182$) and fT3M ($F_{(1,11)} = 0.62$, $P = 0.45$) were found. Accuracy tests produced slopes of 1.18 and 0.98 at working dilution of 1:100 and 1:15 for fGCM and fT3M (Figure 2.2), respectively, suggesting that faecal extracts did not interfere with their metabolite measurement precisions. Intra-assay coefficient of variation (CV) was 3.6 and 8.8, whereas inter-assay CV was 9.0 and 6.2 for fGCM and fT3M, respectively (Table 2.1). Similarly, the Asiatic lion fGCM and fT3M parallelism graphs showed no slope differences ($F_{(1,10)} = 2.06$, $P = 0.182$ and $F_{(1,12)} = 0.69$, $P = 0.42$, respectively). Accuracy graphs showed a slope of 0.92 (at 1:60 dilution for fGCM) and 0.97 (at 1:30 dilution for fT3M), respectively (Figure 2.2). Intra-assay coefficient of variation (CV) was 9.8 and 6.2, whereas inter-assay CV was 9.0 and 9.7 for fGCM and fT3M, respectively (Table 2.1).

2.2.6 Statistical analysis

Tiger and lion fGCM and fT3M data were assessed for normality (both raw as well as log-transformed values) using diagnostic plots (Q-Q plots) and Shapiro-Wilk test. To assess the extent of IOM presence the faecal samples were categorized into five intervals of increasing percentage of IOM as 0-20%, 20-40%, 40-60%, 60-80%, and 80-100 %. IOM percentage was used as a variable to test the strength and direction of association between IOM content and fGCM and fT3M concentrations (expressed as total dry matter) using Pearson’s correlation test. Earlier studies have described the effects of sample mass (biased fGCM measures from less faecal sample amount) (Hayward et al., 2010) and also the impacts of IOM in fGCM measure (Ganswindt et al., 2012). To evaluate any potential impacts of IOM presence in faecal hormone metabolites measure samples with high IOM content (≥80% IOM) were dropped (to reduce sample mass effect). Further, hormone concentrations as per unit of organic dry matter were also calculated. Linear regressions were performed to test for relationships between IOM content and hormone metabolite concentrations for all samples and with both types of corrected data sets (dropping samples with ≥80% IOM and hormone metabolite concentrations expressed as organic dry matter). Finally, for field collected tiger samples the fGCM and fT3M levels were compared among the sampled tiger reserves (RTR, CTR, DTR-PTR and VTR as mentioned above) using one-way ANOVA (separately for total dry matter and organic dry matter, respectively) along with post-hoc comparisons (Tukey's HSD test) to detect any potential alteration in results before and after implementing corrective measures.

For Asiatic lions, post-enrichment fGCM and fT3M data between the control and test groups were compared as it showed significant differences between them (Goswami et al., 2021). Only test group individuals were compared for fT3M levels between pre and post-enrichment period. Independent t-test (for control and test groups) and paired t-test (for pre and post-enrichment period) was used respectively to evaluate any changes in results between two different ways of hormone metabolite concentration expressions (per units of total dry matter vs. total organic matter). The effect sizes (ω^2 for ANOVA, Hedges g for independent t-test and Cohen's d for paired t-test) were also calculated for each method. During all analyses, differences were considered significant at alpha level 0.05. Analyses were performed in GraphPad prism version 5, SPSS version 20 (IBM, 2011) and R v3.5.2 (R Core Team, 2018) using package "ggpubr" (Kassambara, 2020).

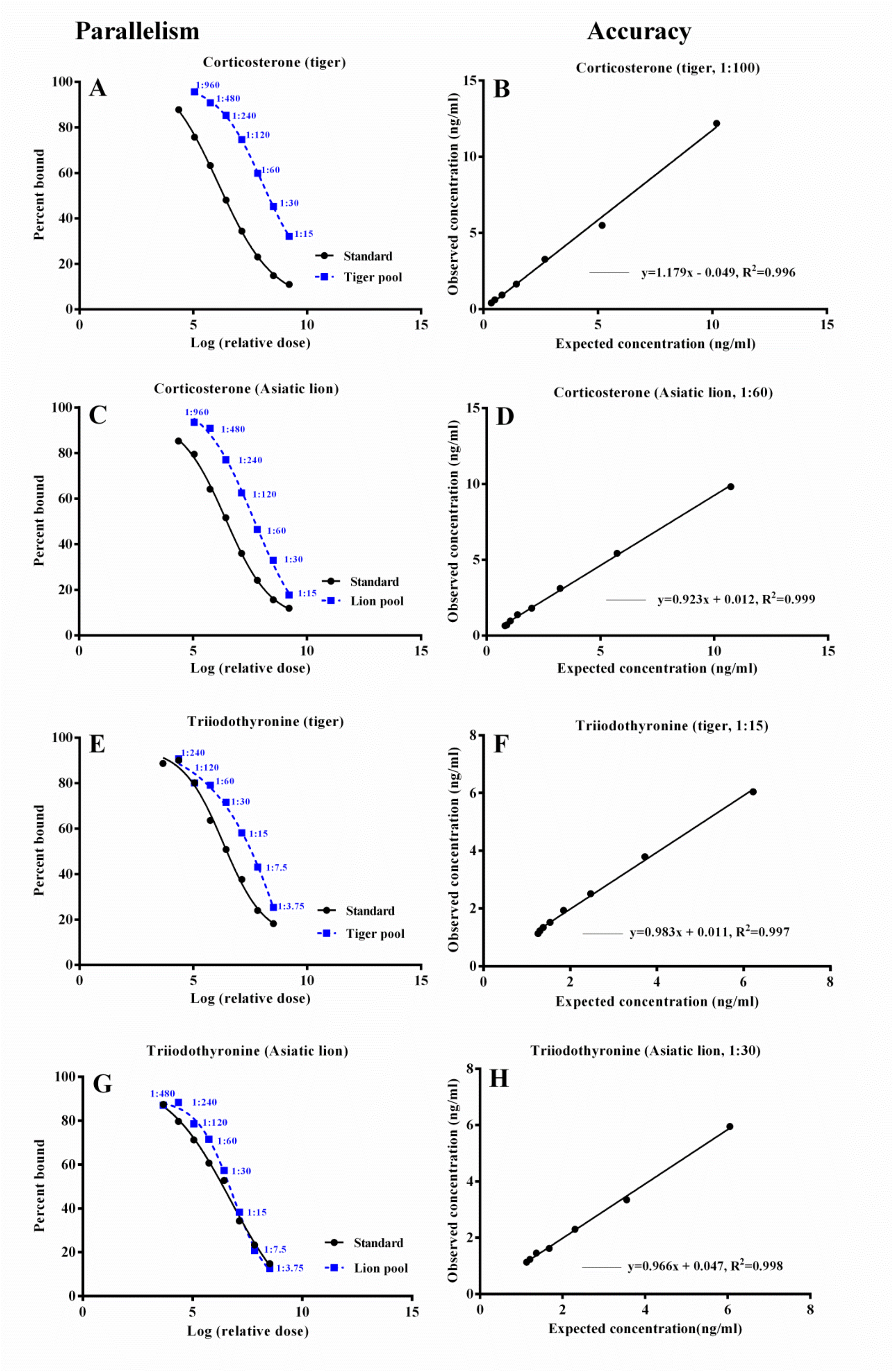
Parallelism
Accuracy
Corticosterone (tiger)
A
Percent bound
Log (relative dose)
1:960
1:480
1:240
1:120
1:60
1:30
1:15
Standard
Tiger pool
Corticosterone (tiger, 1:100)
B
Observed concentration (ng/ml)
Expected concentration (ng/ml)
y=1.179x - 0.049, R²=0.996
Corticosterone (Asiatic lion)
C
Percent bound
Log (relative dose)
1:960
1:480
1:240
1:120
1:60
1:30
1:15
Standard
Lion pool
Corticosterone (Asiatic lion, 1:60)
D
Observed concentration (ng/ml)
Expected concentration (ng/ml)
y=0.923x + 0.012, R²=0.999
Triiodothyronine (tiger)
E
Percent bound
Log (relative dose)
1:240
1:120
1:60
1:30
1:15
1:7.5
1:3.75
Standard
Tiger pool
Triiodothyronine (tiger, 1:15)
F
Observed concentration (ng/ml)
Expected concentration (ng/ml)
y=0.983x + 0.011, R²=0.997
Triiodothyronine (Asiatic lion)
G
Percent bound
Log (relative dose)
1:480
1:240
1:120
1:60
1:30
1:15
1:7.5
1:3.75
Standard
Lion pool
Triiodothyronine (Asiatic lion, 1:30)
H
Observed concentration (ng/ml)
Expected concentration(ng/ml)
y=0.966x + 0.047, R²=0.998

Figure 2.2 (Previous page): *Parallelism and accuracy graphs (8 standard concentrations) for faecal corticosterone and T3 EIA assays.*

2.3 Results

The field-collected tiger samples showed higher variation in IOM content (n=193, 9-98%) than the captive lion samples (n=120, 17-57%) (Figure 2.3). Majority of the tiger samples (n=178, 92.22%) had >40% IOM content, whereas IOM content remained <40% for most of the captive lion samples (n=103, 85.83%). Significant negative correlation was observed between IOM content and tiger fGCM (n=193, r=-0.46, p=0.000) and fT3M (n=187, r=-0.58, p=0.000) concentrations, respectively. The lion samples with less variation in IOM did not show any such pattern for fGCM (n=120, r= -0.05, p=0.579), but were negatively correlated for fT3M (n=46, r= -0.43, p=0.003).

Regression analysis with tiger samples showed strong influence of IOM content on fGCM measures when expressed as per gram of total dry matter (n=193, R^2= 0.21, P= <0.0001) (Figure 2.4A). However, this influence reduced when samples with high IOM contents (≥80%) are removed from the analysis (n=139, R^2= 0.06, P= 0.004) (Figure 2.4B), and reduced further when the data was expressed as organic dry matter (n=139, R^2= 0.05, P= 0.011) (Figure 2.4C). Similarly, the fT3M measures were also influenced strongly by IOM content when expressed as total dry matter (n=187, R^2= 0.34, P= <0.0001) (Figure 2.4D). Removing ≥80% IOM samples reduced the influence (n=138, R^2= 0.09, P= 0.0003) (Figure 2.4E), and subsequent expression of fT3M concentrations as organic dry matter eliminated the influence of IOM (n=138, R^2= 3.24E-4, P= 0.833)

(Figure 2.4F). Protected area-wise mean fGCM and fT3M values are provided in Table 2.2. One-way ANOVA for field collected samples showed no significant differences in mean fGCM levels across the sampled tiger reserves when concentrations were expressed as total dry matter (F (3,189)= 0.52, p=0.671, $\omega^2 = -0.008$), as well as when samples with $\geq 80\%$ IOM content were removed (F (3,135)=1.26, p=0.290, $\omega^2=0.007$) and fGCM measures were expressed as organic dry matter (F (3,135)=1.18, p=0.322, $\omega^2=0.004$) (Figure 2.5A, B & C). However, significant changes were observed in fT3M results among three different datasets. While no significant differences were found in mean fT3M levels in sampled tiger reserves when concentrations were expressed as total dry matter (F (3,183)=1.56, p=0.200, $\omega^2=0.009$), the values were significantly different when samples with $\geq 80\%$ IOM content were removed from analysis (F (3,134)=3.24, p=0.024, $\omega^2=0.046$). The significance increased further when fT3M concentrations were expressed as per gram of organic dry matter (F (3,134) = 4.99, p=0.003, $\omega^2 = 0.08$) (Figure 2.5D, E & F). Subsequent Tukey's HSD test for each of these two datasets shows that CTR population has significantly higher fT3M levels compared to DTR-PTR population (p=0.032, p=0.004, respectively) that has lowest fT3M levels.

However, the captive lion samples showed no influence of IOM content on fGCM measures (Table 2.3) when expressed as per gram total dry matter (n=120, $R^2= 0.003$, P= 0.570) (Figure 2.6A) as well as per gram organic dry matter (n=120, $R^2= 0.017$, P= 0.157) (Figure 2.6B). The fT3M measures in captive lions showed significant influence of IOM when expressed as total dry matter (n=46, $R^2= 0.18$, P= 0.003) (Figure 2.6C), and as found in wild tigers subsequent expression of fT3M concentrations as organic dry matter eliminates the influence (n=46, R^2= 0.02, P= 0.32) (Figure 2.6D). Independent t-test results comparing fGCM levels between the post-enrichment control and test groups of captive lions showed a difference in fGCM titer values but the patterns of the result remained same (total dry matter (t(80)=9.77, P=0.00, g=2.21) and organic dry matter (t(80)=9.51, P=0.00, g=2.14) (Figure 2.7A & B), respectively. No significant difference was found in mean fT3M levels (expressed as total dry matter) during pre and post-enrichment periods (t(11)=0.14, P=0.89, d=0.03) and results remained non-significant even when fT3M concentrations were expressed as organic dry matter (t(11)=0.65, P=0.52, d=0.24) (Figure 2.7C & D).

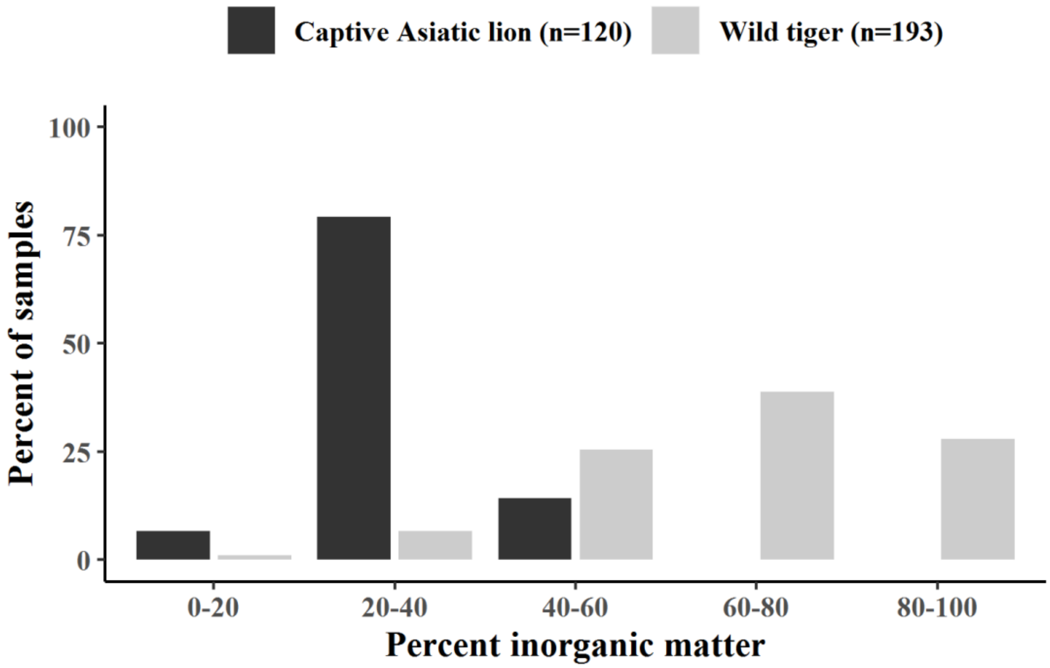

Figure 2.3: *Comparison of Inorganic matter (IOM) content in faecal samples of field-collected wild tigers and captive Asiatic lions. The samples were categorized into five groups.*

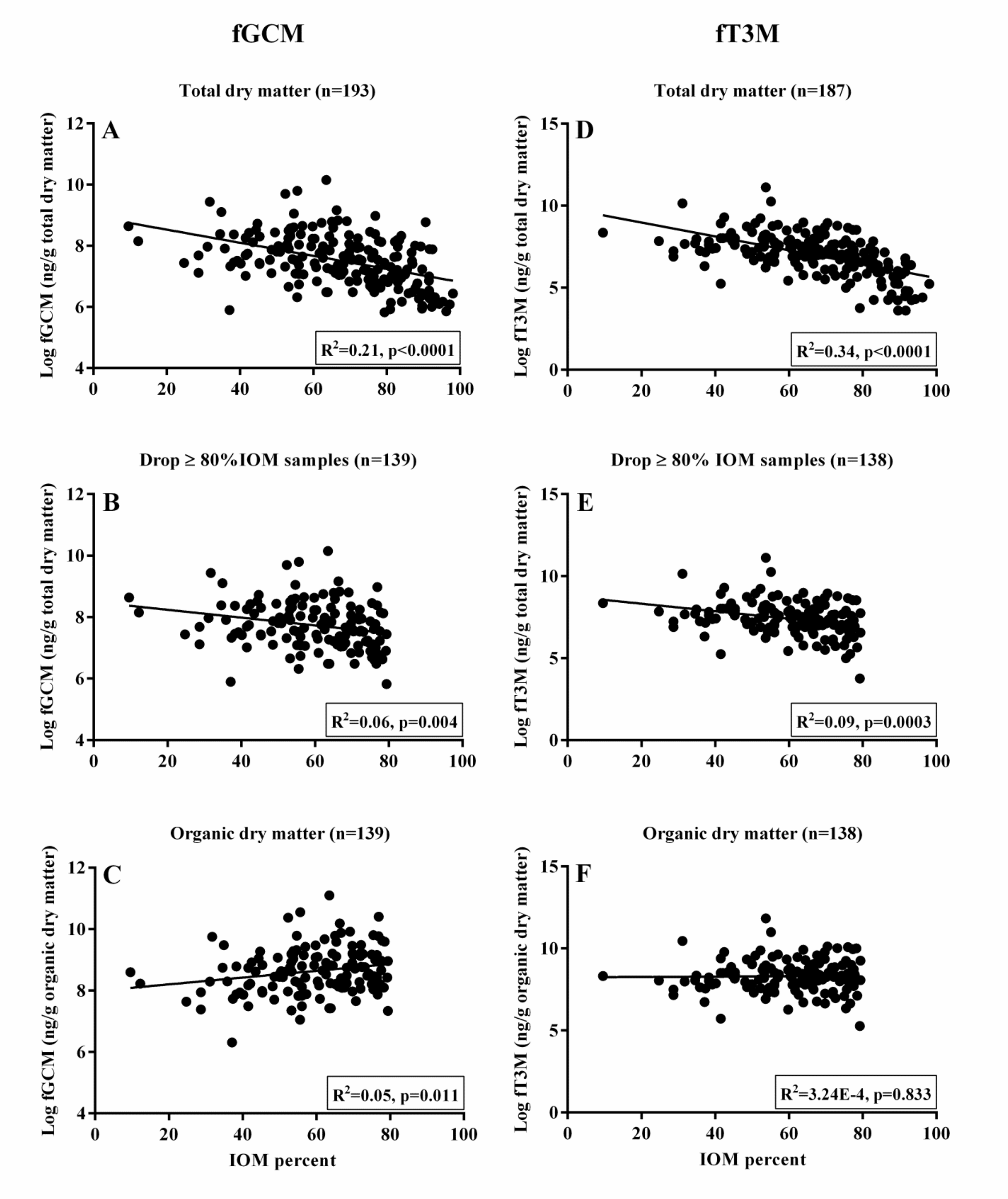

Figure 2.4: *Relationship between percentages of tiger faecal IOM and log-transformed fGCM and fT3M concentrations, when hormone concentrations are expressed as per gram of total dry matter (A and D), when high IOM (>80%) samples are dropped (B and E) and when hormone concentrations are expressed as per gram of organic dry matter (C and F).*

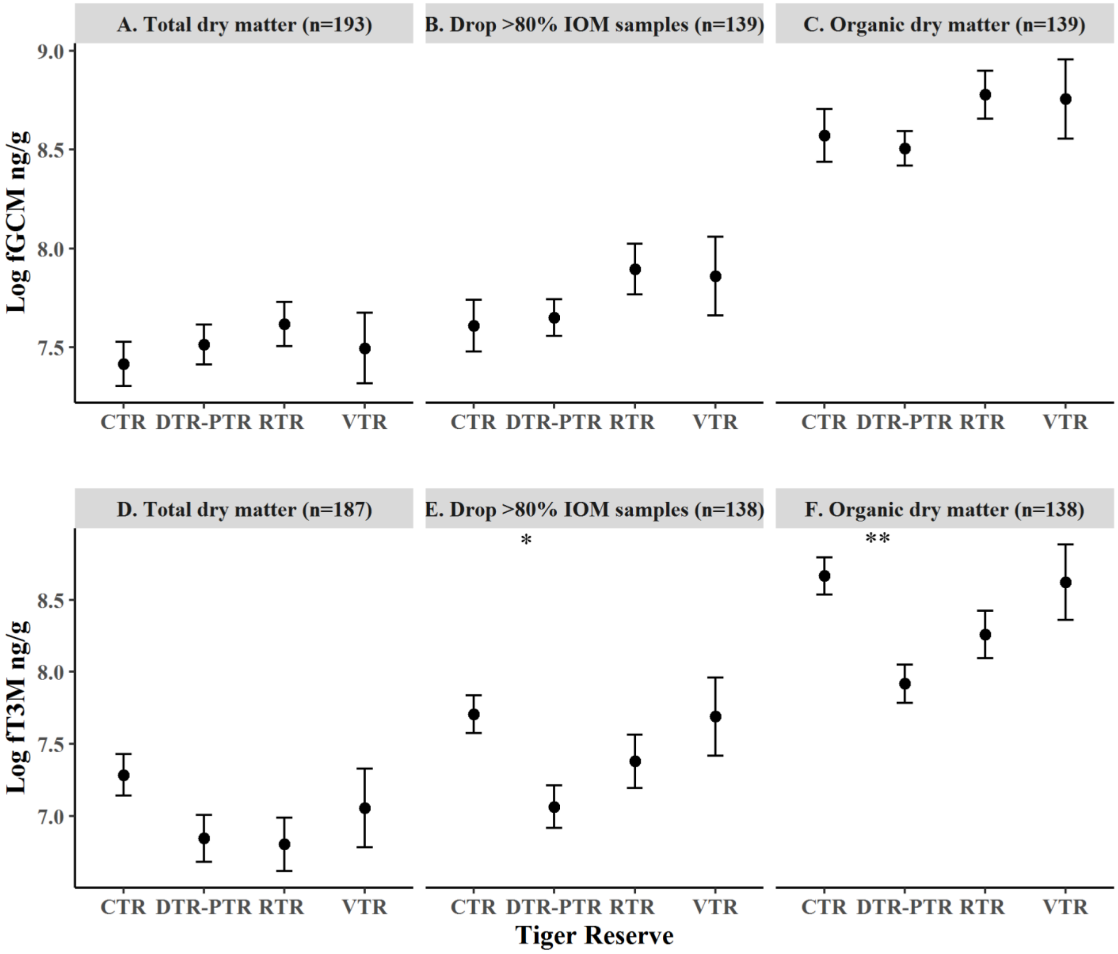

Figure 2.5: *Comparison of mean tiger fGCM and fT3M concentrations across four tiger reserves in Terai-Arc landscape (TAL). The top and bottom panel represents fGCM and fT3M data, respectively. For both hormone metabolites mean values (ng/g; log-transformed) are plotted. The left graphs (A and D) show the concentrations expressed as per gram of total dry matter, the middle graphs (B and E) indicate patterns after dropping samples with high IOM contents (>80%) and the right graphs (C and F) represents patterns when concentrations are expressed as per gram of organic dry matter. Any significant differences are marked with asterisks (*P< 0.05; **P<0.01).*

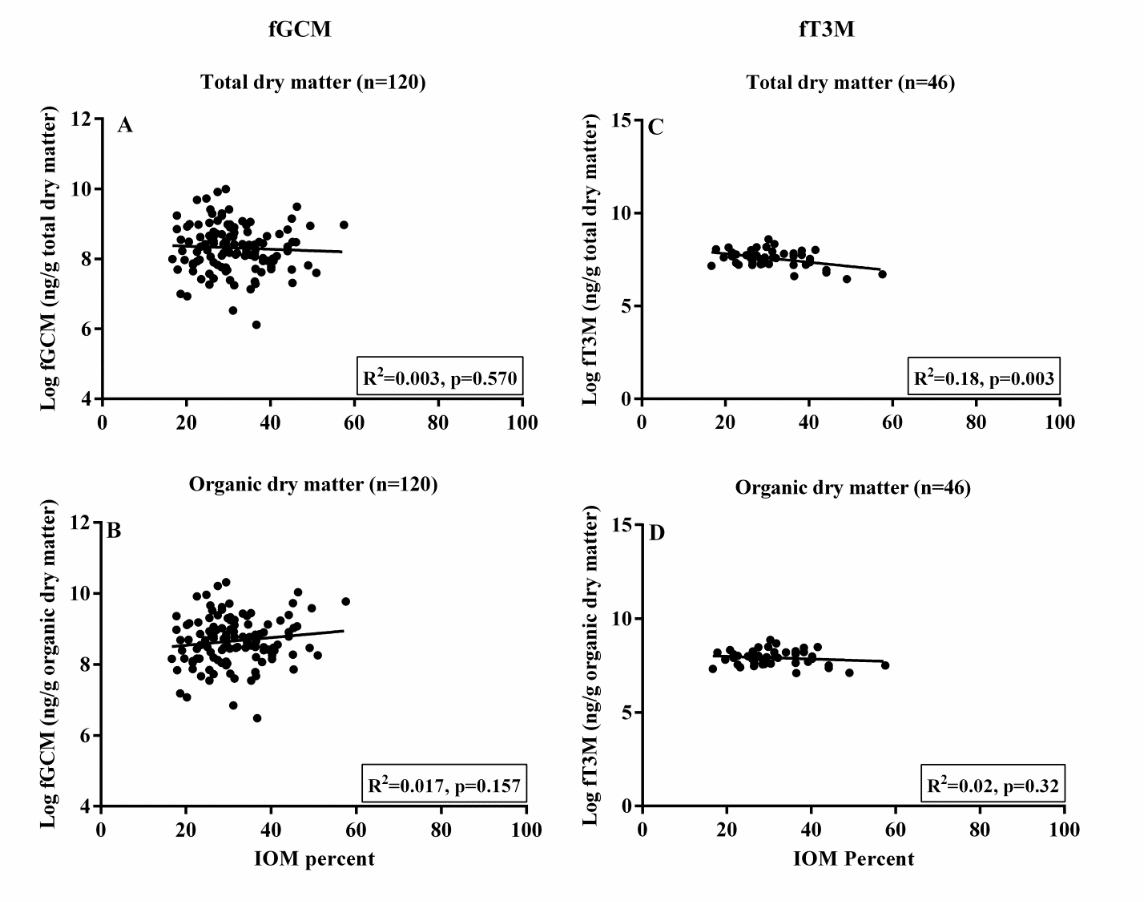

Figure 2.6: *Relationship between inorganic matter (IOM) content and fGCM and fT3M concentration in captive Asiatic lions. The IOM values have been presented as percentages and faecal hormone metabolite values have been log-transformed. The graphs A & C presents the association when hormone metabolites concentration is expressed as per gram of total dry matter, whereas the graphs B & D indicates the association when concentration is expressed as per gram of organic dry matter.*

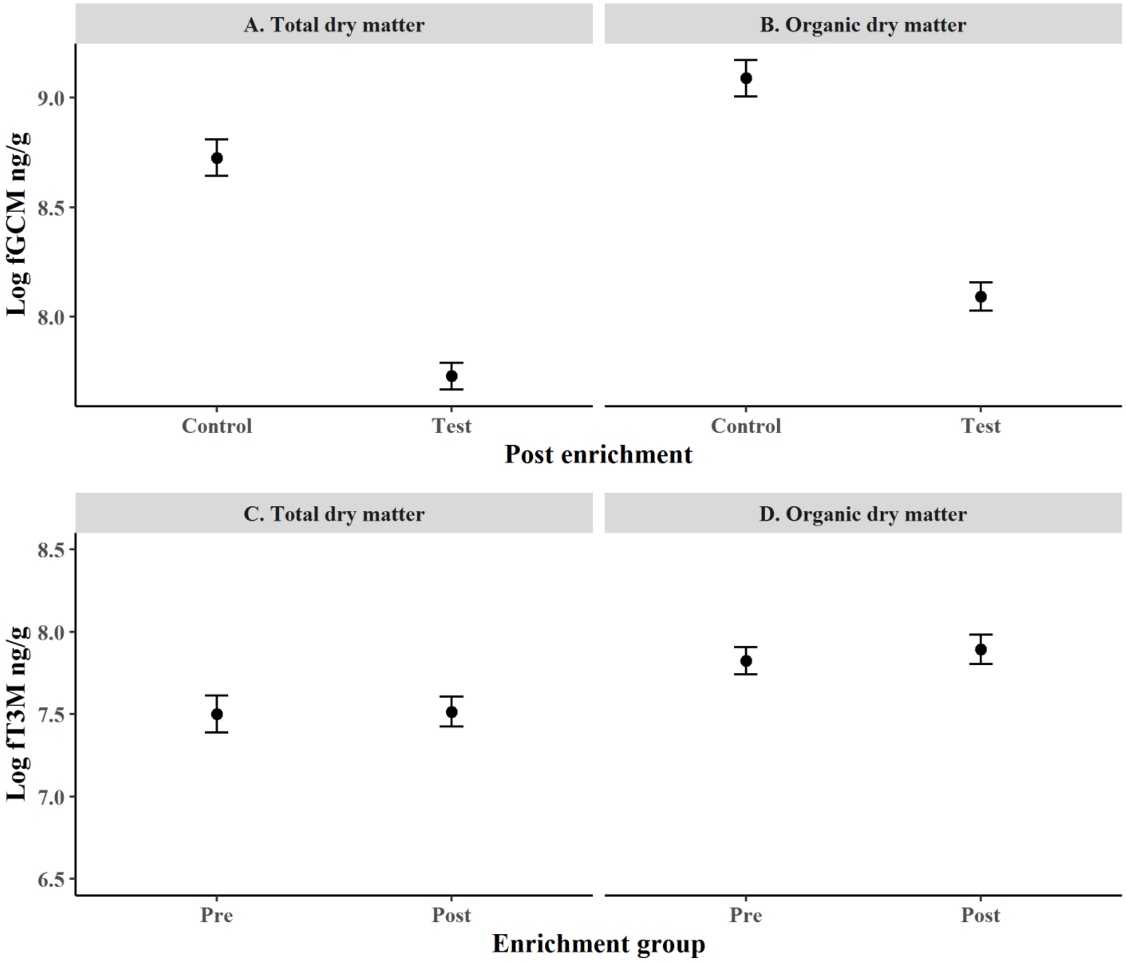

Figure 2.7: *Comparison of captive Asiatic lion mean fGCM (control and test groups) and fT3M (pre and post enrichment groups) level during enrichment interventions. For both hormone metabolites mean values (ng/g; log-transformed) are plotted. Graph A and C shows the mean concentration expressed as per gram of total dry matter, whereas B and D represent pattern when concentration is expressed as per gram of organic dry matter.*

Table 2.2: *Mean fGCM and fT3M levels estimated in compared groups of wild tigers (protected area wise) and captive Asiatic lions across different corrective methods.*

Compared groups	**Concentrations as total dry matter**	**Samples with >80% IOM dropped**	**Concentrations as organic dry matter**
Mean Log fGCM (ng/g) for wild tigers			
RTR	Mean = 7.62, SD = 0.84, n = 57	Mean = 7.90, SD = 0.77, n = 36	Mean = 8.78, SD = 0.72, n = 36
CTR	Mean = 7.42, SD = 0.79, n = 50	Mean = 7.61, SD = 0.77, n = 35	Mean = 8.57, SD = 0.79, n = 35
DTR-PTR	Mean = 7.51, SD = 0.73, n = 52	Mean = 7.65, SD = 0.62, n = 45	Mean = 8.51, SD = 0.59, n = 45
VTR	Mean = 7.49, SD = 1.04, n = 34	Mean = 7.86, SD = 0.95, n = 23	Mean = 8.76, SD = 0.96, n = 23
Mean Log fT3M (ng/g) for wild tigers			
RTR	Mean = 6.80, SD = 1.36, n = 53	Mean = 7.38, SD = 1.11, n = 36	Mean = 8.26, SD = 0.99, n = 36
CTR	Mean = 7.28, SD = 1.02, n = 50	Mean = 7.70, SD = 0.78, n = 35	Mean = 8.67, SD = 0.77, n = 35
DTR-PTR	Mean = 6.84, SD = 1.17, n = 52	Mean = 7.06, SD = 0.99, n = 45	Mean = 7.92, SD = 0.89, n = 45
VTR	Mean = 7.06, SD = 1.55, n = 32	Mean = 7.69, SD = 1.27, n = 22	Mean = 8.62, SD = 1.23, n = 22
Mean Log fGCM (ng/g) for captive Asiatic lions			
Control	Mean = 8.73, SD = 0.47, n = 32	Not applied	Mean = 9.09, SD = 0.47, n = 32
Test	Mean = 7.73, SD = 0.43, n = 50	Not applied	Mean = 8.09, SD = 0.46, n = 50
Mean Log fT3M (ng/g) for captive Asiatic lions			
Pre	Mean = 7.50, SD = 0.39, n = 12	Not applied	Mean = 7.82, SD = 0.29, n = 12
Post	Mean = 7.51, SD = 0.32, n = 12	Not applied	Mean = 7.89, SD = 0.30, n = 12

RTR-Rajaji tiger reserve, CTR-Corbett tiger reserve, DTR-PTR- Dudhwa and Pilibhit tiger reserve, VTR-Valmiki tiger reserve

Table 2.3: *Comparison of faecal hormone data analysis results for wild tigers and captive Asiatic lions. Wild tiger data was analyzed with One-way ANOVA and the captive Asiatic lion data was analyzed using independent t-test and paired t-test.*

	Concentrations as total dry matter	**Samples with >80% IOM dropped**	**Concentrations as organic dry matter**
fGCM analysis for wild tigers			
Sample size	N=193 RTR (n=57), CTR (n=50), DTR-PTR (n=52), VTR (n=34)	N=139 RTR (n=36), CTR (n=35), DTR-PTR (=45), VTR (n=23)	N=139 RTR (n=36), CTR (n=35), DTR-PTR (=45), VTR (n=23)
One-way ANOVA	F (3,189) = 0.52, p=0.671	F (3,135) = 1.26, p=0.290	F (3,135) = 1.18, p=0.322
Within group variance (SS_W)	133.53	77.83	75.16
Effect size (ω^2)	-0.008	0.007	0.004
fT3M analysis for wild tigers			
Sample size	N=187 RTR (n=53), CTR (n=50), DTR-PTR (n=52), VTR (n=32)	N=138 RTR (n=36), CTR (n=35), DTR-PTR (=45), VTR (n=22)	N=138 RTR (n=36), CTR (n=35), DTR-PTR (n=45), VTR (n=22)
One-way ANOVA	F (3,183) = 1.56, p=0.200	F (3,134) = 3.24, p=0.024	F (3,134) = 4.99, p=0.003
Within group variance (SS_W)	290.44	140.86	121.30
Effect size (ω^2)	0.009	0.046	0.08
fGCM analysis for captive Asiatic lions			
Sample size	N=82 Control (n=32), Test (n=50)	Not applied	N=82 Control (n=32), Test (n=50)
Independent t-test	t(80)=9.77, P=0.00	Not applied	t(80)=9.51, P=0.00
Effect size (g)	2.21	Not applied	2.14
fT3M analysis for captive Asiatic lions			
Sample size	N=12 individuals Pre (n=12), Post (n=12)	Not applied	N=12 individuals Pre (n=12), Post (n=12)
Paired t-test	t(11)=0.14, P=0.89	Not applied	t(11)=0.65, P=0.52
Effect size (d)	0.028	Not applied	0.237

2.4 Discussion

In this study the impact of inorganic matter (IOM) in measurement of fGCM and fT3M from field-collected (tiger) and captive (Asiatic lion) animals has been examined. A highly variable amount (9-98%) and a high content of IOM (40 to ≥80%) were found in the wild tiger faecal samples. On the contrary, the samples from the captive Asiatic lions contained comparatively lower IOM amount (20-40%) and variability between samples. Although not quantified in this manner earlier, this pattern of IOM presence was not surprising for tigers. Khan (2004) reported presence of up to ~50% soil in wild tiger scats from the Sunderban landscape, Bangladesh during dry season with a peak in the winters and suggested possible seasonal soil ingestion by tigers. Schaller (1967) has also reported incidences of soil ingestion during winters from wild tigers in Kanha Tiger Reserve. However, such behaviours cannot always be attributed to specific seasons as free-ranging animals often naturally or deliberately ingest soil with food for mineral supplementation, alleviating gastrointestinal disorder or to counteract effects of high endoparasite load (Beyer et al., 1994; Knezevich et al., 1998; Krishnamani and Mahaney, 2000). Sampling of tiger faeces during winters might have influenced the presence of IOM and their variations in the field-collected samples, but it was the best time for faecal sampling due to less leaf litter, availability of fresh samples and better environmental conditions. To the best of my knowledge, this was the first study on any endangered wild large carnivore species where such inter-sample variation in IOM was quantified. Earlier, Ganswindt et al. (2012) did not find significant inter-individual variation in IOM in Aardwolf faeces, possibly due to low sample size (n=2) and similar foraging conditions in the study area. The possible presence of soil in tiger or other large carnivore faeces during sampling season and high variability of IOM makes it important to quantify its content before any downstream processing for hormone metabolite assessment.

Results of fGCM and fT3M measurements from the field-collected tiger faeces (with varied proportions of IOM) indicate a negative impact of IOM on both hormone metabolite concentrations. However, the captive lion samples showed such impact only for fT3M data, indicating that such effects are more pronounced in field-collected samples. This pattern has been earlier described in birds (Hayward et al., 2010) and mammalian species (Ganswindt et al., 2012) and could be explained due to the inflation of sample mass by hormone-inert IOM without any actual increase in metabolite concentration, leading to their inaccurate measures in faeces. Taken together, it can be inferred that the hormone metabolites are mostly contributed by the organic part of the faeces and the use of best quality samples (in terms of organic matter content along with other parameters such as sample freshness) is critical for physiological assessment studies. Overall, the results suggest that the field-collected samples have highly variable IOM contents that influence measures of both fGCM and fT3M. Such effects can be reduced by two possible corrective measures. First approach could be the possibility of physically remove the IOM contents from faeces (as suggested for urates in case of birds by Hayward et al., 2010). However, such physical separation of IOM (soil, sand etc.) from field-collected faeces is difficult for carnivores and thus it would be better to exclude the samples with high IOM content from analyses. The data from tiger samples with high IOM (≥80%) content (and consequently with very low hormone contributing organic matter) showed more variations in hormone concentration and removing them from analyses significantly reduced IOM influence on hormone measures (Figure 2.4B & E). Based on this, it is suggested that samples with 80% or more IOM should not be processed for hormone metabolite assessments. The ideal working protocol would be to first quantify the IOM contents from the field-collected samples, followed by removal of samples with high IOM content and select the best quality samples (fresh faeces with low IOM content) for hormone metabolite assessment study. Future studies should also plan a pilot phase to evaluate the variation in faecal IOM in their respective target study systems before implementing large-scale research projects. Secondly, as these field-collected samples still contain high variation in IOM amount, presenting the hormone titer on total dry matter units (per gram of total dry matter) can potentially be erroneous due to influence from IOM rather than actual biological measures. However, expressing the hormone concentrations as per gram of organic dry matter has reduced the effects significantly in this study (Figure 2.4C & F), and supports the earlier suggestions by Hayward et al. (2010) and Ganswindt et al. (2012). For captive Asiatic lions most of the samples were found with relatively low IOM quantity and the effects of these corrective measures are much less for fGCM when compared to the field collected tiger samples. Interestingly, the lion fT3M measures were significantly affected even with lower IOM content, indicating that fT3M measures are more sensitive to IOM variability. Overall, using a combination of removing very low organic matter-containing samples (≥80% IOM) and expressing the hormone concentration as per unit organic dry matter can help to reduce the negative influences of IOM on fGCM and fT3M measures. Future work should focus on checking the IOM effects on reproductive hormone (progesterone, testosterone etc.) metabolite measures and experimental approaches to assess the utility of the low-quality samples (in terms of IOM presence) for species with low densities and large home ranges, as adequate sampling can often be challenging for them.

Finally, it was investigated if these corrective measures mentioned above bring out any change in the interpretations of the results by comparing the original and corrected tiger hormone data generated from five protected reserves. The mean fGCM level differences among these tiger reserves remained non-significant for original data as well as after both corrective measures. However, the fT3M results across the three situations showed significant differences. After implementing the corrective measures, mean fT3M levels of DTR-PTR group drops to the lowest and the difference with CTR becomes statistically significant, further strengthening the argument of the effects of IOM on the measures on fT3M in field-collected samples. While such pattern of population-level nutritional stress information is relatively new for wild tigers, the results are not surprising given the knowledge of tiger prey dynamics in some of these protected areas. For example, the DTR-PTR habitat in Terai-Arc landscape is known to have relatively low ungulate density with patchy distributions (Bista et al., 2011; Chanchani et al., 2014) than CTR (Jhala et al. 2020). It can thus be hypothesized that the low availability of prey species in DTR-PTR has resulted in lower fT3M concentrations (indicating nutritional stress in this population). However, the fGCM results from the sampled areas were surprising (non-significant differences among the parks) as the GC and T3 hormones are known to rather complement each other (Mondol et al., 2020; Wasser et al., 2011, 2017). Further work involving intensive sampling and collection of site-specific ecological/population parameters from this landscape is required to understand the drivers behind the patterns of physiological indices. However, since none of the Asiatic lion samples exceeded 80% IOM content only one corrective measure was used and the differences in mean fGCM and fT3M concentrations between the groups remained unchanged, differing only in titer values. This was expected as in the captive environment food quality and quantity was controlled, resulting in possibly less IOM variation in lion faeces.

Results of this study show that certain common but under-emphasized factors such as IOM content have the potential to affect faecal hormone measures that in turn can change critical data interpretation and impact conservation decisions. Most of these physiological measures are closely associated with other ecological, behavioral and environmental factors at individual and population levels (Anestis 2010; Busch et al., 2009; Creel et al., 2013), but accurate interpretations should not be affected by measurement errors. I believe that the corrective measures discussed in this study reduce IOM-driven within-group hormone data variations and thus can help in making biologically relevant conservation decisions. Particularly for large, endangered carnivores such as tigers faecal sampling is the only non-invasive approach to assess species physiology in their natural habitats and thus it is essential to reduce the impacts of inorganic materials. These measures will help in accurate physiological assessments and lead us to ecologically relevant interpretations and recommendations, which are valued driving force for species conservation.

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CHAPTER 2

Evaluating the impacts of ecological and anthropogenic factors on tiger stress physiology within Terai-Arc landscape, India

3.1 Introduction

Habitat loss and fragmentation in the age of Anthropocene is a major cause of biodiversity decline (Fahrig 2003; Magioli 2021). Its impact is more drastic for large sized terrestrial mammals both herbivores (Ripple et al., 2015) as well as carnivores (Wolf and Ripple 2017; Ripple et al., 2014) as they require large intact natural spaces with abundant resources to meet their high metabolic need (Carbone et al., 1999; 2007; 2011). Today, distribution of large size predators in various landscapes is restricted to fragmented patches (2002 Crooks; Wolf and Ripple 2017), where their future survival depends on keeping those patches connected (Suraci et al., 2020; Rio-Maio et al. 2019; Zemanova etal., 2017; Kanagaraj et al., 2013). Monitoring large carnivore populations and their responses to landscape changes is essential for maintaining an ecologically effective/viable carnivore population in human modified landscapes (Harihar et al., 2018a; Gubbi et al., 2016; 2017; Ripple 2014).

Landscape changes and resulting impact of environmental stressors on wild animals can be quantified by tracking their physiological responses (Macleod et al., 2018; Romero and Wikelski 2010; Cabezas et al., 2007). Non-invasive physiological biomarkers such as glucocorticoid (GC) metabolites deposited in tissues (hair, blubber, baleen etc. Koren et al., 2018; Champagne et al., 2018; Hunt et al., 2017) or excreted as waste (faeces, urine, mucous etc. see Gholib et al., 2018; Narayan et al., 2010; Santymire et al., 2018) have been found to relate with challenging environmental conditions such as disturbance (Boyle et al., 2021; Tyagi et al., 2019; Tecot et al., 2019), reduced forage (Hunnick et al., 2020b), prey (Patel et al., 2022) or diet quality (Pokharel et al., 2018), temperature (Szott et al., 2020), inter or intraspecies agonistic interactions (Creel 2001; 2005; Creel et al., 2009). Additionally, combining GC measures with triiodothyronine (T3) measures have been found useful in teasing nutritional stress from overall physiological stress (Psychological as well as nutritional stress, Dias et al., 2017; Vynne et al., 2014; Ayres et al., 2012). As vital life processes such as body condition, breeding and reproduction are mediated through physiological process (Macleod et al., 2018; Clinchy et al., 2013), it becomes essential to evaluate physiological conditions against changing habitat factors.

Tigers were once distributed across Asia (Nowell and Jackson 1996), by 2006 their existing population contracted to 7% of their historical range (Dinerstein et al., 2006), largely limited to pockets of discontinuous habitats. Direct poaching and loss of natural habitat and prey are considered to be the prime cause of their decline (Dinerstein et al., 2007). India has a critical place in tiger conservation as it holds approximately 80% of worlds remaining tiger population (Goodrich et al., 2015). Since 2006, India has reported to have doubled its tiger population within existing tiger habitats (Jhala et al., 2020). However, in the face of continued habitat loss future challenge is to manage increasing tiger numbers within these available habitats. As most of the available and potential habitats differ in degree of human interference, habitat composition, prey accessibility and management practices it would be important to quantify tiger responses to these changing conditions. Work on tiger conservation has largely remained focused on their distribution and demography but the underlying physiological causes of changing population dynamics have rarely been addressed and none, so far, at a landscape scale. Tigers are known to respond reproductively to changes in environmental conditions, that is their reproductive success very much depends on availability of sufficient prey biomass (Sunquist 1981; Smith et al., 1987; Smith & MacDougal 1991). In this context, understanding of changing habitat factors and their impact on physiological health that facilitates reproduction can provide important insight into changing population dynamics. In this study we have attempted to quantify physiological responses of tigers towards different habitat factors compared across a landscape.

We used Terai-arc landscape (TAL) as our study area, it is a global priority tiger conservation landscape (Dinerstein et al., 2006) that retains 22% of India's wild tiger population (Jhala et al., 2020). In past, this landscape has witnessed great fall in tiger numbers due to hunting in Mughal period followed by bounty hunting's under British colonial rule (see Rangarajan 2012). In subsequent years heavy forest loss and resulting habitat fragmentation due to establishment of numerous human settlement (TAL is one of most populous landscape of the country, Johnsingh et al., 2004; Chanchani et al., 2016) dwindled tiger population further. In last two decades tigers in TAL have lost their grounds in certain parts (Western Rajaji, Harihar et al., 2009; Suhelwa Wildlife Sanctuary, Chanchani et al., 2016a) but conservation awareness and resulting management interventions have also helped in tiger population recovery in certain other parts (Eastern Rajaji, Harihar et al., 2008; Pilibhit Tiger Reserve, Chanchani et al., 2016a) of this landscape. Currently, TAL contains five major tiger reserves and multiple forest divisions. These areas differ in characters known to heavily impact tiger demography, such as habitat composition (proportion of grasslands, see Chanchani et al., 2016b), tiger abundance

(Bisht et al., 2019), prey abundance (Chanchani et al., 2016b; Kanagaraj et al., 2011; Karanth et al., 2004; Karanth and Stith 1999;), level of human interference (Harihar et al., 2020; Kanagaraj et al., 2011), connectivity and protection status (Thapa et al., 2017; Harihar et al., 2018b). As tiger conservation is most effective when implemented at landscape level (Dinerstein et al., 2006, 2007; Harihar and Pandav 2012; Harihar et al., 2018a), our main objective through this study is to understand the physiological responses of wild tigers when exposed to a gradient of human disturbance at a landscape level. Further, since tiger distribution (and presumably their physiology as well) varies depending on broad and fine-scale spatial factors (Chanchani et al., 2016a), we considered environmental factors at both the scales. Mainly we asked following questions, firstly, if fine scale spatial factors like habitat composition, that is proportion of forest (or non-forest) area, open forest area and extent of disturbance from human activities within tiger's approximate home ranges impacts physiological status of tigers. Also, considering the territorial nature of tigers we asked if local tiger density have any impact on tiger physiological state due to potential intraspecific competition. Secondly, if dietary composition, that is prey size/type (Wild: large, medium and small sized prey; Domestic: livestock) consumed have any impact on hormone responses. Finally, we asked, if broad scale factors (park-based characters) like protection status, prey density, tiger density, accessibility to large prey (by assessing large prey occurrence in diet) and connectivity with surrounding tiger habitats impacts tiger physiological state. As tiger population recovery in a landscape shows high dependence on degree of connectivity between adjacent habitats (Thapa et al., 2017, Kanagaraj et al., 2013; Smith et al., 1999), we tried to summarize our results by understanding the relation of each of fine and broad scale factors with the habitat connectivity. The results of this study could highlight the main factors guiding the physiological health status of tigers in this landscape, at the same time it would provide evidence of declining health in populations that are exposed to limiting factors. We believe that the inferences of this study could be applicable to similar apex predators existing in a human-dominated landscape.

3.2 Material and methods

3.2.1 Study Area

This study was conducted in Terai-arc landscape (TAL), situated at the foot hills of Himalaya, its eastern boundary is delineated by river Yamuna (30°30'N to 77°30'E) and western by Valmiki tiger reserve (27°15'N to 84°45'E). From east to west it stretches 900 km in length occupying an area of 42,700 km2 (Jhala et al., 2020), including several protected areas and forest divisions connected at places with Nepal terai through critical corridors (Chanchani et al., 2014). TAL supports woodlands dominated by sal (*Shorea robusta*) forest and woodland-grassland-wetlands forests (Champion and Seth, 2005).

3.2.2 Field survey and sample collection

To assess physiological stress in wild tigers faecal glucocorticoid (fGCMs) and tri-iodothyronine metabolites (fT3Ms) were measured. For faecal sample collection a team of experienced field personnel surveyed (between 2015-2020, see Biswas et al., 2022a) all potential tiger habitat (protected area: PA and forest divisions: FD) on Indian side of TAL. Forest trails known to have tiger movement (based on knowledge of local forest patrolling team) were surveyed for tiger faecal samples. Fresh faecal samples (intact, dark coloured with strong odour see Vynne et al., 2014) with tigers scrape or pugmarks in vicinity were collected using butter papers and kept in sealed zip lock bags. Location of each sample collected was recorded using handheld GPS devices. Samples were collected only in winter season (November to March) to avoid any inter-seasonal variability. Additionally winter temperatures (ranging from $3^oC$to 15^oC) provides better protection against faster degradation of faecal samples. Samples collected were kept in a cool, dark and dry area inside a steel box. Later samples were transferred to laboratory at Wildlife Institute of India, where they were stored at -20^oC till further processing.

3.2.3 Species identification, fGCM and fT3M analysis

In laboratory faecal samples were first ascertained for species using tiger specific mitochondrial markers (Mukherjee et al., 2007). Faecal samples confirmed for tigers were processed further and hormone metabolite extraction was done following Patel et al., 2021 (Chapter one of this thesis). Briefly, faecal samples were pulverized and dried in a hot air oven at 60^oC for 72 hours. Dried samples were sieved through 0.5 mm steel mesh strainers to obtain fine faecal powder. To control for presence of inorganic matter (IOM) variability (Patel et al., 2021), 0.1 g of each sampled is burned in a muffle furnace at 550^oC for 1 hour, before and after weight are measured to calculate inorganic matter present (IOM = 0.1g-organic matter burned). All samples with >80% of IOM were removed from further analysis. For hormone metabolite extraction from selected samples, 0.1 g of dried faecal powder is mixed in 15 ml of 70% ethanol (1:15 dilution), followed by 30 minute of pulse vortexing and 20 minutes of centrifugation at 2200 rpm. Hormone extract is collected in 2ml cryochill vials and stored at -20^{o}C till final assay. fGCM and fT3M levels was measured using Corticosterone (#K014, Arbor Assays, MI, USA) and Triiodothyronine (T3) (#K056, Arbor Assays, MI, USA) EIA kits, respectively. Earlier, these kits have been validated for tigers by Patel et al., 2021. For fGCM intra and inter assay coefficient of variation (CV) was 3.6 and 9.0 and for fT3M it was 8.8 and 6.2, respectively.

3.2.4 Data acquisition for environmental variables

Environmental variables were selected based on information about conditions that are known to be conducive for free ranging tigers. Land cover data was used to extract forest cover data as tigers are known to prefer dense forest cover (Kanagaraj et al., 2011). Human interference and connectivity between habitats were quantified as tigers avoid disturbance and require good connectivity between habitats (Harihar and Pandav 2012). Accessibility to large sized prey is essential for a large size predator like tiger (Carbone et al., 1999) and hence has been quantified using dietary analysis. Given the territorial nature of these large predators, it is known that intraspecies competition plays an important role in resource acquisition and population dynamics (Chanchani et al., 2018) and resultingly can have an important impact on physiological responses (Armitage 1991; Creel 2001), hence local tiger density has been included as another important variable.

3.2.4.1 Forest cover percentage

Tiger needs good forest cover to hunt, breed and rest (Kanagaraj et al., 2011). Absence of forest cover can lead to an increased encounter with humans causing psychological stress. Reduced forest cover could also be an indicative of increased disturbance in habitat, that could possibly reduce the habitat productivity and thereby prey base for tigers leading to nutritional stress. We retrieved land cover data based on canopy density, courtesy of Forest survey of India (FSI) for year 2017. Since it is biologically meaningful that data should be at spatial scale at which species perceives the landscape (Kanagraj et al., 2011; Schadt et al., 2002), we drew buffer of 3km radius (28.26 km^2) around each collected sample, which approximates to home range of female tigers in Terai-arc landscape (Sunquist 1981). We extracted proportion of different category of land use (dense Forest, moderately dense forest, open forest, non-forest, scrub forest, water bodies) present within this buffer for each sample. Values were extracted using zonal statistic tool (as table 2) in ArcMap 10.5 (ESRI 2016).

3.2.4.2 Human interference index

For evaluation of human interference impact on physiological measures of tigers we downloaded Global Human Modification (gHM) of Terrestrial Systems data set (v1.2016, 1 Km resolution, 0-1 metric) from NASA Socioeconomic Data and Applications Center (SEDAC). This data was derived from five major categories of stressors 1) human settlement (population density, built-up areas), 2) agriculture (cropland, livestock), 3) transportation (major roads, minor roads, two-tracks, and railroads), 4) mining and energy production (mining/industrial areas, oil wells and wind turbines), and 5) electrical infrastructure (powerlines and night-time lights). For each sample buffer (within 3 km radius), mean human interference index was extracted using zonal statistic tool (as table 2) in ArcMap 10.5 (ESRI 2016).

3.2.4.3 Diet assessment

Large carnivores like tigers prefer large sized prey for associated higher energy gain (Carbone et al., 1999). In absence of their availability, they may shift to domestic livestock or relatively medium to small sized wild prey (Chanchani et al., 2016a). This could respectively lead to human-tiger conflict (psychological stress) or lower energy gain (nutritional stress) per unit of energy expenditure and resultingly may cause physiological distress. We assessed tiger diet composition using tiger confirmed scat samples (see Biswas et al., 2022b). Each sample was sieved to separate undigested parts (hairs, bones, hooves) from faecal powder. Following Mukherjee et al., 1994, Primary guard hairs (20-30 hairs) were washed in ethanol and immersed in xylene for maximum of 10 hours. Permanent slides were prepared and medullary structures of primary guard hairs examined using available references (Bahuguna et al. 2010) to identify prey species. Occurrence of a single species was given a frequency of 1, two species for same sample was given 0.5 and so forth. Species rarefaction curve was plotted to ascertain that samples used (total n=510, Biswas et al., 2022b) from each of the sampled area were sufficient for assessment of diet composition (Table 3.1, Annexure I).

Table 3.1: *Relative frequency of occurrence (RFO) and Relative biomass consumed (RBC) calculated for each prey category (Livestock, Large, Medium and Small sized prey) for all sampled protected areas and forest divisions in TAL.*

Sampled Area	**Sample size (n=510)**	**Livestock RFO**	**Livestock RBC**	**Large RFO**	**Large RBC**	**Medium RFO**	**Medium RBC**	**Small RFO**	**Small RBC**
LFD	37	1.35	2.16	64.86	81.2	25.68	14.09	8.11	2.54
RTR	34	4.41	7.48	54.41	71.28	30.88	17.84	10.29	3.39
HLF	37	10.84	17.26	50.95	63.13	33.33	18.13	4.88	1.48
RFD	30	0.00	0.00	46.82	70.21	37.12	23.78	16.05	6
CTR	103	1.94	3.61	42.72	62.32	51.46	32.64	3.88	1.43
TCF	18	13.89	23.23	38.89	49.31	47.22	27.46	0	0
TWF	40	27.50	41.01	36.25	41.04	33.75	17.13	2.5	0.81
PTR	33	12.54	22.64	33.86	45.64	49.53	30.17	4.08	1.54
VTR	46	2.22	4.84	27.78	45.99	67.78	48.13	2.22	1.05
ATR	35	20.00	34.83	25.71	33.47	50	30.15	4.29	1.54
DTR	53	18.87	34.03	25.47	33.85	53.77	31.38	1.89	0.74
FA	26	21.15	38.30	19.23	26.16	55.77	34.03	3.85	1.51
TEF	18	11.11	24.49	11.11	18.02	77.78	57.49	0	0

LFD- Lansdowne forest division, RTR- Rajaji tiger reserve, HLF- Haldwani forest division, RFD- Ramnagar forest division, CTR- Corbett tiger reserve, TCF- Terai central forest division, TWF- Terai west forest division, PTR- Pilibhit tiger reserve, VTR- Valmiki tiger reserve, ATR- Amangarh tiger reserve, DTR- Dudhwa tiger reserve, FA- fragmented areas (includes Najibabad forest division, Pilibhit social forestry, North and South Kheri forest division and Sohagibarwa wildlife sanctuary), TEF- Terai east forest division.

3.2.4.4 Habitat connectivity score

Habitat connectivity gets severely impacted as habitat fragmentation accelerates (Schumaker 1996). We calculated a habitat connectivity score (HCS) for each of the sampled area to determine if connectivity affects the physiological status of tigers inhabiting those areas. For HCS we assigned 8 directions in which an area can have connectivity (East, West, North, South, North-East, North-West, South-East, South-

West), connectivity was established in a particular direction using recent literature (Chanchani et al., 2014; Jhala et al., 2020; Biswas et al., 2022a). A high or a low connectivity in a particular direction was assigned point 1 or 0.5, respectively. Connectivity within TAL-India was based on data from Johnsingh et al., 2004; Qureshi et al., 2014; Anwar & Borah 2020; Chanchani et al., 2016a; Jhala et al., 2020; Biswas et al., 2022a. Transboundary connectivity (with TAL-Nepal) was based on data from Chanchani et al., 2014; Joshi et al., 2016; Thapa et al., 2017; 2018. An additional point of 1 or 0 was given to each sampled area based on their established status as a source or a sink population (Biswas et al., 2022a), respectively. All points for an area were added to get a final HCS score (Annexure II).

3.2.4.5 Tiger and prey density

Tiger and prey density for all sampled area was taken from last all India tiger and prey status report (Annexure III, Jhala et al., 2020, data of year 2018). Areas with 5 or less individuals (hence no density estimate provided) were given a value of 0.5 (relatively lowest tiger density). Since very few faecal samples (1-6) could be obtained from such areas, all data points were clubbed together in a single area category FA (fragmented area). Further we added individual prey species density data (from Jhala et al., 2020) to get a proxy of available prey density in selected areas for which data was available.

3.2.4.6 Local tiger density score

Intraspecific competition and associated agonistic interactions in territorial mammals can be a significant source of physiological stress (Armitage 1991; Creel 2001). We collected local spatial tiger density data to use as an indicator of intraspecies competition pressure at local scale. Data was taken from latest all India tiger report (Jhala et al., 2020) for selected protected areas. As spatial tiger density data (colour gradient) for a particular park is provided for a grid of 5km x 5km, we grided each park in same scale and assigned a value (0-5) to each grid based on colour intensity of respective grid.

3.2.5 Statistical analysis

Total of 492 fresh tiger conformed samples were used for physiological analysis. After removing samples containing >80% IOM, 345 samples were finally assayed for fGCM and fT3M quantification (see figure 3.1). To tease apart overall physiological stress from nutritional stress, separate models were built for fGCM and fT3M while testing impact of single (univariate) or multiple variables (multivariate). To take care of scale of data, analytical framework was divided in three parts: (a) **Fine-scale factors (data from 3km radius buffers and 5x5km grids)**: The effect of proportion of forest area, non-forest area, open forest area, mean human interference on fGCM and fT3M (n = 345) was tested using generalized additive models (GAMs), to account for possible non-linear association. Multicollinearity was assessed using variance inflation factor (VIF), with a cut off value of 3 (Zuur et al., 2010). Forest area and non-forest area (VIF = 4.8) were found to be highly correlated ($r_{(345)} = 0.89$, $P < 0.001$). Models were tested keeping either forest area or non-forest area variable, all models provided better fit with non-forest area, hence it was retained. Generalized linear models (GLMs) were used to explore if these relations with continuous variables changes with protection status (PA or FD). For testing the impact of local tiger density (intraspecies competition) on fGCM and fT3M levels univariate GLMs were used. Additionally to spatially highlight the areas with high GC and T3 levels (possibly areas with high intraspecies competition) surface models were made using nearest neighbour inverse distance weighting (NNIDW) in ArcGIS 10.5 for CTR (Corbett Tiger Reserve), which has the highest reported tiger density of 14 tigers/100km^2 in the world (b) **Diet data**: To understand the effect of diet all detected prey species were categorized into following 4 prey categories (i) Large (≥60 kgs), (ii) Medium (between 16-60 kgs), (iii) Small (≤15 kg) and (iv) Livestock (Harihar et al., 2011). Next, GLMs were used to test the effect of prey category and protection status of sampled area on fGCM and fT3M., an interaction term was also included in models to see if the prey category differences in hormone levels changes with protection status. Relative frequency of occurrence (RFO) and relative biomass consumed (RBC) was calculated for each prey category for each sampled area (using n=510 samples, Table 3.1, Biswas et al., 2022b). (c) **Broad-scale factors (forest division or park specific data)**: Univariate GLMs were used to investigate impact of park-based characters like Livestock RBC (n=345), Large prey RBC (n=345), tiger density (n=345), prey density (n= 205) and habitat connectivity score (HCS, n=345) on fGCM and fT3M levels. As we expected fGCM levels to be higher in most disconnected areas (due to disturbance) and most well-connected better habitats (possible higher stress due to intra-species competition), we used a quadratic term as well for HCS to explain variation in fGCM levels.

All GAMs and GLMs were used with gamma error distribution and a log-link function to take care of the right skew in the hormone concentrations. The fit of the multivariate models was assessed based on lowest AICc value (Burnham and Anderson, 1998). We also investigated association of all continuous and park-based variables with Habitat connectivity score and if those associations change with protection status, using Linear regression models. All statistical analysis were done using R v4.1.1 (R Core Team, 2021) using “mgcv”, “MuMIn”, “readr”, packages, plots were created using “dplyr” and “ggplot2” packages.

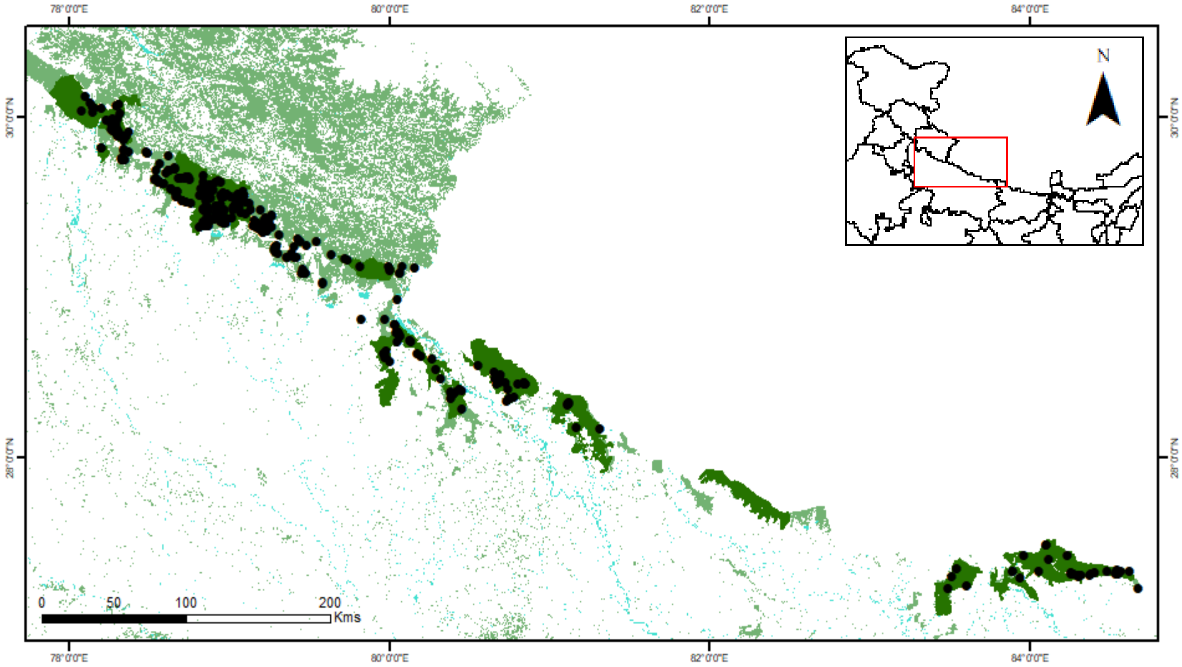


Figure 3.1 *Distribution of faecal samples used for faecal hormone analysis*

3.3 Results

Fine-scale factors: for fGCM, model containing open forest (p= 0.06) and human interference (p = 0.03) was found best for explaining variation in fGCM levels (Table 3.2). fGCM decreases with increase in open forest and increases as human interference increases (see figure 3.2). Interaction of protection status with human interference was best fit but non-significant interaction model for fGCM (Table 3.3). **For fT3M,** model containing non-forest (p= 0.10) and open forest (p = 0.003) was found best for explaining variation in fT3M levels (Table 3.2). fT3M in general decreases with increase in non-forest area. With open forest area fT3M shows a significant polynomial relation (increase followed by a dip and again a rise) (see figure 3.3). Interaction of protection status with open forest was best fit significant interaction model for fT3M (P= 0.002, Table 3.3). Change in fT3M levels follows an opposite pattern based on protection status, in PAs it increases with increase in open forest area, whereas in FDs it decreases with increase in open forest areas (see figure 3.4).

Univariate GLM showed that fGCM levels were significantly positively associated with local spatial tiger density (P= 0.0004) (figure 3.5). Levels of fGCM increased as tiger density score (intraspecific competition) increased locally. Our spatial model for CTR also support these results as most of high fGCM values are concentrated in areas with high local tiger density (figure 3.6).

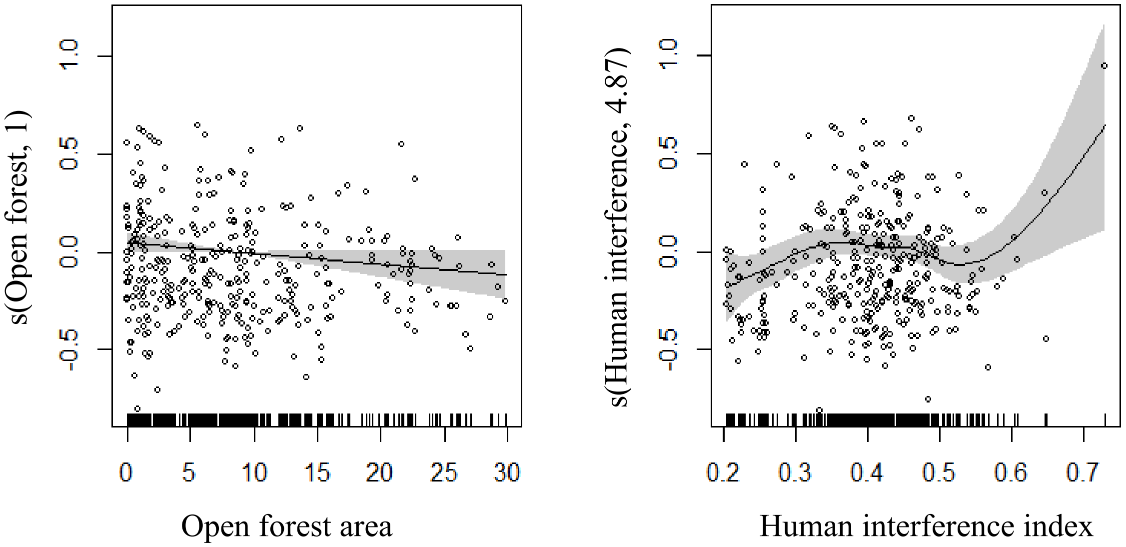


Figure 3.2 *fGCM plot for final GAM (fGCM ~ s(Open forest) + s(Human interference)). Human Interference was statistically significant in the final model, proportion of open forest was not. Grey line indicates 95% CI.*

Table 3.2 *Results of generalized additive model (GAM) for fine scale factors (data from 3km radius buffer around each sample).*

Models tested for fGCM	**AICc**	**delta**	**weight**	**Deviance explained**
fGCM ~ s(Open Forest) + s(Human Interference)	**2123.45**	**0**	**0.64**	**8.04%**
fGCM ~ s(Human Interference)	2125.92	2.47	0.19	7.08%
fGCM ~ s(Non Forest) + s(Open Forest) + s(Human Interference)	2126.85	3.4	0.12	8.60%
fGCM ~ s(Non Forest) + s(Human Interfernce)	2128.26	4.81	0.06	7.12%
fGCM ~ s(Non Forest)	2141.35	17.91	8.27E-05	3.47%
fGCM ~ s(Open Forest)	2142.42	18.98	4.85E-05	4.62%
fGCM ~ 1	2142.43	18.98	4.84E-05	-2.06E-16
fGCM ~ s(Non Forest) + s(Open Forest)	2142.5	19.05	4.66E-05	5.45%
Models tested for fT3M	**AICc**	**delta**	**weight**	**Deviance explained**
fT3M ~ s(Non Forest) + s(Open Forest)	**2022.91**	**0**	**0.77**	**14.10%**
fT3M ~ s(Non Forest) + s(Open Forest) + s(Human Interference)	2025.29	2.38	0.23	14.10%
fT3M ~ s(Open Forest)	2036.21	13.29	0	8.40%
fT3M ~ s(Open Forest) + s(Human Interference)	2036.87	13.96	0	10.60%
fT3M ~ s(Non Forest) + s(Human Interference)	2045.21	22.3	1.10E-05	5.48%
fT3M ~ s(Non Forest)	2048.52	25.61	2.10E-06	4.28%
fT3M ~ 1	2053.43	30.51	1.81E-07	-4.07E-16
fT3M ~ s(Human Interference)	2054.3	31.38	1.17E-07	0.38%

Best model for fGCM	**Level**	**Estimate**	**± SE**	**t value**	**Pr(>|t|)**
fGCM ~ s(Open Forest) + s(Human Interference)	(Intercept)	2.840	0.019	147.700	<2e-16 ***
		edf	**Ref.df**	**F**	**p-value**
	s(Open Forest)	1.000	1.000	3.514	0.0617 .
	s(Human Interference)	**4.867**	**5.807**	**2.347**	**0.0281 ***
Best model for fT3M	**Level**	**Estimate**	**± SE**	**t value**	**Pr(>|t|)**
fT3M ~ s(Non Forest) + s(Open Forest)	(Intercept)	2.68245	0.019	138.6	<2e-16 ***
		edf	**Ref.df**	**F**	**p-value**
	s(Non Forest)	7.22	7.956	1.746	0.09887 .
	s(Open Forest)	**7.142**	**7.97**	**2.961**	**0.00302 ****

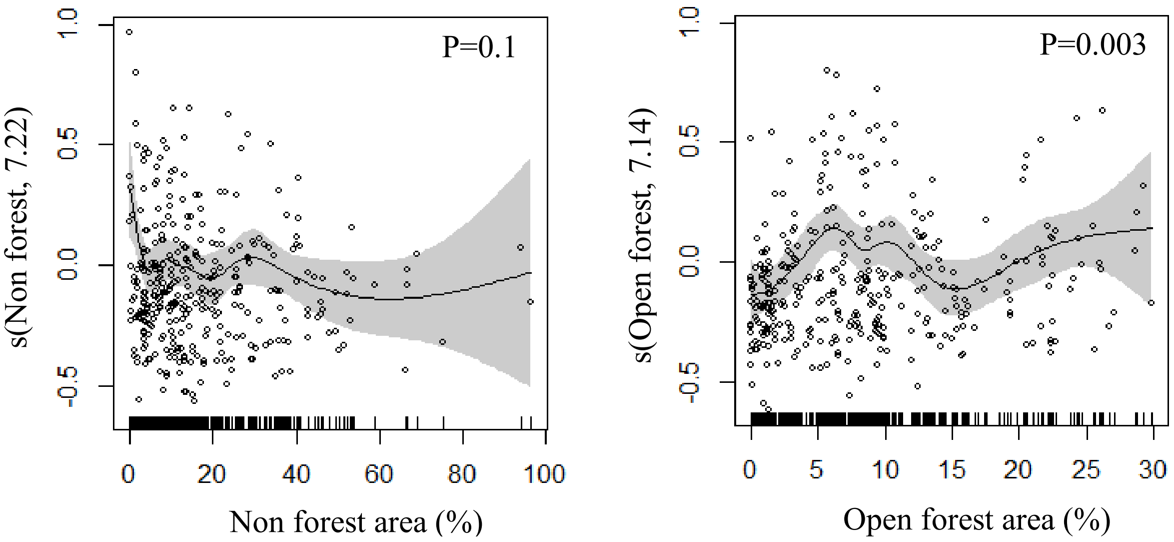


Figure 3.3 *fT3M plot for final GAM (fT3M ~ s(Non forest) + s(Open forest)). Open forest proportion was statistically significant in the final model, proportion of Non forest was not. Grey line indicates 95% CI.*

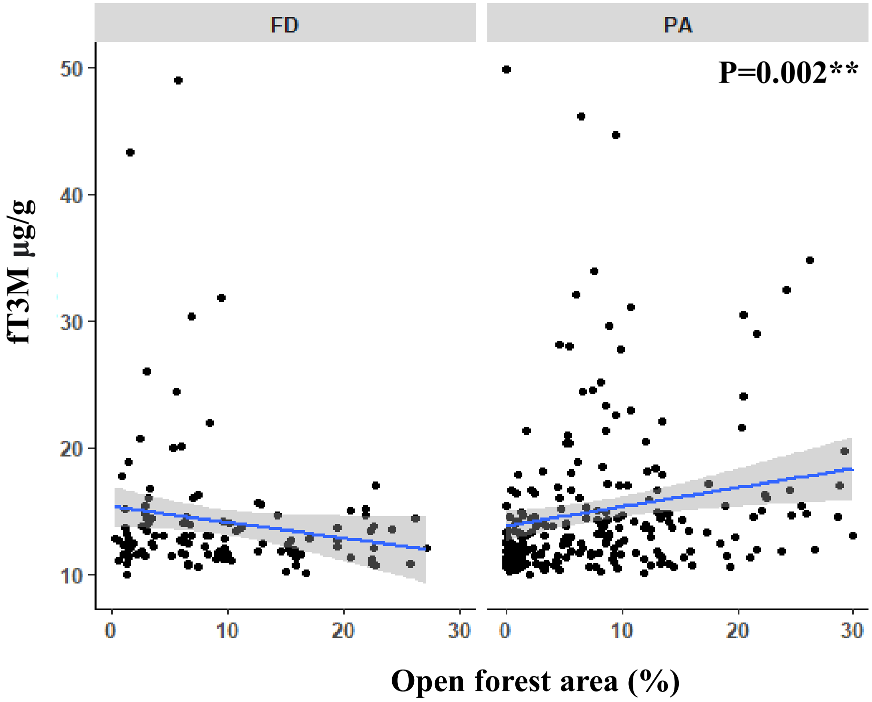


Figure 3.4 *fT3M Interaction plot (fT3M ~ protection status * open forest area), PA is protected area and FD is forest division.*

Table 3.3 *Results of generalized linear model (GLM) testing the interaction of each of fine spatial factor with that of protection status (PA: Protected area; FD: Forest division)*

Interaction model tested for fGCM	**AICc**	**delta**	**weight**
fGCM ~ PA_FD* Human Interference (HI)	**2121.1**	**0**	**0.241**
fGCM ~ 1	2121.9	0.84	0.158
fGCM ~ PA_FD* Open Forest	2122.79	1.74	0.101
fGCM ~ PA_FD	2123.55	2.49	0.069
fGCM ~ PA_FD* Non Forest	2124.44	3.38	0.044
Interaction model tested for fT3M	**AICc**	**delta**	**weight**
fT3M ~ PA_FD* Open Forest (OF)	**2005.4**	**0**	**0.494**
fT3M ~ PA_FD* Non Forest	2014.62	9.24	0.0049
fT3M ~ PA_FD	2018.55	13.2	0.0007
fT3M ~ 1	2019.32	14	0.0005
fT3M ~ PA_FD* Human Interfernce	2019.42	14	0.0004

Best interaction model for fGCM	**Level**	**Estimate**	**± SE**	**t value**	**Pr(>|t|)**
fGCM ~ PA_FD* HI	(Intercept)	3.1742	0.2451	12.95	<2e-16 ***
	PA_FDPA	-0.5023	0.2648	-1.897	0.0587 .
	HI	-0.7187	0.5507	-1.305	0.1927
	PA_FDPA: HI	1.1468	0.6066	1.89	0.0596 .
Best interaction model for fT3M	**Level**	**Estimate**	**± SE**	**t value**	**Pr(>|t|)**
fT3M ~ PA_FD* OF	(Intercept)	2.731013	0.0581	46.995	<2e-16 ***
	PA_FDPA	-0.10533	0.07	-1.504	0.1334
	OF	-0.00902	0.0049	-1.826	0.0687 .
	PA_FDPA:OF	**0.01903**	**0.006**	**3.114**	**0.0020 ****

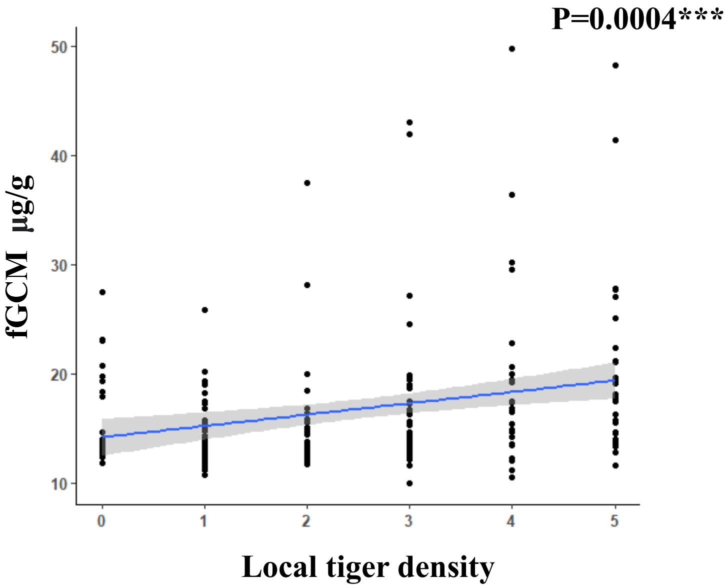


Figure 3.5 *fGCM Univariate GLM plot, testing association of local tiger density score (intraspecific competition) with fGCM level in wild tigers (fGCM ~ tiger local density).*

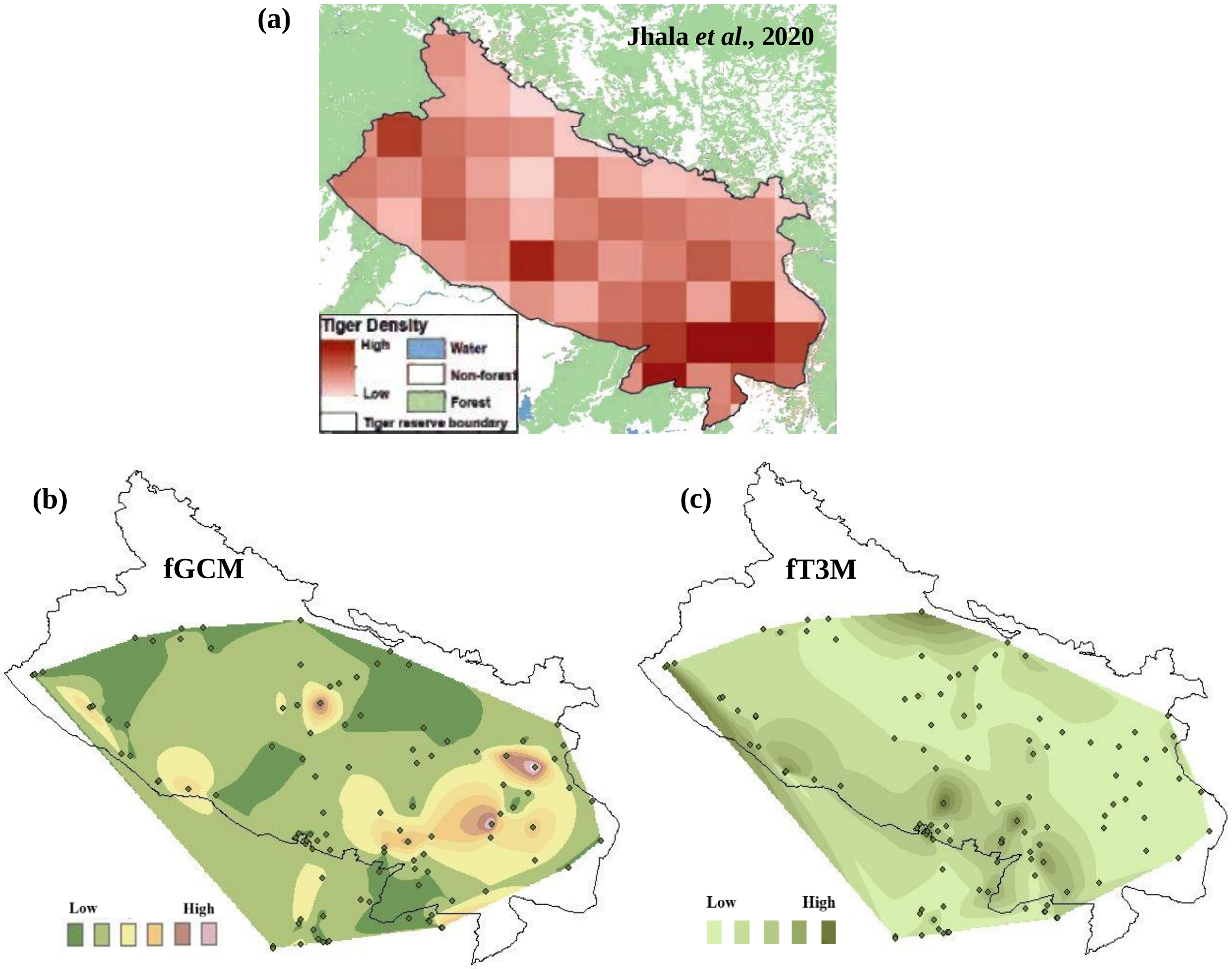


Figure 3.6 *Surface models of CTR a) spatial local tiger density map from Jhala et al., 2020, b) spatially represented fGCM values c) spatially represented fT3M values. Surface models were created using nearest neighbour inverse distance weighting (NNIDW) in ArcGIS 10.5.*

Diet data: for fGCM, best fit model included prey size category only, but no significant differences were found between categories (Table 3.4). Overall, fGCM levels were relatively higher for all prey size categories in FD compared to PA (figure 3.7). **For fT3M,** neither prey size category nor its interaction with protection status could explain fT3M variation better than null mode (Table 3.4).

Broad-scale factors: **for fGCM**, univariate GLM showed that for all park-based variables including the quadratic term for HCS, changes in fGCM levels remained non-significant (Table 3.5, figure 3.8). **For fT3M,** univariate GLM for fT3M showed significant positive association with prey density ($p= 0.001$) and HCS ($p= 0.001$) (see figure 3.8) and significant negative association with livestock RBO ($p= 0.0002$) (see figure 3.10). No significant association was seen with Large RBO and tiger density.

Lastly, linear regression models showed significant positive association of HCS with prey density ($p<0.0001$), large RBC ($p<0.0001$) and tiger density ($p<0.0001$), and significant negative associations with livestock RBC ($p<0.0001$), open forest area ($p<0.0001$), non-forest area ($p<0.0001$) and human interference index ($p=0.049$) (Annexure IV). Interaction term with protection status retains the directionality of the association for all tested variables except for open forest area (Annexure V). Open forest area in FD decreases with increase in HCS, whereas it remains unchanged with HCS increase in PA ($p<0.0001$).

Table 3.4 *Results of generalized linear model testing impact of prey size consumed (diet data) on fGCM and fT3M responses.*

Diet model tested for fGCM	**AICc**	**delta**	**weight**
fGCM ~Preysize	**1733.5**	**0**	**0.56**
fGCM ~1	1734.41	0.882	0.36
fGCMplus ~Preysize*PA_FD	1737.57	4.04	0.07
Diet model tested for fT3M	**AICc**	**delta**	**weight**
fT3M ~1	1655.62	0	0.85
fT3M ~Preysize	1659.57	3.959	0.12
fT3M ~Preysize*PA_FD	1661.87	6.259	0.04

Best model for fGCM	**Level**	**Estimate**	**± SE**	**t value**	**Pr(>|t|)**
fGCMplus ~Preysize	(Intercept)	2.8183	0.0360	78.31	<2e-16 ***
	Preysize_Livestock	-0.0843	0.0727	-1.16	0.247
	Preysize_Medium	0.0573	0.0495	1.16	0.247
	Preysize_Small	0.1279	0.1314	0.97	0.331

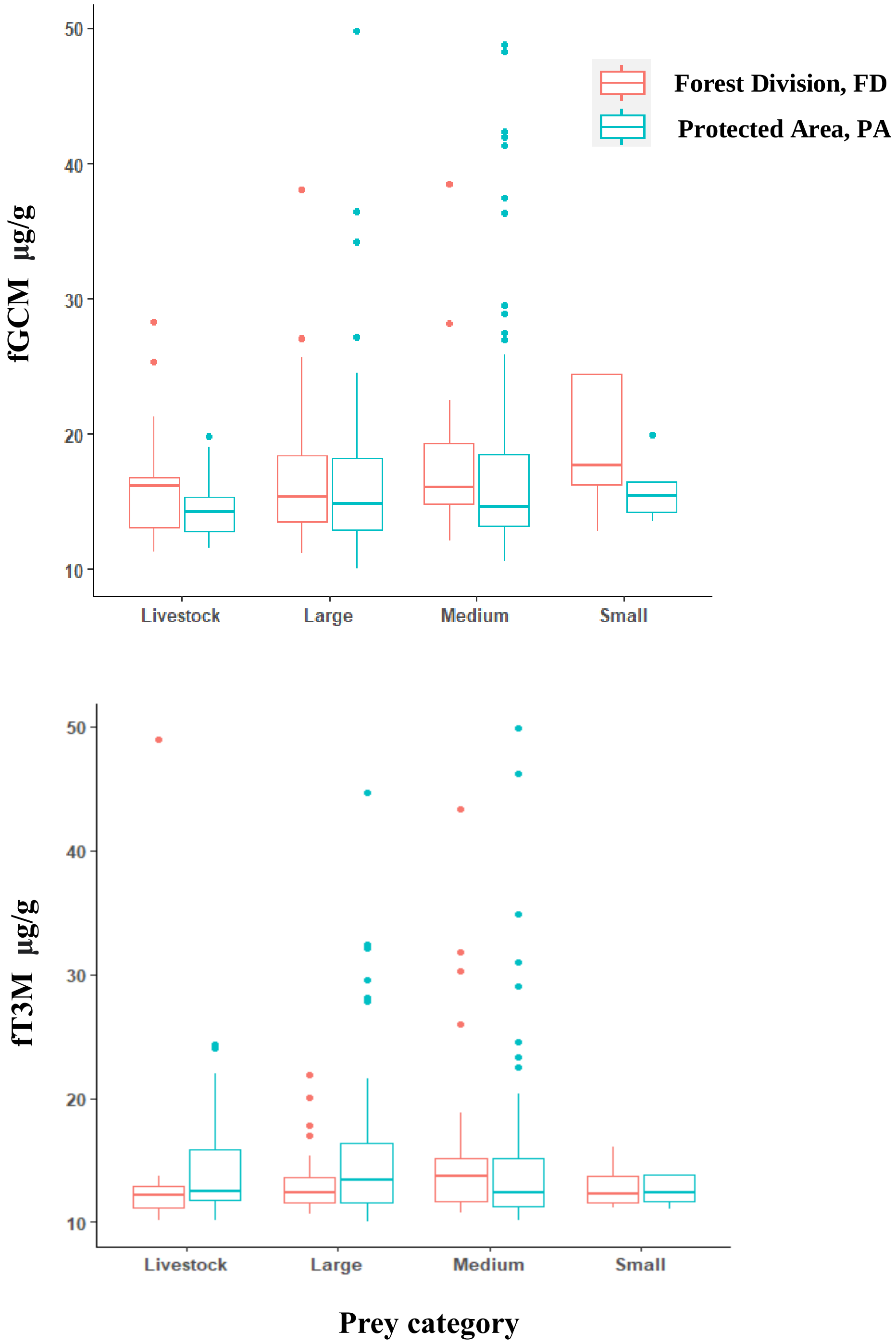


Figure 3.7 *fGCM (above) and fT3M (below) plot for diet data (fGCM/fT3M ~ Prey size * Protection status).*

***Table 3.5** Results of univariate generalized linear model testing association of broad scale factors (park based data) and local tiger density with tiger fGCM and fT3M responses (next page)*

Univariate models for fGCM	**Level**	**Estimate**	**± SE**	**t value**	**Pr(>|t|)**
fGCM ~ large prey	(Intercept)	2.7951	0.0698	40.04	<2e-16 ***
	large prey	0.0009	0.0012	0.729	0.466
fGCM ~ livestock	(Intercept)	2.8623	0.0293	97.76	<2e-16 ***
	livestock	-0.0012	0.0014	-0.873	0.383
fGCM ~ tiger density	(Intercept)	2.8545	0.0397	71.75	<2e-16 ***
	tiger density	-0.0019	0.0061	-0.31	0.757
fGCM ~ prey density	(Intercept)	2.84E+00	6.52E-02	43.482	<2e-16 ***
	prey density	-4.48E-05	6.81E-04	-0.066	0.948
fGCM ~ HCS	(Intercept)	2.8658	0.0608	47.109	<2e-16 ***
	HCS	-0.0072	0.0189	-0.381	0.703
fGCM ~ local tiger density	(Intercept)	2.66633	0.0492	54.193	< 2e-16 ***
	local tiger density	**0.05928**	**0.0166**	**3.574**	**0.0004 *****
Univariate models for fT3M	**Level**	**Estimate**	**± SE**	**t value**	**Pr(>|t|)**
fT3M ~ large prey	(Intercept)	2.5705	0.0734	35.01	<2e-16 ***
	large prey	0.0022	0.0013	1.685	0.0929.
fT3M ~ livestock	(Intercept)	2.761	0.0297	92.858	<2e-16 ***
	livestock	**-0.0049**	**0.0014**	**-3.42**	**7e-04 *****
fT3M ~ tiger density	(Intercept)	2.6458	0.0422	62.608	< 2e-16 ***
	tiger density	0.0078	0.0066	1.193	0.234
fT3M ~ prey density	(Intercept)	2.487	0.0582	42.753	< 2e-16 ***
	prey density	**0.002**	**0.0006**	**3.328**	**0.001 ****
fT3M ~ HCS	(Intercept)	2.494	0.062	40.168	<2e-16 ***
	HCS	**0.0637**	**0.0193**	**3.299**	**0.001****
fT3M ~ local tiger density	(Intercept)	2.776	0.055	50.89	<2e-16 ***
	Local tiger density	-0.021	0.018	-1.138	0.256

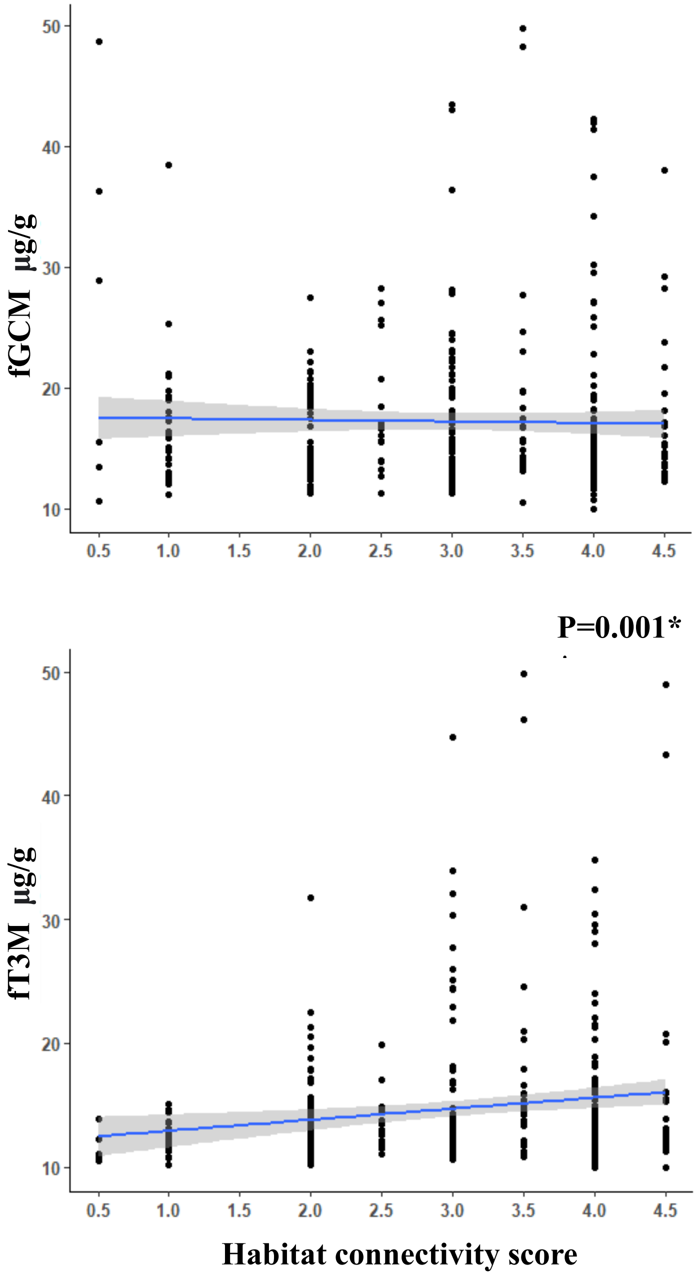


Figure 3.8 *fGCM (above) and fT3M (below) univariate GLM plot for association with habitat connectivity score (fGCM/fT3M ~ Habitat connectivity score).*

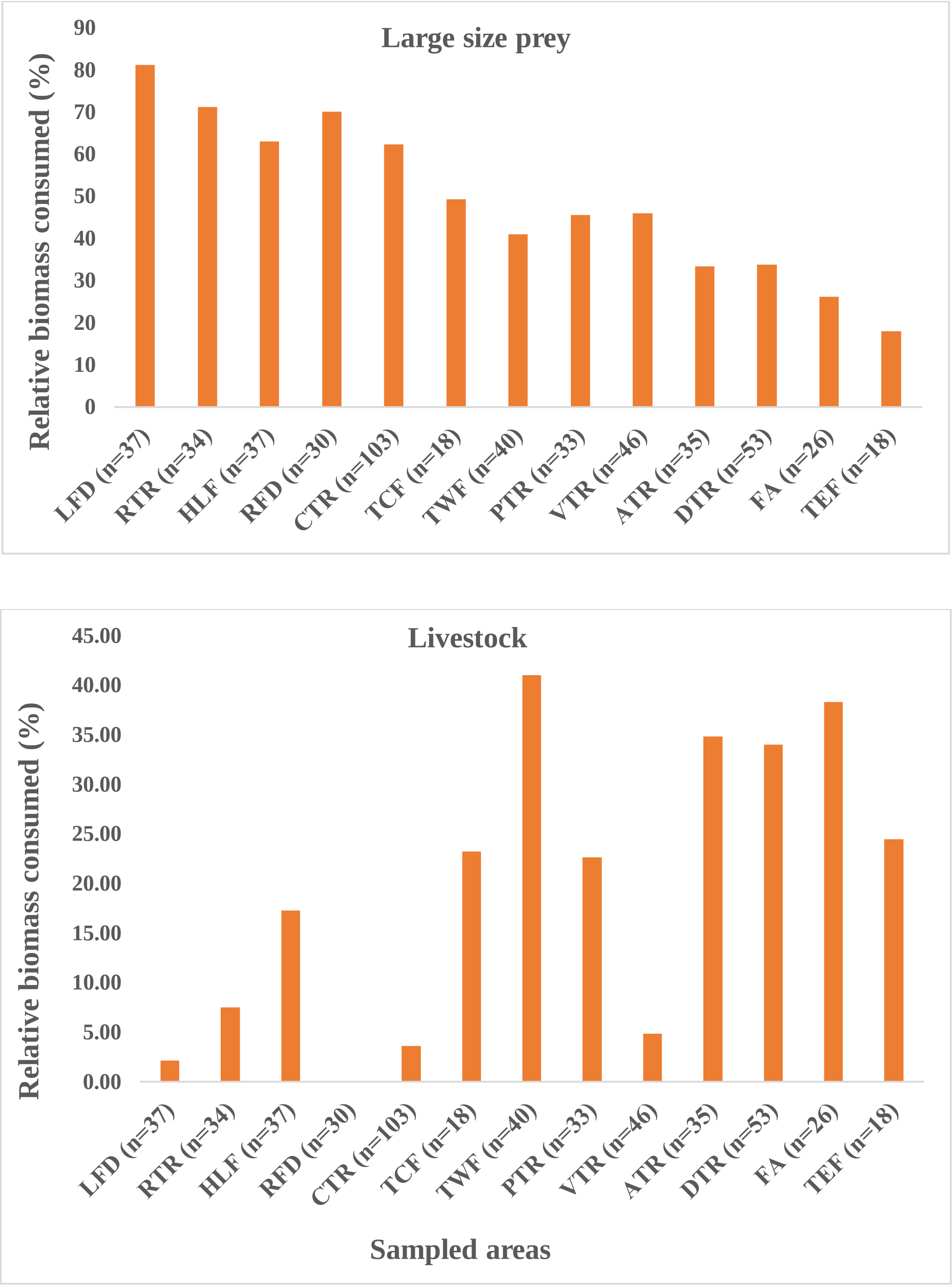


Figure 3.9. *Relative biomass consumed (data from diet assessment using faecal samples) by tiger for large size wild prey (above) and livestock (below).*

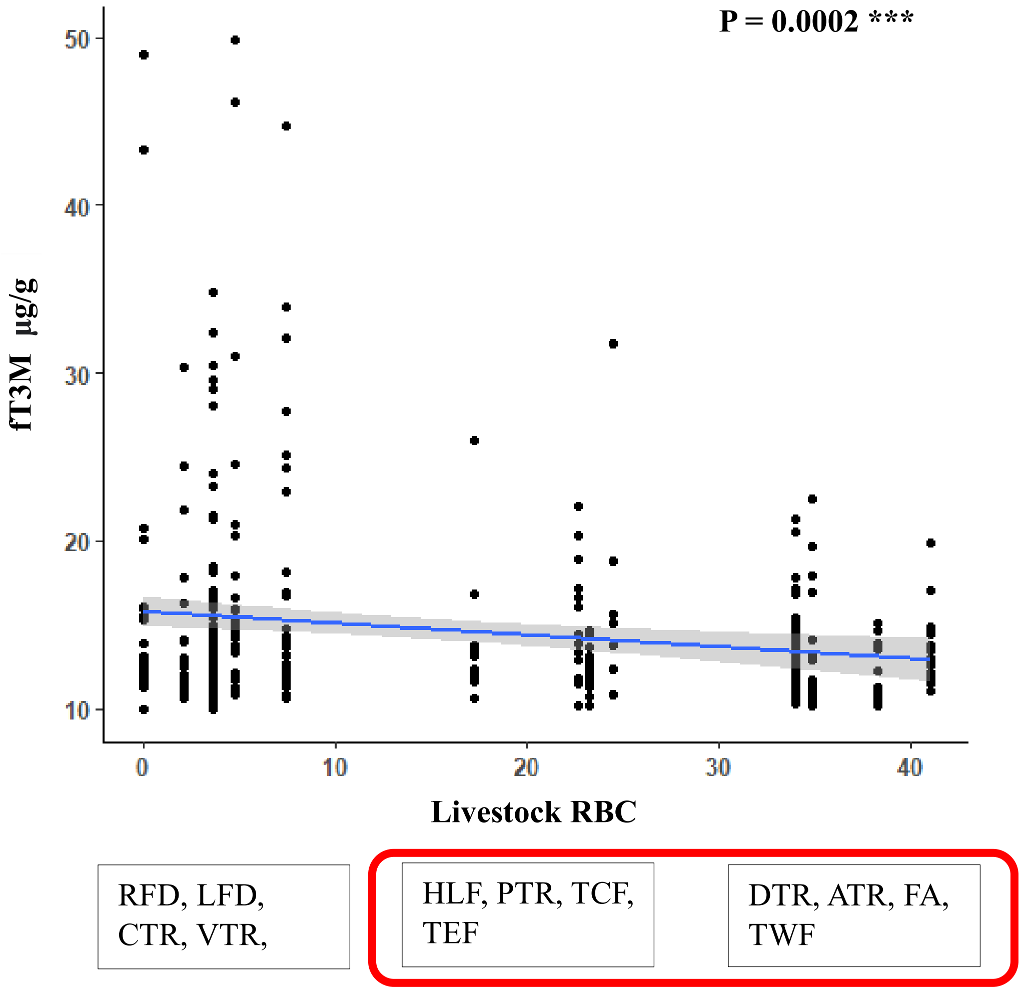
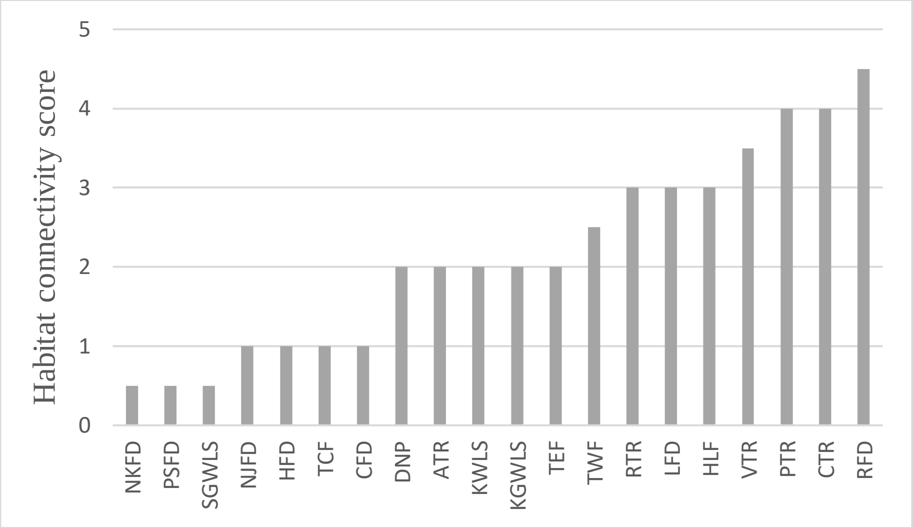


Figure 3.10 *fT3M univariate GLM plot for association with relative biomass consumed (RBC) of Livestock (fT3M ~ Livestock RBC). Red box highlights the areas with high livestock depredation (disturbed areas) containing tigers with relatively poor nutritional status (low fT3M).*

Figure 3.11 (next page) *Summary results showing habitat connectivity scores (above), proportion of non-forest area cover and frequency of occurrence of livestock (middle) and mean fGCM and fT3M values (below) for each of the sampled area.*


Habitat connectivity score
5
4
3
2
1
0
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PSFD
SGWLS
NJFD
HFD
TCF
CFD
DNP
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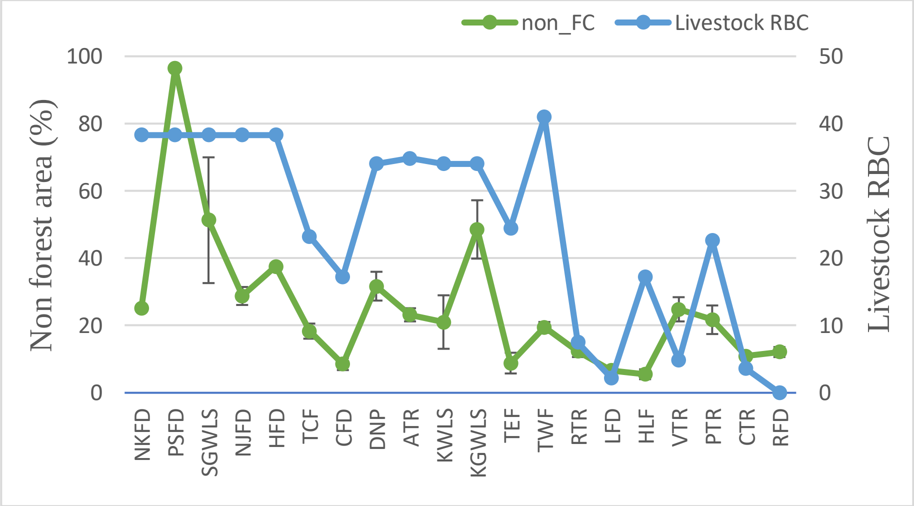
VTR
PTR
CTR
RFD


non_FC
Livestock RBC
Non forest area (%)
Livestock RBC
100
80
60
40
20
0
50
40
30
20
10
0
NKFD
PSFD
SGWLS
NJFD
HFD
TCF
CFD
DNP
ATR
KWLS
KGWLS
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TWF
RTR
LFD
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PTR
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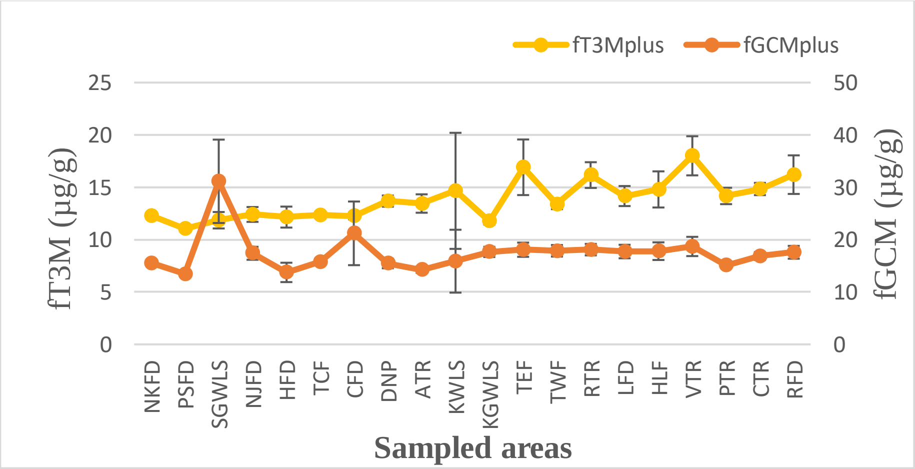
fT3Mplus
fGCMplus
fT3M (µg/g)
fGCM (µg/g)
25
20
15
10
5
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50
40
30
20
10
0
NKFD
PSFD
SGWLS
NJFD
HFD
TCF
CFD
DNP
ATR
KWLS
KGWLS
TEF
TWF
RTR
LFD
HLF
VTR
PTR
CTR
RFD
Sampled areas

3.4 Discussion

In this study we tried to find out the effect of fine and broad scale habitat factors and dietary composition on physiological status of free ranging tigers at a landscape level. We found that increased proportion of open forest area facilitates better physiological health. Results show that as as percentage of open forest area increases within approximate home range area of tiger, nutritional status improves (increased fT3M) in protected areas and over all physiological stress decreases (lower fGCM values) (figure 3.4). Terai-arc landscape supports a mosaic of wooded forest and grasslands (Champion and Seth, 2005). Open alluvial and riparian grasslands of terai are known to be inhabited by large ungulate populations (Chanchani et al., 2016a). Tiger in open forest areas within protected areas have access to such highly productive habitats, which reflects in higher frequency of elevated fT3M individuals in these areas. Same cannot be said for forest divisions, in these areas fT3M pattern is reversed, areas with higher proportion of open forest areas have lower fT3M levels. It seems that in forest divisions, because of lack of required protection or management, open forest areas do not support productive grasslands instead these areas are more like fallow lands or non-forest areas which possibly are largely exploited by livestocks. This reasoning gets support from our data showing that proportion of open forest areas (like non-forest areas) decreases significantly as habitat connectivity improves (Annexure V) for FDs (as opposed to PAs where it remains largely unchanged). This implies that open forest areas in relatively more disturbed or isolated (with lower HCS) forest divisions are qualitatively degraded fallow lands (almost like non-forest areas), and tigers surviving in these areas are nutritionally stressed. In FDs with better connectivity, proportion of open forest area reduces significantly, most probably due to lack of proper grassland management or their conversion into monoculture plantations, which is a common practice in multiple use forest of terai (Johnsingh et al., 2003). Past literature (especially in TAL) repeatedly has emphasized the importance of productive grasslands and their management (Chanchani et al., 2014; 2016a) for tiger conservation. A recent review on afforestation policy of India by Tambe et al., 2022 also emphasizes on ecological restoration (not afforestation) of open habitats. Through this study we provide significant empirical evidence of how tigers physiological status (especially nutritional status) dwindles with decreasing open forest (including grassland) proportion and its quality.

As against our expectations we see little effect of human interference (HI) on physiological stress at fine spatial scale. fGCM levels were found relatively lower in least mean human HI and higher (although with high confidence interval) in very high mean HI areas. For fT3M HI was not a significant predictor. It is known that tigers avoid areas with high human activity (Harihar et al., 2020) with lower/no prey availability (Harihar et al., 2018; Gubbi et al., 2017) and it could be that in our one-time sampling we most probably obtained samples from largely suitable habitats, hence the HI gradient covered might not be enough to bring out significant physiological differences. Considering that non-forest areas (excluding wetlands, riverbeds see FSI glossary) in PAs and FDs are a result of past and current human activities, as hypothesized their increased proportion in tiger approximate home range decreases overall physiological health. Decreased level of fT3M values along with relatively increased fGCM levels shows that habitat composed of more disturbed (non-forest) areas negatively affects tiger physiological health (figure 3.3).

At fine spatial scale, tiger fGCM levels also showed significant positive association with local tiger density (see figure 3.5 and 3.6). As with the prey distribution, tiger local abundance also varies within a park (Jhala et al., 2020; Chanchani et al., 2014). It is possible that throughout their distribution in TAL, certain areas with better resources within each park carry higher local density of tigers. Spatial fGCM model of Corbett tiger reserve (highest tiger density in the world 14 tigers/100km^2) shows that fGCM levels are higher in areas of high local tiger density. Collectively, in such otherwise good habitats, tigers experience more psychological stress, most probably due increased instances of intraspecific agonistic interactions. Many territorial solitary and dominance hierarchies maintaining social animals are known to express elevated levels of fGCM in response to competitive interactions (Armitage 1991; Creel 2001). Such interactions can lead to detrimental fights (even infanticide) which in long run could affect local population dynamics (Chanchani et al., 2018). Further, in our results we have also observed increased frequency of elevated fGCMs in better connected as well as poorly connected habitats (figure 3.8), although a significant quadratic relation could not be established between fGCM and habitat connectivity, we believe this is an important observation. We see that fGCM levels are increased in challenging conditions, which could either be due to competition (in better habitats) or due resource deprivation or conflict with humans (in disturbed habitats). When interpreting fGCM data, it seems, context has to be carefully examined. As done in our study, measures of multiple hormones (or other biological markers) tested against same set of factors, can provide more reliable interpretations.

fGCM and fT3M results of tiger diet data, did not show any significant differences between prey size /type classes as hypothesized. Earlier dietary studies on leopards (another large sized carnivore) showed that samples with remains of large sized prey (compared to medium) showed significantly higher levels of fT3M, representing better nutritional status (Patel et al., 2022). One possible reason of absence of such pattern in this study could be the pooling of samples at landscape level. For example, let’s say we have two samples (from 2 different individuals) with large prey size remain, one coming from better quality habitat (with good chance of having a large sized prey in subsequent hunt) and another coming from deteriorated habitat (with poor chance of having a large sized prey in subsequent hunt), under such scenario, putting those two samples in single category may neutralize the measure of overall long term nutritional state. Data from Patel et al., 2022, although at different spatio-temporal scale is restricted to Rajaji tiger reserve, considered to have good over all prey base. Also, fT3M results with interaction term shows relatively higher fT3M levels for large size (even livestock) prey category in PAs compared to FDs (figure 3.7), supporting our reasoning further.

At broad spatial scale, we see a gradient in occurrence of large and livestock prey in tiger diet. Both have a contrasting relation, occurrence of large prey in diet decreases with increase in livestock (figure 3.9). Physiological response to this gradient was found to be significant for livestock RBC, that is frequency of tigers with high fT3M levels reduces as biomass contribution of livestock in tiger diet increases. This implies that areas with increased instances of livestock depredation largely contains tigers with poor nutritional health. These results are in accordance with the past studies where livestock abundance has been treated as a factor that contributes to decrease in natural prey, directly by competing with them for forage and indirectly by degrading habitat quality (Wolf and Ripple 2017). Our results also showed tiger fT3M responses to be positively associated with prey density and increased habitat connectivity, further affirming the pattern. Overall, we would say habitats with poor connectivity are degraded with more non-forest areas and higher abundance of livestock (see figure 3.11). These low-quality habitats have lower prey density and supports a smaller number of tigers with overall poor nutritional state. Our results, successfully illustrates the intricate interdependency of various habitat factors and their cumulative impact on tiger physiological health. Additionally direct conflict with humans (due to livestock depredation in such areas) can also be a potential source of physiological distress for tigers. Future studies can include area specific conflict quantification and its effect on tigers physiology in those areas.

In light of current increase in tiger population in certain areas of TAL and given that there is only limited natural habitat available to accommodate increasing population, it would be advisable to improve on already available sub-optimal habitats by increasing their protection, so that they recover and provide suitable grounds for dispersing tigers. While devising plans for habitat recovery, special attention must be given to maintain and protect natural open forest areas which support highly productive alluvial or riparian grasslands. Further, as shown, poorly connected habitats (such as DTR complex, TCFD, SGWLS, NJFD, PSFD see annexure ii) are more degraded and could only support individuals of relatively poor health (significantly lower fT3Ms), it is essential to devise management plans that can preserve and improve connectivity between existing habitats in human dominated landscapes. A related finding was that poor physiological parameters (lower fT3Ms accompanied with lower fGCM) of relatively more fragmented central TAL (tiger genetic habitat block, TGB II) were in concordance with the its earlier found lower genetic heterozygosity compared to better connected western and eastern TAL (Biswas et al., 2022a). These, re-occurring indication of perilous condition of tigers in fragmented tiger habitats of central TAL (for occupancy and space use data see Chanchani 2016a; 2018; for genetic data see Biswas et al., 2022a and current study for physiological health data) should receive immediate attention of management planning in this landscape. We expect that this study would encourage future studies (at fine or broad scale) to include physiological biomarkers (wherever possible accompanied with behavioural and space use data as well) to have a more wholesome and real-time understanding of how external environment affects individual or population fitness of large carnivores.

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CHAPTER 3

Cost of co-existence: a spatio-temporal analyses of leopard physiological responses to varying tiger density in Rajaji Tiger Reserve, Uttarakhand, India

4.1 Introduction

Apex predators are critical in maintaining the structure and control of the local ecosystem functioning through prey-predator dynamics (Ritchie and Johnsan 2009; Ritchie *et al.*, 2012; Ripple *et al.,* 2014; Gaynor *et al.,* 2019) and their limiting effects on subordinates (Ritchie and Johnsan 2009; Ripple *et al.,* 2014; Suraci *et al.,* 2016; Feit *et al.,* 2019). Intensity of such limiting effects within a large carnivore guild is more pronounced for species competing for similar resources (Palomares and Caro 1999; Donadio and Buskirk 2006; Ritchie and Johnson 2009). Dominant species within the guild exercise interference competition through aggression (Linnell and strand 2000; Merkle *et al.,* 2009), harassment (Merkle *et al.,* 2009), kleptoparasitism (Merkle *et al.,* 2009; Périquet *et al.,* 2015), delaying intraspecific communication (Cornhill and Kerley 2020) and direct killing (Palomares and Caro 1999; Ritchie and Johnsan 2009; Donadio and Buskirk 2006; Laurenson 1994; Merkle *et al.,* 2009). Subordinate members, on the other hand employ various tactics (for example, spatio-temporal and dietary separation) to minimize interference and maximize resource acquisition (Hayward *et al.*, 2009, Vanak *et al.*, 2013) to achieve a balance for successful co-existence. Local ecological factors are known to drive such behavioral strategies, which has been an area of extensive research interest in various carnivore guilds globally (Karanth and Sunquist 1995; 2000; Carlsson *et al.,* 2010; Broekhuis *et al.,* 2013; Steinmetz *et al.,* 2013; Vanak *et al.,* 2013; Périquet *et al.,* 2014; López-Bao *et al.,* 2016).

Dominant-subordinate agonistic interactions exerts two different kinds of negative impacts on the subordinate species: (a) direct impacts such as getting killed or displacement from the best-quality habitats (Mitchell and Banks 2005; Merkle *et al.,* 2009; Newsome *et al.,* 2017; Ramesh *et al.,* 2017) and (b) indirect effects from increased pressures from competitions, inadequate food resources and resulting energy deficits (Creel *et al.,* 2008, 2017; Suraci *et al.,* 2016; Sheriff *et al.,* 2020) affecting survival, growth, body condition, reproduction and parental provisioning (Creel *et al.,* 2007; 2008; 2009; Parker *et al.,* 2009; LaManna and Martin 2016). As many of these indirect effects are mediated through physiological processes (Clinchy *et al.,* 2013; Macleod *et al.,* 2018), quantification of the physiological responses is essential to understand changes in various population parameters of the subordinate species (Creel *et al.,* 2009; Gaynor *et al.,* 2019). Recent advances in physiological measurements of environmental stressors, particularly in combination with non-invasive sampling approaches have made it easier to link the environmental effects with their respective physiological responses (Dantzer *et al.,* 2014; Sopinka *et al.,* 2015; Ames *et al.,* 2020; Palme 2019). For example, a number of inter-species (predator-prey- see Ylönen *et al.,* 2006; Creel *et al.,* 2009, dominant-subordinate dynamics-see Van Meter *et al.,* 2009 etc.) and intra-species (social hierarchy-see Sapolsky 1983; Armitage 1991; Creel *et al.,* 1996; Creel 2001; Van Meter *et al.,* 2009, competition-see Armitage 1991; Creel 2001) interactions have been addressed using glucocorticoid (GC) measures, demonstrating its use. Further, recent addition of thyroid hormone (T3, in particular) (Eales, 1988; Flier *et al.,* 2000; Wasser *et al.,* 2010; Behringer *et al.,* 2018) measure is allowing us to separate the impacts of dietary resource availability from overall stress measures (through GC) as shown in marine (Ayres *et al.,* 2012; Wasser *et al.,* 2017; McCormley *et al.,* 2018) and terrestrial mammals (Wasser *et al.,* 2011; Vynne *et al.,* 2014;

Joly *et al.,* 2015; Dias *et al.,* 2017; Szott *et al.,* 2020), including large carnivores (Vynne *et al.,* 2014; Patel *et al.,* 2021).

The sympatric tiger (*Panthera tigris*) and leopard (*Panthera pardus*) are one of the most well-studied model systems to understand the dominant-subordinate intraguild competition (Seidensticker 1976; Mcdougal 1988; Mondal *et al.,* 2012; Steinmetz *et al.,* 2013; Carter *et al.,* 2015; Pokheral and Wegge 2019; Kafley *et al.,* 2019; Kumar *et al.,* 2019; Thapa *et al.,* 2021). Leopards, when co-existing with tigers, are often dominated by their larger counterpart in terms of resources (both space and food) (Seidensticker 1976). Large number of studies have focused on exploring different strategies adopted by leopards such as spatio-temporal (Carter *et al.,* 2015; Kafley *et al.,* 2019; Kumar *et al.,* 2019; Pokheral and Wegge 2019; Thapa *et al.,* 2021) and dietary niche segregation (Karanth and Sunquist 1995; Andheria *et al.,* 2007; Harihar *et al.,* 2011; Pokheral and Wegge 2019) for successful co-existence with tigers, but the physiological consequences of such interactions have received less attention. Here I address leopard physiological and dietary responses in the context of competition with tigers within Rajaji Tiger Reserve (RTR), western Terai-Arc landscape, India. RTR is a major source tiger population (estimated density of 8 ±1.4/100 km^2 in 2018) and retains one of the highest density of leopards (16.90 ±1.44/100km^2) in the landscape (Jhala *et al.,* 2021). The park is physically separated by the river Ganges in two parts: eastern and western RTR (henceforth ERTR and WRTR) that are structurally connected by a narrow corridor, heavily affected by anthropogenic activities (Johnsingh *et al.,* 1990; Harihar *et al.,* 2018; Biswas *et al.,* 2022a) (Figure 4.1). Both sites are similar in terms of wild prey densities and vegetation structure

(Harihar *et al.*, 2009b) but differ in the extent of tiger competition intensity. Almost the entire tiger population of RTR is found in the ERTR whereas leopard, in the absence of tiger, is functionally the apex predator in the WRTR. This unique situation provides an ideal, natural system to assess the physiological impacts of inter-species competition in a control-test framework (WRTR is a control site with no inter-species competition when compared with ERTR). Leopard faecal hormone metabolite measurements (fGCM and fT3M) in 2015 and 2020 were used to address spatio-temporal differences in physiological and dietary responses against a tiger competition gradient. More specifically following questions were asked: (i) how leopard dietary profiles, fGCM and fT3M measures vary with changing tiger competition intensities over space and time, and (ii) how local ecological factors (habitat productivity and prey body size) explain such differences in leopard physiology. I believe that results of this study have larger implications in understanding the physiological costs for subordinate carnivores co-existing within a guild and their long-term conservation.

4.2 Material and methods

4.2.1 Study area

This study was conducted in Rajaji Tiger Reserve (RTR) (Figure 4.1), the westernmost part of Rajaji-Corbett Tiger Conservation Unit (RCTCU, Johnsingh and Negi 2003), in the Indian part of the Terai-Arc landscape (TAL). Located at base of Himalayan foothills and starting of Indo-Gangetic plains, RTR has an undulating topography with a mosaic of woodlands and grasslands, drained by multiple rivers and streams. The forest type is broadly classified as northern Indian moist deciduous, dominated by Sal (*Shorea robusta*) (Champion and Seth, 2005). The park is naturally separated by the river Ganges in two parts, connected by a narrow corridor called as Chilla-Motichur corridor (3 km length and 1 km width) (Johnsingh *et al.*, 1990; Harihar *et al.*, 2018). The eastern part (covering 579 km^2 area of core and buffer zones), situated in the east bank of Ganges maintains structural as well as functional connectivity with major tiger populations (such as Corbett Tiger Reserve) of the landscape (Harihar *et al.*, 2020; Biswas *et al.*, 2022a). However, the western part (571 km^2 area on the west bank of Ganges) has become isolated over years from the ERTR. Historically, the entire study area (RTR) was inhabited by large numbers of agro-pastoralist community settlements (Toungya and Gujjars, respectively) which were primarily dependent on forest resources (Berkmuller *et al.*, 1987), leading to forest degradation. In 1983, Rajaji was declared as a national park and significant efforts towards tiger and prey population recovery were undertaken. As part of this, a plan for relocation and rehabilitation of local communities was prepared to create inviolate space for wildlife (Roy 2003), and accordingly all settlements from the core areas of ERTR were relocated by 2003 (Harihar *et al.*, 2009b). Subsequently, the conditions of the natural habitat improved and the densities of the wild prey species increased and facilitated population recovery of wild tigers in ERTR (from 2.08 in 2004-5 to 7.05 individuals/100km^2 in 2016-17, Harihar *et al.*, 2020). However, in the WRTR, the human rehabilitation was completed in periodical manner where by 2005 some ranges were made inviolate (Harihar *et al.*, 2009b) and others were completed by 2016. As WRTR remained functionally

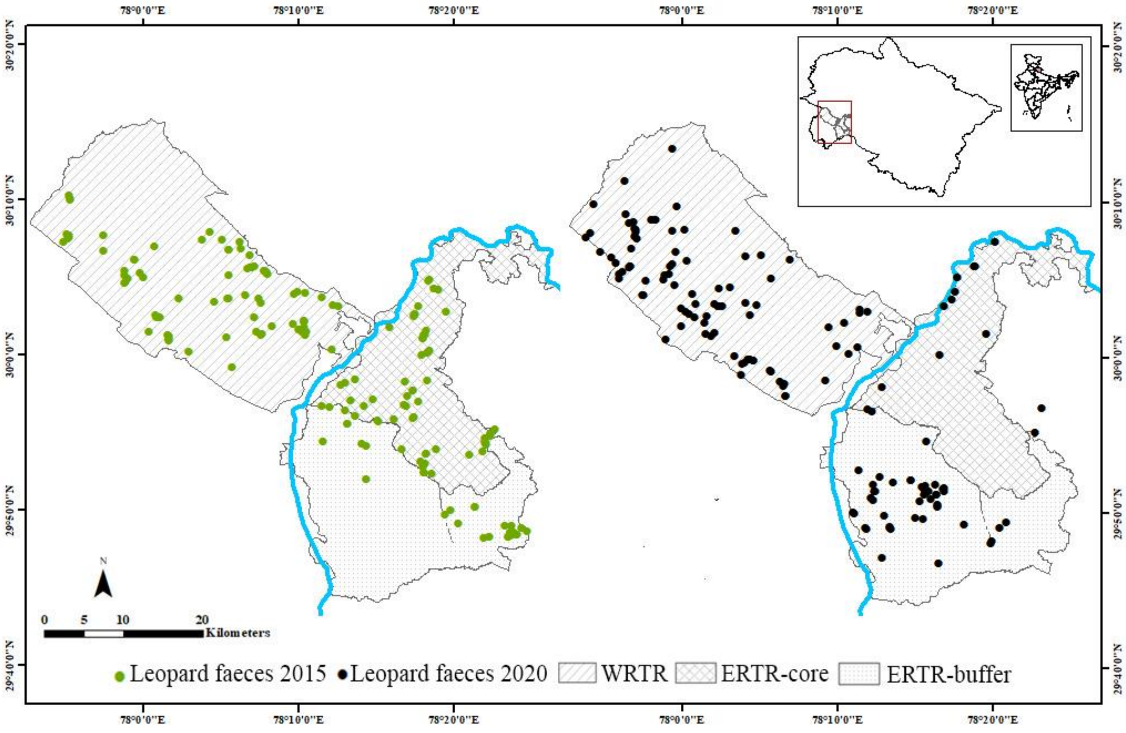

***Figure 4.1** Spatio-temporal locations of field-collected leopard faecal samples from Rajaji Tiger Reserve (RTR) used in this study. The core and buffer zones are differentially marked. The left pane represents samples collected in 2015, whereas the right pane shows 2020 samples.*

disconnected from eastern counterpart during this period, the tiger population reduced drastically (from 5-10 individuals in 2000 to two females in 2006, Johnsingh *et al.*, 2006, Jhala *et al.*, 2020) despite comparable prey density (41.22±6.65 individuals/km^2 in WRTR and 39.23±4.76 individuals/km^2 in ERTR, Harihar *et al.*, 2020). The leopard population, on the other hand, showed a reverse trend where ERTR recorded a decrease in their density (9.76 to 2.07 individuals/100km^2 between 2004 to 2008, respectively, Harihar *et al.*, 2011) (possibly due to inter-species competition) and WRTR showed an increasing trend in leopard density (Jhala *et al.,* 2021) (due to less competition from tigers). Such contrasting population patterns under a scenario of inter-species competition provided an ideal 'natural experimental setup' to understand leopard physiological responses under low and high tiger density in adjacent and similar habitats. Further, this study was conducted over a five-year temporal framework (2015 and 2020) during which the ERTR has seen a significant increase in tiger population (2.90±0.87 to 8±1.4/100 km^2, Jhala *et al.*, 2015 and 2020), therefore providing an opportunity to test the effects of competition against a tiger density gradient (high tiger density in 2020 than 2015 within ERTR).

4.2.2 Study design

The unique situation of naturally high (ERTR) vs. low (WRTR) tiger density and reverse patterns of leopard density in adjacent and similar habitats provided opportunity to ask some important questions regarding various effects of inter-species competition. From a spatial difference perspective we hypothesize that: (1) there would be a dietary niche separation between ERTR and WRTR, where the eastern leopard population would show higher frequency of medium and small sized prey in their diet than the western population (where they don't face competition from tigers); (2) corresponding high fGCM in ERTR (due to inter-species competition) than western population; and (3) lower fT3M in ERTR leopards (due to possible dietary niche separation) than their western counterpart. In a temporal data perspective, I expect that (4) there would be no significant difference in prey relative frequency of occurrence (RFO) in diet, as well as in fGCM and fT3M measures in WRTR; whereas (5) significant difference in prey RFO in diet, fGCM and fT3M is expected in ERTR resulting from increased competition from tigers between 2015 and 2020 (tiger density of 2.90±0.87/100 km^2 in 2015 and 8±1.4/100 km^2 in 2020, Jhala *et al.*, 2020). Apart from the inter-species competition habitat productivity-related differences were also expected in leopard dietary and physiological responses. Better habitat was expected to be associated with higher frequency of large sized prey in diet, lower fGCM and higher fT3M levels.

4.2.3 Faecal sampling, species confirmation and prey identification

Due to the spatio-temporal nature of the study, it was critical to establish a standard sampling framework for faecal collection from the entire study area. Some of the major concerns were identification of sampling trails across RTR, seasonal effects, uniform field efforts, constant sampling team etc. As RTR has been a long-term study site for photographic monitoring of tigers (Harihar *et al.*, 2009a; 2009b; 2012; 2020) and earlier genetic studies have used already identified forest trails and tracks (Biswas *et al.,* 2022a), the information was used for faeces collection in this study during both sampling periods (2015 and 2020). Two experienced research and tracking team (each consisting of 3-4 members) surveyed all identified forest trails during 2015 and 2020 and collected fresh faecal samples of large carnivores. Both sampling (in 2015 and 2020) were conducted in winter (December-January) to counter seasonal variations and make use of better environmental conditions (mean temperature of 4-6^{o}C) that maintain relatively long-term sample freshness. In field, sample freshness was determined based on intactness, minimal insect activity and strong odor (Vynne *et al.,* 2014). All fresh faecal samples were collected in wax paper with location details and stored in zip-lock bags (Biswas *et al.*, 2019) before transporting them to the laboratory, where they were stored -20 °C until laboratory analysis.

In the laboratory, the samples were genetically ascertained using leopard-specific molecular markers (Mondol *et al.,* 2014) to ensure only confirmed leopard samples were used in downstream diet and physiological analyses. In brief, DNA extraction was performed using a modified Qiagen DNA extraction protocol (Biswas *et al.*, 2019) for all samples and leopard-specific mitochondrial DNA markers (TigParND4-F and ParND4-R, Mondol *et al.*, 2014) were used to ascertain leopard faeces. Confirmed leopard samples were further dried at 60 °C for 72 hours in an oven (#Unilab-112HO, Haryana, India) to control for moisture (Wasser *et al.*, 1993). The undigested parts (prey hair, broken bones, hoof etc.) were separated by sieving the dried samples through sterile 0.5 mm stainless steel mesh and the faecal powders were collected and stored in -20 °C. The primary guard hairs (20-30 hairs/sample) were used to prepare permanent slides and were examined for medulla structures (Mukherjee *et al.*, 1994) using available references (Bahuguna *et al.*, 2010, Biswas *et al.*, 2022b) to identify leopard prey species. Sample size estimation for diet analyses was conducted through a sample rarefaction curve (Magurran 2004), where the species diversity in leopard diet was estimated using Shannon diversity index (Magurran 2004) with EstimateS (Colwell 2006).

4.2.4 Habitat productivity assessment

For leopards or for large carnivores in general a good quality habitat is one with good prey availability (Carbone and Gittleman 2002). Prey availability is associated with forage availability that is often quantified in terms of vegetation cover or green cover (Pettorelli *et al.,* 2005a, b, 2011). Vegetation cover was used as a proxy of habitat productivity (Pettorelli *et al.,* 2011), that would facilitate higher prey base for leopards and quantified it by extracting Normalized Difference Vegetation Index (NDVI) values. I used 16-day composite NDVI values recorded by NASA's MODIS (Moderate Resolution Imaging Spectroradiometer, MOD13Q1 version 6.1 at 250m resolution), downloaded for RTR (for the month of December, corresponding to winter sampling season) for year 2015 and 2020. The analyses were conducted at two scales: (a) For overall habitat productivity assessment, study area was divided into three zones: WRTR, ERTR-core and ERTR-buffer (see figure 4.1). Each zone was further divided into 3km X 3km grids (9 km^2 area, approximate leopard home range see Seidensticker 1990); and (b) For sample-based assessment, leopard faecal sample locations were used as center and buffers of 2 km radius (12 km^2 area) were drawn around each faecal sample. Mean NDVI values were extracted for each grid and buffer using MODIS raster images of year 2015 and 2020, where extraction was done using zonal statistics tool (as table for grids and table 2 for buffers) in ArcMap 10.5 (ESRI 2016).

4.2.5 Hormone metabolite extraction and assays

Recent study on wild tigers in the same landscape showed highly variable contents of inorganic matter (IOM) in the faeces that negatively impacted fGCM and fT3M measures (Patel *et al.,* 2021). As leopards share the same space, environmental conditions and prey base in RTR, the field-collected samples were processed for percent IOM measures using the same approach described in Patel *et al* (2021). In brief, 0.1g of faecal powder was ashed in a muffle furnace (#NSW-101, NSW, New-Delhi, India) at 550˚C for 2 h, reweighed and the amount of IOM was calculated. As suggested in the earlier study (Patel *et al.,* 2021), leopard samples with <80% IOM content were used for hormone assays.

For hormone metabolite extractions, each dried faecal powder was thoroughly mixed and 0.1 grams of powder was weighed. The extraction procedure involved pulse-vortexing the weighed amount of faecal powder in 15 ml of 70% ethanol for 30 minutes, followed by centrifugation at 2200 rpm for 20 min (Mondol *et al.,* 2020; Wasser *et al.,* 2010). The hormone extracts were collected in 2 ml cryochill vials (1:15 dilution) and stored at -20 °C in freezer until assays. Leopard fGCM and fT3M were measured using Corticosterone (#K014, Arbor Assays, MI, USA) and Triiodothyronine (T3) (#K056, Arbor Assays, MI, USA) EIA kits. These kits were earlier successfully validated in wild tigers (from TAL) and captive lions (Patel *et al.,* 2021, Goswami *et al.,* 2021) and thus were considered suitable for this study. Further, parallelism and accuracy tests were conducted for leopard faecal extracts in the laboratory. Serial dilutions of faecal extracts paralleled well with standard curves of fGCM (Figure 4.2a) as well as fT3M (Figure 4.2c). F ratio test showed no differences between slopes of standard and pooled extract curves for fGCM (F(1,10) =

1.89, P = 0.2) and fT3M (F(1,11) = 1.34, P = 0.27). Accuracy tests using regression analysis produced slopes of 1.09 and 1.02 at working dilution of 1:120 and 1:7.5 for fGCM and fT3M (Figure 4.2b and 3.2d). Intra-assay coefficient of variation (CV) was 7.15 and 8.36, whereas inter-assay CV was 10.35 and 7.86 for fGCM and fT3M, respectively. During assays, hormone extracts were dried and reconstituted in assay buffers at required dilution (1:120 for fGCM and 1:7.5 for fT3M). Samples were assayed in duplicate using kit protocols and optical density (at 450 nm) was measured with ELISA plate reader (#GMB-580, Genetix Biotech Asia, New Delhi, India). Cross-reactivities of respective antibodies are presented in table 4.1.

Table 4.1 *Details of the faecal hormone assays conducted for leopards in this study*

Hormone	**Assay method**	**Dilution**	**Slope (R^2)**	**Inter-assay CV**	**Intra-assay CV**	**Cross-reactivity**
Corticosterone (Leopard pool)	EIA	1:120	1.09 (0.98)	10.35	7.15	100% with corticosterone, 12.30% with Desoxycorticosterone, 2.30% with Tetrahydrocorticosterone and <1% with Aldosterone, Cortisol, Progesterone, Dexamethasone, Corticosterone-21-Hemisuccinate, Cortisone and Estradiol
Triiodothyronine (Leopard Pool)	EIA	1:7.5	1.02 (0.99)	7.86	8.36	100% with T3, 0.88% with thyroxine and less than 0.1% with reverse T3 (3,3’,5’-Triiodo-L-thyronine)

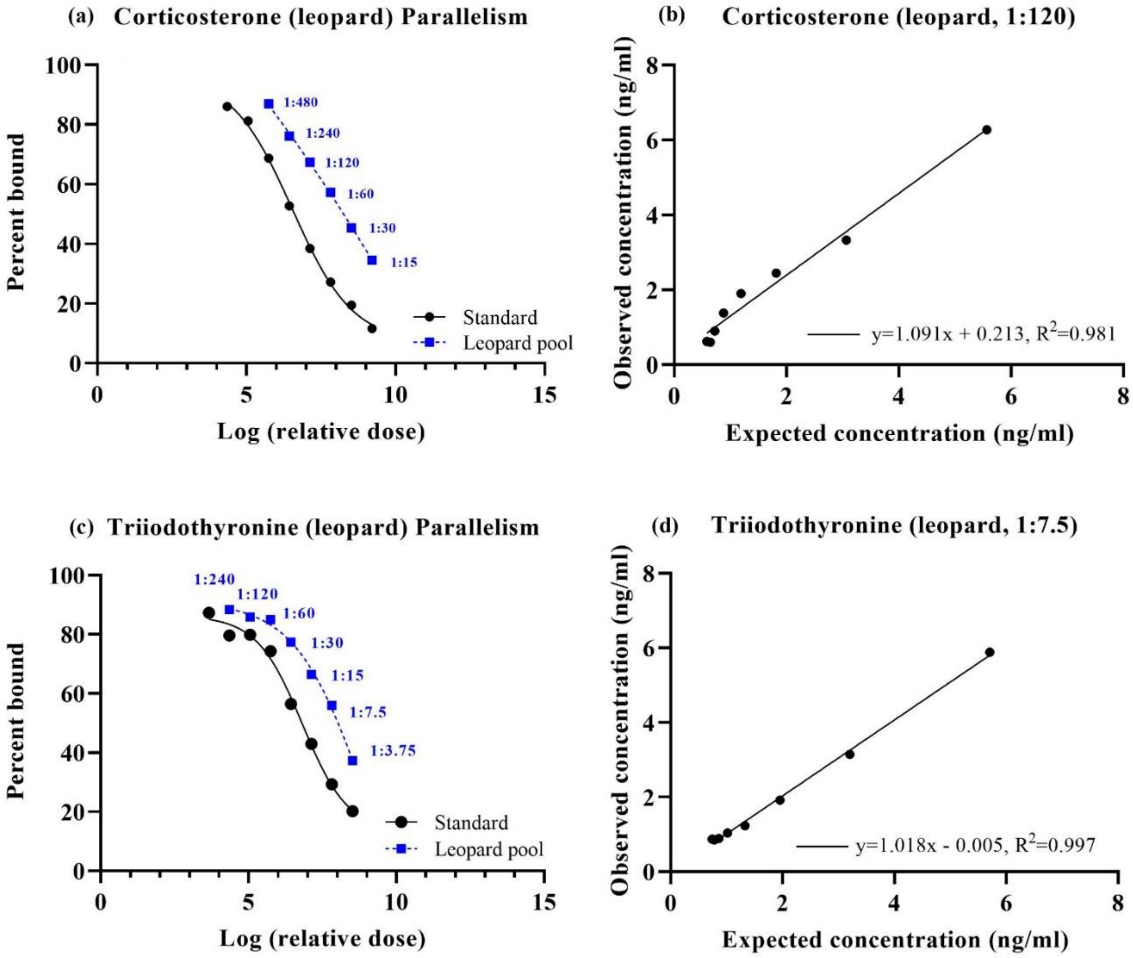

Figure 4.2 *Parallelism and accuracy plots for faecal corticosterone and T3 EIA assays.*

4.2.6 Statistical analysis

The analytical framework was established based on the hypothesis proposed in this study, where comparisons were made at two scales: 1) at spatial level, prey and hormone metabolite (fGCM and fT3M) data between ERTR vs. WRTR (individually the 2015 and 2020 data); and 2) at temporal scale where comparisons were made with each part of RTR (2015 vs 2020 for ERTR and WRTR, respectively). While reporting the methods and results following terms have been used to describe the sampled groups: ERTR in 2015- ERTR_{2015}, ERTR in 2020- ERTR_{2020}, WRTR in 2015- WRTR_{2015} and WRTR in 2020- WRTR_{2020}.

To ascertain leopard food habit, data on relative frequencies of occurrences (RFO) for each prey species was calculated using formula $i/j*100$, where 'i' represents the frequency of number of samples in which a specific prey occurs and 'j' represents the total frequency count of all prey species (Mukherjee *et al.,* 1994, Kruuk 1989). Further, Relative prey biomass consumed was calculated using formula $D= (A*Y)/\sum(A*Y)*100$ where, 'A' represents the RFO of each prey species and 'Y' represents weight of consumed prey in each faeces. 'Y' is calculated using Ackerman's equation: $Y=1.980 + 0.035X$, where X=mean body weight of a particular prey species (Ackerman *et al.,* 1984, Karanth and Sunquist 1995). The mean body weight of prey was taken from Harihar *et al* (2011), Rathore (2015) and Upadhyaya *et al* (2018) (Table 4.2). All the prey species data was categorized into three major classes: (a) large (≥60 kgs), (b) medium (between 16-60 kgs); and (c) small (≤15 kg) and absolute frequency of occurrence (AFO) was calculated for these classes using formula s_k*100/n, where 's_k' is the number of samples containing class 'k' and 'n' is total number of faeces analyzed (Harihar *et al.,* 2011). Any difference in AFO percentage in sampled groups were tested using chi-square analyses, followed by f-test for pair wise comparison at spatial and temporal scales (mentioned above). For overall assessment of the habitat productivity, mean NDVI values of three zones (WRTR, ERTR-core and ERTR-buffer) were compared spatially using one-way ANOVA (with subsequent post hoc Tukey's HSD test) and temporally (between 2015 and 2020) using paired t-test. All analyses were conducted using SPSS version 20 (IBM, 2011).

During hormone data analyses, the leopard fGCM and fT3M data (raw as well as log transformed) were assessed for normality using diagnostic plots (density plots) and Shapiro-wilk test. Generalized linear models (GLMs) with log link and gamma distribution errors were used to explain the variation in fGCM and fT3M data. To assess any possible changes in fGCM and fT3M levels across spatial (ERTR vs WRTR) and temporal (2015 vs 2020) scales, an interaction term 'Area*Year' (as the tiger density in ERTR was lower in 2015 than in 2020, that may impact fGCM and fT3M levels) and the prey size class (large, medium and small, as prey size may impact fGCM and fT3M levels)

Table 4.2 *Details of the various leopard diet parameters for all nine prey species identified in this study. Results are presented for percentage of relative frequency of occurrence (% RFO), and relative biomass of the consumed prey species in ERTR and WRTR for both 2015 and 2020, respectively.*

Prey species	**Mean body weight of prey (in kg)**	**ERTR$_{2015}$ (n=86)**		**WRTR$_{2015}$ (n=76)**		**ERTR$_{2020}$ (n=53)**		**WRTR$_{2020}$ (n=89)**	
		RFO (%)	**Relative biomass (%)**	**RFO (%)**	**Relative biomass (%)**	**RFO (%)**	**Relative biomass (%)**	**RFO (%)**	**Relative biomass (%)**
Livestock	250	5.81	12.50	0.00	0	1.89	4.32	0.00	0
Sambar	185	19.19	32.51	20.39	37.54	19.81	35.75	31.46	48.39
Nilgai	169	6.98	11.04	4.61	7.91	5.66	9.54	10.67	15.33
Chital	50	38.72	28.95	48.68	39.53	47.17	37.55	43.26	29.35
Wild pig	35	4.65	2.99	5.26	3.67	5.66	3.87	3.93	2.29
Hog deer	33	7.56	4.75	7.24	4.94	0.00	0	2.81	1.60
Langur	10	0.58	0.27	0.66	0.33	0.00	0	0.00	0
Hare	4	13.72	5.83	13.16	6.07	19.81	8.96	7.30	2.82
Indian peafowl	5	2.67	1.16	0.00	0	0.00	0	0.56	0.22

were used as explanatory variables. Likelihood ratio test (LRT) was used to determine if the explanatory variables explain the data independently or in combination. Finally, post-hoc Tukey's HSD test was employed to assess any pair-wise differences in fGCM and fT3M levels for all sampled groups (mentioned above) and prey size classes. To evaluate the relationship of fGCM and fT3M with habitat productivity (NDVI values derived from sample buffers), two separate linear models (function 'lm') were used. Additionally, we also performed a multivariate GLM including all three predictor variables (Area*Year, Prey size and NDVI change) and compared the resulting models with null model (Annexure vi) to discern the effect of each of the variable on fGCM and FT3M data. All analyses were conducted in R v4.1.1 (R Core Team, 2021) with the following packages: 'ggpubr' (Kassambara 2020), and 'multcomp' (Hothorn *et al.*,2022).

4.3 Results

During the study period, a total of 564 large carnivore faeces was collected (n=276: ERTR-172 and WRTR-104 samples in 2015 and n=288: ERTR-178 and WRTR-110 samples in 2020) from the entire study area. After species confirmation, 324 leopard faecal samples were further processed for dietary and hormone analyses. The distribution of these samples was as followed: $ERTR_{2015}$- 92, $WRTR_{2015}$- 81, $ERTR_{2020}$- 60 and $WRTR_{2020}$- 91. However, prey species could be identified from 304 samples (93.82% success rate, $ERTR_{2015}$- 86, $WRTR_{2015}$- 76, $ERTR_{2020}$- 53 and $WRTR_{2020}$- 89, respectively; Table 4.2). The remaining samples (n=20, 6.18%) contained damaged hairs for accurate species identification and were excluded from further dietary analyses. For hormone analyses, samples with >80% IOM were discarded (n=121) and finally 203 faecal hormone extracts (ERTR$_{2015}$- 56, WRTR$_{2015}$- 49, ERTR$_{2020}$- 42 and WRTR$_{2020}$- 56) were used in physiology analyses.

4.3.1 Food habits of leopard

Overall, a total of nine prey species (large-bodied- Sambar, Nilgai and Livestock; medium-bodied- Chital, Wild pig and Hog deer and small-bodied- Langur, Hare and Peafowl) were detected. The large and medium-bodied prey species contributed 85.38% (RFO) of leopard diet whereas small prey species comprised only 14.62% (RFO). RFO of Chital (44.49%) and Sambar (22.71%) were highest followed by others (Table 4.2). However, biomass of sambar was highest (38.55%), closely followed by chital (33.85%) (Table 4.2). Majority of the samples (N=273, 89.8%) contained single prey species. All prey species except livestock (identified only in the ERTR) was found across all sampled groups. The rarefaction curve saturated beyond 40 samples within each group and no new prey species was identified further (Figure 4.3). Comparative analyses (Chi-square test with prey body-size groups) revealed large-sized prey frequencies differed significantly among sampled groups (χ^2=8.62, P=0.035). F-test showed that at spatial scale, there were no significant differences in frequencies of different prey classes between ERTR$_{2015}$ and WRTR$_{2015}$ (p = 0.445, p = 0.476 and p = 1.00 for large, medium and small prey classes, respectively) (Table 4.3). However, compared to ERTR$_{2020,}$ WRTR$_{2020}$ showed significantly higher large-bodied (p = 0.018) and lower small-bodied prey species (p =0.019). Temporally, the ERTR$_{2015}$ and ERTR$_{2020}$ showed no difference in prey class occurrences (p = 0.445 (large), p = 1.00 (medium) and p = 0.335 (small), whereas WRTR$_{2020}$ showed a significant increase in large prey occurrences (p = 0.018) with no differences in medium and smaller-bodied prey classes (p = 0.156 and p = 0.238, respectively) compared to WRTR$_{2015}$.

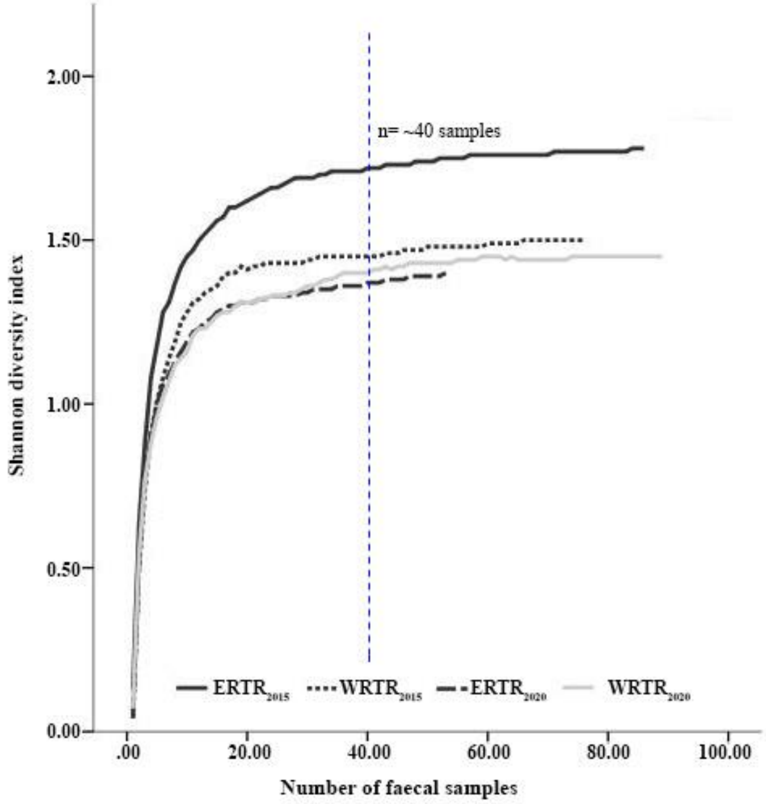

__Figure 4.3__ The rarefaction curves indicating sample sufficiency for leopard food habit assessments. Different line types represent separate spatial (ERTR and WRTR) and temporal (2015 and 2020) sample combinations. The curves stabilize at a sample size of ~40, represented by dashed blue line.

4.3.2 NDVI values at spatio-temporal scales

NDVI comparison using one-way ANOVA showed significant differences between zones (WRTR, ERTR-core and ERTR-buffer) in both 2015 (F (2,279) = 5.81, P = 0.003) as well as 2020 (F (2,279) = 7.74, P = 0.001), at spatial scale. Subsequent post hoc test showed that WRTR and ERTR-core zones did not differ significantly in their mean NDVI values in year 2015 (P = 0.47) as well as in 2020 (P = 0.93). However, ERTR-buffer zone continuously showed significantly lower NDVI values in both the years compared to WRTR (2015: P = 0.03, 2020: P = 0.001) and ERTR-core (2015: P = 0.004, 2020: P = 0.003) zones (Figure 4.4c). At temporal scale, paired t-test showed that mean NDVI values improved significantly in year 2020 for WRTR (t(134) = 25.06, P < 0.0001), ERTR-core (t(66) = 9.39, P < 0.0001) as well as ERTR-buffer (t(79) = 12.45, P < 0.0001) zones compared to year 2015 (Figure 4.4c).

4.3.3 Physiological responses of the sampled groups at spatial and temporal scales

Likelihood ratio test selected the individual GLM explanatory variables (Area*Year and Prey size, respectively) over combined model as significant factors to explain the physiological response patterns (Table 4.4). GLM results with Area*Year model indicated year is a significant factor (p=0.05) for fGCM data (Table 4.5). At spatial scale, both $ERTR_{2015}$-$WRTR_{2015}$ and $ERTR_{2020}$-$WRTR_{2020}$ comparisons showed no significant differences in mean fGCM levels (2015- z = 0.834, p = 0.838; and 2020- z = 0.253, p = 0.994) (post-hoc test, Table 4.6). However, temporal scale comparisons showed contrasting results where mean fGCM levels between $ERTR_{2015}$ and $ERTR_{2020}$ showed non-significant differences (z = -1.97, p = 0.2) but $WRTR_{2020}$ had significantly low fGCM values than $WRTR_{2015}$ (z = -2.73, p = 0.032) (Figure 4.5b(iv)). For fT3M data, GLM results showed that the area and year interaction factor (Area*Year) is a significant factor

(Table 4.5). At spatial scale, the fT3M levels between $ERTR_{2015}$-$WRTR_{2015}$ showed no difference ($z = -1.163$, $p = 0.65$), but $WRTR_{2020}$ fT3M levels were significantly higher than the $ERTR_{2020}$ ($z = 2.644$, $p = 0.041$) (Figure 4.5a(iii)). At temporal scale, $ERTR_{2015}$-$ERTR_{2020}$ comparisons revealed no significant differences in fT3M levels ($z = -1.303$, $p = 0.56$) but $WRTR_{2020}$ showed significantly higher values of fT3M than $WRTR_{2015}$ ($z = 2.602$, $p = 0.046$) (Figure 4.5b(iii)).

GLM outputs with Preysize model indicated no significant differences between prey size class (large, medium and small body-sized prey) for fGCM levels (Table 4.5, Figure 4.6a). However, the fT3M levels showed strong relationship with prey size classes, where the fT3M levels from the leopard samples with large prey remains were higher than small prey class ($z =1.15$, $p =0.5$) and significantly higher than medium prey class ($z = 2.61$, $p = 0.02$) (Table 4.5, Figure 4.6b). Linear models showed a marginally significant positive association between leopard fGCM and habitat NDVI values (t-value = 2.003, $F(1, 201) = 4.01$, $P = 0.05$) (Figure 4.7a), and no significant association between leopard FT3M and habitat NDVI values (t-value = -1.11, $F(1, 201) = 1.23$, $P = 0.27$) (Figure 4.7b). GLM with all three variables (Area*Year, Prey size and NDVI change) shows that the interaction factor of Area and Year (indicating towards unique physiological responses of leopards in space and time) is included in the best models. NDVI and Prey size best explains the variation in the data only when included with Area*Year, but not alone, for both fGCM as well as fT3M (Annexure vi).

Table 4.3 *Spatio-temporal comparison of differences in absolute frequency of prey occurrence in leopard diet at Rajaji Tiger Reserve, India. The comparisons were calculated using Fisher's Exact test (2x2).*

Prey category	Detection	Spatial comparison						Temporal comparison					
		ERTR$_{2015}$	WRTR$_{2015}$	P	ERTR$_{2020}$	WRTR$_{2020}$	P	ERTR$_{2015}$	ERTR$_{2020}$	P	WRTR$_{2015}$	WRTR$_{2020}$	P
Large	Yes	33.72	27.63	0.445	28.30	44.94	**0.018***	33.72	28.30	0.445	27.63	44.94	**0.018***
	No	66.28	72.37		71.70	55.06		66.28	71.70		72.37	55.06	
Medium	Yes	53.49	59.21	0.476	52.83	48.31	0.572	53.49	52.83	1.000	59.21	48.31	0.156
	No	46.51	40.79		47.17	51.69		46.51	47.17		40.79	51.69	
Small	Yes	12.79	13.16	1.000	18.87	6.74	**0.019***	12.79	18.87	0.335	13.16	6.74	0.238
	No	87.21	86.84		81.13	93.26		87.21	81.13		86.84	93.26	

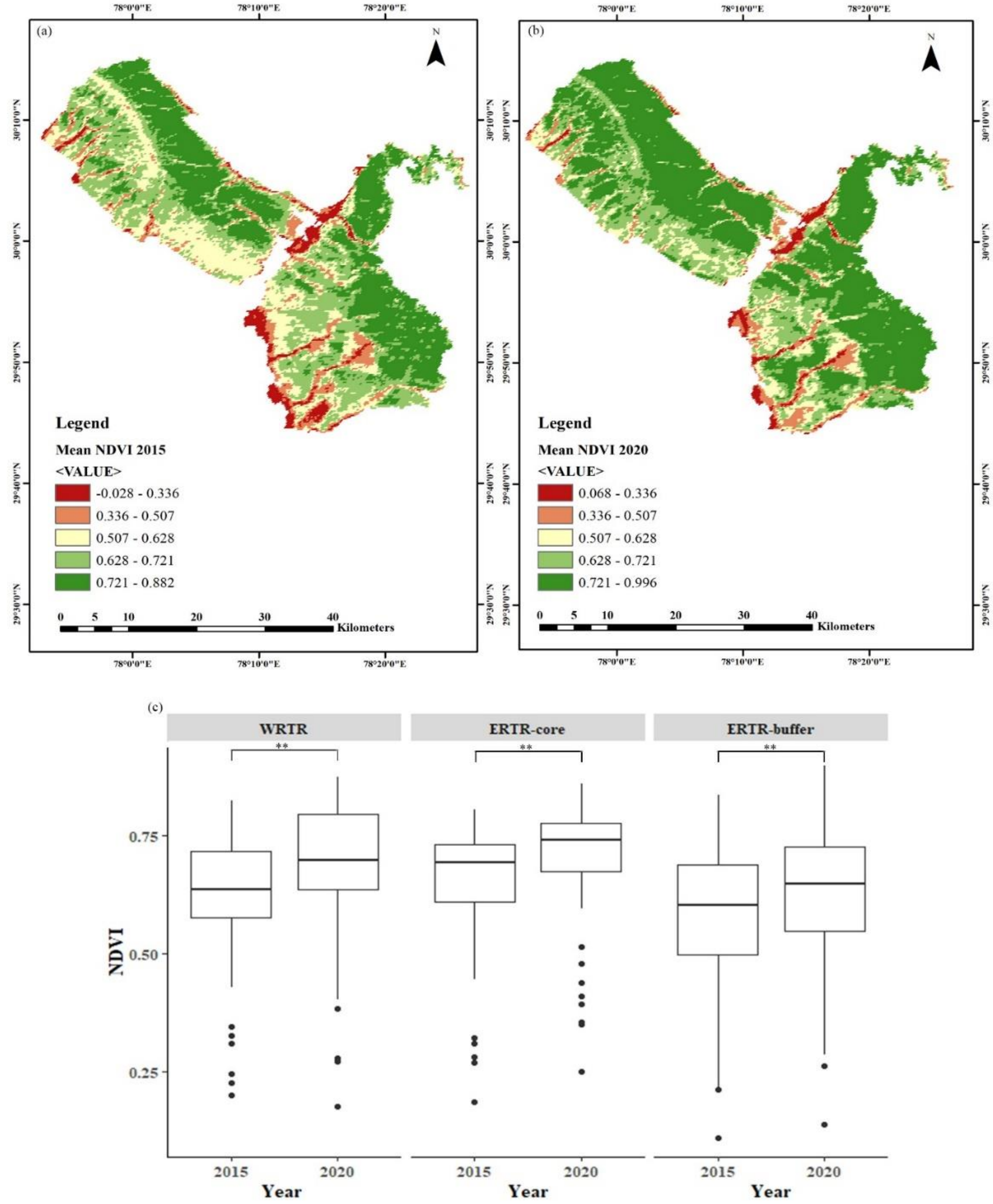

***Figure 4.4** Assessment and comparison of habitat quality (through mean NDVI values) across core and buffer zones in two time points (winter 2015 and winter 2020). Panel (a) and (b) represents the mean NDVI gradients in 2105 and 2020, respectively. Panel (c) shows the temporal differences in mean NDVI values for WRTR, ERTR-core and ERTR-buffer areas. ** indicate significance value at P<0.005.*

***Table 4.4** Results of Likelihood ratio test (LRT).*

Likelihood ratio test	
Models	**Pr(>Chi)**
fGCM ~ Prey size	0.006**
fGCM ~ Prey size + Area * Year	
fGCM ~ Area*Year	0.117
fGCM ~ Prey size + Area * Year	
fT3M ~ Prey size	0.092
fT3M ~ Prey size + Area * Year	
fT3M ~ Area*Year	0.164
fT3M ~ Prey size + Area * Year	

Table 4.5 *Results showing the association between the predictor (prey size categories and interaction of area and year) and response variables (fGCM and fT3M) based on GLM analyses. Models were fitted using log link and gamma error distributions.*

Response variable	**Predictor variable**	**Level**	**Estimate**	**± SE**	**t value**	**Pr(>|t|)**
fGCM	Area*Year	(Intercept)	162.256	78.035	2.079	0.0389 *
		AreaWest	41.441	105.873	0.391	0.696
		Year	-0.076	0.039	-1.972	0.0500 *
		AreaWest:Year	-0.020	0.052	-0.390	0.697
fGCM	Prey size (L, M and S)	(Intercept)	8.377	0.109	76.946	<2e-16 ***
		Prey size (Medium)	-0.042	0.142	-0.294	0.769
		Prey size (small)	0.341	0.214	1.592	0.113
fT3M	Area*Year	(Intercept)	133.090	96.622	1.377	0.170
		AreaWest	-356.293	131.091	-2.718	0.00718 **
		Year	-0.062	0.048	-1.303	0.194
		AreaWest:Year	0.177	0.065	2.719	0.00716 **
fT3M	Prey size (L, M and S)	(Intercept)	7.538	0.135	55.876	< 2e-16 ***
		Prey size (Medium)	-0.458	0.175	-2.612	0.00972 **
		Prey size (small)	-0.306	0.266	-1.154	0.250

Table 4.6 *Results of Post hoc pair wise comparisons.*

	Post hoc (Tukey HSD)	**Estimate**	**Std. Error**	**z value**	**Pr(>|z|)**
fGCM (Area*Year)	ERTR$_{2020}$ - ERTR$_{2015}$	-0.382	0.193	-1.972	0.198
	WRTR$_{2020}$ - WRTR$_{2015}$	-0.484	0.177	-2.729	0.0317 *
	WRTR$_{2015}$ - ERTR$_{2015}$	0.151	0.181	0.834	0.838
	WRTR$_{2020}$ - ERTR$_{2020}$	0.048	0.190	0.253	0.994
fGCM (Prey size)	Medium - Large	-0.042	0.142	-0.294	0.952
	Small - Large	0.341	0.214	1.592	0.243
	Small - Medium	0.383	0.206	1.863	0.146
fT3M (Area*Year)	ERTR$_{2020}$ - ERTR$_{2015}$	-0.312	0.240	-1.303	0.560
	WRTR$_{2020}$ - WRTR$_{2015}$	0.571	0.220	2.602	0.0456 *
	WRTR$_{2015}$ - ERTR$_{2015}$	-0.260	0.224	-1.163	0.649
	WRTR$_{2020}$ - ERTR$_{2020}$	0.623	0.236	2.644	0.0408 *
fT3M (Prey size)	Medium - Large	-0.458	0.175	-2.612	0.024 *
	Small - Large	-0.306	0.266	-1.154	0.475
	Small - Medium	0.152	0.255	0.596	0.819

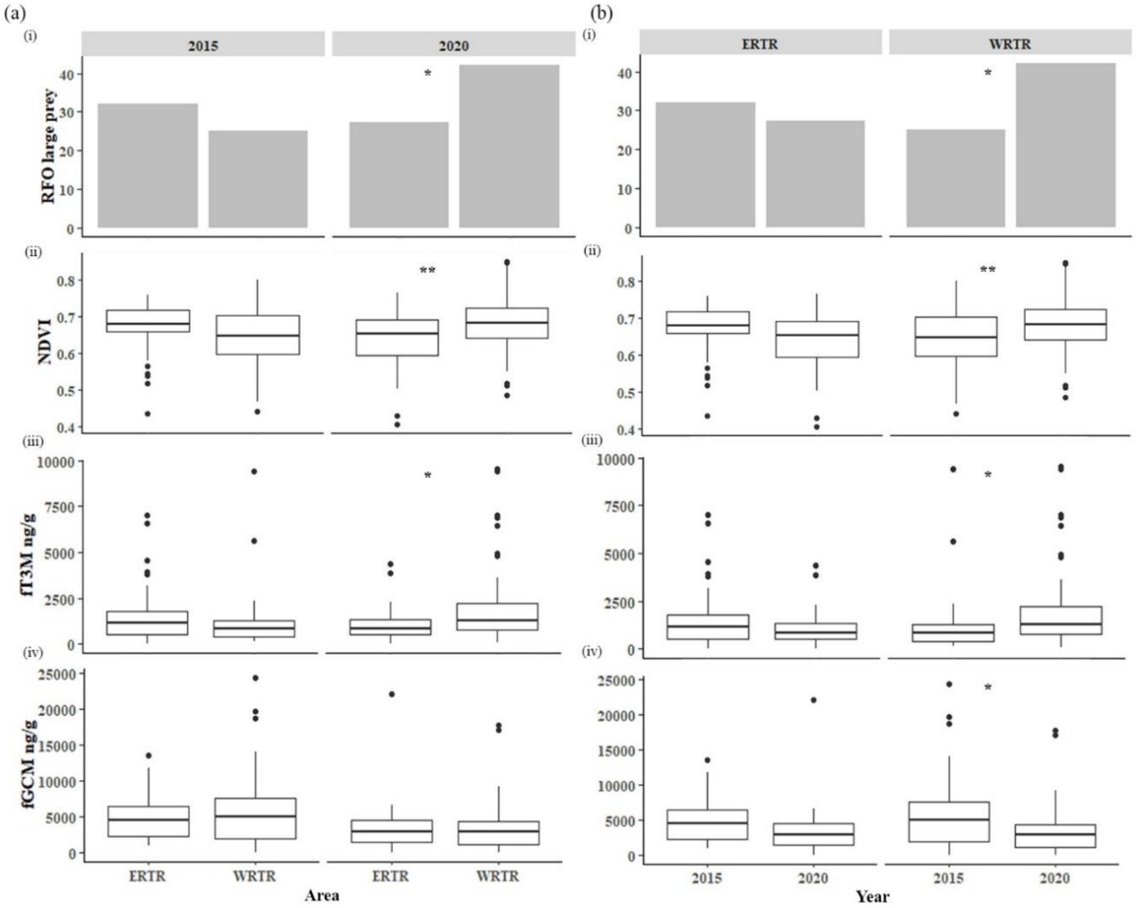

Figure 4.5 *Spatio-temporal comparisons of the major variables (habitat variable- (i) large prey RFO, (ii) NDVI and physiological variable- (iii) fT3M and (iv) fGCM) used in this study. Panel (a) shows the temporal-scale differences and panel (b) indicate spatial differences. Significant differences are depicted by * (indicating* $P<0.05$*) and ** (indicating* $P<0.005$*).*

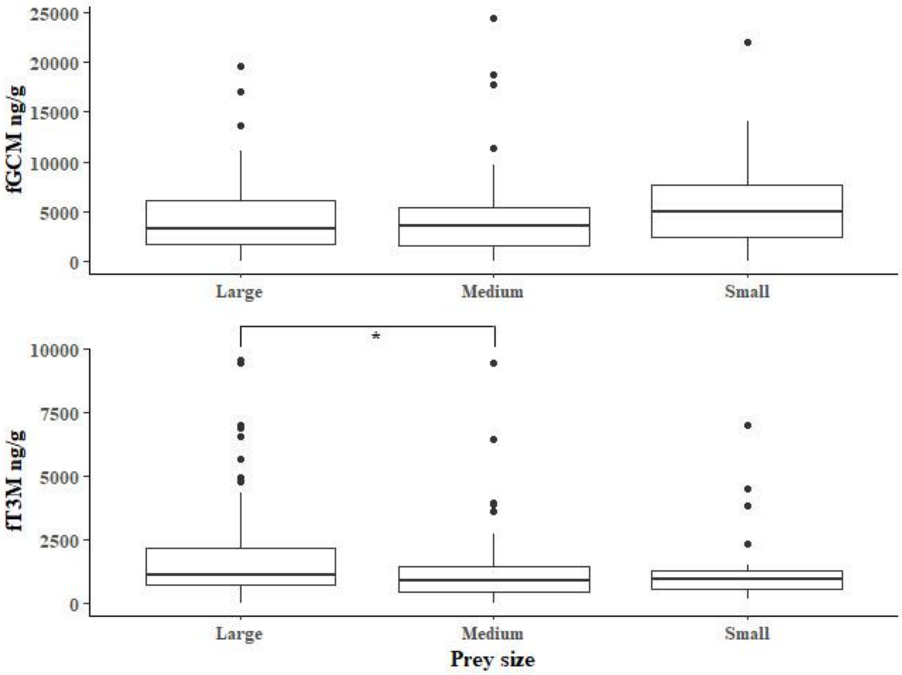

Figure 4.6 *Comparison of (a) fGCM and (b) fT3M measures from faecal samples consisting large, medium and small body-sized prey. Significant differences are depicted by * (indicating* $P<0.05$*).*

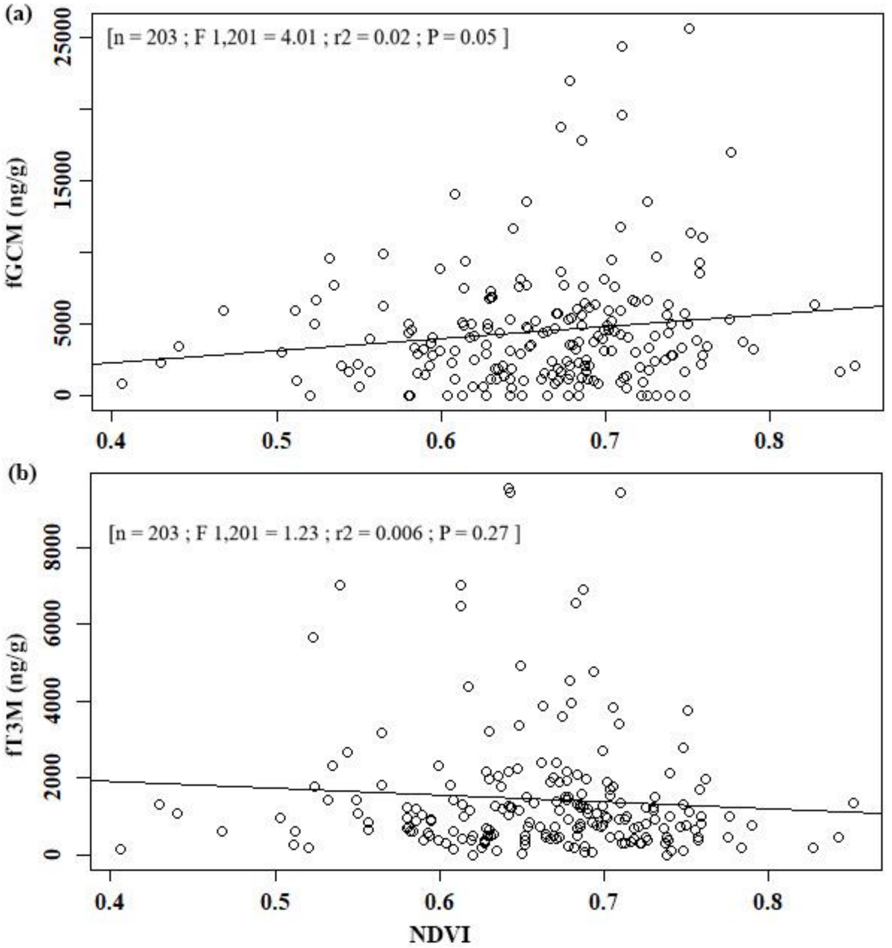

Figure 4.7 *Relationship of habitat NDVI values with leopard fGCM and fT3M levels. Scatter-plots showing (a) the positive association between NDVI values and fGCM levels and b) no significant association between NDVI values and fT3M levels.*

4.4 Discussion

As expected, results of this study provide strong support for certain known patterns of tiger-leopard dominance dynamics. For example, one such dominance hierarchy was observed in the form of dietary niche separation within and between ERTR and WRTR at spatio-temporal scale. In 2015, relatively higher chital and similar sambar frequencies (two major prey species in the study area) were seen in WRTR compared to ERTR (Table 4.2) but overall distribution of different prey size class (large, medium and small) remained similar. However, in 2020 the WRTR area showed significantly higher large-bodied and lower small-bodied prey species compared to ERTR (as hypothesized in this study). For ERTR (in 2020, spatially as well as temporally), relatively higher frequency of small-bodied prey in leopard diet can be explained through possible competitive spatial exclusion of leopards. During 2020 surveys majority of the leopard faecal samples were obtained from the buffer regions of the park, which are not prime habitats in terms of prey abundances (Harihar *et al.,* 2020). The southern buffer and Gohri range of ERTR (see Figure 4.1) supports lower prey densities (25.24 individuals/ km^2) than the core areas (39.23 individuals/ km^2) (Harihar *et al.,* 2020) and still hosts human settlements and livestock that exert heavy pressure on forest resources (Johnsingh *et al.,* 1994; Harihar *et al.,* 2014; Harihar *et al.,* 2020) and affects the ungulates population density by hindering the forest resource availability to wild prey species (Pozo *et al.,* 2021; Rasal *et al.,* 2022; Salvatori *et al.,* 2022). An alternate explanation behind such pattern could be leopard prey preferences for the medium to small bodied prey size class (Karanth and Sunquist 1995; Pokheral and Wegge 2019), but the natural experimental setup (presence of tiger in ERTR and not in WRTR) allowed us to confirm the effects of competition. The results indicate that leopard consumption of medium to small sized prey in ERTR is not driven by their preference towards these particular classes but rather is a consequence of competition from tiger. Significantly high consumption of large body-sized prey in WRTR (in absence of tiger) supports that such pattern is driven by competition pressure. Earlier Vanak *et al.*, (2013) has also suggested similar pattern of competition (instead of preference) driven dietary niche separation.

These patterns can also be explained through the results of the NDVI analyses presented here. Since the relocation of majority of the human settlements from the eastern and western RTR (from 2003 onwards) and creation of inviolate space the habitat productivity of the park has improved significantly (Harihar *et al.,* 2008). Our NDVI analyses supported this by showing significant increase in vegetation cover across the park (WRTR, ERTR-core and ERTR-buffer) (Figure 4.4a and b). We feel that the habitat improvement (and subsequent increase in prey density, Jhala *et al.,* 2015; 2020) along with no-competition from tigers has resulted in availability of large-bodied prey species in the WRTR. In the ERTR, despite the habitat improvement the population faced competition pressure from tigers over years (tiger density doubled in 2020 in ERTR, Jhala *et al.,* 2020), resulting in their potential displacement to the buffer regions (sub-optimal habitats with medium-sized prey availability). This can be substantiated by observing the faecal location patterns in this study and earlier reports of tiger and leopard occupancy in the park (Harihar *et al.,* 2011; Rathore *et al.,* 2015). Spatial projection of the confirmed ERTR leopard samples showed that most of the 2015 samples were distributed in the core areas whereas majority of the 2020 samples were from the buffer areas (southern boundaries of the core) (Figure 4.1). Given that our sampling was conducted in the same trails in the core region during both years it can be inferred that the leopard presence in ERTR core decreased in 2020, possibly due to competition from socially-dominant tigers (as seen in other Terai habitats, McDougal 1988; Wegge *et al.,* 2009; Odden *et al.,* 2010; Kafley *et al.,* 2019; Thapa *et al.,* 2021). Further, the tiger and leopard population estimation data (Jhala *et al.,* 2020) also suggest more photographic captures of leopards at northern and southern boundaries of the ERTR-core during 2018-19. Earlier, Harihar et al (2011) and Rathore (2015) also reported a decline in leopard density in ERTR-core and their activity hotspots were concentrated towards the peripheral areas of the park as compared to the tiger activity hotspots towards the core areas. On the contrary, leopard faeces were found across more uniformly in WRTR in both sampling periods in the absence of any competition from tigers in this side of the park.

The results of the physiological impacts of dietary differences and habitat productivity largely mimicked the earlier mentioned patterns. Measurements of fGCM and fT3M largely followed the NDVI (proxy for habitat productivity) and prey size class patterns across ERTR and WRTR, respectively (Figure 4.5 a and b). At spatial scale, no significant differences were observed in both fGCM and fT3M between ERTR and WRTR in 2015. This pattern did not support study hypothesis where higher fGCM measures (from tiger competition) and lower fT3M (from possibly reduced prey accessibility) were expected in ERTR. However, in 2020 data show slightly different pattern in physiological measures where the fT3M levels in WRTR was significantly higher than ERTR, but no difference was found in fGCM titers. When looked at temporal scale, these patterns provide contrasting patterns for the physiological measures. Within ERTR no significant difference in fGCM and fT3M values were found between 2015 and 2020 (similar to the spatial patterns) but differences were significant for both measures in WRTR. The temporal-scale data provides strong support to explain the patterns in the light of ecological variables (habitat productivity and resource availability). From the observed data the influences of prey availability (associated with habitat productivity) on fT3M (nutritional hormone) can be inferred as these measures showed very similar patterns. Considering WRTR (both 2015 and 2020) as an example, this habitat showed low NDVI values in 2015 (indicating low habitat productivity at that time due to still recovering phase after human relocation events) and corresponding lower frequency of large-body sized prey in the leopard faeces. However, during 2020 drastic changes were observed in both ecological factors where both NDVI and large prey frequencies have significantly increased. The fT3M measures exactly follow the same patterns (low in 2015 and significantly high in 2020), indicating a clear correlation between ecological variables and associated physiological responses (fT3M in this case). It is important to point out that absence of tiger in WRTR and stark differences in ecological variables in temporal scale made this inference easy compared to ERTR where more complex ecological interactions are seen. Apart from the other two ecological factors mentioned above, competition from tigers play a major role in the patterns of physiological responses in ERTR resulting in different outcomes. Here the data suggests decreasing habitat productivity and large prey frequency in leopard diet from 2015 to 2020, corroborating with a decreasing (but non-significant) trend in fT3M values between these two time periods. During 2015 the tiger density in ERTR was 2.90 (±0.87)/100 km^2 (Jhala *et al.*, 2015) which increased to 8 (± =1.4)/100 km^2 (Jhala *et al.*, 2020) during 2020, and the resulting increase in competition would explain the nutritional stress (low fT3M value) during 2020. Given the complex interactions among various ecological variables (habitat productivity, prey availability (in terms of size class) and various levels of competition pressures (from tigers)), I feel that it might take more time to observe significant differences in physiological parameters in ERTR. A similar study after 3-5 years may provide further clarification on any possible differences in fT3M values in ERTR.

Another possible factor that could play important role in leopard physiology in this landscape is intra-species competition. It is well-documented that the WRTR has experienced an increase in the leopard numbers during the study duration (Jhala et al., 2021), possibly due to absence of wild tigers whereas increasing tiger population was associated with decreasing leopard density in ERTR-core (Harihar et al., 2011). Such increase in population density in WRTR thus can cause some impact in leopard physiology. However, our data do not support this as any such intra-species competition should have resulted in higher fGCM (stress from competition) and lower fT3M (decrease nutritional status in a growing population) in WRTR in 2020 compared to 2015. Combined together, we can interpret that intra-species competition probably has no/less effects in the physiological pattern found during this study duration. However, it is important to point out that presenting the data as a whole (average value of fGCM from all samples collected) might also mask any possible local effects of such competitions. Future studies with fine-scale, grid-based sampling effort with leopard density information within each grid can help to address any effects of such competition.

One of the critical considerations in this study is the significant association of prey body size classes (large, medium and small) with fT3M measures. This was considered based on available knowledge that large terrestrial predator distribution and abundance is significantly driven by higher biomass availability (Carbone *et al.*, 2011) and they are known to prefer large-bodied prey due to higher energy gains (Carbone *et al.,* 1999; 2002; 2007; 2011; Radloff and Du Toit 2004). Work on leopard energetics has also reported increased energy expenditure between meals when meal size from previous kill is large (Wilmers 2017). Data from this study was used to test the effect of prey body size on fT3M measures, where leopard faeces with evidences of only specific body-size classes were identified and the fT3M measures were correlated (Figure 4.6b). In absence of any physiological validation of fT3M measures in leopard (see Mondol *et al.,* 2020 for tiger) this result can also be considered as biological validation of fT3M under field conditions.

The fGCM analyses showed slightly different patterns than the resource-driven fT3M data. Spatially, the ERTR and WRTR did not show any significant differences in fGCM levels in both 2015 and 2020 (Figure 4.5a(iv)) although 2020 fGCM values were relatively lower than 2015 (probably due to increased habitat productivity in 2020, at least for WRTR). However, at temporal scale the ERTR and WRTR showed contrasting patterns (in terms of study hypotheses). In ERTR, a lower value of fGCM was observed in 2020 (non-significant) when compared to 2015. This is surprising when considered that in 2020 the NDVI as well as large prey proportion was lower in ERTR (with corresponding low fT3M indicating higher nutritional stress) (Figure 4.5b). Physiologically this should result in high fGCM (Wasser *et al.,* 2011; 2017; Ayres *et al.,* 2012; Vynne *et al.,*2014; Joly *et al.,*2015; Dias *et al.,*2017; McCormley *et al.,*2018) values but the data shows an opposite pattern. While it is difficult to point out the exact mechanism behind such pattern, one possible reason could be much less competition from tigers in the buffer areas (in 2020). This could be a preferred bargain for the spatially displaced leopards from the core areas of ERTR in 2020 (tiger density 8 (±1.4)/100 km^2 in 2020) at the cost of better nutritional status. Such behavior has been earlier documented in many anti-predatory response studies (Ylönen *et al.,* 2006; Creel *et al.,* 2009; Wasser *et al.,* 2011). WRTR leopards, in absence of any competition exhibited expected physiological responses at temporal scale where higher fGCM (and corresponding high nutritional stress/ low fT3M) in 2015 and low nutritional stress (high fT3M) and low fGCM in 2020 was observed. Both these patterns are also corroborating with the habitat quality and prey size class information in respective time frames (Figure 4.5b). It is important to point out that recent studies have cautioned regarding careful interpretations of GC/fGCM data due to contrasting pattens of directionality in GC response to chronic stress (Dickens and Romero 2013), where both increase and decrease in GC titer has been observed as a consequence of chronic stress. Therefore, this study provides strong evidence of combining additional hormones (such as T3 used here for nutritional stress) along with GC as biomarkers to reveal different physiological regulatory responses to the environment under different contexts. Multidisciplinary approaches, as used in this study would also bring out more comprehensive and ecologically meaningful outcomes that can be used in making appropriate conservation interventions.

Finally, the unique natural experimental habitat scenario, spatio-temporal sampling strategy and the patterns of fGCM and fT3M levels bring out some important conservation perspectives for leopards. Results of this study suggest that from a physiological perspective prey body size (large, medium and small) and availability (driven by habitat productivity) directly affect the dominant-subordinate dynamics, which is further compounded by the competition between both species (resulting in competitive exclusion). This has critical conservation implications for areas surrounding majority of the tiger landscapes across India. India has recently declared doubling its tiger numbers (population estimate of 1411 (1165-1675) in 2006 to 2967 (2603-3346) in 2018) across the country (Jhala *et al.*, 2020). Such increase in tiger population is expected to increase pressure on sympatric leopard populations pushing them towards buffers or more sub-optimal habitats, further exacerbating chances of human-leopard conflict. Therefore, expansion of leopard monitoring and population estimation efforts to buffers, their management in the context of conflicts and understanding of local factors driving the changes in population pattern would be critical for their future conservation. These results highlight the importance of good-quality habitats and prey base for this species and future conservation efforts should ensure availability of the same for their persistence. This study also emphasizes the importance of similar work on other carnivore guilds particularly in the context of the ongoing Anthropocene, which is affecting inter-species dynamics globally.

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Conclusion

Conclusion

Major threat to terrestrial free ranging animals in the age of Anthropocene is loss or fragmentation of their natural habitat (Murphy and Romanuk, 2013; Wolf et al., 2021; Magioli 2021), whose direct and indirect cascading effects on loss of ecological functions along with loss of species have evidently been proven in many research studies (Andrén 1994; Tracewski et al., 2016; Betts et al., 2017; Wolf et al., 2021). Species like tiger and leopards are apex predators and owing to their large size and trophic position require large intact natural spaces with good prey base (Ripple et al., 2014; Wolf and Ripple 2017). They are among top 10 large terrestrial carnivores who have experienced major range contraction (95% of tigers and 79% of leopard historical range lost, see Ripley and Wolf 2017). Their existing populations are restricted to disconnected habitat patches in human modified landscapes (Dinerstein et al., 2006). As natural habitat shrinks these animals face multiple stressors like decreased prey densities, increased negative interspecies interactions, increased chance of losing young ones and increased overall energy demand that may result in physiological dysregulation (Ellis et al., 2012). As growth, body condition, survival and reproduction are regulated by physiological processes it important to measure physiological responses to understand the changing population dynamics of these large carnivores. This thesis attempted to understand impact of disturbance and inter-species competition on stress physiology of tigers and leopards in the Terai-Arc landscape.

Faecal hormone analysis has become a powerful tool due to the variety of stress, nutrition and reproductive measures that can be used to evaluate the magnitude and direction of physiological responses of disturbances (Creel et al., 2002; Gobush et al., 2008; Keay et al., 2006; Wasser et al., 1997, 2005, 2011, 2017). Non-invasive faecal hormone analysis allows easy access of samples at spatio-temporal scales and provide a cumulative and feedback-free measure of circulating hormone concentrations over a longer time period. More importantly it is the only feasible method when dealing with elusive large carnivores in wild. Despite such potential there are many factors that can impact hormone metabolite quantification from faecal samples. Controlling these factors are important to achieve reliable measurements that can be useful for informing management decisions.

First chapter of this thesis mainly deals with standardizing the method for fGCM and fT3M quantification from large felines faeces using enzyme immunoassays. More specifically I looked in if inorganic matter content impacts faecal hormone measurements. It was found that corticosterone and triiodothyronine EIA (arbor assay) can reliably be used to measure fGCM and fT3M from large feline faecal samples (for tiger see Patel et al., 2021, for Asiatic lion see Goswami et al., 2021 and for leopard see Patel et al., 2022). High and variable amount of faecal inorganic matter was found to be a significant factor that confounds hormone metabolite measurements and if not controlled can lead to wrong data interpretations. For large felines I found that dropping samples with high IOM content (>80% IOM) and subsequent expressing of concentrations in per gram of organic dry matter (not total dry matter) eliminates (for fT3M) and reduces (for fGCM) IOM impact. The extent of IOM variability impact on hormone metabolites measures can be species or area specific (free raging or animals from enclosure), hence studies should plan a pilot phase to evaluate the variation in faecal IOM in their respective target study systems before implementing large-scale research projects. Future studies can also explore the extent to which IOM can impact faecal reproductive hormone metabolite measures. The methodological consideration discussed in this chapter will help in accurate physiological assessments (specifically for free ranging large felines) and lead us to ecologically relevant interpretations and recommendations, which are valued driving force for species conservation.

Once the methods for hormone metabolite measurements were standardized, next in chapter two I explored the question of how free ranging tigers physiologically respond to environmental factors in the Terai-Arc landscape. In terms of habitat composition proportion of open forest system in approximate home range of tigers was found to be significantly impacting the nutrition status (fT3M levels). Increased proportion of open forest area facilitates better physiological health. Terai-arc landscape supports a mosaic of wooded forest and grasslands, open alluvial and riparian grasslands of terai are known to support large ungulate populations (Chanchani et al., 2016a). Tiger in open forest areas within protected areas have access to such highly productive habitats, which reflects in higher frequency of elevated fT3M individuals in these areas. However, a reverse pattern was found in forest divisions. It seems that in forest divisions, because of lack of required protection or management, open forest areas do not support productive grasslands instead these areas are more like fallow lands or non-forest areas which possibly are largely exploited by livestocks. Past literature (especially in TAL) repeatedly has emphasized the importance of productive grasslands and their management (Chanchani et al., 2014; 2016a; 2016b) for tiger conservation. In this chapter I provide significant empirical evidence of how tigers physiological status (especially nutritional status) dwindles with decreasing open forest proportion. In this context this study highlights the need of management effort in protection and restoration of open forest systems in forest divisions or buffer areas.

Habitat connectivity was another important factor impacting physiological status. Areas with poor connectivity are degraded with higher proportion of non-forest area and higher occurrence of livestock in tiger diet. These low-quality habitats have lower prey density and supports a smaller number of tigers with overall poor nutritional state. This chapter provides quantitative evidence linking disturbance with physiological health in tigers. Management effort would be required to improve overall habitat condition (by reducing potential sources of disturbance) and efficacy of those practices could be tested using physiological measures in future studies.

Another important result is that tigers expressed higher fGCM levels (showing high psychological stress) in areas of high local tiger density indicating impact of intraspecies competition. It is possible that in such high tiger density areas, tigers experience increased instances of intraspecies agonistic interactions. Many solitary as well as social animals that maintain territory and/or social hierarchy are known to express elevated levels of fGCM in response to competitive interactions (Armitage 1991; Creel 2001). Such interactions can lead to detrimental fights (even infanticide) which in long run could affect local population dynamics (Chanchani et al., 2018). Future studies can look into sex specific physiological responses of intraspecies competition, identify the areas of local pressure and use that information for management planning.

Competition from a dominant intraguild co-predator can be a significant limitation for the socially subordinate species. Leopards, when co-existing with tigers, are often dominated by their larger counterpart in terms of resources (both space and food) (Seidensticker 1976). Large number of studies have focused on exploring different strategies adopted by leopards such as spatio-temporal (Carter *et al.*, 2015; Kafley *et al.*, 2019; Kumar *et al.*, 2019; Pokheral and Wegge 2019; Thapa *et al.*, 2021) and dietary niche segregation (Karanth and Sunquist 1995; Andheria *et al.*, 2007; Harihar *et al.*, 2011; Pokheral and Wegge 2019) for successful co-existence with tigers, but the physiological consequences of such interactions have received less attention. In third chapter of this thesis, I used a large carnivore intraguild system involving tiger (*Panthera tigris*) and leopard (*Panthera pardus*) to understand the dietary and physiological responses under a spatio-temporal gradient of tiger competition pressures in Rajaji Tiger Reserve (RTR) between 2015-2020. The park provided a natural experimental set-up for tiger competition with the eastern part (ERTR) having high tiger density and the western (WRTR) part with functionally no competition from tigers. It was found that leopards in WRTR-2020 showed higher large prey occurrence in diet compared to WRTR-2015 and ERTR-2020. Physiological responses followed dietary responses with better health status (high fT3M and low fGCM) of leopards in WRTR-2020. In presence of competition from tigers, ERTR leopards (largely from suboptimal buffers) showed poor nutritional status. Findings of this chapter indicates that access to large prey has a direct impact on physiological status of leopards and dominant tigers restricts accessibility to these resources. These patterns strongly indicate a physiological cost of sympatry where competition with dominant tigers resulted in elevated nutritional stress. This has critical conservation implications for areas surrounding majority of the tiger landscapes across India. India has recently declared doubling its tiger numbers (population estimate of 1411 (1165-1675) in 2006 to 2967 (2603-3346) in 2018) across the country (Jhala et al. 2020). Such increase in tiger population is expected to increase pressure on sympatric leopard populations pushing them towards buffers or more sub-optimal habitats. We recommend that tiger recovery planning should include cascading impact on local leopard population. Protection and habitat management efforts should be extended to buffers areas which are expected to receive more leopard population as core areas get more occupied by dominant tigers. This study emphasizes the importance of good-quality habitats and prey base for leopard and future conservation efforts should ensure availability of the same for their persistence.

This thesis also highlights the importance of using fT3M measures along with fGCM that has conventionally being more popular as a stress measure in conservation physiology studies. In this study for wild tigers and leopards in TAL, I found fT3M results to be more conclusive than fGCM results, also fT3M measures helped in better interpretation of fGCM results in any given context. This thesis provides strong evidence of combining additional hormones (such as T3 used here for nutritional stress) along with GC as biomarkers to reveal different physiological regulatory responses to the environment under different contexts. Further, multidisciplinary approaches would also bring out more comprehensive and ecologically meaningful outcomes that can be used in making appropriate conservation interventions.

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ANNEXURE- I

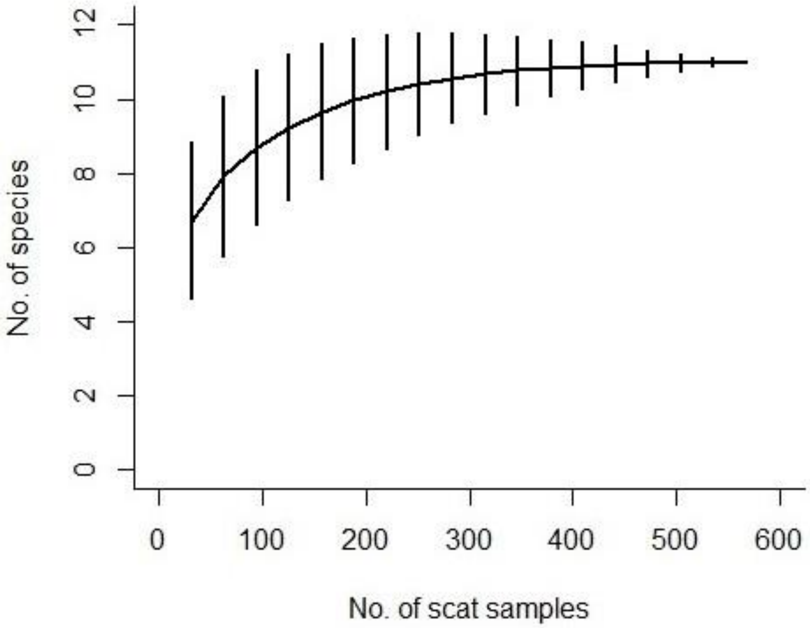

Figure annexure 1. *Rarefaction curve indicating sample sufficiency for diet assessment (Biswas et al., 2022).*

Annexure II

***Table Annexure ii**: Details of Habitat connectivity score calculation for each sampled protected area and forest division of TAL.*

Area	Direction	Connected Area	Corridor conductance	Reference	Connectivity	Connectivity score	Source/ sink	Habitat connectivity score (HCS)
Rajaji TR	E	Lansdowne FD	High**	Jhala et al., 2020; Biswas et al., 2022a	1	2	1	3
	S	Haridwar FD	High**	Biswas et al., 2022a	1			
Lansdowne FD	E	Corbett TR	High**	Jhala et al., 2020; Biswas et al., 2022a	1	2	1	3
	W	Rajaji TR	High**	Jhala et al., 2020; Biswas et al., 2022a	1			
Corbett TR	E	Ramnagar FD	High**	Jhala et al., 2020; Biswas et al., 2022a	1	3	1	4
	W	Lansdowne FD	High**	Jhala et al., 2020; Biswas et al., 2022a	1			
	SW	Amangarh TR, Terai West FD	High**	Jhala et al., 2020; Biswas et al., 2022a	1			
Amangarh TR	W	Terai West FD	High**	Jhala et al., 2020; Biswas et al., 2022a	1	2	0	2
	N	Corbett TR	High**	Jhala et al., 2020; Biswas et al., 2022a	1			
Terai West FD	E	Terai Central FD	Low*	Johnsingh et al., 2004; Biswas et al., 2022a	0.5	2.5	0	2.5
	N	Corbett TR, Ramnagar FD	High**	Jhala et al., 2020; Biswas et al., 2022a	1			
	NW	Amangarh TR	High**	Jhala et al., 2020; Biswas et al., 2022a	1			

Area	Direction	Connected Area	Corridor conductance	Reference	Connectivity	Connectivity score	Source/ sink	Habitat connectivity score (HCS)
Ramnagar FD	E	Haldwani FD	Low*	Biswas et al., 2022a	0.5	3.5	1	4.5
	W	Corbett TR	High**	Jhala et al., 2020; Biswas et al., 2022a	1			
	N	Almora FD and Nainital FD	High**	Jhala et al., 2020	1			
	S	Terai West FD	High**	Jhala et al., 2020; Biswas et al., 2022a	1			
Haldwani FD	E	Suklaphanta NP (Boom-Brahmadev corridor)	Occasionally used*	Johnsingh et al., 2004; Chanchani et al., 2014	0.5	3	0	3
	W	Ramnagar FD, Terai Central FD	Low*	Biswas et al., 2022a	0.5			
	S	Terai East FD	High**	Jhala et al.,2020; Biswas et al., 2022a	1			
	NE	Champawat FD	High**	Jhala et al., 2020; Biswas et al., 2022a	1			
Champawat FD	S	Haldwani FD	High**	Jhala et al., 2020; Biswas et al., 2022a	1	1	0	1
Terai Central FD	W	Terai West FD	Low*	Johnsingh et al., 2004; Biswas et al., 2022a	0.5	1	0	1
	NW	Ramnagar FD	Low*	Johnsingh et al., 2004; Biswas et al., 2022a	0.5			
Terai East FD	N	Haldwani FD	High**	Biswas et al., 2022a	1	2	0	2

Area	**Direction**	**Connected Area**	**Corridor conductance**	**Reference**	**Connectivity**	**Connectivity score**	**Source/ sink**	**Habitat connectivity score (HCS)**
	SE	Pilibhit TR	High**	Anwar and Borah 2020; Jhala et al., 2020; Biswas et al., 2022a	1			
Pilibhit TR	NE	Suklaphanta NP (Laggabagga-Tatarganj corridor)	frequently used**	Chanchani et al., 2014; Thapa et al., 2017	1	3	1	4
	NW	Terai East FD	High**	Anwar and Borah 2020; Jhala et al., 2020; Biswas et al., 2022a	1			
	SW	Kishanpur WLS	frequently used**	Chanchani et al., 2014	1			
Dudhwa NP	N	Suklaphanta NP (Laljihadi corridor)	Occasionally used*	Chanchani et al., 2014	0.5	1	1	2
	NE	Bardia NP (Basanta corridor)	Occasionally used*	Chanchani et al., 2014; Joshi et al., 2016	0.5			
Kishanpur WLS	N	Pilibhit TR	High**	Qureshi et al., 2014; Chanchani et al., 2016a; Biswas et al., 2022a	1	1	1	2
Katerniaghat WLS	N	Bardia NP (Khata corridor)	frequently used**	Chanchani et al., 2014; Thapa et al., 2017; Joshi et al., 2016	1	1	1	2
Sohagibarwa WLS	E	Valmiki TR	Occasionally used*	Chanchani et al., 2014	0.5	0.5	0	0.5
Valmiki TR	E	Parsa NP	frequently used**	Chanchani et al., 2014; Thapa et al., 2017	1	2.5	1	3.5

Area	Direction	Connected Area	Corridor conductance	Reference	Connectivity	Connectivity score	Source/ sink	Habitat connectivity score (HCS)
	W	Sohagibarwa WLS	Occasionally used*	Chanchani et al., 2014	0.5			
	N	Chitwan NP	frequently used**	Chanchani et al., 2014; Thapa et al., 2017	1			
Haridwar FD		Rajaji TR	High**	Biswas et al., 2022a	1	1	0	1
Najibabad FD		Corbett TR	High**	Biswas et al., 2022a	1	1	0	1
Pilibhit SFD		Pilibhit TR	Low*	Biswas et al., 2022a	0.5	0.5	0	0.5
North Kheri FD		Kisanpur WLS	Low*	Biswas et al., 2022a	0.5	0.5	0	0.5
South Kheri FD		Kisanpur WLS	Low*	Biswas et al., 2022a	0.5	0.5	0	0.5

TR- Tiger Reserve, NP- National Park, FD- Forest Division, SFD- Social Forestry Division, WLS- Wildlife Sanctuary, N-North, S-South, E-East, W-West, NE-North east, NW-North west, SE-South east, SW-South west, ** High conductance or frequently used area, * low conductance or occasionally used area.

Annexure III

Table annexure iii: *Tiger and prey density of protected areas and forest divisions of TAL as reported in latest all India tiger report (Jhala et al., 2020).*

Area	**Tiger density (tigers/100km2)**	**Unique tigers captured**	**Prey density (individuals/km2)**
RTR	8	37	107.83
LFD	4.36	34	
CTR	14	231	117.91
ATR	2.06	20	
TWFD	6.23	39	
RFD	4.13	37	
HLF	3.18	23	
CFD		9	
TCFD		5	
TEFD	3.3	23	
PTR	6.6	57	68.86
DNP	2.3	20	66.77
Kishanpur (KWLS)	4.21	33	
Katerniaghat (KGWLS)	4.6	29	74.98
SKFD		2	
NKFD			10.35
Sohagibarwa (SGWLS)		5	
VTR	2.5	33	

Annexure IV

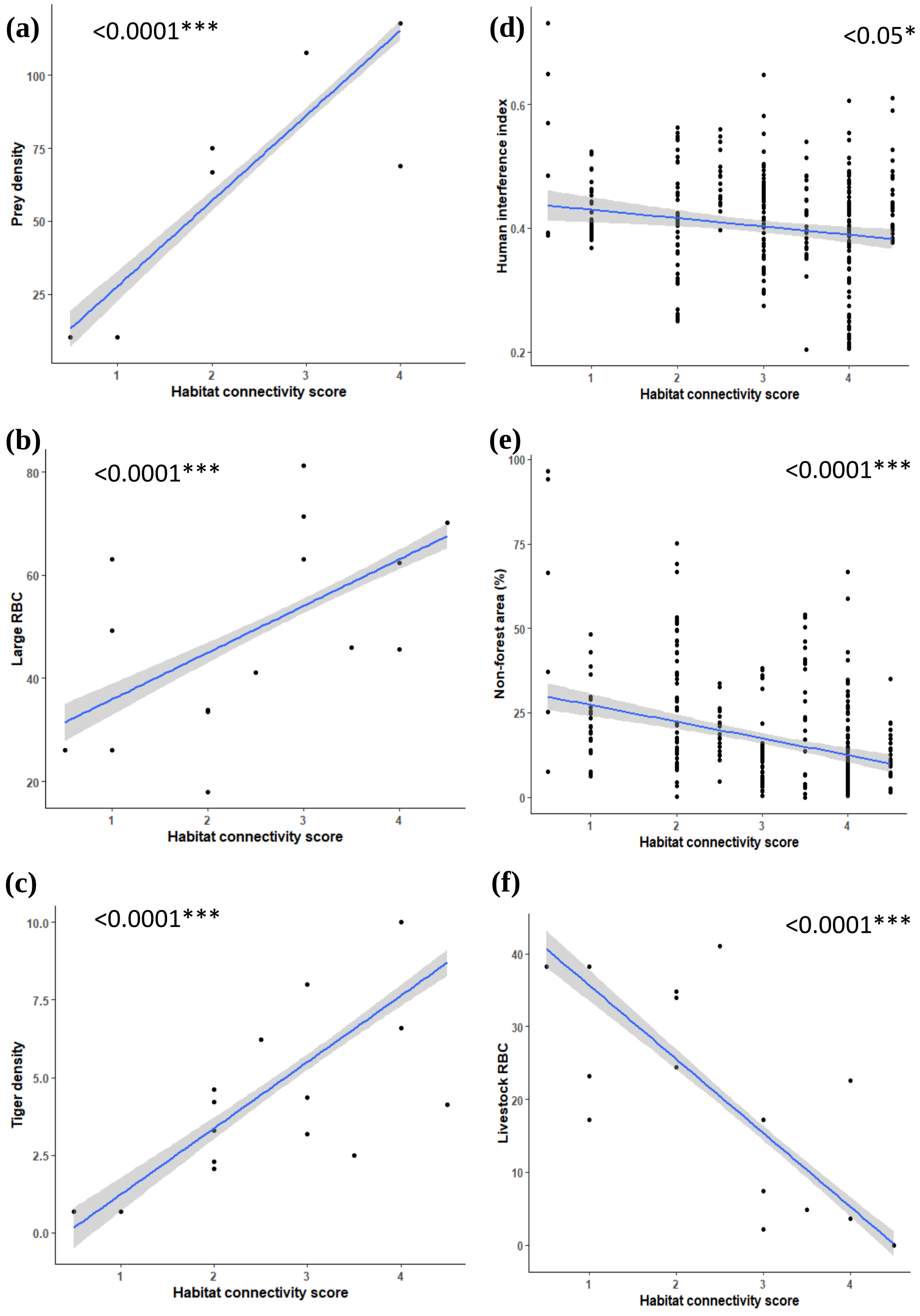

Figure annexure iv. *Plots of linear regression models showing positive association (left panel) of habitat connectivity score with prey density (a), relative biomass consumed of large prey (b), tiger density (c) and negative association with human interference index (d), non-forest area (e) and relative biomass consumed of livestock (f).*

Annexure V

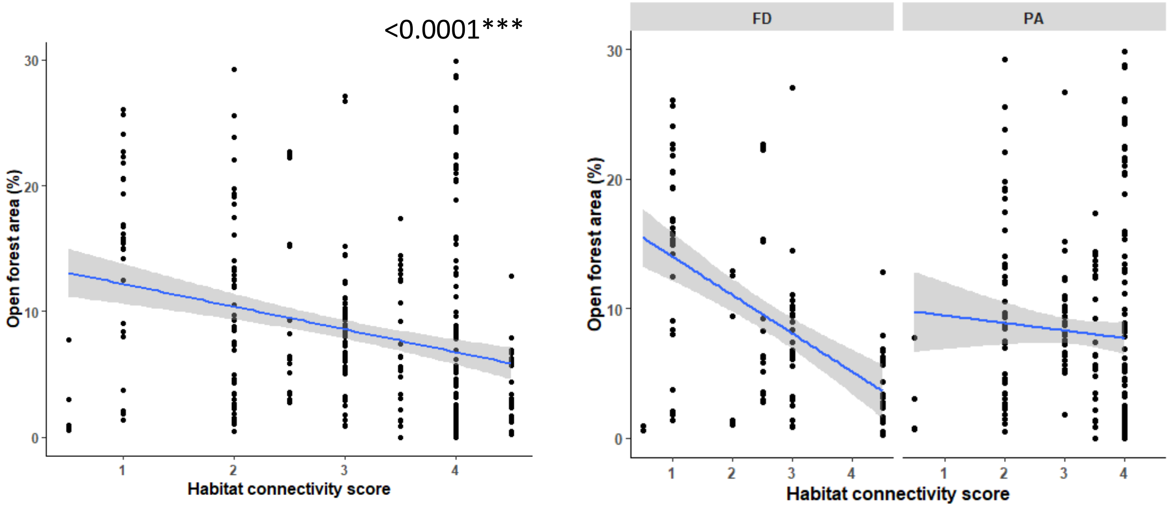

Figure annexure v. *Plots of linear regression model (left) and with an interaction factor (right) showing negative association of habitat connectivity score with open forest area (left) in forest divisions (FD).*

Annexure VI

Table annexure vi. *Results showing the output of multivariate GLM, showing the best selected models.*

Models tested for fGCM	**(Intercept)**	**family**	**df**	**logLik**	**AICc**	**delta**	**weight**
Area*Year + mean NDVI	153.827	Gamma(log)	6.00	-1902.531	3817.491	**0.000**	**0.616**
Area*Year	192.982	Gamma(log)	5.00	-1904.893	3820.091	**2.600**	**0.168**
Area*Year + mean NDVI + Prey size	161.675	Gamma(log)	9.00	-1901.083	3821.098	3.607	0.101
mean NDVI	7.116	Gamma(log)	3.00	-1908.311	3822.742	5.251	0.045
Area*Year + Prey size	198.106	Gamma(log)	8.00	-1903.325	3823.392	5.900	0.032
Null	8.409	Gamma(log)	2.00	-1909.977	3824.014	6.522	0.024
mean NDVI + Prey size	7.160	Gamma(log)	6.00	-1906.692	3825.813	8.322	0.010
Prey size	8.573	Gamma(log)	5.00	-1908.458	3827.220	9.728	0.005
Models tested for fT3M	**(Intercept)**	**family**	**df**	**logLik**	**AICc**	**delta**	**weight**
Area*Year + mean NDVI + Prey size	167.658	Gamma(log)	9.00	-1663.959	3346.851	**0.000**	**0.431**
Area*Year + mean NDVI	173.442	Gamma(log)	6.00	-1667.307	3347.042	**0.191**	**0.391**
Area*Year + Prey size	136.794	Gamma(log)	8.00	-1666.385	3349.513	2.662	0.114
Area*Year	143.632	Gamma(log)	5.00	-1670.437	3351.178	4.327	0.049
Prey size	6.737	Gamma(log)	5.00	-1672.294	3354.893	8.042	0.008
mean NDVI + Prey size	7.662	Gamma(log)	6.00	-1671.347	3355.123	8.272	0.007
mean NDVI	8.296	Gamma(log)	3.00	-1678.505	3363.130	16.279	0.000
Null	7.278	Gamma(log)	2.00	-1679.688	3363.437	16.586	0.000

Annexure VII

Permits and ethical clearance:

All required permissions for field surveys and biological sampling were provided by Forest Departments of Uttarakhand (Permit no: 90/ 5–6 and Permit no: 3707/ 5–6), Uttar Pradesh (Permit no: 1127/23–2-12(G) and 1891/23–2-12) and Bihar (Permit no: Wildlife-589), respectively. Due to non-invasive nature of work, no further ethical clearance was required for this study.

Annexure VIII

Conference certificates:

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Society for Conservation Biology

CERTIFICATE OF

PRESENTATION

THIS CERTIFICATE IS AWARDED TO

Shiv Patel

For presenting: Cost of Co-existence: behavioral and physiological constraints on leopards in presence and absence of competition from tigers

on December 15, 2021
at the 2021 International Congress for Conservation Biology

Antony J. Lynam

Tony Lynam
President, Society for Conservation Biology

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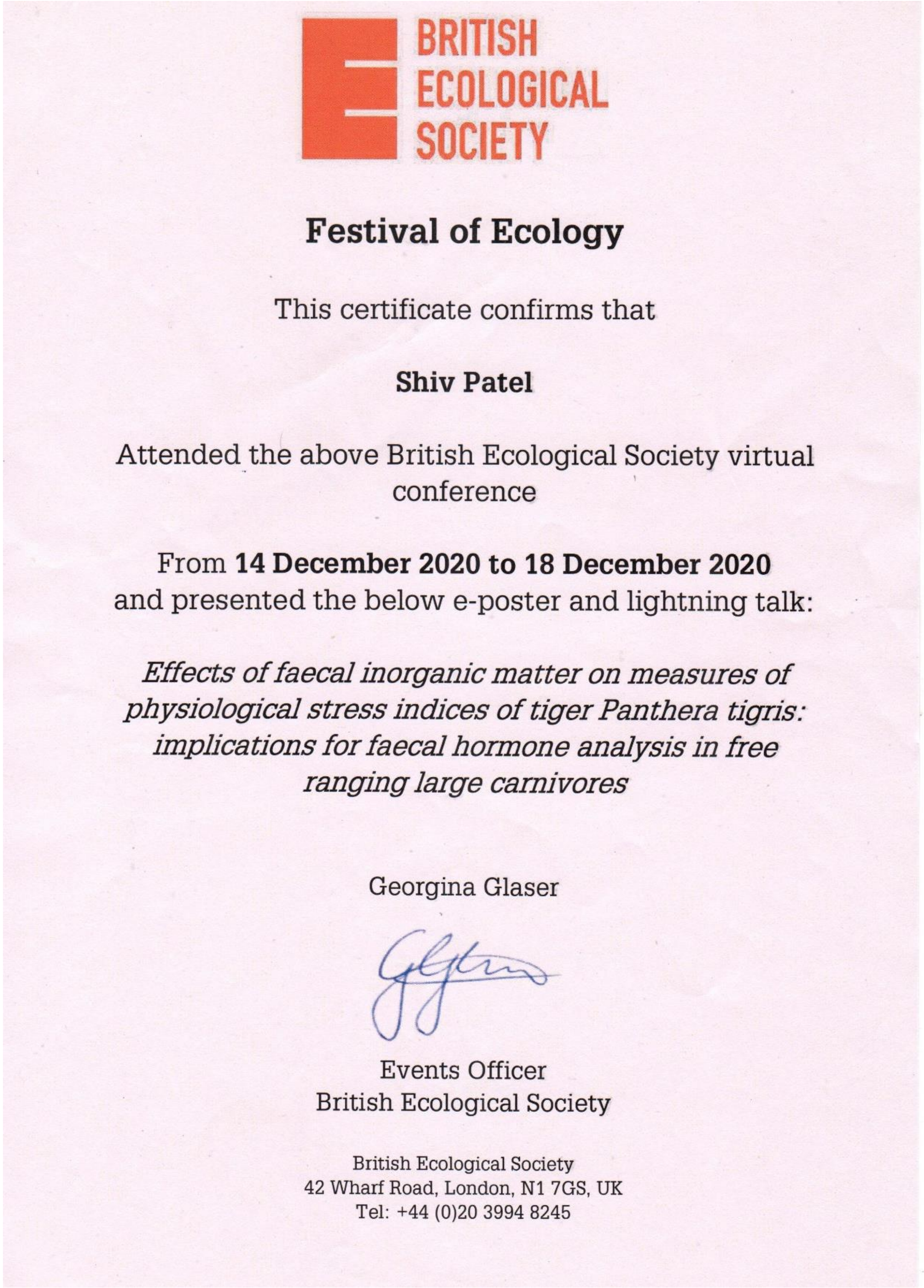

BRITISH
ECOLOGICAL
SOCIETY

Festival of Ecology

This certificate confirms that

Shiv Patel

Attended the above British Ecological Society virtual conference

From **14 December 2020 to 18 December 2020** and presented the below e-poster and lightning talk:

Effects of faecal inorganic matter on measures of physiological stress indices of tiger Panthera tigris: implications for faecal hormone analysis in free ranging large carnivores

Georgina Glaser

Events Officer
British Ecological Society

British Ecological Society
42 Wharf Road, London, N1 7GS, UK
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CERTIFICATE OF PRESENTATION

This is to certify that

Shiv Kumari Patel

participated with a Poster at the 7th Conference of the International Society of Wildlife Endocrinology, 13-16 October 2019, Skukuza, Kruger National Park, hosted by the Mammal Research Institute of the University of Pretoria and South African National Parks

"Understanding stress physiology of tigers in the Terai-Arc landscape (TAL), India - a pilot study."

PROF A. GANSWINDT
Director Mammal Research Institute
University of Pretoria
Chair (ISWE)
Vice President (ZSSA)

DR D. KOESTER
Research Curator
Cleveland Metroparks Zoo
Conference Chair (ISWE)

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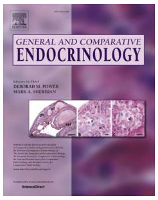

Research paper

Effects of faecal inorganic content variability on quantifying glucocorticoid and thyroid hormone metabolites in large felines: Implications for physiological assessments in free-ranging animals

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ABSTRACT

Faecal glucocorticoid (GC) and triiodothyronine (T3) metabolites and their interactions are increasingly used to monitor perceived stress and nutritional challenges in free-ranging animals. However, a number of extrinsic and intrinsic factors including hormone-inert dietary materials, inorganic matters etc. are known to affect reliable hormone metabolite quantifications. In this study, the impacts of inorganic matter (IOM) on faecal GC (fGCMs) and T3 (fT3Ms) metabolite measure were addressed in wild tiger ($n = 193$ from Terai Arc landscape, India) and captive lion ($n = 120$ from Sakkarbaug Zoological Garden, Gujarat, India) and possible corrective measures were evaluated. The wild tiger samples contained highly variable IOM content (9–98%, mostly with $> 40\%$ IOM) compared to captive Asiatic lion (17–57%, majority with $< 40\%$ IOM). Significant correlations were observed between IOM content and tiger fGCM ($r = -0.46$, $p = 0.000$), fT3M ($r = -0.58$, $p = 0.000$) and lion fT3M measures ($r = -0.43$, $p = 0.003$). Two corrective measures viz. removing samples with $\geq 80\%$ IOM and subsequently expressing concentrations as per gram of organic dry matter (instead of total dry matter) reduced IOM influence on tiger fGCM, fT3M and lion fT3M, without affecting lion fGCM measures. The corrective measures changed the interpretations of fT3M data of field-collected tiger samples with no significant changes in fGCM (both tiger and lion) and fT3M (lion) data. As faecal IOM content is common in many wild species, the results emphasize the need to reduce IOM-driven hormone data variation for ecologically relevant interpretations towards species conservation.

1. Introduction

The ever-increasing impacts of humans on wildlife through habitat destruction and other environmental alterations call for various reliable indices that can monitor temporal and spatial variations in physiological responses of free ranging animals. Faecal hormone analysis has become a powerful tool in this regard due to the variety of stress, nutrition and reproductive measures that can be used to evaluate the magnitude and direction of physiological responses of disturbances (Creel et al., 2002; Gobush et al., 2008; Keay et al., 2006; Wasser et al., 1997, 2011, 2017; Wasser and Hunt, 2005). Such non-invasive approach allow easy access of samples at spatial and temporal scales and provide a cumulative and feedback-free measure of circulating hormone concentrations over a longer time period (Brown et al., 1996; Graham and Brown, 1996; Palme et al., 1996, 2005) when compared with blood (Sheriff et al., 2010). The interactive effects of these stress, nutrition and reproductive hormones also allow relating wildlife health measures and adaptations to environmental changes in response to various disturbances (Ayres et al., 2012; Corkeron et al., 2017; Gobush et al., 2008; Hayward et al., 2011; Hunt et al., 2006; Mondol et al., 2020). For example, glucocorticoid (GC) concentrations rapidly increase in response to wide range of short or long-term psychophysiological stressors (Dallman and Bhatnagar, 2001; Wingfield and Romero, 2010), whereas the bioactive thyroid hormone triiodothyronine (T3) acts more slowly, altering metabolism in response to acute or chronic nutritional stress (Eales, 1988; Flier et al., 2000). When studied together, psychological and nutritional hormone metabolites provide complementary information on an animal's coping mechanism under different ecological or environmental conditions at different scales. Despite such potential of the non-invasive endocrinology approaches in wildlife conservation, reliable measurements of

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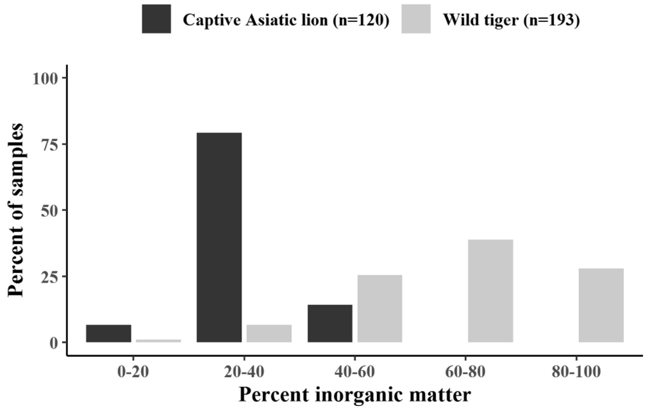

Fig. 1. Comparison of Inorganic matter (IOM) content in faecal samples of field-collected wild tigers and captive Asiatic lions. The samples were categorized into five groups.

faecal hormone metabolites are often challenging. A myriad of extrinsic and intrinsic factors are known to affect measures of various faecal hormone metabolites including sampling and storage methods (Hunt and Wasser, 2003; Khan et al., 2002; Lafferty et al., 2019; Millspaugh et al., 2003; Wilkening et al., 2016), species and sex-specific hormone metabolite compositions (Brown et al., 1994; Goymann, 2012; Young et al., 2004), and appropriate laboratory protocols for hormone extraction and assays (Davidian et al., 2015; Gholib et al., 2018; Millspaugh and Washburn, 2003; Nugraha et al., 2017; Pappano et al., 2010; Watson et al., 2013). The influences of these major factors in physiological assessments and their technical solutions have been the major focus of research over years (Millspaugh and Washburn, 2004; Palme et al., 2013; Palme, 2019; Sopinka et al., 2015; Touma and Palme, 2005). However, alterations introduced by dietary differences in free ranging animals that changes faecal composition and output have received less attention (Goymann, 2012; Von der Ohe and Servheen, 2002). To this end, undigested parts of the diet (e.g., dietary fiber) and other inorganic matters (e.g., urates, soil) that do not contribute hormone metabolites have been recognized to influence estimates of multiple steroid hormones in various species (Ganswindt et al., 2012; Wasser and Hunt, 2005). Studies suggest that these hormone-inert parts of faecal matter decreases hormone metabolite concentrations during extraction (Wasser and Hunt, 2005) and introduce errors in measuring physiological biomarkers for stress (for example, see Ganswindt et al., 2012 for aardwolves; Goymann, 2005 for stone chats; Wasser and Hunt, 2005, Hayward et al., 2010 for owls etc.) and reproductive status (for example, see Goymann, 2005 for stone chats; Wasser et al., 1993 for baboons; Wasser and Hunt, 2005 for owls etc.). Such effects seem to be more pronounced in free-ranging animals, possibly due to large variations in species-specific food quantity and quality in the wild compared to captivity (Dierenfeld et al., 1994; Tilson et al., 2016). If unaccounted, these variations can result in inaccurate assessment of stress- (GC) and nutrition-related (T3) hormones, resulting in misguided conservation efforts.

In this paper, we addressed key methodological issues to examine the impacts of hormone-inert inorganic matters (henceforth IOM) on measures of stress (GC) and nutritional (T3) hormones in wild tigers in the Terai-Arc landscape, north India. The tiger (*Panthera tigris*) typifies global international conservation efforts across their range countries (Sanderson et al., 2010). Decades of intense conservation efforts have resulted in doubling their population in India (Jhala et al., 2015; 2020), and their future persistence will depend on managing these increasing population within the existing habitat (Gubbi et al., 2016, 2017). It is expected that the increasing tiger density will have distinct physiological impacts, particularly perceived stress (from intra-specific competition and anthropogenic disturbances), nutritional challenges (wild prey depletion) and possibly reproductive effects. Tigers occupy a wide variety of land cover types (Jhala et al., 2020) with varying dietary regimes (Basak et al., 2018; Harihar et al., 2011; Kumar et al., 2008) and their faeces is known to contain significant amount of soil and other inorganic matter (Khan, 2004; Schaller, 1967), thereby making this an informative system to study impacts of these materials on hormone quantification. While other studies have focused on stress status measurements in wild tigers in India (Bhattacharjee et al., 2015; Malviya et al., 2018; Naidenko et al., 2019; Tyagi et al., 2019), they have not looked at the impacts of inorganic matters on measurement of faecal glucocorticoid (fGCMs) and T3 metabolites (fT3Ms). In addition, we used another large feline carnivore species, the Asiatic lions (*Panthera leo persica*) housed under captive environment where differences in dietary regimes are generally negligible and hence variations in inorganic matter content and its resulting influence on hormone measurements are expected to be low. Our main objectives were: (1) estimate the amount of IOM present in faecal samples from wild tigers and captive lions and evaluate their variations; (2) assess the impacts of IOM in measures of fGCMs and fT3Ms and explore approaches to minimize the effects; and (3) evaluate how errors introduced by IOM variability, if any, can affect the ecological interpretations of fGCM and fT3M measures in both species. We believe that our results have wider relevance for non-invasive endocrinology studies of wild animals, particularly carnivores with variable inorganic matter contents in their faeces.

2. Materials and methods

2.1. Research permissions and ethical considerations

All required permissions for field surveys and biological sampling were provided by Forest Departments of Uttarakhand (Permit no: 90/5–6), Uttar Pradesh (Permit no: 1127/23–2-12(G) and 1891/23–2-12) and Bihar (Permit no: Wildlife-589), respectively. Due to non-invasive nature of work, no further ethical clearance was required for this study.

2.2. Study area

Wild tiger faeces have been collected from five major protected areas in the Terai-Arc landscape (TAL) in India. This linear landscape of TAL contains a total of 20800 km^2 of potential tiger habitat, covering the states of Uttarakhand, Uttar Pradesh and Bihar (Qureshi et al., 2006). Situated at the foothills of the Himalayas, this region supports tropical moist deciduous forests dominated by Sal (*Shorea robusta*), tall Terai swamp grasslands and permanently moist reed swamps (Champion and Seth, 2005). This landscape is identified as 'Global priority' tiger conservation landscape (Sanderson et al., 2010) and retains about 22% of the India's wild tiger population (Jhala et al., 2020). Our sampling was mostly concentrated inside five main tiger reserves in this landscape: Rajaji and Corbett Tiger Reserves in Uttarakhand (western TAL), Pilibhit and Dudhwa Tiger Reserves in Uttar Pradesh (central TAL) and Valmiki Tiger Reserve in Bihar (eastern TAL).

The captive Asiatic lion faecal sampling was conducted at the largest Asiatic lion conservation-breeding center at Sakkarbaug Zoological Garden (SZG), Gujarat. SZG hosts the largest captive population (N = 60) with the highest reported number of wild founders (Srivastav et al., 2018). We collected faeces from 35 individuals housed at the off-display conservation-breeding center of SZG. The same samples were used in an earlier study to evaluate the effects of enrichment interventions on behaviour and stress measures (see details in Goswami et al., 2021). During the enrichment intervention, the animals were randomly assigned to control (n = 16) and test (n = 19) group to ascertain the physiological stress status during pre- and post-enrichment periods.

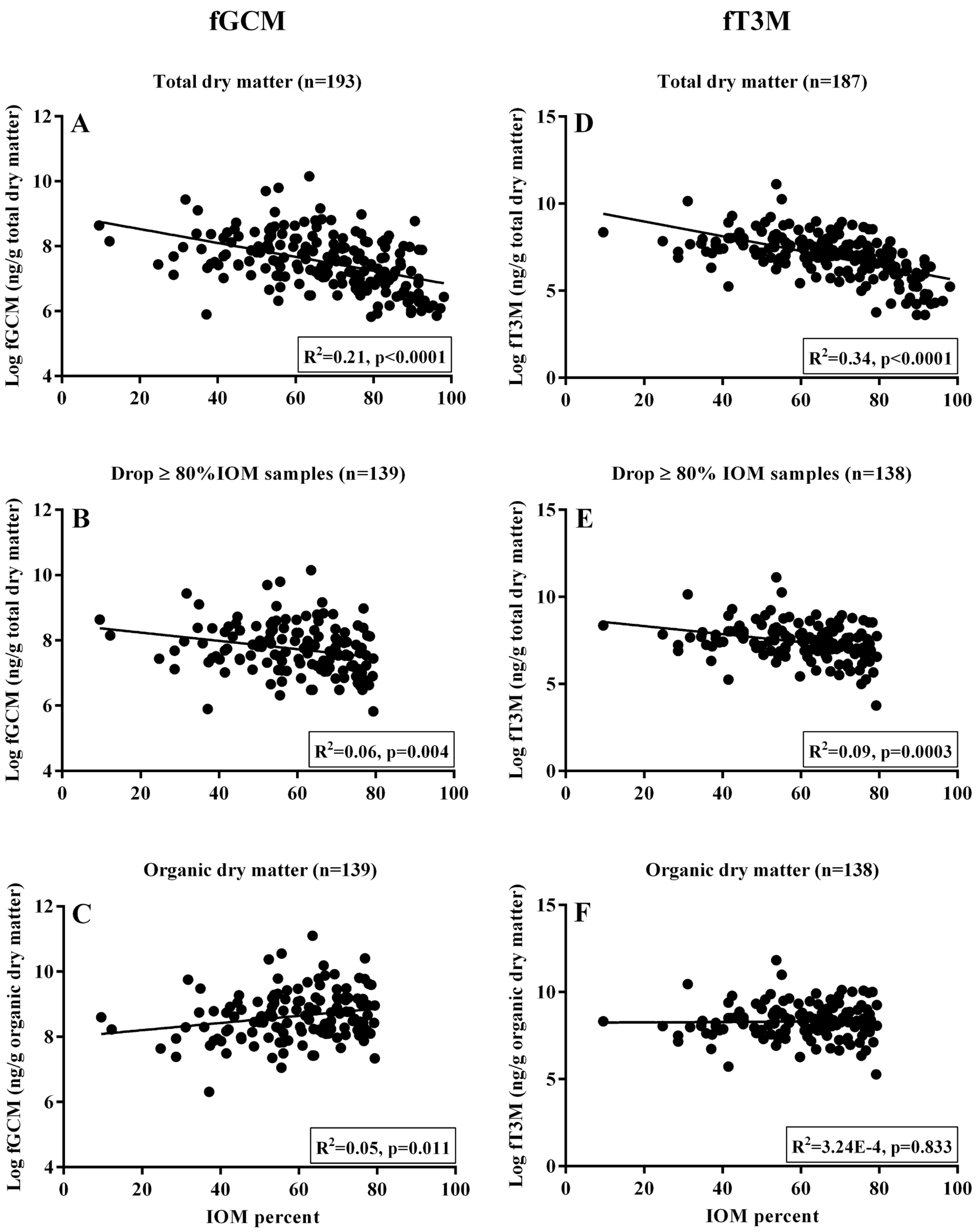

Fig. 2. Relationship between percentages of faecal IOM and log-transformed fGCM and fT3M concentrations. Figure A and D represents results when hormone concentrations are expressed as per gram of total dry matter. Figure B and E shows results after dropping samples with high IOM contents (>80%). Figure C and F shows results when subsequently hormone concentrations are expressed as per gram of organic dry matter.

2.3. Faecal sample collection, species identification and hormone extraction

A team of experienced field personnel surveyed forest trails of aforementioned protected areas (see Bhatt et al., 2020) and collected tiger faecal samples that were visibly fresh, intact with minimal insect activities and contained strong odour (Vynne et al., 2014), during winter seasons of 2016–2018. Faecal samples were stored in −20 °C freezer till DNA extraction (Biswas et al., 2019). Tiger-specific mitochondrial DNA markers (Mukherjee et al., 2007) were used to identify tiger faeces and only confirmed tiger faecal samples were used for downstream hormone and inorganic matter analyses. A total of 193 tiger faecal samples were

Table 1
Mean fGCM and fT3M levels estimated in compared groups of wild tigers (protected area wise) and captive Asiatic lions across different corrective methods.

Compared groups	Concentrations as total dry matter	Samples with > 80% IOM dropped	Concentrations as organic dry matter
Mean Log fGCM (ng/g) for wild tigers			
RTR	Mean = 7.62, SD = 0.84, n = 57	Mean = 7.90, SD = 0.77, n = 36	Mean = 8.78, SD = 0.72, n = 36
CTR	Mean = 7.42, SD = 0.79, n = 50	Mean = 7.61, SD = 0.77, n = 35	Mean = 8.57, SD = 0.79, n = 35
DTR-PTR	Mean = 7.51, SD = 0.73, n = 52	Mean = 7.65, SD = 0.62, n = 45	Mean = 8.51, SD = 0.59, n = 45
VTR	Mean = 7.49, SD = 1.04, n = 34	Mean = 7.86, SD = 0.95, n = 23	Mean = 8.76, SD = 0.96, n = 23
Mean Log fT3M (ng/g) for wild tigers			
RTR	Mean = 6.80, SD = 1.36, n = 53	Mean = 7.38, SD = 1.11, n = 36	Mean = 8.26, SD = 0.99, n = 36
CTR	Mean = 7.28, SD = 1.02, n = 50	Mean = 7.70, SD = 0.78, n = 35	Mean = 8.67, SD = 0.77, n = 35
DTR-PTR	Mean = 6.84, SD = 1.17, n = 52	Mean = 7.06, SD = 0.99, n = 45	Mean = 7.92, SD = 0.89, n = 45
VTR	Mean = 7.06, SD = 1.55, n = 32	Mean = 7.69, SD = 1.27, n = 22	Mean = 8.62, SD = 1.23, n = 22
Mean Log fGCM (ng/g) for captive Asiatic lions			
Control	Mean = 8.73, SD = 0.47, n = 32	Not applied	Mean = 9.09, SD = 0.47, n = 32
Test	Mean = 7.73, SD = 0.43, n = 50	Not applied	Mean = 8.09, SD = 0.46, n = 50
Mean Log fT3M (ng/g) for captive Asiatic lions			
Pre	Mean = 7.50, SD = 0.39, n = 12	Not applied	Mean = 7.82, SD = 0.29, n = 12
Post	Mean = 7.51, SD = 0.32, n = 12	Not applied	Mean = 7.89, SD = 0.30, n = 12

RTR-Rajaji tiger reserve, CTR-Corbett tiger reserve, DTR-PTR- Dudhwa and Pilibhit tiger reserve, VTR-Valmiki tiger reserve

used in this study: Rajaji Tiger Reserve (RTR, n = 57), Corbett Tiger Reserve (CTR, n = 50), Dudhwa-Pilibhit region (DTR-PTR, n = 52) and Valmiki Tiger Reserve (VTR, n = 34). For lions, a total of 120 fresh faecal samples were collected from the SZG conservation breeding center over a period of three months. Two fresh faecal samples per week from each individual were collected during the enrichment experiment duration (covering both pre- and post-enrichment periods). The samples were stored in −20 °C freezer till further processing.

For hormone extraction from tiger (n = 193) and lion (n = 120), a modified hormone extraction protocol described in Wasser et al. (2010) was followed. Each frozen sample was broken down to smaller pieces and dried up to 72 h in an oven (#Unilab-112HO, Haryana, India) at 60 °C prior to hormone extraction to control for moisture (Wasser et al., 1993). Dried samples were then pulverized and sieved through a 0.5 mm steel mesh strainers to remove prey remains/other hard parts and obtain faecal powder. The dried faecal powder was thoroughly mixed and subsequently hormone extraction was performed by pulse-vortexing 0.1 g of powder in 15 ml of 70% ethanol, followed by centrifugation at 2200 rpm for 20 min (Mondol et al., 2020; Wasser et al., 2010). The hormone extracts were collected in 2 ml cryochill vials (1:15 dilution) and stored in −20 °C freezer till further analyses. A total of 193 and 187 samples were used for tiger fGCM and fT3M analyses, respectively. For captive Asiatic lions, a total of 120 (from 35 individuals) and 46 samples (from 12 individuals) were used in fGCM and fT3M analyses, respectively.

2.4. Estimation of inorganic/organic content

To estimate inorganic/organic content in each faeces (both tiger as well as Asiatic lion), a slightly modified protocol described in Ganswindt et al. (2012) was used. About 0.1 g (same weight as used for extraction) of dried faecal powder was measured in a crucible and ashed the powder in a muffle furnace (#NSW-101, NSW, New-Delhi, India) at 550 °C for 2 h. Post-combustion, the residual IOM was weighed and amount of organic matter (OM) combusted in each sample was calculated using the formula: Organic matter = (0.1- weight of inorganic matter) grams.

2.5. fGCM and fT3M assays

Corticosterone (#K014, Arbor Assays, MI, USA) and Triiodothyronine (T3) (#K056, Arbor Assays, MI, USA) EIA kits were used to measure fGCMs and fT3Ms from tiger and lion faecal extracts. Physiological validation for the corticosterone EIA kit was conducted by quantifying fGCMs in sample extracts of tiger faeces collected before and after ACTH challenge study conducted in Mondol et al. (2020). Assays showed 2.5-fold increase in fGCM titers post ACTH challenge followed by gradual decrease in subsequent samples (see Supplementary Fig. 1). For lions, biological validation of fGCM was earlier conducted by Goswami et al. (2021) where the levels of fGCM were measured during pre- and post-enrichment interventions under captive conditions (see Supplementary Fig. 2). Although the T3 EIA kit has not been physiologically/biologically validated for tiger and lion, it has been earlier successfully used in many terrestrial and marine mammals (Ajó et al., 2020; Kozlowski et al., 2020; Szott et al., 2020).

During assays, sample extracts were air-dried inside an incubator (#ATI-117, Obromax, Delhi, India) and re-suspended in assay buffer as per required dilutions. Each sample was assayed in duplicate using respective kit protocols and the optical density was measured at 450 nm using GMB-580 automatic microplate ELISA plate reader (#GMB-580, Genetix Biotech Asia, New Delhi, India). Hormone metabolite concentration is interpolated using four parametric logistic (4PL) regression function of GraphPad prism version 5 (GraphPad Software, California, USA). Cross-reactivities of respective antibodies are listed in Supplementary table 1.

2.6. Parallelism and accuracy

Parallelism and accuracy tests were used for laboratory validation of GC and T3 hormone antibodies for both species. Parallelism tests for fGCM and fT3M were conducted using dilutions of pooled tiger and lion faecal extracts from multiple random samples (n = 20) to assess reliable quantification of respective hormone metabolites at different concentrations and to find optimal dilutions for final assays (at 50% binding). For both hormones, species-wise sigmoid curves were generated as relative dose vs. percent bound hormones for the pools and the standards, where parallel slopes indicate better antibody binding at different concentrations. Subsequently, accuracy tests were performed for both assays to determine any interference in faecal extracts during antibody interactions. Independently both hormone standards were spiked with equal volumes of diluted faecal extract of known concentration (dilution level close to 50% bound from parallelism test) and assayed with standards. Results were plotted as regression lines using observed and expected concentrations from accuracy tests to show that faecal components were not interfering with assay accuracy at the tested dilution. Inter- and intra- assay coefficients of variation were determined using repeated measures of same-pooled extract. Parallelism results were examined using an F ratio test for differences in slopes. Accuracy results were evaluated using analysis of linear regression. All analysis for parallelism and accuracy were performed in GraphPad prism version 5 (GraphPad Software, California, USA).

Parallelism and accuracy tests for tiger fGCM and fT3M indicated reliable measures across different concentration ranges. Serial dilutions

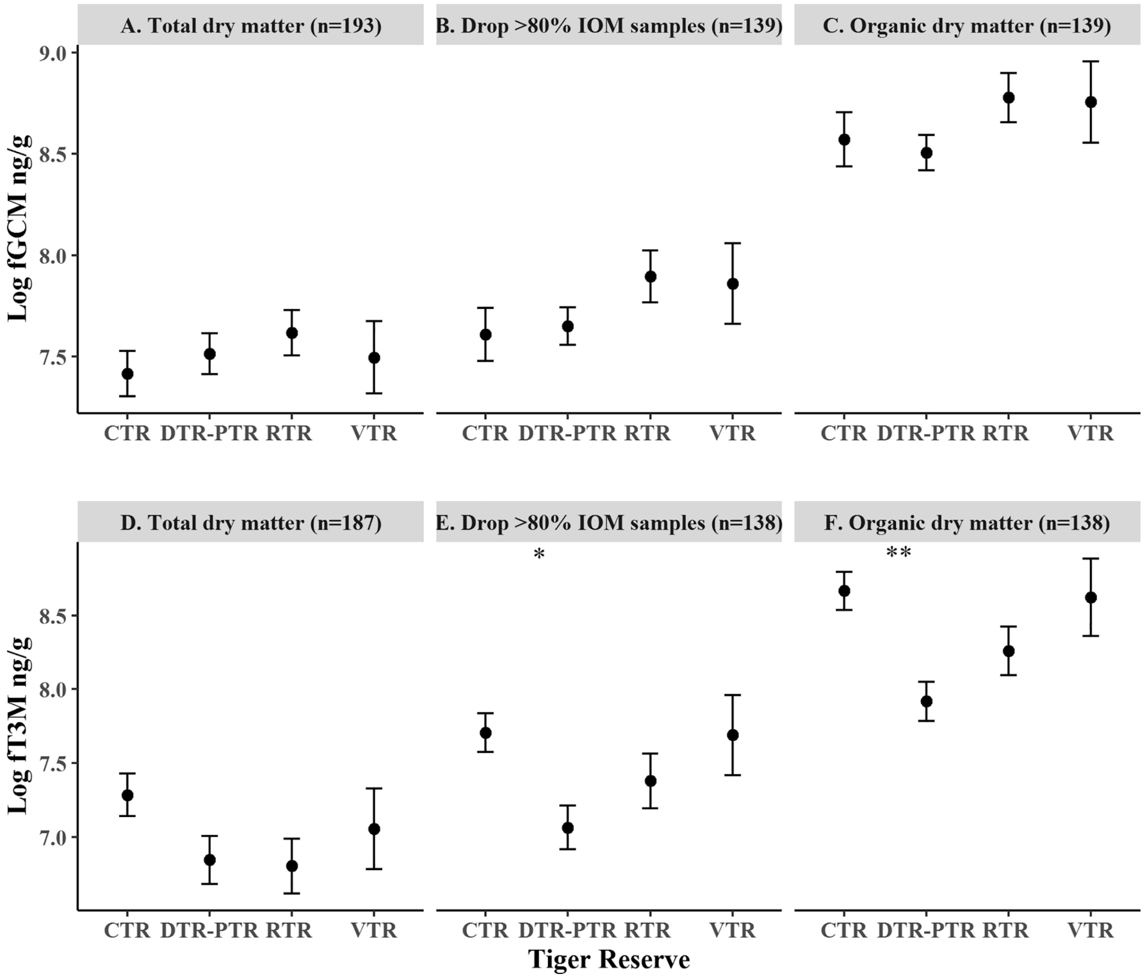

Fig. 3. Comparison of mean tiger fGCM and fT3M concentrations across four tiger reserves in Terai-Arc landscape (TAL). The top and bottom panel represents fGCM and fT3M data, respectively. For both hormone metabolites mean values (ng/g; log-transformed) are plotted. The left graphs (A and D) show the concentrations expressed as per gram of total dry matter, the middle graphs (B and E) indicate patterns after dropping samples with high IOM contents (>80%) and the right graphs (C and F) represents patterns when concentrations are expressed as per gram of organic dry matter. Any significant differences are marked with asterisks (*$P < 0.05$; **$P < 0.01$).

of faecal extracts paralleled the standard curves (Supplementary Fig. 3). No differences between slopes of standard and pooled extract curves for fGCM ($F_{(1,12)} = 2.01$, $P = 0.182$) and fT3M ($F_{(1,11)} = 0.62$, $P = 0.45$) were found. Accuracy tests produced slopes of 1.18 and 0.98 at working dilution of 1:100 and 1:15 for fGCM and fT3M (Supplementary Fig. 3), respectively, suggesting that faecal extracts did not interfere with their metabolite measurement precisions. Intra-assay coefficient of variation (CV) was 3.6 and 8.8, whereas inter-assay CV was 9.0 and 6.2 for fGCM and fT3M, respectively (Supplementary Table 1). Similarly, the Asiatic lion fGCM and fT3M parallelism graphs showed no slope differences ($F_{(1,10)} = 2.06$, $P = 0.182$ and $F_{(1,12)} = 0.69$, $P = 0.42$, respectively). Accuracy graphs showed a slope of 0.92 (at 1:60 dilution for fGCM) and 0.97 (at 1:30 dilution for fT3M), respectively (Supplementary Fig. 3). Intra-assay coefficient of variation (CV) was 9.8 and 6.2, whereas inter-assay CV was 9.0 and 9.7 for fGCM and fT3M, respectively (Supplementary Table 1).

2.7. Statistical analysis

Tiger and lion fGCM and fT3M data were assessed for normality (both raw as well as log-transformed values) using diagnostic plots (Q-Q plots) and Shapiro-Wilk test. To assess the extent of IOM presence the faecal samples were categorized into five intervals of increasing percentage of IOM as 0–20%, 20–40%, 40–60%, 60–80%, and 80–100%. IOM percentage was used as a variable to test the strength and direction of association between IOM content and fGCM and fT3M concentrations (expressed as total dry matter) using Pearson's correlation test. Earlier studies have described the effects of sample mass (biased fGCM measures from less faecal sample amount) (Hayward et al., 2010) and also the impacts of IOM in fGCM measure (Ganswindt et al., 2012). To evaluate any potential impacts of IOM presence in faecal hormone metabolites measure samples with high IOM content (≥80% IOM) were dropped (to reduce sample mass effect). Further, hormone concentrations as per unit of organic dry matter were also calculated. Linear regressions were performed to test for relationships between IOM content and hormone metabolite concentrations for all samples and with both types of corrected data sets (dropping samples with ≥ 80% IOM and hormone metabolite concentrations expressed as organic dry matter). Finally, for field collected tiger samples the fGCM and fT3M levels were compared among the sampled tiger reserves (RTR, CTR, DTR-PTR and VTR as mentioned above) using one-way ANOVA (separately for total dry matter and organic dry matter, respectively) along with post-hoc comparisons (Tukey's HSD test) to detect any potential alteration in results before and after implementing corrective measures.

For Asiatic lions, post-enrichment fGCM and fT3M data between the control and test groups were compared as it showed significant differences between them (Goswami et al., 2021). Only test group individuals were compared for fT3M levels between pre and post-enrichment

Table 2
Comparison of faecal hormone data analysis results for wild tigers and captive Asiatic lions. Wild tiger data was analyzed with One-way ANOVA and the captive Asiatic lion data was analyzed using independent *t*-test and paired *t*-test.

	Concentrations as total dry matter	Samples with > 80% IOM dropped	Concentrations as organic dry matter
fGCM analysis for wild tigers			
Sample size	N = 193 RTR (n = 57), CTR (n = 50), DTR-PTR (n = 52), VTR (n = 34)	N = 139 RTR (n = 36), CTR (n = 35), DTR-PTR (=45), VTR (n = 23)	N = 139 RTR (n = 36), CTR (n = 35), DTR-PTR (=45), VTR (n = 23)
One-way ANOVA	F (3,189) = 0.52, p = 0.671	F (3,135) = 1.26, p = 0.290	F (3,135) = 1.18, p = 0.322
Within group variance (SS_W)	133.53	77.83	75.16
Effect size (ω^2)	−0.008	0.007	0.004
fT3M analysis for wild tigers			
Sample size	N = 187 RTR (n = 53), CTR (n = 50), DTR-PTR (n = 52), VTR (n = 32)	N = 138 RTR (n = 36), CTR (n = 35), DTR-PTR (=45), VTR (n = 22)	N = 138 RTR (n = 36), CTR (n = 35), DTR-PTR (n = 45), VTR (n = 22)
One-way ANOVA	F (3,183) = 1.56, p = 0.200	F (3,134) = 3.24, p = 0.024	F (3,134) = 4.99, p = 0.003
Within group variance (SS_W)	290.44	140.86	121.30
Effect size (ω^2)	0.009	0.046	0.08
fGCM analysis for captive Asiatic lions			
Sample size	N = 82 Control (n = 32), Test (n = 50)	Not applied	N = 82 Control (n = 32), Test (n = 50)
Independent *t*-test	t(80) = 9.77, P = 0.00	Not applied	t(80) = 9.51, P = 0.00
Effect size (g)	2.21	Not applied	2.14
fT3M analysis for captive Asiatic lions			
Sample size	N = 12 individuals Pre (n = 12), Post (n = 12)	Not applied	N = 12 individuals Pre (n = 12), Post (n = 12)
Paired *t*-test	t(11) = 0.14, P = 0.89	Not applied	t(11) = 0.65, P = 0.52
Effect size (d)	0.028	Not applied	0.237

period. Independent *t*-test (for control and test groups) and paired *t*-test (for pre and post-enrichment period) were used respectively to evaluate any changes in results between two different ways of hormone metabolite concentration expressions (per unit of total dry matter vs. total organic matter). The effect sizes (ω2 for ANOVA, Hedges g for independent *t*-test and Cohen's d for paired *t*-test) were also calculated for each method. During all analyses, differences were considered significant at alpha level 0.05. Analyses were performed in GraphPad prism version 5, SPSS version 20 (IBM, 2011) and R v3.5.2 (R Core Team, 2018) using package "ggpubr" (Kassambara, 2020).

3. Results

The field-collected tiger samples showed higher variation in IOM content (n = 193, 9–98%) than the captive lion samples (n = 120, 17–57%) (Fig. 1). Majority of the tiger samples (n = 178, 92.22%) had > 40% IOM content, whereas IOM content remained < 40% for most of the captive lion samples (n = 103, 85.83%). Significant negative correlation was observed between IOM content and tiger fGCM (n = 193, r = -0.46, p = 0.000) and fT3M (n = 187, r = −0.58, p = 0.000) concentrations, respectively. The lion samples with less variation in IOM did not show any such pattern for fGCM (n = 120, r = -0.05, p = 0.579), but were negatively correlated for fT3M (n = 46, r = -0.43, p = 0.003).

Regression analysis with tiger samples showed strong influence of IOM content on fGCM measures when expressed as per gram of total dry matter (n = 193, $R^2 = 0.21$, P = <0.0001) (Fig. 2A). However, this influence reduced when samples with high IOM contents (≥80%) are removed from the analysis (n = 139, $R^2 = 0.06$, P = 0.004) (Fig. 2B), and reduced further when the data was expressed as organic dry matter (n = 139, $R^2 = 0.05$, P = 0.011) (Fig. 2C). Similarly, the fT3M measures were also influenced strongly by IOM content when expressed as total dry matter (n = 187, $R^2 = 0.34$, P = <0.0001) (Fig. 2D). Removing ≥ 80% IOM samples reduced the influence (n = 138, $R^2 = 0.09$, P = 0.0003) (Fig. 2E), and subsequent expression of fT3M concentrations as organic dry matter eliminated the influence of IOM (n = 138, $R^2 = 3.24\text{E-}4$, P = 0.833) (Fig. 2F). Protected area-wise mean fGCM and fT3M values are provided in Table 1. One-way ANOVA for field collected samples showed no significant differences in mean fGCM levels across the sampled tiger reserves when concentrations were expressed as total dry matter (F (3,189) = 0.52, p = 0.671, $\omega^2 = -0.008$), as well as when samples with ≥ 80% IOM content were removed (F (3,135) = 1.26, p = 0.290, $\omega^2 = 0.007$) and fGCM measures were expressed as organic dry matter (F (3,135) = 1.18, p = 0.322, $\omega^2 = 0.004$) (Fig. 3A, B & C). However, significant changes were observed in fT3M results among three different datasets. While no significant differences were found in mean fT3M levels in sampled tiger reserves when concentrations were expressed as total dry matter (F (3,183) = 1.56, p = 0.200, $\omega^2 = 0.009$), the values were significantly different when samples with ≥ 80% IOM content were removed from analysis ((F (3,134) = 3.24, p = 0.024, $\omega^2 = 0.046$). The significance increased further when fT3M concentrations were expressed as per gram of organic dry matter (F (3,134) = 4.99, p = 0.003, $\omega^2 = 0.08$) (Fig. 3D, E & F). Subsequent Tukey's HSD test for each of these two datasets shows that CTR population has significantly higher fT3M levels compared to DTR-PTR population (p = 0.032, p = 0.004, respectively) that has lowest fT3M levels.

However, the captive lion samples showed no influence of IOM content on fGCM measures (Table 2) when expressed as per gram total dry matter (n = 120, $R^2 = 0.003$, P = 0.570) (Fig. 4A) as well as per gram organic dry matter (n = 120, $R^2 = 0.017$, P = 0.157) (Fig. 4B). The fT3M measures in captive lions showed significant influence of IOM when expressed as total dry matter (n = 46, $R^2 = 0.18$, P = 0.003) (Fig. 4C), and as found in wild tigers subsequent expression of fT3M concentrations as organic dry matter eliminates the influence (n = 46, $R^2 = 0.02$, P = 0.32) (Fig. 4D). Independent *t*-test results comparing fGCM levels between the post-enrichment control and test groups of captive lions showed a difference in fGCM titer values but the patterns of the result remained same (total dry matter (t(80) = 9.77, P = 0.00, g = 2.21) and organic dry matter (t(80) = 9.51, P = 0.00, g = 2.14) (Fig. 5A & B), respectively. No significant difference was found in mean fT3M levels (expressed as total dry matter) during pre and post-enrichment periods (t(11) = 0.14, P = 0.89, d = 0.03) and results remained non-significant even when fT3M concentrations were expressed as organic dry matter (t (11) = 0.65, P = 0.52, d = 0.24) (Fig. 5C & D).

4. Discussion

In this study the impact of inorganic matter (IOM) in measurement of fGCM and fT3M from field-collected (tiger) and captive (Asiatic lion) animals has been examined. A highly variable amount (9–98%) and a high content of IOM (40 to ≥ 80%) were found in the wild tiger faecal samples. On the contrary, the samples from the captive Asiatic lions contained comparatively lower IOM amount (20–40%) and variability between samples. Although not quantified in this manner earlier, this pattern of IOM presence was not surprising for tigers. Khan (2004) reported presence of up to ~ 50% soil in wild tiger scats from the Sunderban landscape, Bangladesh during dry season with a peak in the winters and suggested possible seasonal soil ingestion by tigers. Schaller (1967) has also reported incidences of soil ingestion during winters from

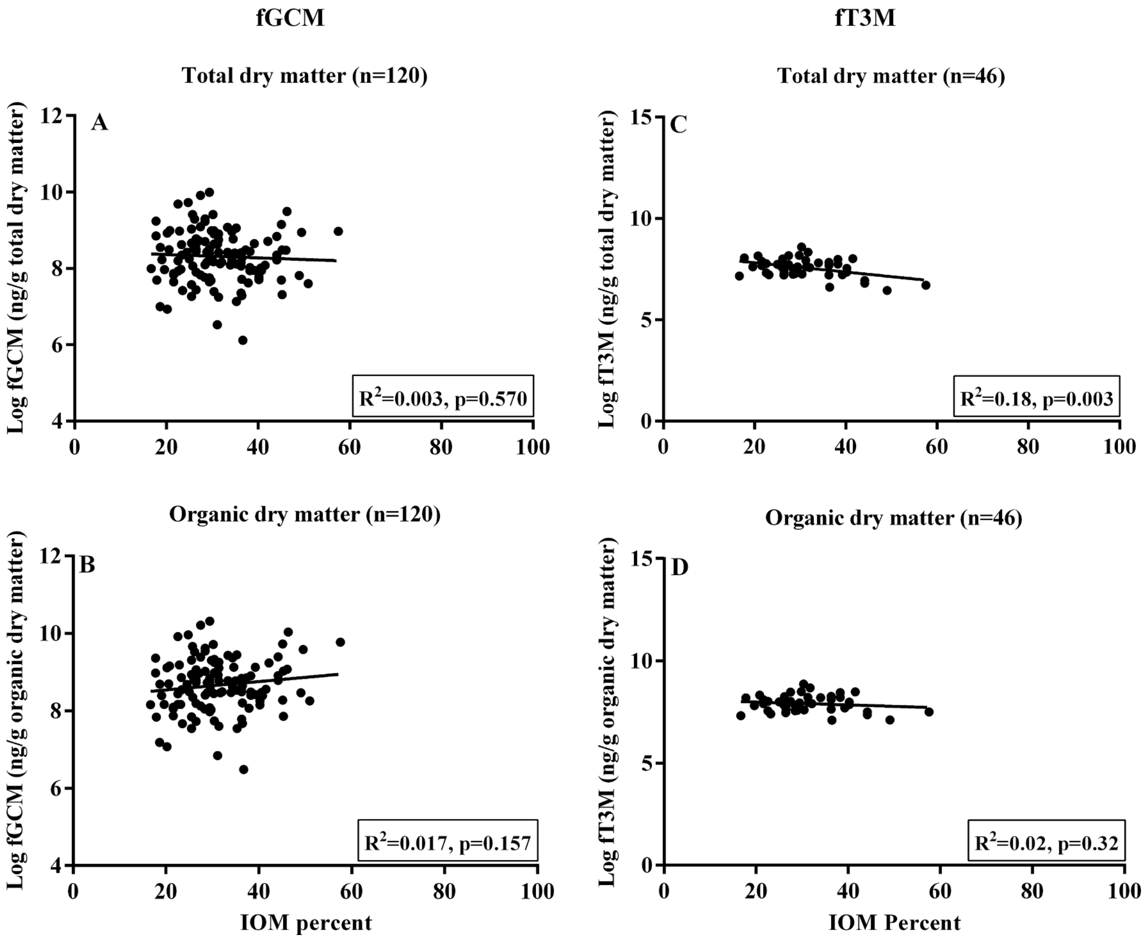

Fig. 4. Relationship between inorganic matter (IOM) content and fGCM and fT3M concentration in captive Asiatic lions. The IOM values have been presented as percentages and faecal hormone metabolite values have been log-transformed. The graphs A & C presents the association when hormone metabolites concentration is expressed as per gram of total dry matter, whereas the graphs B & D indicates the association when concentration is expressed as per gram of organic dry matter.

wild tigers in Kanha Tiger Reserve. However, such behaviours cannot always be attributed to specific seasons as free-ranging animals often naturally or deliberately ingest soil with food for mineral supplementation, alleviating gastrointestinal disorder or to counteract effects of high endoparasite load (Beyer et al., 1994; Knezevich et al., 1998; Krishnamani and Mahaney, 2000). Our sampling of tiger faeces during winters might have influenced the presence of IOM and their variations in the field-collected samples, but it was the best time for faecal sampling due to less leaf litter, availability of fresh samples and better environmental conditions. To the best of our knowledge, this was the first study on any endangered wild large carnivore species where such inter-sample variation in IOM was quantified. Earlier, Ganswindt et al. (2012) did not find significant inter-individual variation in IOM in Aardwolf faeces, possibly due to low sample size (n = 2) and similar foraging conditions in the study area. The possible presence of soil in tiger or other large carnivore faeces during sampling season and high variability of IOM makes it important to quantify its content before any downstream processing for hormone metabolite assessment.

Results of fGCM and fT3M measurements from the field-collected tiger faeces (with varied proportions of IOM) indicate a negative impact of IOM on both hormone metabolite concentrations. However, the captive lion samples showed such impact only for fT3M data, indicating that such effects are more pronounced in field-collected samples. This pattern has been earlier described in birds (Hayward et al., 2010) and mammalian species (Ganswindt et al., 2012) and could be explained due to the inflation of sample mass by hormone-inert IOM without any actual increase in metabolite concentration, leading to their inaccurate measures in faeces. Taken together, it can be inferred that the hormone metabolites are mostly contributed by the organic part of the faeces and the use of best quality samples (in terms of organic matter content along with other parameters such as sample freshness) is critical for physiological assessment studies. Overall, the results suggest that the field-collected samples have highly variable IOM contents that influence measures of both fGCM and fT3M. Such effects can be reduced by two possible corrective measures. First approach could be the possibility of physically remove the IOM contents from faeces (as suggested for urates in case of birds by Hayward et al., 2010). However, such physical separation of IOM (soil, sand etc.) from field-collected faeces is difficult for carnivores and thus it would be better to exclude the samples with high IOM content from analyses. The data from tiger samples with high IOM (≥80%) content (and consequently with very low hormone contributing organic matter) showed more variations in hormone concentration and removing them from analyses significantly reduced IOM influence on hormone measures (Fig. 2B & E). Based on this, it is suggested that samples with 80% or more IOM should not be processed for hormone metabolite assessments. The ideal working protocol would be to first quantify the IOM contents from the field-collected samples, followed by removal of samples with high IOM content and select the best quality samples (fresh faeces with low IOM content) for hormone metabolite assessment study. Future studies should also plan a pilot phase to evaluate the variation in faecal IOM in their respective target study systems before implementing large-scale research projects. Secondly, as these field-collected samples still contain high variation in IOM amount, presenting the hormone titer on total dry matter units (per gram of total

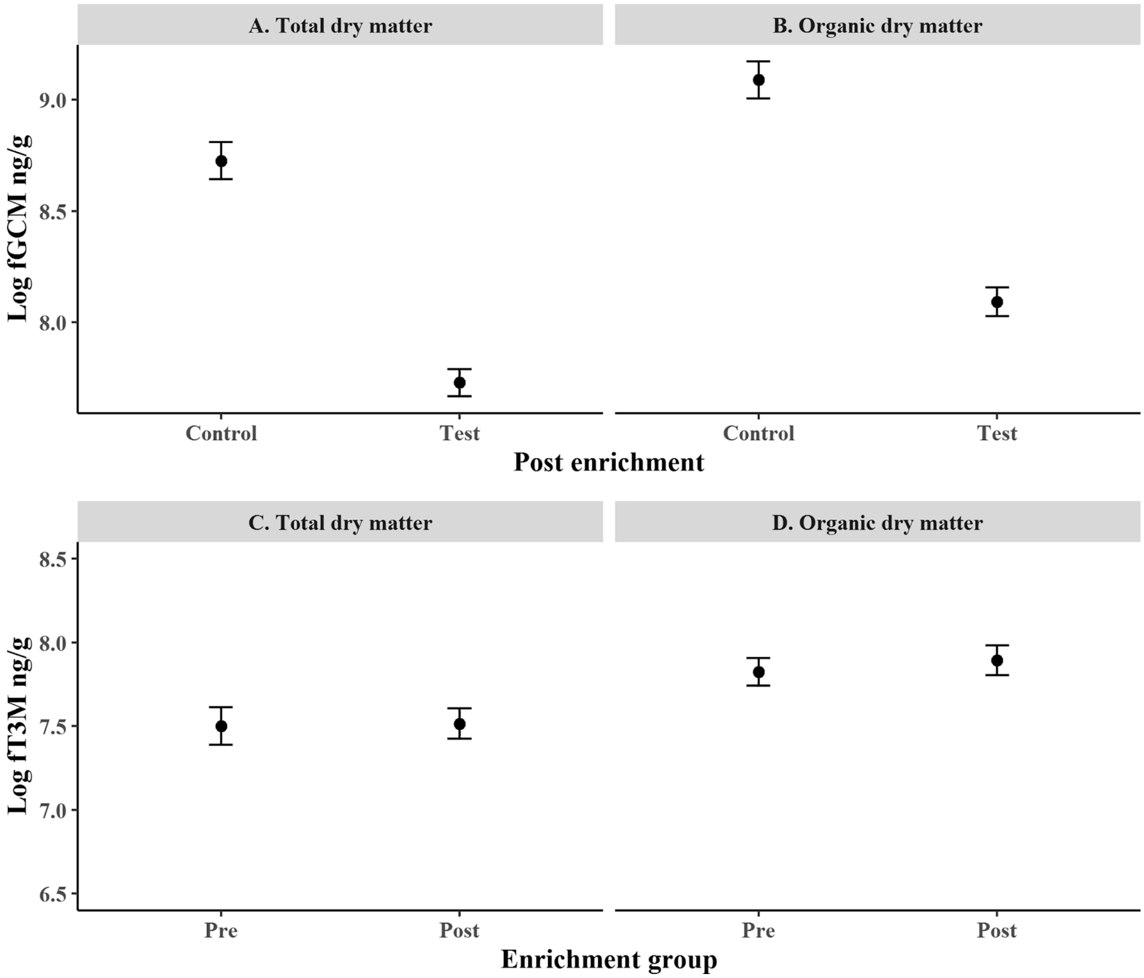

Fig. 5. Comparison of captive Asiatic lion mean fGCM (control and test groups) and fT3M (pre and post enrichment groups) level during enrichment interventions. For both hormone metabolites mean values (ng/g; log-transformed) are plotted. Graph A and C shows the mean concentration expressed as per gram of total dry matter, whereas B and D represent pattern when concentration is expressed as per gram of organic dry matter.

dry matter) can potentially be erroneous due to influence from IOM rather than actual biological measures. However, expressing the hormone concentrations as per gram of organic dry matter has reduced the effects significantly in this study (Fig. 2C & F), and supports the earlier suggestions by Hayward et al. (2010) and Ganswindt et al. (2012). For captive Asiatic lions most of the samples were found with relatively low IOM quantity and the effects of these corrective measures are much less for fGCM when compared to the field collected tiger samples. Interestingly, the lion fT3M measures were significantly affected even with lower IOM content, indicating that fT3M measures are more sensitive to IOM variability. Overall, using a combination of removing very low organic matter-containing samples (≥80% IOM) and expressing the hormone concentration as per unit organic dry matter can help to reduce the negative influences of IOM on fGCM and fT3M measures. Future work should focus on checking the IOM effects on reproductive hormone (progesterone, testosterone etc.) metabolite measures and experimental approaches to assess the utility of the low-quality samples (in terms of IOM presence) for species with low densities and large home ranges, as adequate sampling can often be challenging for them.

Finally, we investigated if these corrective measures mentioned above bring out any change in the interpretations of the results by comparing the original and corrected tiger hormone data generated from five protected reserves. The mean fGCM level differences among these tiger reserves remained non-significant for original data as well as after both corrective measures. However, the fT3M results across the three situations showed significant differences. After implementing the corrective measures, mean fT3M levels of DTR-PTR group drops to the lowest and the difference with CTR becomes statistically significant, further strengthening the argument of the effects of IOM on the measures on fT3M in field-collected samples. While such pattern of population-level nutritional stress information is relatively new for wild tigers, the results are not surprising given the knowledge of tiger prey dynamics in some of these protected areas. For example, the DTR-PTR habitat in Terai-Arc landscape is known to have relatively low ungulate density with patchy distributions (Bista et al., 2011; Chanchani et al., 2014) than CTR (Jhala et al. 2020). It can thus be hypothesized that the low availability of prey species in DTR-PTR has resulted in lower fT3M concentrations (indicating nutritional stress in this population). However, the fGCM results from the sampled areas were surprising (non-significant differences among the parks) as the GC and T3 hormones are known to rather complement each other (Mondol et al., 2020; Wasser et al., 2011, 2017). Further work involving intensive sampling and collection of site-specific ecological/population parameters from this landscape is required to understand the drivers behind the patterns of physiological indices. However, since none of the Asiatic lion samples exceeded 80% IOM content only one corrective measure was used and the differences in mean fGCM and fT3M concentrations between the groups remained unchanged, differing only in titer values. This was expected as in the captive environment food quality and quantity was controlled, resulting in possibly less IOM variation in lion faeces.

Our results show that certain common but under-emphasized factors such as IOM content have the potential to affect faecal hormone measures that in turn can change critical data interpretation and impact conservation decisions. Most of these physiological measures are closely associated with other ecological, behavioral and environmental factors at individual and population levels (Anestis, 2010; Busch and Hayward, 2009; Creel et al., 2013), but accurate interpretations should not be affected by measurement errors. We believe that the corrective measures discussed in this study reduce IOM-driven within-group hormone data variations and thus can help in making biologically relevant conservation decisions. Particularly for large, endangered carnivores such as tigers faecal sampling is the only non-invasive approach to assess species physiology in their natural habitats and thus it is essential to reduce the impacts of inorganic materials. These measures will help in accurate physiological assessments and lead us to ecologically relevant interpretations and recommendations, which are valued driving force for species conservation.

CRediT authorship contribution statement

Shiv Kumari Patel: Conceptualization, Data curation, Formal analysis, Validation, Visualization, Writing - original draft, Writing - review & editing. **Suvankar Biswas:** Data curation, Writing - review & editing. **Sitendu Goswami:** Data curation, Writing - review & editing. **Supriya Bhatt:** Data curation, Writing - review & editing. **Bivash Pandav:** Conceptualization, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Samrat Mondol:** Conceptualization, Data curation, Resources, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2021.113833.

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SOCIETY FOR EXPERIMENTAL BIOLOGY

Research article

The cost of sympatry: spatio-temporal patterns in leopard dietary and physiological responses to tiger competition gradient in Rajaji Tiger Reserve, Uttarakhand, India

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Apex predators have critical roles in maintaining the structure of ecosystem functioning by controlling intraguild subordinate populations. Such dominant–subordinate interactions involve agonistic interactions including direct or indirect impacts on the subordinates. As these indirect effects are often mediated through physiological processes, it is important to quantify such responses to better understand population parameters. We used a large carnivore intraguild system involving tiger (*Panthera tigris*) and leopard (*Panthera pardus*) to understand the dietary and physiological responses under a spatio-temporal gradient of tiger competition pressures in Rajaji Tiger Reserve (RTR) between 2015 and 2020. We conducted systematic faecal sampling in the winters of 2015 and 2020 from the park to assess diet and physiological measures. Analyses of leopard-confirmed faeces suggest a dietary-niche separation as a consequence of tiger competition. In 2020, we found an increased occurrence of large-bodied prey species without tiger competition in western-RTR. Physiological measures followed the dietary responses where leopards with large-sized prey in the diet showed higher fT3M and lower fGCM measures in western-RTR. In contrast, eastern-RTR leopards showed lower levels of fT3M and fGCM in 2020, possibly due to intense competition from tigers. Overall, these patterns strongly indicate a physiological cost of sympatry where competition with dominant tigers resulted in elevated nutritional stress. We recommend expansion of leopard monitoring and population estimation efforts to buffers, developing appropriate plans for human–leopard conflict mitigation and intensive efforts to understand leopard population dynamics patterns to ensure their persistence during the ongoing Anthropocene.

Key words: stress and nutrition hormones, physiological cost, inter-species competition, habitat recovery, dominance hierarchy, Carnivore conservation

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Introduction

Apex predators are critical in maintaining the structure and control of the local ecosystem functioning through prey–predator dynamics (Ritchie and Johnson, 2009; Ritchie *et al.*, 2012; Ripple *et al.*, 2014; Gaynor *et al.*, 2019) and their limiting effects on subordinates (Ritchie and Johnson, 2009; Ripple *et al.*, 2014; Suraci *et al.*, 2016; Feit *et al.*, 2019). Intensity of such limiting effects within a large carnivore guild is more pronounced for species competing for similar resources (Palomares and Caro, 1999; Donadio and Buskirk, 2006; Ritchie and Johnson, 2009). Dominant species within the guild exercise interference competition through aggression (Linnell and Strand, 2000; Merkle *et al.*, 2009), harassment (Merkle *et al.*, 2009), kleptoparasitism (Merkle *et al.*, 2009; Périquet *et al.*, 2015), delaying intraspecific communication (Cornhill and Kerley, 2020) and direct killing (Laurenson, 1994; Palomares and Caro, 1999; Donadio and Buskirk, 2006; Merkle *et al.*, 2009; Ritchie and Johnson, 2009). Subordinate members, on the other hand employ various tactics (for example, spatio-temporal and dietary separation) to minimize interference and maximize resource acquisition (Hayward and Slotow, 2009; Vanak *et al.*, 2013) to achieve a balance for successful co-existence. Local ecological factors are known to drive such behavioral strategies, which has been an area of extensive research interest in various carnivore guilds globally (Karanth and Sunquistt, 1995; Karanth and Sunquist, 2000; Carlsson *et al.*, 2010; Broekhuis *et al.*, 2013; Steinmetz *et al.*, 2013; Vanak *et al.*, 2013; Périquet *et al.*, 2014; López-Bao *et al.*, 2016). Dominant–subordinate agonistic interactions exerts two different kinds of negative impacts on the subordinate species: (a) direct impacts such as getting killed or displacement from the best-quality habitats (Mitchell and Banks, 2005; Merkle *et al.*, 2009; Newsome *et al.*, 2017; Ramesh *et al.*, 2017) and (b) indirect effects from increased pressures from competitions, inadequate food resources and resulting energy deficits (Creel and Christianson, 2008; Suraci *et al.*, 2016; Creel *et al.*, 2017; Sheriff *et al.*, 2020) affecting survival, growth, body condition, reproduction and parental provisioning (Creel *et al.*, 2007, 2009; Creel and Christianson, 2008; Parker *et al.*, 2009; Lamanna and Martin, 2016). As many of these indirect effects are mediated through physiological processes (Clinchy *et al.*, 2013; Macleod *et al.*, 2018), quantification of the physiological responses is essential to understand changes in various population parameters of the subordinate species (Creel *et al.*, 2009; Gaynor *et al.*, 2019). Recent advances in physiological measurements of environmental stressors, particularly in combination with non-invasive sampling approaches, have made it easier to link the environmental effects with their respective physiological responses (Dantzer *et al.*, 2014; Sopinka *et al.*, 2015; Palme, 2019; Ames *et al.*, 2020). For example, a number of inter-species (predator–prey—see Ylönen *et al.*, 2006; Creel *et al.*, 2009, dominant–subordinate dynamics-see Van Meter *et al.*, 2009 etc.) and intra-species (social hierarchy-see Sapolsky, 1983; Armitage, 1991; Creel *et al.*, 1996; Creel, 2001; Van Meter *et al.*, 2009, competition-see Armitage, 1991; Creel, 2001) interactions have been addressed using glucocorticoid (GC) measures, demonstrating its use. Further, recent addition of thyroid hormone (T3, in particular) (Eales, 1988; Flier *et al.*, 2000; Wasser *et al.*, 2010; Behringer *et al.*, 2018) measure is allowing us to separate the impacts of dietary resource availability from overall stress measures (through GC) as shown in marine (Ayres *et al.*, 2012; Wasser *et al.*, 2017; McCormley *et al.*, 2018) and terrestrial mammals (Wasser *et al.*, 2011; Vynne *et al.*, 2014; Joly *et al.*, 2015; Dias *et al.*, 2017; Szott *et al.*, 2020), including large carnivores (Vynne *et al.*, 2014; Patel *et al.*, 2021).

The sympatric tiger (*Panthera tigris*) and leopard (*Panthera pardus*) are one of the most well-studied model systems to understand the dominant–subordinate intraguild competition (Seidensticker, 1976; McDougal, 1988; Mondal *et al.*, 2012; Steinmetz *et al.*, 2013; Carter *et al.*, 2015; Pokheral and Wegge, 2019; Kafley *et al.*, 2019; Kumar *et al.*, 2019; Thapa *et al.*, 2021). Leopards, when co-existing with tigers, are often dominated by their larger counterpart in terms of resources (both space and food) (Seidensticker, 1976). Large number of studies have focused on exploring different strategies adopted by leopards, such as spatio-temporal (Carter *et al.*, 2015; Kafley *et al.*, 2019; Kumar *et al.*, 2019; Pokheral and Wegge, 2019; Thapa *et al.*, 2021) and dietary niche segregation (Karanth and Sunquistt, 1995; Andheria *et al.*, 2007; Harihar *et al.*, 2011; Pokheral and Wegge, 2019) for successful co-existence with tigers, but the physiological consequences of such interactions have received less attention. Here we address leopard physiological and dietary responses in the context of competition with tigers within Rajaji Tiger Reserve (RTR), western Terai-Arc landscape, India. RTR is a major source tiger population (estimated density of 8 ± 1.4/100 km^2 in 2018) and retains one of the highest density of leopards (16.90 ± 1.44/100km^2) in the landscape (Jhala *et al.*, 2021). The park is physically separated by the river Ganges in two parts: eastern and western RTR (henceforth ERTR and WRTR) that are structurally connected by a narrow corridor, heavily affected by anthropogenic activities (Johnsingh *et al.*, 1990; Harihar *et al.*, 2018; Biswas *et al.*, 2022a) (Figure 1). Both sites are similar in terms of wild prey densities and vegetation structure (Harihar *et al.*, 2009b) but differ in the extent of tiger competition intensity. Almost the entire tiger population of RTR is found in the ERTR whereas leopard, in the absence of tiger, is functionally the apex predator in the WRTR. This unique situation provides an ideal, natural system to assess the physiological impacts of inter-species competition in a control-test framework (WRTR is a control site with no inter-species competition when compared with ERTR). We used leopard faecal hormone metabolite measurements (fGCM and fT3M) in 2015 and 2020 to address spatio-temporal differences in physiological and dietary responses against a tiger competition gradient. More specifically, we asked the following questions: (i) how leopard dietary profiles, fGCM and fT3M measures vary with changing tiger competition intensities over space and time, and (ii) how local

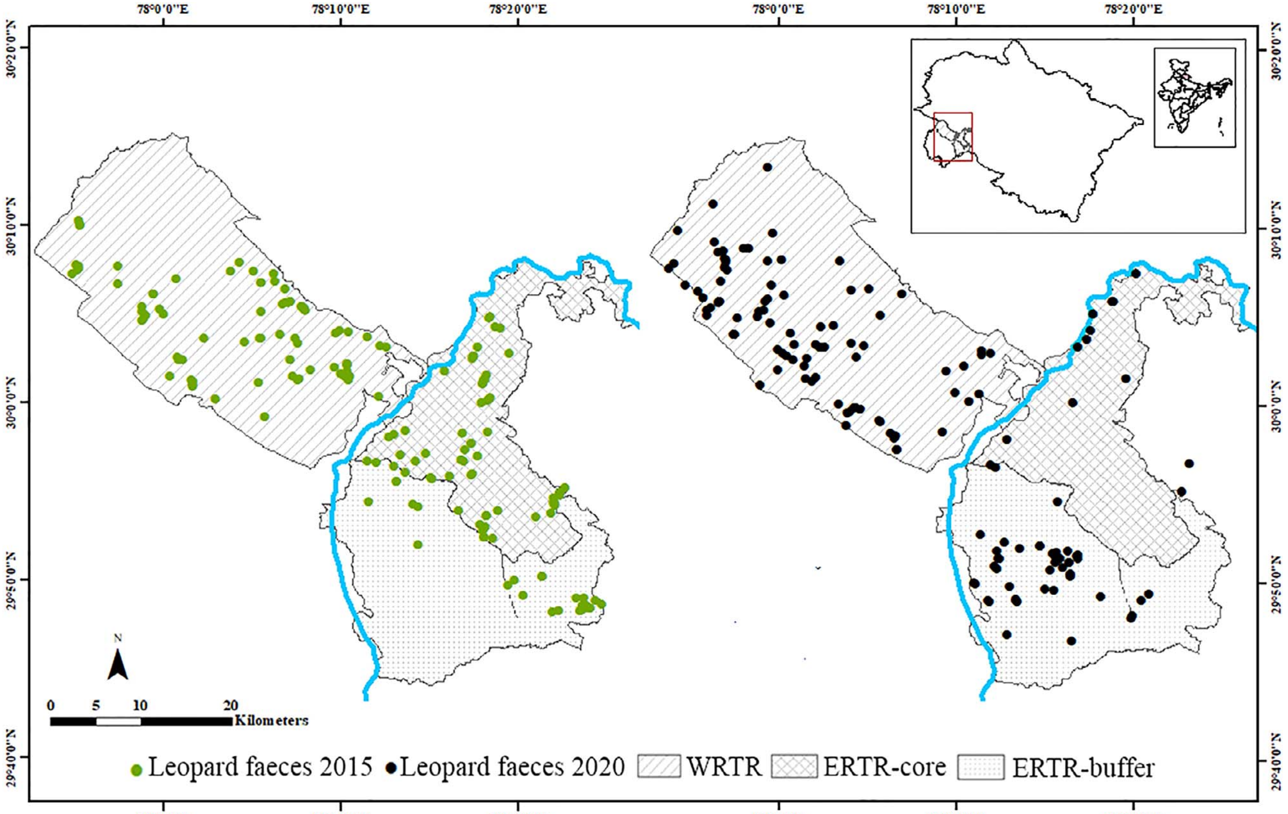

Figure 1: Spatio-temporal locations of field-collected leopard faecal samples from Rajaji Tiger Reserve (RTR) used in this study. The core and buffer zones are differentially marked. The left pane represents samples collected in 2015, whereas the right pane shows 2020 samples.

ecological factors (habitat productivity and prey body size) explain such differences in leopard physiology. We believe that results of this study have larger implications in understanding the physiological costs for subordinate carnivores co-existing within a guild and their long-term conservation.

Materials and Methods

Research permissions and ethical considerations

All required permissions for field surveys and faecal sampling were provided by Forest Departments of Uttarakhand (permit no: 90/5-6 and permit no: 3707/5-6). Due to the non-invasive nature of this study, no further ethical clearance was required.

Study area

This study was conducted in Rajaji Tiger Reserve (RTR) (Figure 1), the westernmost part of Rajaji-Corbett Tiger Conservation Unit (RCTCU, Johnsingh and Negi, 2003), in the Indian part of the Terai-Arc landscape (TAL). Located at base of Himalayan foothills and starting of Indo-Gangetic plains, RTR has an undulating topography with a mosaic of woodlands and grasslands, drained by multiple rivers and streams. The forest type is broadly classified as northern Indian moist deciduous, dominated by Sal (*Shorea robusta*) (Champion and Seth, 2005). The park is naturally separated by the river Ganges in two parts, connected by a narrow corridor called as Chilla-Motichur corridor (3 km length and 1 km width) (Johnsingh *et al.*, 1990; Harihar *et al.*, 2018). The eastern part (covering 579 km^2 area of core and buffer zones), situated in the east bank of Ganges maintains structural, as well as functional connectivity with major tiger populations (such as Corbett Tiger Reserve) of the landscape (Harihar *et al.*, 2020; Biswas *et al.*, 2022a). However, the western part (571 km^2 area on the west bank of Ganges) has become isolated over years from the ERTR. Historically, the entire study area (RTR) was inhabited by large numbers of agro-pastoralist community settlements (Toungya and Gujjars, respectively), which were primarily dependent on forest resources (Berkmuller *et al.*, 1987), leading to forest degradation. In 1983, Rajaji was declared as a national park and significant efforts towards tiger and prey population recovery were undertaken. As part of this, a plan for relocation and rehabilitation of local communities was prepared to create inviolate space for wildlife (Roy, 2003), and accordingly, all settlements from the core areas of ERTR were relocated by 2003 (Harihar *et al.*, 2009b). Subsequently, the conditions of the natural habitat improved and the densities of the wild prey species increased and facilitated population recovery of wild tigers in ERTR (from 2.08 in 2004–5 to 7.05 individuals/100 km^2 in 2016–17, Harihar *et al.*, 2020). However, in the WRTR, the human rehabilitation was completed in periodical manner where by 2005 some ranges were made inviolate (Harihar *et al.*, 2009b) and others were completed by 2016. As WRTR remained functionally disconnected from eastern counterpart during this period, the tiger population reduced drastically (from 5 to 10 individuals in 2000 to two females in 2006, Johnsingh, 2006, Jhala *et al.*, 2020) despite comparable prey density (41.22 ± 6.65 individuals/km^2 in WRTR and 39.23 ± 4.76 individuals/km^2 in ERTR, Harihar *et al.*, 2020). The leopard population, on the other hand, showed a reverse trend where ERTR recorded a decrease in their density (9.76 to 2.07 individuals/100 km^2 between 2004 and 2008, respectively, Harihar *et al.*, 2011) (possibly due to inter-species competition) and WRTR showed an increasing trend in leopard density (Jhala *et al.*, 2021) (due to less competition from tigers). Such contrasting population patterns under a scenario of inter-species competition provided an ideal ‘natural experimental setup’ to understand leopard physiological responses under low and high tiger density in adjacent and similar habitats. Further, we conducted this study over a 5-year temporal framework (2015 and 2020) during which the ERTR has seen a significant increase in tiger population (2.90 ± 0.87 to 8 ± 1.4/100 km^2, Jhala *et al.*, 2015 and 2020), therefore providing an opportunity to test the effects of competition against a tiger density gradient (high tiger density in 2020 than 2015 within ERTR).

Study design

The unique situation of naturally high (ERTR) vs. low (WRTR) tiger density and reverse patterns of leopard density in adjacent and similar habitats allowed us to ask some important questions regarding various effects of inter-species competition. From a spatial difference perspective we hypothesize that: (1) there would be a dietary niche separation between ERTR and WRTR, where the eastern leopard population would show higher frequency of medium and small sized prey in their diet than the western population (where they do not face competition from tigers); (2) corresponding high fGCM in ERTR (due to inter-species competition) than western population; and (3) lower fT3M in ERTR leopards (due to possible dietary niche separation) than their western counterpart. In a temporal data perspective, we expect that (4) there would be no significant difference in prey relative frequency of occurrence (RFO) in diet, as well as in fGCM and fT3M measures in WRTR, whereas (5) significant difference in prey RFO in diet, fGCM and fT3M is expected in ERTR resulting from increased competition from tigers between 2015 and 2020 (tiger density of 2.90 ± 0.87/100 km^2 in 2015 and 8 ± 1.4/100 km^2 in 2020, Jhala *et al.*, 2020). Apart from the inter-species competition we also expected habitat productivity-related differences in leopard dietary and physiological responses. We expected better habitat to be associated with higher frequency of large sized prey in diet, lower fGCM and higher fT3M levels.

Faecal sampling, species confirmation and prey identification

Due to the spatio-temporal nature of the study, it was critical to establish a standard sampling framework for faecal collection from the entire study area. Some of the major concerns were identification of sampling trails across RTR, seasonal effects, uniform field efforts, constant sampling team etc. As RTR has been a long-term study site for photographic monitoring of tigers (Harihar *et al.*, 2009a, 2009b, 2020; Harihar and Pandav, 2012) and earlier genetic studies have used already identified forest trails and tracks (Biswas *et al.*, 2022a), the information was used for faeces collection in this study during both sampling periods (2015 and 2020). Two experienced research and tracking team (each consisting of three to four members) surveyed all identified forest trails during 2015 and 2020 and collected fresh faecal samples of large carnivores. Both sampling (in 2015 and 2020) were conducted in winter (December–January) to counter seasonal variations and make use of better environmental conditions (mean temperature of 4–6°C) that maintain relatively long-term sample freshness. In field, sample freshness was determined based on intactness, minimal insect activity and strong odor (Vynne *et al.*, 2014). All fresh faecal samples were collected in wax paper with location details and stored in zip-lock bags (Biswas *et al.*, 2019) before transporting them to the laboratory, where they were stored −20°C until laboratory analysis.

In the laboratory, the samples were genetically ascertained using leopard-specific molecular markers (Mondol *et al.*, 2014) to ensure only confirmed leopard samples were used in downstream diet and physiological analyses. In brief, DNA extraction was performed using a modified Qiagen DNA extraction protocol (Biswas *et al.*, 2019) for all samples and leopard-specific mitochondrial DNA markers (TigParND4-F and ParND4-R, Mondol *et al.*, 2014) were used to ascertain leopard faeces. Confirmed leopard samples were further dried at 60°C for 72 hours in an oven (Unilab-112HO, Haryana, India) to control for moisture (Wasser *et al.*, 1993). The undigested parts (prey hair, broken bones, hoof etc.) were separated by sieving the dried samples through sterile 0.5 mm stainless steel mesh and the faecal powders were collected and stored in −20°C. The primary guard hairs (20–30 hairs/sample) were used to prepare permanent slides and were examined for medulla structures (Mukherjee *et al.*, 1994) using available references (Bahuguna *et al.*, 2010; Biswas *et al.*, 2022b) to identify leopard prey species. Sample size estimation for diet analyses was conducted through a sample rarefaction curve (Magurran, 2004), where the species diversity in leopard diet was estimated using Shannon diversity index (Magurran, 2004) with EstimateS (Colwell, 2006).

Habitat productivity assessment

For leopards or for large carnivores in general a good quality habitat is one with good prey availability (Carbone and Gittleman, 2002). Prey availability is associated with forage availability that is often quantified in terms of vegetation cover or green cover (Pettorelli *et al.,* 2005a, 2005b, 2011). We used vegetation cover as a proxy of habitat productivity (Pettorelli *et al.,* 2011) that would facilitate higher prey base for leopards and quantified it by extracting Normalized Difference Vegetation Index (NDVI) values. We used 16-day composite NDVI values recorded by NASA's MODIS (Moderate Resolution Imaging Spectroradiometer, MOD13Q1 version 6.1 at 250 m resolution), downloaded for RTR (for the month of December, corresponding to winter sampling season) for year 2015 and 2020. The analyses were conducted at two scales: (a) For overall habitat productivity assessment, we divided study area into three zones: WRTR, ERTR-core and ERTR-buffer (see Fig. 1). Each zone was further divided into 3 × 3 km grids (9 km^2 area, approximate leopard home range see Seidensticker *et al.,* 1990); and (b) for sample-based assessment, we used leopard faecal sample location as center and drew buffers of 2 km radius (12 km^2 area) around each faecal sample. We extracted mean NDVI values for each grid and buffer using MODIS raster images of year 2015 and 2020, where extraction was done using zonal statistics tool (as table for grids and table 2 for buffers) in ArcMap 10.5 (ESRI 2016).

Hormone metabolite extraction and assays

Recent study on wild tigers in the same landscape showed highly variable contents of inorganic matter (IOM) in the faeces that negatively impacted fGCM and fT3M measures (Patel *et al.,* 2021). As leopards share the same space, environmental conditions and prey base in RTR, the field-collected samples were processed for percent IOM measures using the same approach described in Patel *et al.* (2021). In brief, 0.1 g of faecal powder was ashed in a muffle furnace (NSW-101, NSW, New-Delhi, India) at 550°C for 2 hours, reweighed and the amount of IOM was calculated. As suggested in the earlier study (Patel *et al.,* 2021), leopard samples with $< 80\%$ IOM content were used for hormone assays.

For hormone metabolite extractions, each dried faecal powder was thoroughly mixed and 0.1 g of powder was weighed. The extraction procedure involved pulse-vortexing the weighed amount of faecal powder in 15 ml of 70% ethanol for 30 minutes, followed by centrifugation at 2200 rpm for 20 min (Wasser *et al.,* 2010; Mondol *et al.,* 2020). The hormone extracts were collected in 2 ml cryochill vials (1:15 dilution) and stored at −20°C in freezer until assays. Leopard fGCM and fT3M were measured using corticosterone (K014, Arbor Assays, MI, USA) and triiodothyronine (T3) (K056, Arbor Assays, MI, USA) EIA kits. These kits were earlier successfully validated in wild tigers (from TAL) and captive lions (Goswami *et al.,* 2021; Patel *et al.,* 2021) and thus were considered suitable for this study. Further, parallelism and accuracy tests were conducted for leopard faecal extracts in the laboratory. Serial dilutions of faecal extracts paralleled well with standard curves of fGCM (Supplementary Material, Fig. S1a), as well as fT3M (Supplementary Material, Fig. S1c). *F* ratio test showed no differences between slopes of standard and pooled extract curves for fGCM ($F(1,10) = 1.89$, $P = 0.2$) and fT3M ($F(1,11) = 1.34$, $P = 0.27$). Accuracy tests using regression analysis produced slopes of 1.09 and 1.02 at working dilution of 1:120 and 1:7.5 for fGCM and fT3M (Supplementary Material, Fig. S1b and S1d). Intra-assay coefficient of variation (CV) was 7.15 and 8.36, whereas inter-assay CV was 10.35 and 7.86 for fGCM and fT3M, respectively. During assays, hormone extracts were dried and reconstituted in assay buffers at required dilution (1:120 for fGCM and 1:7.5 for fT3M). Samples were assayed in duplicate using kit protocols and optical density (at 450 nm) was measured with ELISA plate reader (GMB-580; Genetix Biotech Asia, New Delhi, India). Cross-reactivities of respective antibodies are presented in Supplementary Material, Table S1.

Statistical analysis

The analytical framework was established based on the hypothesis proposed in this study, where comparisons were made at two scales: 1) at spatial level, prey and hormone metabolite (fGCM and fT3M) data between ERTR and WRTR (individually the 2015 and 2020 data); and 2) at temporal scale where comparisons were made with each part of RTR (2015 vs 2020 for ERTR and WRTR, respectively). While reporting the methods and results following terms have been used to describe the sampled groups: ERTR in 2015—ERTR_{2015}, ERTR in 2020—ERTR_{2020}, WRTR in 2015—WRTR_{2015} and WRTR in 2020—WRTR_{2020}.

To ascertain leopard food habit, data on relative frequencies of occurrences (RFO) for each prey species were calculated using formula $i/j*100$, where 'i' represents the frequency of number of samples in which a specific prey occurs and 'j' represents the total frequency count of all prey species (Kruuk, 1989; Mukherjee *et al.,* 1994). Further, relative prey biomass consumed was calculated using formula $D = (A*Y)/\sum(A*Y)*100$ where 'A' represents the RFO of each prey species and 'Y' represents weight of consumed prey in each faeces. 'Y' is calculated using Ackerman's equation: $Y = 1.980 + 0.035X$, where $X =$ mean body weight of a particular prey species (Ackerman *et al.,* 1984; Karanth and Sunquistt, 1995). The mean body weight of prey was taken from Harihar *et al.* (2011), Rathore (2015) and Upadhyaya *et al.* (2018) (Table 1). Two-way ANOVA was used to test any significant differences in prey RFO and biomass among sampled groups (spatial scale: ERTR_{2015} vs. WRTR_{2015}, ERTR_{2020} vs. WRTR_{2020}; temporal scale: ERTR_{2015} vs. ERTR_{2020}, WRTR_{2015} vs. WRTR_{2020}). Additionally, all the prey species data were categorized into three major classes: (a) large (≥60 kg), (b) medium (between 16 and 60 kg); and (c) small (≤15 kg) and absolute frequency of occurrence (AFO) was calculated for these classes using formula s_k*100/n, where 's_k' is the number of samples containing class k and n is total number of faeces analysed (Harihar *et al.*, 2011). Any difference in AFO percentage in sampled groups were tested using chi-square analyses, followed by f test for pair wise comparison at spatial and temporal scales (mentioned above). For overall assessment of the habitat productivity, we compared mean NDVI values of three zones (WRTR, ERTR-core and ERTR-buffer) spatially using one-way ANOVA (with subsequent post hoc Tukey's HSD test) and temporally (between 2015 and 2020) using paired t test. All analyses were conducted using SPSS version 20 (IBM, 2011).

During hormone data analyses, the leopard fGCM and fT3M data (raw as well as log transformed) were assessed for normality using diagnostic plots (density plots) and Shapiro–Wilk test. Generalized linear models (GLMs) with log link and gamma distribution errors were used to explain the variation in fGCM and fT3M data. To assess any possible changes in fGCM and fT3M levels across spatial (ERTR vs WRTR) and temporal (2015 vs 2020) scales, an interaction term 'Area∗Year' (as the tiger density in ERTR was lower in 2015 than in 2020, that may impact fGCM and fT3M levels) and the prey size class (large, medium and small, as prey size may impact fGCM and fT3M levels) were used as explanatory variables. Likelihood ratio test (LRT) was used to determine if the explanatory variables explain the data independently or in combination. Finally, post-hoc Tukey's HSD test was employed to assess any pair-wise differences in fGCM and fT3M levels for all sampled groups (mentioned above) and prey size classes. To evaluate the relationship of fGCM and fT3M with habitat productivity (NDVI values derived from sample buffers), we used two separate linear models (function 'lm'). Additionally, we also performed a multivariate GLM including all three predictor variables (Area∗Year, Prey size and NDVI change) and compared the resulting models with null model (Supplementary Material, Table S6) to discern the effect of each of the variable on fGCM and FT3M data. All analyses were conducted in R v4.1.1 (R Core Team, 2021) with the following packages: 'ggpubr' (Kassambara, 2020) and 'multcomp' (Hothorn *et al.*, 2008).

Results

During the study period, a total of 564 large carnivore faeces was collected ($n = 276$: ERTR-172 and WRTR-104 samples in 2015 and $n = 288$: ERTR-178 and WRTR-110 samples in 2020) from the entire study area. After species confirmation, 324 leopard faecal samples were further processed for dietary and hormone analyses. The distribution of these samples was as followed: $ERTR_{2015}$–92, $WRTR_{2015}$–81, $ERTR_{2020}$–60 and $WRTR_{2020}$–91. However, prey species could be identified from 304 samples (93.82% success rate, $ERTR_{2015}$–86, $WRTR_{2015}$–76, $ERTR_{2020}$–53 and

Table 1: Details of the various leopard diet parameters for all nice prey species identified in this study. Results are presented for percentage of relative frequency of occurrence (% RFO), and relative biomass of the consumed prey species in ERTR and WRTR for both 2015 and 2020, respectively

Prey species	Mean body weight of prey (in kg)	$ERTR_{2015}$ ($n = 86$)		$WRTR_{2015}$ ($n = 76$)		$ERTR_{2020}$ ($n = 53$)		$WRTR_{2020}$ ($n = 89$)	
		RFO (%)	Relative biomass (%)	RFO (%)	Relative biomass (%)	RFO (%)	Relative biomass (%)	RFO (%)	Relative biomass (%)
Livestock	250	5.81	12.50	0.00	0	1.89	4.32	0.00	0
Sambar	185	19.19	32.51	20.39	37.54	19.81	35.75	31.46	48.39
Nilgai	169	6.98	11.04	4.61	7.91	5.66	9.54	10.67	15.33
Chital	50	38.72	28.95	48.68	39.53	47.17	37.55	43.26	29.35
Wild pig	35	4.65	2.99	5.26	3.67	5.66	3.87	3.93	2.29
Hog deer	33	7.56	4.75	7.24	4.94	0.00	0	2.81	1.60
Langur	10	0.58	0.27	0.66	0.33	0.00	0	0.00	0
Hare	4	13.72	5.83	13.16	6.07	19.81	8.96	7.30	2.82
Indian peafowl	5	2.67	1.16	0.00	0	0.00	0	0.56	0.22

$WRTR_{2020}$–89, respectively; Table 1). The remaining samples ($n = 20$, 6.18%) contained damaged hairs for accurate species identification and were excluded from further dietary analyses. For hormone analyses, samples with > 80% IOM were discarded ($n = 121$) and finally 203 faecal hormone extracts ($ERTR_{2015}$–56, $WRTR_{2015}$–49, $ERTR_{2020}$–42 and $WRTR_{2020}$–56) were used in physiology analyses.

Food habits of leopard

Overall, a total of nine prey species (large-bodied—Sambar, Nilgai and Livestock; medium-bodied—Chital, Wild pig and Hog deer and small-bodied—Langur, Hare and Peafowl) were detected. The large and medium-bodied prey species contributed 85.38% (RFO) of leopard diet whereas small prey species comprised only 14.62% (RFO). RFO of Chital (44.49%) and Sambar (22.71%) were highest followed by others (Table 1). However, biomass of Sambar was highest (38.55%), closely followed by chital (33.85%) (Table 1). Majority of the samples ($n = 273$, 89.8%) contained single prey species. All prey species except livestock (identified only in the ERTR) was found across all sampled groups. The rarefaction curve saturated beyond 40 samples within each group, and no new prey species was identified further (Supplementary Material, Fig. S2).

The two-way ANOVA analyses with all leopard prey species among the sampled groups (spatial: $ERTR_{2015}$ vs. $WRTR_{2015}$, $ERTR_{2020}$ vs. $WRTR_{2020}$; temporal: $ERTR_{2015}$ vs. $ERTR_{2020}$, $WRTR_{2015}$ vs. $WRTR_{2020}$) showed no significant differences in both RFO and biomass. However, overall comparisons within habitats across all compared groups indicated significant differences in both RFO and biomass (Supplementary Material, Table S5). Comparative analyses (Chi-square test with prey body-size groups) revealed large-sized prey frequencies differed significantly among sampled groups ($\chi^2 = 8.62$, $P = 0.035$). *F* test showed that at spatial scale, there were no significant differences in frequencies of different prey classes between $ERTR_{2015}$ and $WRTR_{2015}$ ($P = 0.445$, $P = 0.476$ and $P = 1.00$ for large, medium and small prey classes, respectively) (Table 2). However, compared to $ERTR_{2020}$, $WRTR_{2020}$ showed significantly higher large-bodied ($P = 0.018$) and lower small-bodied prey species ($P = 0.019$). Temporally, the $ERTR_{2015}$ and $ERTR_{2020}$ showed no difference in prey class occurrences ($P = 0.445$ (large), $P = 1.00$ (medium) and $P = 0.335$ (small), whereas $WRTR_{2020}$ showed a significant increase in large prey occurrences ($P = 0.018$) with no differences in medium and smaller-bodied prey classes ($P = 0.156$ and $P = 0.238$, respectively) compared to $WRTR_{2015}$.

NDVI values at spatio-temporal scales

NDVI comparison using one-way ANOVA showed significant differences between zones (WRTR, ERTR-core and ERTR-buffer) in both 2015 ($F(2,279) = 5.81$, $P = 0.003$), as well as 2020 ($F(2,279) = 7.74$, $P = 0.001$), at spatial scale. Subsequent post hoc test showed that WRTR and ERTR-core zones

Table 2: Spatio-temporal comparison of differences in absolute frequency of prey occurrence in leopard diet at Rajaji Tiger Reserve, India. The comparisons were calculated using Fisher's Exact test (2x2)

Prey category	Detection	Spatial comparison						Temporal comparison					
		$ERTR_{2015}$	$WRTR_{2015}$	*P*	$ERTR_{2020}$	$WRTR_{2020}$	*P*	$ERTR_{2015}$	$ERTR_{2020}$	*P*	$WRTR_{2015}$	$WRTR_{2020}$	*P*
Large	Yes	33.72	27.63	0.445	28.30	44.94	**0.018***	33.72	28.30	0.445	27.63	44.94	**0.018***
	No	66.28	72.37		71.70	55.06		66.28	71.70		72.37	55.06	
Medium	Yes	53.49	59.21	0.476	52.83	48.31	0.572	53.49	52.83	1.000	59.21	48.31	0.156
	No	46.51	40.79		47.17	51.69		46.51	47.17		40.79	51.69	
Small	Yes	12.79	13.16	1.000	18.87	6.74	**0.019***	12.79	18.87	0.335	13.16	6.74	0.238
	No	87.21	86.84		81.13	93.26		87.21	81.13		86.84	93.26	

did not differ significantly in their mean NDVI values in year 2015 ($P = 0.47$), as well as in 2020 ($P = 0.93$). However, ERTR-buffer zone continuously showed significantly lower NDVI values in both the years compared to WRTR (2015: $P = 0.03$, 2020: $P = 0.001$) and ERTR-core (2015: $P = 0.004$, 2020: $P = 0.003$) zones (Figure 2c). At temporal scale, paired t test showed that mean NDVI values improved significantly in year 2020 for WRTR ($t(134) = 25.06$, $P < 0.0001$), ERTR-core ($t(66) = 9.39$, $P < 0.0001$), as well as ERTR-buffer ($t(79) = 12.45$, $P < 0.0001$) zones compared to year 2015 (Figure 2c).

Physiological responses of the sampled groups at spatial and temporal scales

Likelihood ratio test selected the individual GLM explanatory variables (Area∗Year and Prey size, respectively) over combined model as significant factors to explain the physiological response patterns (Supplementary Material, Table S3). GLM results with Area∗Year model indicated year is a significant factor ($P = 0.05$) for fGCM data (Table 3). At spatial scale, both ERTR_{2015}-WRTR_{2015} and ERTR_{2020}-WRTR_{2020} comparisons showed no significant differences in mean fGCM levels (2015—$z = 0.834$, $P = 0.838$; and 2020—$z = 0.253$, $P = 0.994$) (post-hoc test, Supplementary Material, Table S2). However, temporal scale comparisons showed contrasting results where mean fGCM levels between ERTR_{2015} and ERTR_{2020} showed non-significant differences ($z = -1.97$, $P = 0.2$) but WRTR_{2020} had significantly low fGCM values than WRTR_{2015} ($z = -2.73$, $P = 0.032$) (Figure 3b(iv)). For fT3M data, GLM results showed that the area and year interaction factor (Area∗Year) is a significant factor (Table 3). At spatial scale, the fT3M levels between ERTR_{2015}-WRTR_{2015} showed no difference ($z = -1.163$, $P = 0.65$), but WRTR_{2020} fT3M levels were significantly higher than the ERTR_{2020} ($z = 2.644$, $P = 0.041$) (Figure 3a(iii)). At temporal scale, ERTR_{2015}-ERTR_{2020} comparisons revealed no significant differences in fT3M levels ($z = -1.303$, $P = 0.56$) but WRTR_{2020} showed significantly higher values of fT3M than WRTR_{2015} ($z = 2.602$, $P = 0.046$) (Figure 3b(iii)).

GLM outputs with Preysize model indicated no significant differences between prey size class (large, medium and small body-sized prey) for fGCM levels (Table 3, Figure 4a). However, the fT3M levels showed strong relationship with prey size classes, where the fT3M levels from the leopard samples with large prey remains were higher than small prey class ($z = 1.15$, $P = 0.5$) and significantly higher than medium prey class ($z = 2.61$, $P = 0.02$) (Table 3, Figure 4b). Linear models showed a marginally significant positive association between leopard fGCM and habitat NDVI values (t value $= 2.003$, $F(1, 201) = 4.01$, $P = 0.05$) (Supplementary Material, Fig. S4a), and no significant association between leopard FT3M and habitat NDVI values (t value $= -1.11$, $F(1, 201) = 1.23$, $P = 0.27$) (Supplementary Material, Fig. S4b). GLM with all three variables (Area∗Year, Prey size and NDVI change) shows that the interaction factor of Area and Year (indicating towards unique physiological responses of leopards in space and time) is included in the best models. NDVI and Prey size best explains the variation in the data only when included with Area∗Year, but not alone, for both fGCM as well as fT3M (Supplementary Material, Table S6).

Discussion

As expected, our results provide strong support for certain known patterns of tiger-leopard dominance dynamics. For example, we observed one such dominance hierarchy in the form of dietary niche separation within and between ERTR and WRTR at spatio-temporal scale. In 2015, our results show relatively higher chital and similar Sambar frequencies (two major prey species in the study area) in WRTR compared to ERTR (Table 1) but overall distribution of different prey size class (large, medium and small) remained similar. However, in 2020, the WRTR area showed significantly higher large-bodied and lower small-bodied prey species compared to ERTR (as hypothesized in this study). For ERTR (in 2020, spatially as well as temporally), relatively higher frequency of small-bodied prey in leopard diet can be explained through possible competitive spatial exclusion of leopards. During 2020 surveys majority of the leopard faecal samples were obtained from the buffer regions of the park, which are not prime habitats in terms of prey abundances (Harihar *et al.*, 2020). The southern buffer and Gohri range of ERTR (see Figure 1) supports lower prey densities (25.24 individuals/km^2) than the core areas (39.23 individuals/km^2) (Harihar *et al.*, 2020) and still hosts human settlements and livestock that exert heavy pressure on forest resources (Johnsingh and Joshua, 1994; Harihar *et al.*, 2014; Harihar *et al.*, 2020) and affects the ungulates population density by hindering the forest resource availability to wild prey species (Pozo *et al.*, 2021; Rasal *et al.*, 2022; Salvatori *et al.*, 2022). An alternate explanation behind such pattern could be leopard prey preferences for the medium to small bodied prey size class (Karanth and Sunquistt, 1995; Pokheral and Wegge, 2019), but the natural experimental setup (presence of tiger in ERTR and not in WRTR) allowed us to confirm the effects of competition. The results indicate that leopard consumption of medium to small sized prey in ERTR is not driven by their preference towards these particular classes but rather is a consequence of competition from tiger. Significantly high consumption of large body-sized prey in WRTR (in absence of tiger) supports that such pattern is driven by competition pressure. Earlier Vanak *et al.* (2013) has also suggested similar pattern of competition (instead of preference) driven dietary niche separation.

These patterns can also be explained through the results of the NDVI analyses presented here. Since the relocation of majority of the human settlements from the eastern and western RTR (from 2003 onwards) and creation of inviolate space the habitat productivity of the park has improved significantly (Harihar *et al.*, 2008). Our NDVI analyses supported this by showing significant increase in vegetation

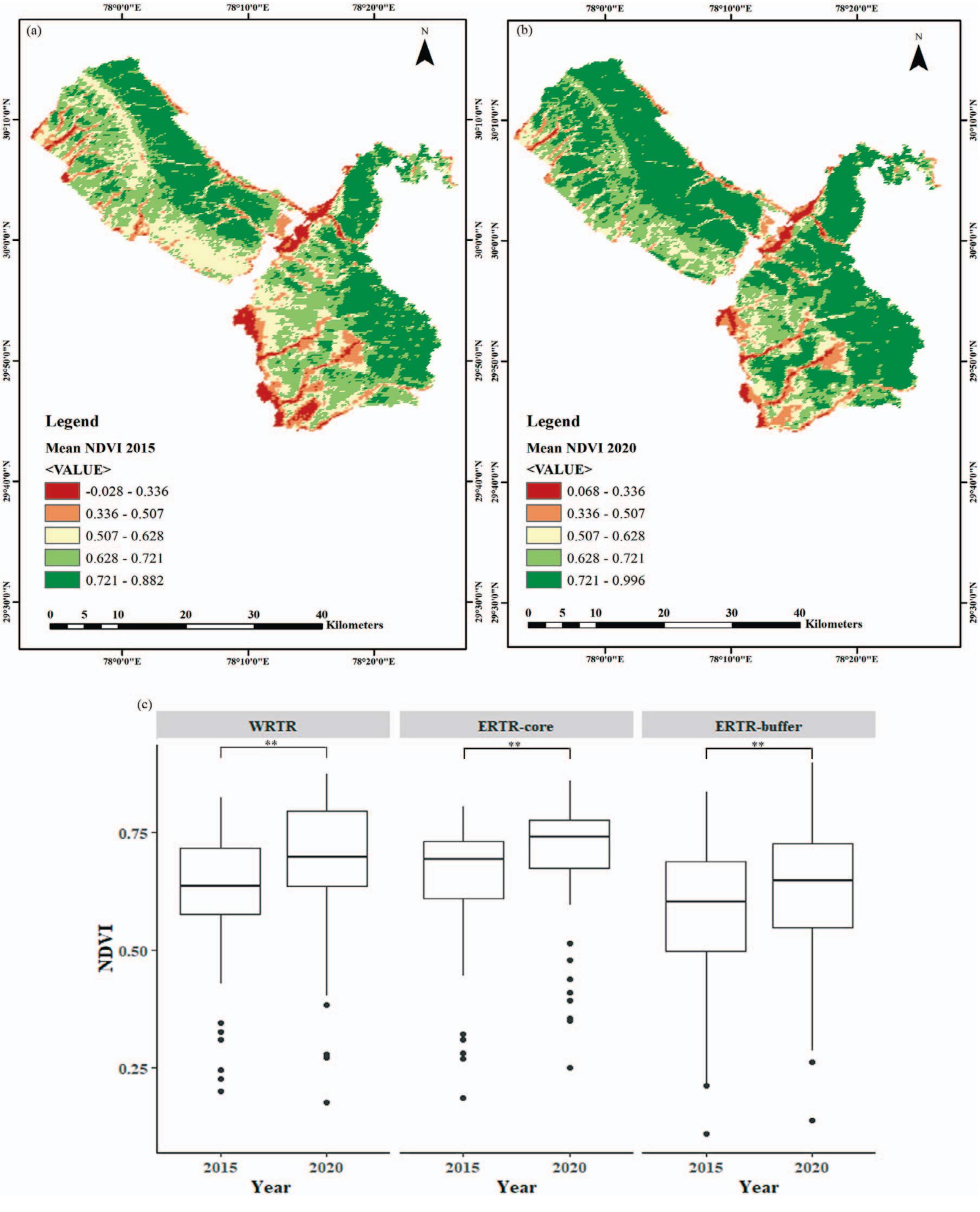

Figure 2: Assessment and comparison of habitat quality (through mean NDVI values) across core and buffer zones in two time points (winter 2015 and winter 2020). Panel (a) and (b) represents the mean NDVI gradients in 2105 and 2020, respectively. Panel (c) shows the temporal differences in mean NDVI values for WRTR, ERTR-core and ERTR-buffer areas. ** indicate significance value at P < 0.005.

cover across the park (WRTR, ERTR-core and ERTR-buffer) (Figure 2a and b). We feel that the habitat improvement (and subsequent increase in prey density, Jhala *et al.*, 2015, 2020) along with no-competition from tigers has resulted in availability of large-bodied prey species in the WRTR. In the ERTR, despite the habitat improvement, the population faced

Table 3: Results showing the association between the predictor (prey size categories and interaction of area and year) and response variables (fGCM and fT3M) based on GLM analyses. Models were fitted using log link and gamma error distributions

Response variables	Predictor variable	Level	Estimate	± SE	t value	Pr(>|*t*|)
fGCM	Area*Year	(Intercept)	162.256	78.035	2.079	0.0389*
		AreaWest	41.441	105.873	0.391	0.696
		Year	−0.076	0.039	−1.972	0.0500*
		AreaWest:Year	−0.020	0.052	−0.390	0.697
fGCM	Prey size (L, M and S)	(Intercept)	8.377	0.109	76.946	<2e-16***
		Prey size (Medium)	−0.042	0.142	−0.294	0.769
		Prey size (small)	0.341	0.214	1.592	0.113
fT3M	Area*Year	(Intercept)	133.090	96.622	1.377	0.170
		AreaWest	−356.293	131.091	−2.718	0.00718**
		Year	−0.062	0.048	−1.303	0.194
		AreaWest:Year	0.177	0.065	2.719	0.00716**
fT3M	Prey size (L, M and S)	(Intercept)	7.538	0.135	55.876	< 2e-16***
		Prey size (Medium)	−0.458	0.175	−2.612	0.00972**
		Prey size (small)	−0.306	0.266	−1.154	0.250

competition pressure from tigers over years (tiger density doubled in 2020 in ERTR, Jhala *et al.*, 2020), resulting in their potential displacement to the buffer regions (sub-optimal habitats with medium-sized prey availability). This can be substantiated by observing the faecal location patterns in this study and earlier reports of tiger and leopard occupancy in the park (Harihar *et al.*, 2011; Rathore, 2015). Spatial projection of the confirmed ERTR leopard samples showed that most of the 2015 samples were distributed in the core areas whereas majority of the 2020 samples were from the buffer areas (southern boundaries of the core) (Figure 1). Given that our sampling was conducted in the same trails in the core region during both years it can be inferred that the leopard presence in ERTR core decreased in 2020, possibly due to competition from socially-dominant tigers (as seen in other Terai habitats, McDougal, 1988; Wegge *et al.*, 2009; Odden *et al.*, 2010; Kafley *et al.*, 2019; Thapa *et al.*, 2021). Further, the tiger and leopard population estimation data (Jhala *et al.*, 2020) also suggest more photographic captures of leopards at northern and southern boundaries of the ERTR-core during 2018–19. Earlier, Harihar *et al.* (2011) and Rathore (2015) also reported a decline in leopard density in ERTR-core and their activity hotspots were concentrated towards the peripheral areas of the park as compared with the tiger activity hotspots towards the core areas. On the contrary, leopard faeces were found across more uniformly in WRTR in both sampling periods in the absence of any competition from tigers in this side of the park.

The results of the physiological impacts of dietary differences and habitat productivity mirrored the earlier mentioned patterns. Our measurements of fGCM and fT3M largely followed the NDVI (proxy for habitat productivity) and prey size class patterns across ERTR and WRTR, respectively (Figure 3 a and b). At spatial scale, we did not observe any significant differences in both fGCM and fT3M between ERTR and WRTR in 2015. This pattern did not support our hypothesis where we expected higher fGCM measures (from tiger competition) and lower fT3M (from possibly reduced prey accessibility) in ERTR. However, in 2020, our data show slightly different pattern in physiological measures where the fT3M levels in WRTR was significantly higher than ERTR, but no difference was found in fGCM titers. When looked at temporal scale, these patterns provide contrasting patterns for the physiological measures. Within ERTR no significant difference in fGCM and fT3M values were found between 2015 and 2020 (similar to the spatial patterns) but we observed significant differences in both measures in WRTR. The temporal-scale data provide strong support to explain the patterns in the light of ecological variables (habitat productivity and resource availability). From the observed data, the influences of prey availability (associated with habitat productivity) on fT3M (nutritional hormone) can be inferred as these measures showed very similar patterns. If we consider WRTR (both 2015 and 2020) as an example, this habitat showed low NDVI values in 2015 (indicating low habitat productivity at that time due to still recovering phase after human relocation events) and corresponding lower frequency of large-body sized prey in the leopard faeces. However, during 2020 we observed drastic changes in both ecological factors where both NDVI and large prey frequencies have significantly increased. The fT3M measures exactly follow the

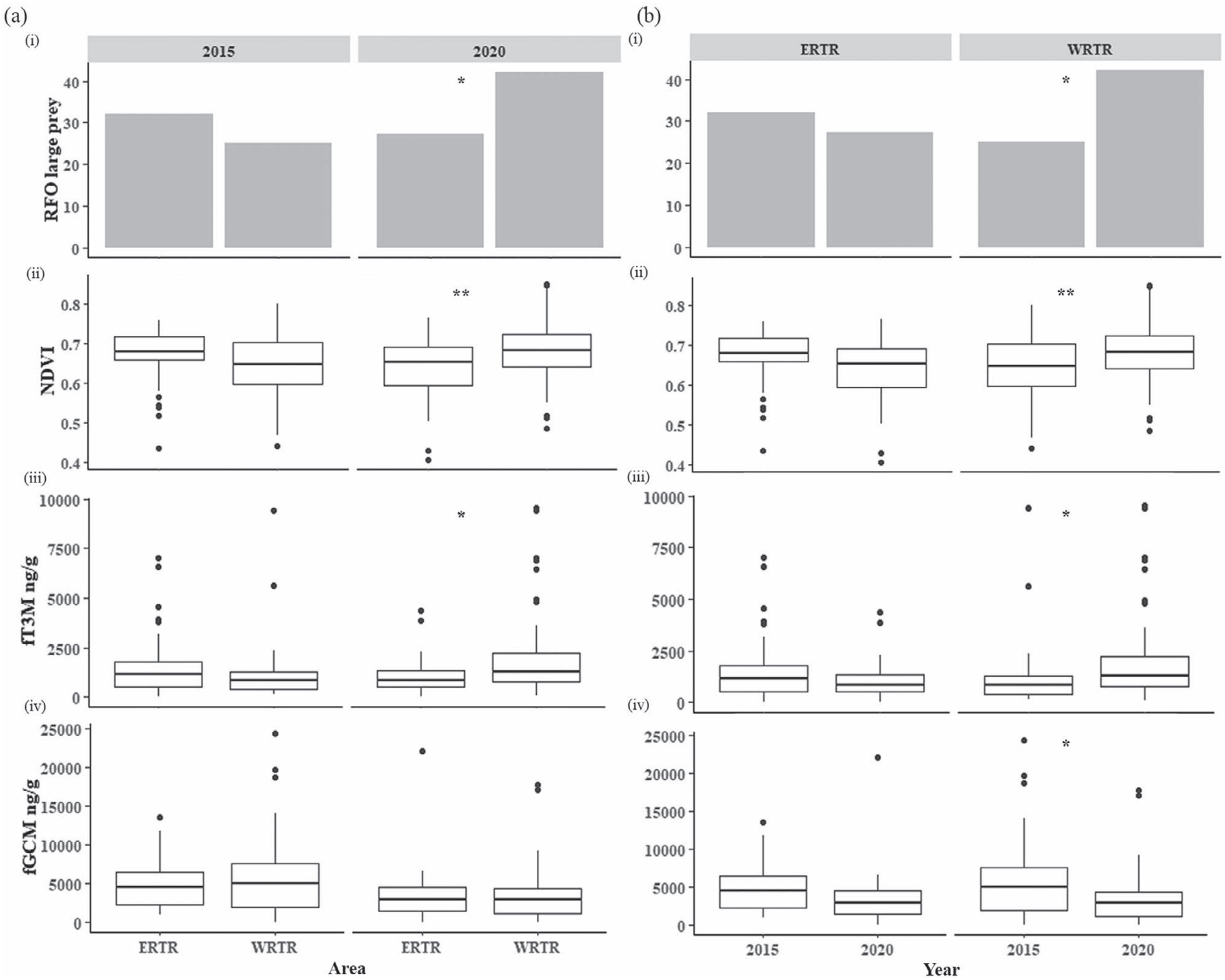

Figure 3: Spatio-temporal comparisons of the major variables (habitat variable—(i) large prey RFO, (ii) NDVI and physiological variable—(iii) fT3M and (iv) fGCM) used in this study. Panel (a) shows the temporal-scale differences and panel (b) indicate spatial differences. Significant differences are depicted by * (indicating $P < 0.05$) and ** (indicating $P < 0.005$).

same patterns (low in 2015 and significantly high in 2020), indicating a clear correlation between ecological variables and associated physiological responses (fT3M in this case). It is important to point out that absence of tiger in WRTR and stark differences in ecological variables in temporal scale made this inference easy compared to ERTR where more complex ecological interactions are seen. Apart from the other two ecological factors mentioned above, competition from tigers plays a major role in the patterns of physiological responses in ERTR resulting in different outcomes. Here, the data suggest decreasing habitat productivity and large prey frequency in leopard diet from 2015 to 2020, corroborating with a decreasing (but non-significant) trend in fT3M values between these two time periods. During 2015 the tiger density in ERTR was 2.90 (±0.87)/100 km^2 (Jhala *et al.*, 2015) which increased to 8 (± =1.4)/100 km^2 (Jhala *et al.*, 2020) during 2020, and the resulting increase in competition would explain the nutritional stress (low fT3M value) during 2020. Given the complex interactions among various ecological variables (habitat productivity, prey availability (in terms of size class) and various levels of competition pressures (from tigers)), we feel that it might take more time to observe significant differences in physiological parameters in ERTR. A similar study after 3 to 5 years may provide further clarification on any possible differences in fT3M values in ERTR.

Another possible factor that could play important role in leopard physiology in this landscape is intra-species competition. It is well-documented that the WRTR has experienced an increase in the leopard numbers during the study duration (Jhala *et al.*, 2021), possibly due to absence of wild tigers whereas increasing tiger population was associated

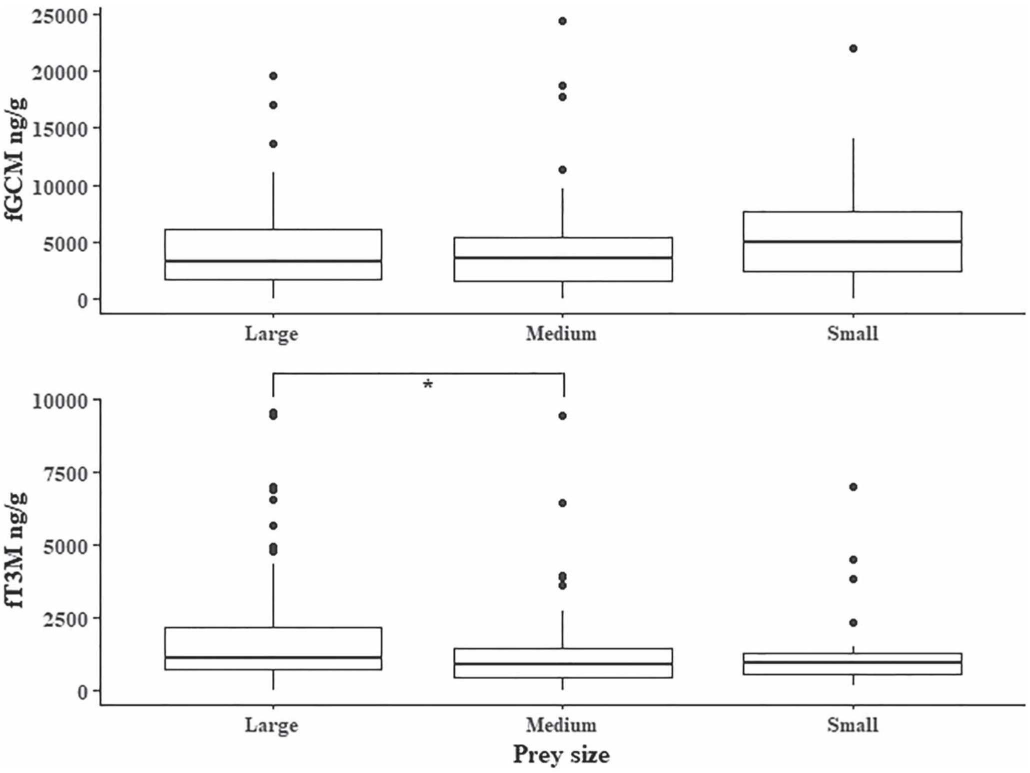

Figure 4: Comparison of (a) fGCM and (b) fT3M measures from faecal samples consisting large, medium and small body-sized prey. Significant differences are depicted by * (indicating $P < 0.05$).

with decreasing leopard density in ERTR-core (Harihar *et al.*, 2011). Such increase in population density in WRTR thus can cause some impact in leopard physiology. However, our data do not support this as any such intra-species competition should have resulted in higher fGCM (stress from competition) and lower fT3M (decrease nutritional status in a growing population) in WRTR in 2020 compared to 2015. Combined together, we can interpret that intra-species competition probably has no/less effects in the physiological pattern found during this study duration. However, it is important to point out that presenting the data as a whole (average value of fGCM from all samples collected) might also mask any possible local effects of such competitions. Future studies with fine-scale, grid-based sampling effort with leopard density information within each grid can help to address any effects of such competition.

One of the critical considerations in this study is the significant association of prey body size classes (large, medium and small) with fT3M measures. This was considered based on our available knowledge that large terrestrial predator distribution and abundance is significantly driven by higher biomass availability (Carbone *et al.*, 2011), and they are known to prefer large-bodied prey due to higher energy gains (Carbone *et al.*, 1999, 2007, 2011; Carbone and Gittleman, 2002; Radloff and Du Toit, 2004). Work on leopard energetics has also reported increased energy expenditure between meals when meal size from previous kill is large (Wilmers *et al.*, 2017). We used our data to test the effect of prey body size on fT3M measures, where leopard faeces with evidences of only specific body-size classes were identified and the fT3M measures were correlated (Figure 4b). In absence of any physiological validation of fT3M measures in leopard (see Mondol *et al.*, 2020 for tiger), this result can also be considered as biological validation of fT3M under field conditions.

The fGCM analyses showed slightly different patterns than the resource-driven fT3M data. Spatially, the ERTR and WRTR did not show any significant differences in fGCM levels in both 2015 and 2020 (Figure 3a(iv)), although 2020 fGCM values were relatively lower than 2015 (probably due to increased habitat productivity in 2020, at least for WRTR). However, at temporal scale, the ERTR and WRTR showed contrasting patterns (in terms of our hypotheses). In ERTR, we observed a lower value of fGCM in 2020 (non-significant) when compared to 2015. This is surprising when considered that in 2020 the NDVI as well as large prey proportion was lower in ERTR (with corresponding low fT3M indicating higher nutritional stress) (Figure 3b). Physiologically, this should result in high fGCM (Wasser *et al.*, 2011, 2017; Ayres *et al.*, 2012; Vynne *et al.*, 2014; Joly *et al.*, 2015; Dias *et al.*, 2017; McCormley *et al.*, 2018) values but the data show an opposite pattern. While it is difficult to point out the exact mechanism behind such pattern, one possible reason could be much less competition from tigers in the buffer areas (in 2020). This could be a preferred bargain for the spatially displaced leopards from the core areas of ERTR in 2020 (tiger density 8 (±1.4)/100 km^2 in 2020) at the cost of better nutritional status. Such behavior has been earlier documented in many anti-predatory response studies (Ylönen *et al.*, 2006; Creel *et al.*, 2009; Wasser *et al.*, 2011). WRTR leopards, in absence of any competition exhibited expected physiological responses at temporal scale where we observed higher fGCM (and corresponding high nutritional stress/low fT3M) in 2015 and low nutritional stress (high fT3M) and low fGCM in 2020. Both these patterns are also corroborating with the habitat quality and prey size class information in respective time frames (Figure 3b). It is important to point out that recent studies have cautioned regarding careful interpretations of GC/fGCM data due to contrasting patterns of directionality in GC response to chronic stress (Dickens and Romero, 2013), where both increase and decrease in GC titer has been observed as a consequence of chronic stress. Therefore, this study provides strong evidence of combining additional hormones (such as T3 used here for nutritional stress) along with GC as biomarkers to reveal different physiological regulatory responses to the environment under different contexts. Multidisciplinary approaches, as used in this study, would also bring out more comprehensive and ecologically meaningful outcomes that can be used in making appropriate conservation interventions.

Finally, the unique natural experimental habitat scenario, spatio-temporal sampling strategy and the patterns of fGCM and fT3M levels bring out some important conservation perspectives for leopards. Our results suggest that from a physiological perspective prey body size (large, medium and small) and availability (driven by habitat productivity) directly affect the dominant–subordinate dynamics, which is further compounded by the competition between both species (resulting in competitive exclusion). This has critical conservation implications for areas surrounding majority of the tiger landscapes across India. India has recently declared doubling its tiger numbers (population estimate of 1411 (1165–1675) in 2006 to 2967 (2603–3346) in 2018) across the country (Jhala *et al.*, 2020). Such increase in tiger population is expected to increase pressure on sympatric leopard populations pushing them towards buffers or more sub-optimal habitats, further exacerbating chances of human–leopard conflict. Therefore, expansion of leopard monitoring and population estimation efforts to buffers, their management in the context of conflicts and understanding of local factors driving the changes in population pattern would be critical for their future conservation. Our results highlight the importance of good-quality habitats and prey base for this species and future conservation efforts should ensure availability of the same for their persistence. This study also emphasizes the importance of similar work on other carnivore guilds particularly in the context of the ongoing Anthropocene, which is affecting inter-species dynamics globally.

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Conflict of Interest

The authors declare no conflict of interests.

Data availability statement

The data will be available upon request.

Authors' contributions

Conceptualization, Data generation, Data curation, Formal analysis, Validation, Visualization, Writing-Original Draft, Writing-Review and Editing: [**Shiv Kumari Patel**]; Data generation, Data curation, Writing-Review and Editing: [**Sourabh Ruhela, Suvankar Biswas and Supriya Bhatt**]; Conceptualization, Writing-Review and Editing, Supervision: [**Bivash Pandav**]; Conceptualization, Data Curation, Analysis, Resources, Writing-Original draft, Writing-Review and Editing, Supervision, Project administration, Funding acquisition: [**Samrat Mondol**].

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