UNIVERSIDADE DE LISBOA

Faculdade de Medicina Veterinária

Parasite ecology in spotted hyena in Serengeti National Park in Tanzania

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“You'll never know everything about anything, especially something you love.”

—Julia Child

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Parasite ecology in spotted hyena in Serengeti National Park in Tanzania

Abstract: Allostatic load is the energetic cost required to maintain homeostasis. A significant increase in allostatic load which cannot be fulfilled by increased food intake would be expected to result in resource allocation trade-offs, i.e., reduced allocation of resources to one life-process so that allocation of resources to another, more critical process can be maintained. In young animals, maintenance of growth is essential, and when food intake is insufficient, other life processes such as components of the immune system may be down regulated, leading to increased susceptibility to infections. This study aimed to investigate the impact of allostatic load, indicated by faecal glucocorticoid metabolite concentrations (fGCM), on the susceptibility to parasite infections as a result of resource allocation trade-offs, in juvenile spotted hyenas (*Crocuta crocuta*) in the Serengeti National Park, Tanzania. Therefore, I measured the allostatic load using a cortisol-3-CMO enzyme immunoassay verified for this species (Benhaiem et al., 2012) and assessed the parasite burden using faecal egg counts (FEC) of the three most abundant parasite species (*Ancylostoma, Spirometra* and *Cystoisospora*) with the expectation that FECs would increase with allostatic load. In general, the results indicated that juvenile spotted hyenas have an overall high prevalence of gastrointestinal parasites (98%, n = 104), with a mean of 3.1± 1.6 parasite genera per juvenile. The genus *Ancylostoma*, *Cystoisospora*, *Spirometra*, *Trichuris*, *Dipylidium* and parasites from the family Taeniidae and Spirurida were found. The fGCM concentration ranged between 4.9 and 503.2 ng/g with a mean of 55.8 ± 72.4 ng/g. I demonstrated that fGCM concentrations were significantly correlated to FECs of *Ancylostoma* spp., *Spirometra* sp. and *Cystoisospora* spp. in relation to fGCM (Spearman's rank correlation test, $\rho=0.371$, $p<0.001$, $\rho=0.272$, $p<0.05$, $\rho=0.287$, $p<0.01$ respectively). In addition, I investigated the factors modulating infection intensity of *Ancylostoma* spp. and revealed that age and co-infecting interactions are key factors of infection intensity. Furthermore, a preliminary phylogenetic analysis of the coccidian parasites from several carnivores living in the Serengeti National Park is provided, indicating that several coccidian are present in the carnivores living in the Serengeti ecosystem. This study provides important information on the mechanisms shaping parasite infections in a free-ranging carnivore.

Key words: Spotted hyena; Parasitology; Wildlife; *Ancylostoma*; Serengeti National Park; Allostatic load; Allostasis; faecal Glucocorticoid Metabolites;

Ecologia dos parasitas da hiena malhada do Parque Nacional do Serengeti na Tanzânia.

Resumo: A carga alostática refere-se ao desgaste associado aos mecanismos que mantêm a homeostase. Quando há um aumento significantivo da carga alostática que não seja compensado por um aumento de recursos disponíveis, é espectável que haja alocação de recursos de um sistema fisiológico para outro, para que processos críticos possam ser mantidos. Em juvenis, o crescimento é essêncial e quando há uma diminuição de recursos disponíveis, outros processos, como componentes do sistema imunitário, podem diminuir a sua atividade para que o crescimento seja mantido, consequentemente aumentando a suscetibilidade a infeções. Este estudo tem como objectivo avaliar o impacto da carga alostática, por intermédio da mensuração de metabolitos de glucocorticóides fecais (fGCM) na susceptibilidade a infeções parasitárias como resultado de "trade-offs" na alocação de recursos, em juvenis de hienas malhadas (*Crocuta crocuta*) do Parque Nacional do Serengeti, Tanzânia. Para a medição da carga alostática foi aplicado um teste imunoenzimático, cortisol-3-CMO, verificado para esta espécie (Benhaiem et al., 2012). A carga parasitária de hienas malhadas juvenis (<24 meses) é acedida através de contagens fecais de formas parasitárias (FEC) das espécies mais abundantes (*Ancylostoma, Spirometra* and *Cystoisospora*) com a expectativa que FEC aumente com a carca alostática. Os resultados indicam uma prevalência elevada de parasitas gastrointestinais (98%, n = 104), com uma média de 3.1± 1.6 géneros de parasitas por juvenil. Foram encontrados os géneros *Cystoisospora*, *Spirometra*, *Trichuris*, *Dipylidium* e as famílias Taeniidae e Spirurida. A concentração de fGCM varia entre 4.9 e 503.2 ng/g com uma média de 55.8 ± 72.4 ng/g. Foi demonstrada uma correlação significativa entre FEC de *Ancylostoma* spp., *Spirometra* sp. e *Cystoisospora* spp. com fGCM (teste de correlação de Spearman, $\rho=0.371$, $p<0.001$, $\rho=0.272$, $p<0.05$, $\rho=0.287$, $p<0.01$ respetivamente). Adicionalmente foram analisados possíveis fatores que influenciam a intensidade de infeção com *Ancylostoma* spp. e foi demonstrado que a idade e interações entre parasitas presentes são fatores chave na intensidade de infeção. Ademais foi feita uma análise filogenética preliminar dos coccidias presentes em vários carnívoros que co-habitam no Parque Nacional do Serengeti, revelando vários coccidias presentes no ecosistema. Este estudo providência informações relevantes dos mecanismos que modulam infeções num carnívoro de vida livre.

Palavras-chave: Hiena malhada; Parasitologia; Vida-selvagem; *Ancylostoma* spp; Coccídia; Parque Nacional do Serengeti; Carga alostática, Alostase, Metabolitos de Glucocorticóides fecais

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List of Abbreviations:

AIC	Akaike Information Criterion (AIC)
BLAST	Basic local alignment search tool (BLAST).
bp	base pair
dNTP	Deoxyribonucleotide triphosphate
EIA	Enzyme Immunoassay
EPG	Eggs per gram
FEC	Faecal egg counts
fGCM	Faecal glucocorticoids metabolites
FOC	Faecal oocyst counts
HPA	hypothalamic–pituitary–adrenal
i.e.	that is
L3	third stage larvae
LSU	Large subunit
MHC	major histocompatibility complex
OPG	oocyst per gram
PCR	Polymerase Chain Reactions
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SNS	Sympathetic Nervous System
sp.	Species (singular)
spp.	Species (plural)
SSU	Small subunit
Th1	T helper 1
Th2	T helper 2

CHAPTER 1. INTRODUCTION

The spotted hyena (*Crocuta crocuta*) in Serengeti National Park, which is situated in northern Tanzania, form large, stable social groups, termed clans, with separate dominance hierarchies between adult females and reproductively active (mostly immigrant) males (East & Hofer, 1993a). Juveniles stay in communal dens for the first year of their lives, and are not weaned until the 12-18 months old (Hofer & East, 1993c). Some information is available on parasites infecting spotted hyenas (Engh et al. 2003; Berentsen, 2012; East et al, 2013, East et al., 2008) but studies on factors modulating parasite infections in this species are scarce (East et al, 2013; East el al., 2015).

Allostasis is the term used to describe the process of maintaining homeostasis through change. Its cumulative cost, arising from the frequent activation of allostasis' mediators or its inefficient management, and the repair of "wear and tear of the body" is called allostatic load (McEwen, 1998; McEwen & Wingfield, 2003; Romero & Dickens, 2009; Hofer & East, 2012). In other words, allostatic load reflects the variation in the cost to an individual of maintaining homeostasis during its daily and seasonal routines. Moreover, life history theory predicts that when food intake and stored body resources available to an individual at a given life stage are insufficient to sustain all body functions required at that life stage, then available resources must be divided among key processes such as growth, immunity, maintenance, and reproduction in a manner that should optimize fitness (Fabian & Flatt 2012; Archie 2013).

In the spotted hyena, it is known that the rate of juvenile growth during this early life history stage has important fitness consequences (Hofer & East, 1993c; Hofer & East, 2003). Juveniles with higher growth rates have better survival to adulthood (at 24 months of age) and also start reproduction earlier than those with slower growth (Hofer & East, 2003; Höner et al. 2010). It is expected that (all else being equal) juvenile spotted hyenas with a high allostatic load that cannot compensate for this by increasing their resource intake, would need to trade-off one life process against another. For example, resources that otherwise should go to maintaining the immune system could be reallocated to maintain growth (Schmid-Hempel, 2003). If this trade-off was sufficiently high to cause a reduction in immune function then increased susceptibility to infection would be expected. Furthermore, parasite infections are costly to the host, increasing the host's allostatic load in several ways, such as by consuming host nutrients, activating immune system cells, and using body resources to repair tissue damaged by parasites (Colditz, 2008). Spotted hyenas normally give birth to singleton or twin litters. In twin litters, a dominance relationship between littermates emerges soon after birth (Benhaiem et al., 2013)

and litter size significantly impacts growth in that members of singleton litters have higher growth than those of twin litters, and dominant members of twin litters grow faster than subordinates (Hofer & East 2008).

Combining knowledge of spotted hyena biology with the theories of life-history trade-offs and allostatic load, I predicted that: 1) insufficient intake of food would be most likely to lead to a reduction in the allocation of resources to immune processes in subordinate members of twin litters and least likely to do so in singleton cubs during the first 12 months of life when young hyenas are dependent on milk from their mothers. I used parasite eggs/oocysts per gram faeces (termed hereafter as parasite egg load) as a proxy measure of immune function; 2) that the cost of maintaining allostasis should be higher in subordinate members of twin litters than in members of singleton litters, all else being equal. I used the concentration of faecal glucocorticoid metabolites (fGCM) as a measure of allostatic load (McEwen & Wingfield, 2003); 3) parasite egg load would decrease with increasing age, i.e. younger juveniles (<6m) would harbour higher parasite egg loads than older juvenile (>6 and < 24 month of age), because they have higher allostatic load (Benhaiem, 2013) and are less likely to have acquired immunity to intestinal parasites (Ardia, Parmentier & Vogel, 2011; Tinsley et al., 2012; Hayward, 2013; Obata-Ninomiya et al., 2013; East et al., 2013). Finally, both positive and negative synergistic interactions have been described between various species of parasites (reviewed by Maizels et al., 2004; Riet, Hartgers & Yazdanbakhsh, 2007; Graham, a. L. 2008; Helmby, 2009; reviewed by Råberg, Graham, & Read, 2009), and specifically in spotted hyena an increase in the likelihood of infection with the opportunistic protozoan *Cystoisospora sp*., as infection with tween *Ancylostoma sp*. increased, I expected to find evidence of similar interaction between intestinal parasites in juvenile spotted hyenas.

In this thesis a modification of the McMaster flotation technique was performed using samples collected from individually known juvenile spotted hyenas in order to quantify parasite egg load. For the assessment of allostatic load, faecal glucocorticoid metabolites (fGCM) were measured using enzyme immunoassays (EIA) previously validated for the spotted hyena (Benhaiem et al, 2012). Additionally, I aimed to genetically describe coccidian parasites present in the faeces from several African carnivores in Serengeti National Park in Tanzania and to determine the phylogenetic relationship between these parasites.

The aims of this thesis are the following:

1) Estimate the prevalence of intestinal parasites that infected individually know juvenile spotted hyenas;

2) Investigate the factors modulating the infection intensity with the hookworm *Ancylostoma* spp. in juvenile spotted hyenas

3) Provide a phylogenetic analysis of the protozoa *Cystoisospora* spp. in carnivores living in Serengeti National Park in Tanzania.

1.1 Internship activities:

This study resulted from an internship at Leibniz Institute for Zoo and Wildlife research in Berlin, starting from the 18th August until 19th of December, 2014, under the supervision of Dr. Marion East and co-supervision of Prof. Dr. Luis Madeira de Carvalho.
During this period I performed the parasite screening, extraction and the measuring of faecal glucocorticoid metabolites and genetic, phylogenetic and statistical analysis of all the samples available for the study, described in the method section of the present thesis.

CHAPTER 2. LITERATURE REVIEW

2.1. Spotted hyena (*Crocuta crocuta*)

The spotted hyena (figure 1) is the largest member of the family Hyaenidae. They have a spotted coat, round ears and in the Serengeti National Park, males weight around 45 kg and females 55 kg. They are unique among mammals due to their social behaviour and outwardly similar genitalia (Frank, 1986a; Frank, Glickman, and Pouch, 1990; Mills & Hofer, 1998). This species exists in several countries and their conservation status is considered as lower risk: least concern (IUCN, 2014), even though they are widely persecuted, most often in farming areas. In some areas spotted hyena populations are considered to be declining (Mills & Hofer, 1998).

Figure 1. Spotted hyena (*Crocuta crocuta*) juvenile from the Serengeti National Park, Tanzania. Courtesy of Dagmar Thierer.

2.1.1. Social behaviour

The spotted hyenas in the Serengeti National Park form a large, stable social unit called clans (Kruuk, 1972) with strict intra-sexual hierarchies, in which the lowest ranking females at the bottom of the female hierarchy is socially dominant over top ranking male in the immigrant male hierarchy (East & Hofer, 1993a). Maternal rank is inherited, i.e. juveniles acquire the social status below their mothers (Holekamp & Smale, 1993; East et al. 2009). Female hyenas stay in their natal clan, whereas most (but not all) natal males disperse at approximately 3 yrs

of age and eventually join other clans (Frank, 1986b; East & Hofer, 1993a). Litters are born throughout the year (Frank, 1986a), and all females attempt to breed, regardless of their social status (Hofer & East, 1993a).

Clans are "fission-fusion" societies in which individuals frequently operate alone or in small groups and join to cooperate in defence of their territory, food resources and at the communal den (Mills & Hofer, 1998).

In juvenile spotted hyenas, aggression between siblings is associated with competition over access to maternal milk (Golla et al., 1999, Hofer & East, 1997; Benhaiem et al., 2012b). The resulting agonistic encounters between siblings may result in wounds, both in the dominant and in the subordinate sibling (Hofer & East, 1997). If frequent, they can lead to significant growth differences between littermates, ultimately causing the death by starvation of the subordinate member of the litter (Hofer & East, 1997). Wounds inflicted during sibling conflict may be debilitating for undernourished cubs. Death resulting from the monopolization of food resources, occurs in mostly before litters are 3 months of age (Hofer & East, 1997; Golla et all, 1999) and it is facultative, meaning it is dependent on maternal input in the form of milk (Hofer & East, 1997; Golla, Hofer & East, 1999; Hofer & East, 2008). Facultative siblicide in the spotted hyena is likely to be due to the high investment in lactation by mothers, which favoured selection for neonatal aggression (Hofer & East 1997; Hofer & East 2008).

2.1.2. Communal dens

Most female spotted hyenas give birth in private birth dens, and when about two week old, juveniles are moved to communal dens (East, Hofer & Turk, 1989) The use by juveniles of underground burrows is probably an adaptation to reduce juvenile predation by other carnivores, particularly the lion (*Panthera leo*). The first few weeks after birth they only emerge in the den entrance in the presence of their mother (East, Hofer & Turk, 1989). Juveniles stay in communal dens for the first year of their lives (Hofer & East, 1993c), and are dependent on their mother milk for the first 12 months of life and may not be weaned until 18 month of age. Mothers suckle their offspring at dawn and dusk, at the den or in its vicinity (Hofer & East, 1993c; Hofer and East; 1995) and they visit their offspring almost every morning and evening, in the first 4 weeks of life, independently of prey abundance. After this period, mother attendance decreases. Moreover, communal dens are the centre of the clan's social activity and individuals of both sexes visit the den (Hofer & East, 1993c, Smale et al., 1993). During the day, adults and sub adults (i.e. animals between 12 and 24 months of age) rest in other sites than

the communal den, and in the evening they forage inside the clan's territory or go on commuting trips (Hofer & East, 1993c).

2.1.3. Demography

The group size in the Serengeti is estimated to be about 59 individuals, with a highly variable ratio of juvenile per female (0.4-0.84) (Hofer & East, 1993a). The clans occupy and defend territories where the communal dens are located, used for breeding and feeding purpose (Hofer & East, 1993a).

2.1.4. Feeding behaviour

The spotted hyenas are opportunistic hunters, preying several species, switching to feed on the most abundant prey and can also feed on carrion (Cooper, Holekamp & Smale, 1999). In the Serengeti National Park, the main prey of spotted hyenas are all migratory species, namely wildebeests (*Connochaetes taurinus*), Thomson's gazelles (*Gazella thomsoni*) and zebras (*Equus burchelli*) (Hofer & East, 1993a). Because prey availability is highly variable in the Serengeti National Park, due to migratory movement of these ungulates, spotted hyena not only feed inside their territory, but when food availability is insufficient, they go on commuting trips outside their territories, to feed on large migratory herds in a non-synchronized pattern (Hofer & East, 1993 a, b).

Individuals that go on commuting trips usually either go alone or as small groups. Females can be accompanied by their older offspring, and the straight line distances travelled from the clan 'home' territory is between 40 km and 70 km (Hofer & East, 1993b). Prey availability in the clan territory influences maternal milk provisioning patterns, as when prey availability declines, the frequency in which mothers visit the communal den to suckle their offspring decreases (Hofer & East, 1993c). Another factor influencing maternal input is the mother's social status. Because females at the top of the hierarchy have priority in access to large food resources (Hofer & East, 1993a; Frank, 1986b), when prey availability is medium, dominant females feed within the clan territory and nurse their offspring more often, compared to low ranking females that go on costly commuting trips and suckle their offspring less often (Hofer & East, 2003).

2.2. Introduction to life history theory

Life history theory analyses how environmental factors, trade-offs among life history traits (size at weaning, lactation period, longevity, etc.) and limiting constraints affect measures of fitness, such as lifetime reproductive success and longevity (Fabian & Flatt 2012). As resources are often limited, when nutrients are insufficient to maintain all life processes, individuals must allocate their resources accordingly to their specific life stage so that fitness is optimized. For instance, young animals should invest in growth, lactating mother should bias investment in lactation, provided this should increase the offspring chances of surviving without compromising the survival of the mother (M. East, personal communication, April 13, 2015).

Maintenance of the immune system is a plastic trait that can be adjust by individual decisions, reflecting the environment context and life history trade-offs (Schmid-Hempel, 2003; Ardia, Parmentier & Vogel, 2011; reviewed by Horrocks, Matson & Tieleman, 2011). However, constrains can arise and limit the flexibility of the immune variability, such as genetic or physiological constrains. For example, elevated stress hormones, such as glucocorticoids constrain immunity due to limitation on an integrated physiological system (Ardia, Parmentier & Vogel, 2011). Even though it can be adaptive for an individual to shift resources to immunity in a particular context, individuals with chronic elevation on glucocorticoids may not have the ability to do so (Ardia, Parmentier & Vogel, 2011).

There is a broad range of studies, (reviewed by Lochmiller & Deerenberg, 2000; Verhulst, Riedstra & Wiersma, 2005; Colditz; 2008) demonstrating that the immune system has important nutritional cost. So, a trade-off between immunity and growth is expected when the organism cannot compensate for the extra demand on resources (Schmid-Hempel, 2003). Moreover, there is clear evidence of trade-offs between immunity and reproduction (French, DeNardo & Moore, 2007; Verhulst, Riedstra & Wiersma, 2005) and it depends on the availability of energetic resources. For instance, in female tree lizards, when food intake is restricted during reproductive stages, the immune system is down regulated, but when there is no restriction of food resources, females are able to mount an immune response similar to their pre-reproductive counterparts (French, DeNardo & Moore, 2007).

Growth is an important life history stage in spotted hyenas, because of the relatively slow rate of juvenile development, and the dependence on maternal milk for the first year of their life (Hofer & East, 1993c). Moreover, the significant fitness consequences of female social status in the spotted hyena matrilineal society, in terms of cub growth rates and survival to adulthood are linked to high maternal input in terms of the production of nutritious milk over a long

nursing period and how the pattern of milk delivery to offspring is determined by rank related access to food resources in the clan territory (Hofer & East, 1993c; Hofer & East, 2003).

Spotted hyena juveniles with higher long term growth are more likely to survive to the age of adulthood (2 year old) and females with higher growth rates have their first litter at a younger age (Hofer & East, 1993c ; Hofer & East, 2003). Thus, the females which have their first litter at younger age are likely to raise more juveniles during their lifetime than females which have their first parturition later in their life. Growth rates are higher in offspring that are members of a singleton litter, followed by dominant and lastly subordinate members of a twin litter (Hofer & East, 2008). Offspring growth and survival to adulthood increases as maternal social status increases (Hofer & East, 2003). Hence, maternal social status, litter size and within-litter social status are all factors that have an important influence in juvenile survival to adulthood, and thus are expected to affect lifetime reproductive success.

So, it is expected that hunger or insufficient food increases cortisol (an indicator of allostatic load). When these circumstances apply to juveniles, it should result in allocation trade-offs so that growth can be maintained, likely to result in insufficient nutrients to fully maintain all immune processes, sufficient to increase parasite infections. However, parasite infections have shown to be costly to the host by consumption of nutrients, damaged caused by parasites and activation of host's immune responses (Lochmiller & Deerenberg, 2000; Colditz, 2008), increasing its allostatic load. As consequence, it may lead to a decrease in body condition and therefore predisposing to infections (Beldomico et al., 2008). In accordance with this idea, experimental manipulation of parasite levels in the European shag and North American red squirrels, respectively, have shown an increase of offspring survival, demonstrating the costs of parasitism (Reed et al, 2008; Patterson et al, 2013). Moreover, female spotted hyenas have higher incidence of the hookworm *Ancylostoma* spp. and higher egg loads during energy costly period of lactation than during non-lactating period, consistent with the idea of decrease resource allocation into the immune system during this period (East et al., 2015).

2.3. Allostatic load

The concept of stress has been used in different contexts, generating confusion. Stress can imply an environmental perturbation, the response an individual makes in order to adapt or the resulting pathologies, and does not take into account the individual differences of responses to the environment (McEwen, 1998; McEwen & Wingfield, 2003). Hence, in my thesis I have used the allostasis concept is adopted, and stress refers to the unpredictable events that lead to

allostatic load, hence application of the term helps avoid ambiguity (McEwen & Wingfield, 2003).

2.3.1 General concepts

Allostasis is the process that maintains homeostasis, for example through physiology and behaviour adjustments made to prevailing environmental conditions (McEwen & Wingfield, 2003; McEwen & Wingfield, 2010). It covers all changes which allows individuals to maintain stability during predictable events, such as lactation or migration, and unpredictable events, such as environmental perturbations or hierarchical conflicts in order to adapt.

Allostatic state refers to a chronic alteration of the activity of regulatory systems, i.e. allostasis mediators such as glucocorticoids, resulting in an imbalance of these mediators (McEwen & Wingfield, 2003; Korte, Koolhaas, Wingfield & McEwen, 2005). It can result due to dysregulation or dysfunction of primary mediator's production due to frequent challenges, failure to adapt to the challenges, prolonged or inadequate response (McEwen, 2002). A sustained activation of the allostatic state leads to allostatic load.

Allostatic load is a term used to describe the cumulative wear and tear of the body, which is "the gradual breakdown in responsiveness of an organism's physiological system because its maintenance for the purpose of enabling *physiological mediators* is costly" (Hofer & East, 2012). Allostatic load depends in the energy supply and demand. Thus, the ability of an individual to maintain these changes depend on social status, diseases, parasite load, etc. (McEwen, 1998; McEwen & Wingfield, 2003). In other words, allostatic load reflects the ease individuals have in their daily and seasonal activities in relation to social organization and environmental conditions plus unpredictable environmental events.

When there is an increase in allostatic load, such as in the presence of a disease or social interaction, and the energy requirements are higher than the energy intake, a type I allostatic overload emerges (McEwen, 1998; McEwen & Wingfield 2003). If the energy requirements are not exceeded by the energy intake, and allostatic load is continuously elevated, it is considered type II allostatic overload and it is independent of seasonal changes in the environment (McEwen & Wingfield 2003; McEwen & Wingfield 2004).

Unpredictable events can prejudice an individual by decreasing the availability of resources, increasing energetic requirements or restricting access to resources, reflected in an elevated glucocorticoid concentration, it may result in type I allostatic overload and an emergency life history stage is activated (Wingfield et al., 1998; Goymann & Wingfield, 2004). This response is characterised by changes of behaviours that lead to suspension of life history stages, but also

social hierarchies may be suspended and emergency behaviours such as changing habitat and mobilization of stored energy (Wingfield et al., 1998). This response helps the individual to cope with unpredictable events and it is a strategy to avoid and resist stress (Goymann & Wingfield, 2004). On the other hand, permanent imbalances that may lead to allostatic overload, such as social conflicts, which do not exceed available resources to provide energy demands do not activate the emergence life history stage and are termed type II allostatic overload (McEwen &Wingfield 2003; Goymann & Wingfield, 2004).

2.3.2 Allostatic load and immunity

The response to predictable (e.g., growth, lactation, migration) and unpredictable challenges (e.g., disease, storms), are through the release of mediators of allostasis. Most changes in the immune system are through the activation of the hypothalamic–pituitary–adrenal (HPA) axis and activation of the sympathetic nervous system (SNS) (Padgett & Glaser, 2003; Martin, 2009; Crespi, Williams, Jessop, Delehanty, 2013). These hormones modulate immune responses act either directly, by biding to cellular receptors, such as lymphocytes, monocytes or macrophages, or indirectly by dysregulation of the cytokine production (Padgett & Glaser, 2003; Glaser & Kiecolt-Glaser, 2005). Glucocorticoids play an important role in regulating energy during challenges, because they regulate energetic allocation between life processes (Crespi, Williams, Jessop, Delehanty, 2013).

The duration of the stimuli leading to an increase in allostasis modulates the animal responses. The immediate physiological consequence is a tendency for increasing immunity. However, when the challenge persist for a longer period of time (days to months), immune responses tends to be suppressed (Sapolsky, Romero & Munck 2000; Martin, 2009; Ardia, Parmentier & Vogel, 2011). Suppression of the immune system may reflect an adaptive response in terms of energy savings or the consequence of the persistence of high allostatic load for too long might result in allostatic overload, which creates pathological problems such as bacterial and viral pathogenesis exacerbation, slower wound healing, etc. (McEwen, 2002; Padgett & Glaser, 2003; Martin, 2009, Romero, Dickens & Cyr, 2009).

Immune responses to the release of allostasis mediators are characterized by a tendency to elevate the innate immunity, within hours following the challenge, followed by the elevation firstly of the T helper 1 response, and then elevation of the T helper 2 response if the stimulus persists days to weeks. After this, a general decrease in the immune responses occurs, such as a decrease of neutrophils, macrophages, antigen-presenting cells, and circulating levels of natural killer cells and suppression of pro-inflammatory cytokines and chemokines (Padgett &

Glaser, 2003; Korte, Koolhas, Wingfield & McEwen, 2005; Martin, 2009; Ardia, Parmentier & Vogel, 2011). Neuropeptide mediated responses are rapidly effective, acting through second messenger cascades within minutes to hours after the challenge and glucocorticoids take longer to exert its effects, hours to months after the challenge (Sapolsky et al., 2000; Martin, 2009).

2.3.3. Allostatic load in the spotted hyena

Sibling aggression is associated with competition over access to maternal milk (Hofer & East, 1997; Golla, Hofer & East, 1999; Hofer & East, 2008), and it appears to be the result of competition and frequent exclusion from mother resources, i.e. milk (Benhaiem et al, 2013). However, during low prey abundance periods, twins had higher allostatic load but not singletons compared to when they were feed daily, leading to the assumption that sibling competition has a stronger effect on allostatic load than hunger in the spotted hyena (Benhaiem et al., 2013). Thus, in young siblings, subordinates have higher allostatic load than dominants (Benhaiem et al., 2013). Moreover, sibling aggression increases as maternal social status declines, because mothers with a higher social rank have priority of access to food resources and less often go on commuting trips, consequently less often needing to leave their cubs unattended for several days. Additionally there may be a decrease in the ability of mothers to lactate, due to the lack of food, when they go on commuting trips (Golla et al, 1999). Female dominants have higher allostatic load than male dominants, especially if their sibling is a male (Benhaiem et al, 2013), likely because subordinate siblings with a dominant sister are more likely to suffer siblicide than subordinates with a dominant brother (Hofer & East, 1997; Benhaiem et al., 2012). When resource availability is low, the subordinate brother is more likely to suffer siblicide, thus responds less subordinately (Hofer & East 1997; Benhaiem et al, 2012). It is also shown (Golla et all, 1999) that all female and all male litters have higher aggression rates compared with mixed sex litters. Furthermore, sibling competition is not only through aggressive behaviour. It is likely that females are better competitors than males, use more effective counter tactics and gain more from being dominant than males (Benhaiem et al, 2012). Therefore, dominant siblings do not exert complete control over their subordinate littermates, so in a low resource setup, hungry subordinates respond less submissively to the dominant one, leading to escalated conflicts (Benhaiem et al, 2012).

2.4. Immune responses of parasite infections

The distribution of nematode parasites is typically aggregated (Poulin, 2013). That means most animals have light infection load, or are uninfected and few hosts harbour heavy infection load and are the principal source of environmental contamination (reviewed by Hayward, 2013). Moreover, some individuals carry a parasite load and show no signs while other carry the same load and become ill (Maizels et al., 2004).

These differences amongst individuals may arise due to variation in immune responses, but there are other factors, such as constraints that limit the flexibility of immune investment, co-infections and parasite tactics to avoid host defences (Ardia, Parmentier, Vogel, 2011, Graham, 2013). There are multiple choices in immune responses, and these are influenced by many factors, including sex, nutritional status, social status, life history stages and trade-offs. Differences in immune responses are thought to reflect interaction between environment and life history trade-offs (Ardia, Parmentier, Vogel, 2011; reviewed by Horrocks, Matson & Tieleman, 2011).

Host defences against parasites can adopt distinct strategies, that include avoidance, to reduce the risk of infection, resistance, in order to reduce the parasite burden and tolerance, to reduce the negative impacts of the parasite infection on host fitness (reviewed by Råberg, Graham, & Read, 2009, reviewed by Medzhitov, Scnheider & Soares, 2012). Typically, when transmission rates are high, it is better to tolerate infection, leading to chronicity (Cressler, Graham & Day, 2015). Resistance is measured as the inverse of infection intensity and tolerance as the rate of change in fitness caused by an increase in parasite burden (Råberg, Graham, & Read, 2009).

Helminth infections generally induce a skewed T helper 2 type (Th2) response and regulatory immune modulation that becomes evident in chronically or highly infected hosts (Maizels et al., 2004; Riet, Hartgers & Yazdanbakhsh, 2007; Helmby, 2009). The Th2 response leads to cytokine production, eosinophilia, mastocytosis, goblet cell hyperplasia and IgE antibodies elevation, however is not always successful to eliminate the parasites. The results are chronic infections, enhanced by parasite modulation of immune responses mechanisms, such as regulatory T cells, in order to create an anti-inflammatory environment, beneficial to the parasite feeding and reproduction. This modulation is beneficial to the parasite and likely to the host as well, because it can have a protective effect by preventing excessive realise of cytokines that may lead to damage in the host's tissues (Maizels et al., 2004; Riet, Hartgers & Yazdanbakhsh, 2007; Helmby, 2009). This immune modulation has potential impacts on co-infections, described below.

2.4.1. Genetic influence in immune responses

Genetic diversity plays an important role in the immune responses towards pathogens via antigens recognition. This is particularly true for major histocompatibility complex (MHC) diversity. MHC molecules recognize and present antigens to T-cell receptors, resulting in the onset of a cascade of immune responses. MHC class I molecules are important in intracellular pathogens recognition and MHC class II molecules in extracellular pathogens, such as helminths. Genes coding MHC molecules are highly polymorphic and its variability is thought to reflect pathogen selection pressure (Meyer-Lucht & Sommer, 2005; Schwensow, Fietz, Dausmann, & Sommer, 2007; Ardia, Parmentier, Vogel, 2011; Castro-Pietro et al., 2012). There are different hypotheses to explain pathogen mediated selection mechanisms: 1) heterozygote advantage, which assumes that heterozygotes have the ability to resist to a broader range of pathogens than homozygotes, thus having advantage in pathogen resistance; and 2) Rare allele advantage in which pathogens have selective pressures to evade recognition by the common MHC alleles, changing the antigenicity, providing advantage to select rare MHC alleles that recognize the new antigens, which consequently spread throughout the population; 3) The fluctuation selection pressure that maintains diversity at the MHC genes due to the spatial and temporal pathogen heterogeneity. However the role of this mechanism is not yet clarified (Meyer-Lucht, 2005; Spurgin & Richardson, 2010; Castro-Pietro et al., 2012)

2.4.2. Sex influence in immune responses

Males generally have weaker immune responses toward high parasite loads than females, and it is hypothesised that the reasons for that are different behaviour and resource allocation trade-offs, in which males maximize fitness by increasing paternity and females by increasing longevity (Hillegass, Waterman & Roth 2008; Love, Salvante, Dale & Williams, 2008; reviewed by Hayward, 2013). The key for this process seems to be the neuroendocrine system, in which sex hormones play a role in the sexual dimorphism in the immune responses towards parasites.

2.4.3. Age influence in immune responses

Age related variation in immune system can be explained by resource allocation concepts explained above and immunity development. This latter is dependent on previous exposure and the level of exposure, protection acquired from the mother and nutritional access (reviewed by Hayward, 2013). Once infected, immunity can be acquired by the host towards the same type

of parasites, providing immunity protection in the following infections. Affinity of antigen-antibody increases with exposure and is an example of adaptive changes, however, immune responses are constrained with age, for example due to a decrease of function in the T-cell population due to thymic involution. Acquired immunity usually increases with age, to a peak until stabilization or decline, influencing parasite load (Ardia, Parmentier & Vogel, 2011; Tinsley et al., 2012; Hayward, 2013; Obata-Ninomiya et al., 2013).

2.4.4. Co-infections influence on parasite load

Parasite community dynamics are driven by several mechanisms. Parasite densities and host health are influenced by different co-existing parasites and parasite-host interactions, such as downregulation of inflammatory immune responses or resource competition (reviewed by Maizels et al., 2004; Riet, Hartgers & Yazdanbakhsh, 2007; Graham, a. L. 2008; Helmby, 2009; reviewed by Råberg, Graham, & Read, 2009). Helminths regulate the host immune responses, in order to create a favourable environment so that they cannot only survive but also successfully feed and reproduce (Maizels et al., 2004; Riet, Hartgers & Yazdanbakhsh, 2007), thus altering the host overall tolerance to parasites. Moreover, acquired immunity for one parasite species can also give protection to other parasite species (Schmidt-Hempel, 2003; Bourke, Maizels & Mutapi, 2011). The interaction between co-infecting parasites depends on several factors, unlikely to act separately, such as the type of parasite, infection intensity, host age, etc. Thus, the presence of a parasite can be protective, have a neutral effect or increase susceptibility to other parasites.

2.5. Parasites previously described in spotted hyena population and general description

Several parasite taxa were previously described in spotted hyenas (East et al., 2008; Engh et al, 2003; Berentsen et al., 2012; East et al., 2013). In this study I applied similar methods to those described for a coprologic survey of spotted hyenas in the Masai Mara National Reserve in Kenya (Engh et al., 2003) which is the northern extension of the Serengeti ecosystem. Engh et al. (2003) presented the prevalence and intensity of intestinal parasites infecting spotted hyenas in one study clan. The most prevalent parasite species in spotted hyenas in the Masai Mara were considered important for my study and hence are briefly described.

2.5.1. Genus *Ancylostoma*

Ancylostoma sp. was the most prevalent intestinal parasite species found by Engh et al.(2003). Also known as hookworms, this parasite belongs to the family of Ancylostomatidae, has a direct life cycle and live attached to the mucous layer of the small intestine. In some *Ancylostoma* species a small percentage of adult worms may also be found in the large intestine (Schmidt & Roberts, 1996; Bowman, 1999; Sowemimo & Asaolu, 2008). Hookworms feed on their host's blood and tissue fluids. Eggs morphology is typically of strongyle worms (figure 2) - with a smooth surface, elliptic shape, containing an embryo in the morula stage of development when shred in the faeces - and are shred in the faeces by the adult hookworm who lives and mates in the small intestine. They hatch in an environment with suitable substrate, temperature and moisture, and once they develop into the third stage larvae (L3), they become infective to the host. Infections can occur with the L3 by penetration of the skin, ingestion, transmammary or ingestion of paratenic hosts. Once in the definite host they undergo tissue migration until they reach adulthood in the small intestine (Schmidt & Roberts, 1996; Urqhhart et al. 1996; Bowman, 1999). They ingest blood, rupture erythrocytes and degrade haemoglobin. This loss of blood leads to iron deficiency anaemia and protein malnutrition (reviewed by Hotez et al, 2005). In children, a high burden of hookworm infection is associated with growth retardation, intellectual and cognitive impairments (reviewed by Hotez et al, 2005).

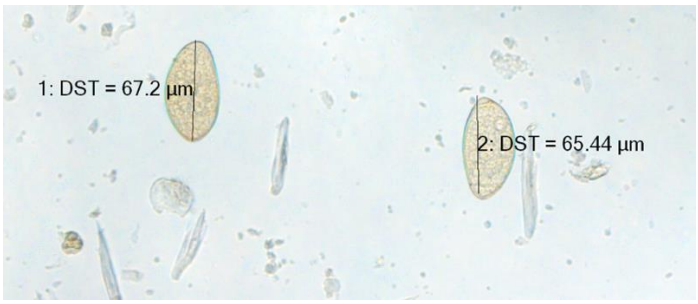
Figure 2. *Ancylostoma* sp. egg from a spotted hyena juvenile under light microscope (original).

2.5.2. Genus *Spirometra*

Spirometra sp. is a cestode which belongs to the family Diphyllobothriidae and requires at least two intermediate hosts, the first being a copepode crustacean and the second can be any vertebrate (Bowman, 1999). The eggs (figure 3), once released through the parasites uterine pore from the gravid segments are present in the host's faeces and develop into a coracidium (oncosphere with ciliated embryophore). Once ingested by a copepod, the first intermediate host, the coracidium develops into a procercoid, the first parasitic larval stage. When the second

intermediate host, such as an amphibian or bird, ingests the infected copepod, the procercoid is released and passes through the intestinal wall, migrates to body muscles or connective tissue and develops into a plerocercoid, the second larval stage. The intermediate host having plerocercoids can be ingested by a predatory paratenic host and/or by a suitable definitive host and mature into an adult tapeworm (Urquhart et al. 1996; Bowman, 1999).

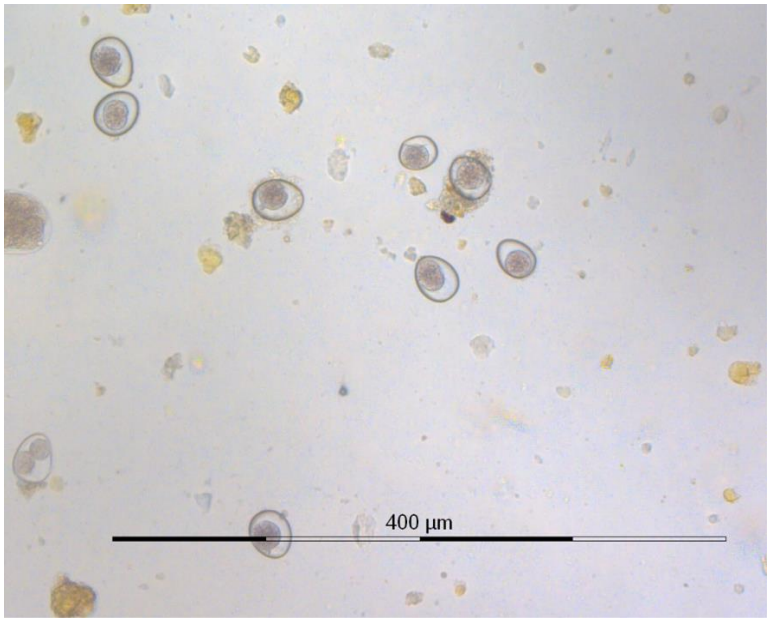
Figure 3. *Spirometra* sp. eggs from a spotted hyena juvenile under light microscope (original).



2.5.3. Genus *Cystoisospora*

The genus *Cystoisospora,* (formerly *Isospora*) includes protozoans that belong to the phylum Apicomplexa and are small, obligatory intracellular parasites, members of the coccidian group (figure 4). In contrast to the genus *Eimeria*, the sporulated oocyst of the genus *Cystoisospora* have two sporocysts per oocyst containing four sporozoites in each sporocyst. This configuration is termed the isosporoid oocyst type, which differs from that of Eimeria which has four sporocysts each having two sporozoites (Lindsay, Dubey, & Blagburn, 1997; Bowman, 1999). The infective stage for the host cell is the sporozoite, from a sporulated oocyst. Once sporozoites have invaded the host cells, they divide and form merozoites. If they divide fast they are named tachyizoite and if slowly, bradizoite (Bowman, 1999). The life-cycle of the genus *Cystoisospora* is complex, having a life stage inside the host's body (endgenous) and outside the hot's body (exogenous) . Additionally, it has the possibility of using paratenic hosts as a transport host (Lindsay et al., 1997; Bowman, 1999).

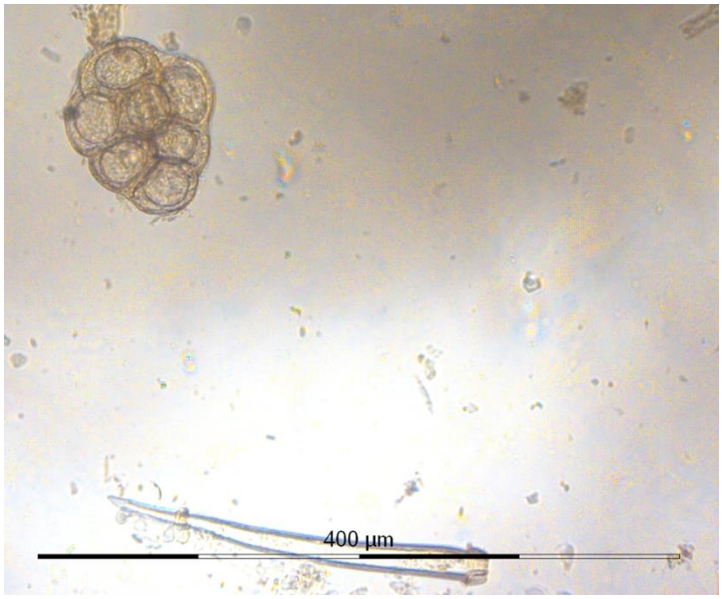
Figure 4. *Cystoisospora* sp. oocysts from the faeces of a juvenile spotted hyena under light microscope (original).



2.5.4. Genus *Dipylidium*

Dipylidium sp. is distributed worldwide and it is very common intestinal parasite of domestic dogs (*Canis familiaris*) and cats (*Felis catus*), and through contact with these domestic animals humans can be infected as well (Urquhart et al. 1996). It has prevalence in the spotted hyena of 55 % in juveniles and 15.8 % in adults living in the Serengeti (East et al., 2013). It has an indirect life cycle, with one intermediate host, flees or lice. The definitive host ingests the intermediate host while grooming and the cysticercoids develop into a tapeworm which releases gravid proglottids containing egg packages (figure 5) with oncospheres. These eggs are ingested by the intermediate host and the cysticercoid larvae stage matures in the abdominal cavity (Urquhart et al. 1996; Bowman, 1999).

Figure 5. *Dipylidium* sp. egg package from a spotted hyena juvenile under light microscope (original).



Dipylidium infection prevalence in the spotted hyena is higher in juveniles than in adults, suggesting that acquired immunity is effective and increases with age, and/or the presence of other cestodes in adults causes cross immunity with *Dipylidium* (East et al., 2013). Because female clan members give birth throughout the addition of susceptible juveniles to communal den throughout the year maintains *Dipylidium* infections in clans. During low prey abundance, infection prevalence decreases, probably as a result of fewer clan members visiting dens leading to a decrease in the flea population in the den. Furthermore, reduced nutrients in the digestive system of infected juvenile might also lead to decreased fecundity of *Dipylidium* and as a consequent, lower environmental contamination and a decreased prevalence of *Dipylidium* infected fleas in the communal den. It is also possible that a decline in *Dipylidium* fecundity during periods of low prey may result in an increase in juveniles being falsely scored as negative in terms of infection (East et al., 2013).

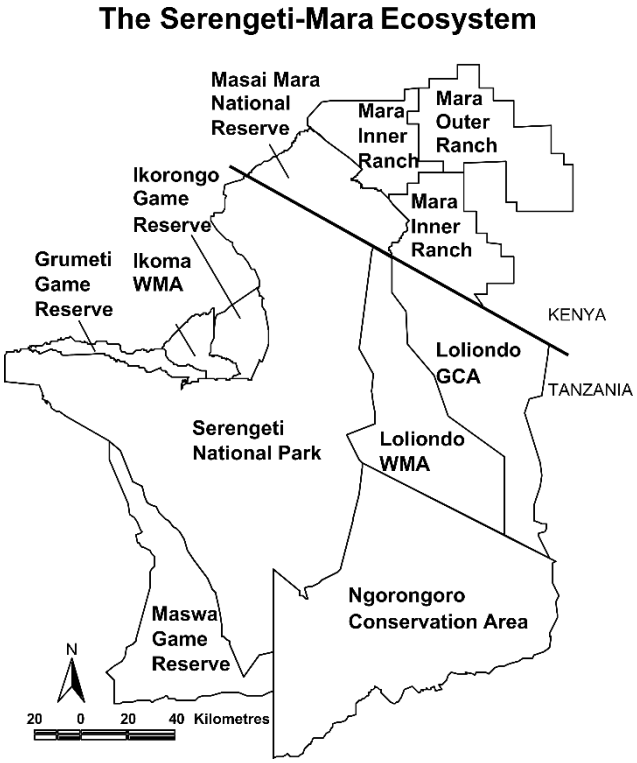
2.6. Serengeti ecosystem characterization

2.6.1. Geography

The Serengeti National Park in Tanzania is part of the Serengeti Mara ecosystem, together with the Ngorongoro Conservation Area, the Masai Mara National Reserve, Maswa Game Reserve, Grumeti Game Reserve, Ikorongo Game Reserve, and Loliondo Controlled Area (Figure 6). It is defined with the seasonal migratory movements of large herbivores of wildebeest (*Connochaetes taurinus*), Thomson gazelle (*Gazella thomsoni*) and zebra (*Equus burchelli*) (Frame, 1986; Boone, Thirgood & Hopcraft, 2006).

Temperature is relatively constant with maximum of 27-28 ºC and minimum around 16 ºC in the hot months (October-March) to 13 ºC during May-August. The wet season goes from November to May, and it is bimodal with a peak in December and another one in April, followed by the dry season (Frame, 1986; Sinclair, 1995).

Figure 6. Map of the Serengeti-Mara Ecosystem showing the location and status of the protected areas. GCA, game controlled area; WMA, wildlife management area (Thirgood et al., 2004).



2.6.2. Carnivores living in Serengeti ecosystem

Large herds of migratory ungulates and resident ungulates live in the Serengeti ecosystem, together with large predators. There are 27 known species of the order Carnivora described to live in the Serengeti ecosystem, represented in the table 1 (Frame, 1986) with the addition of domestic carnivores associated with human communities living outside the Serengeti National Park (Durant et al., 2011). The spotted hyenas are the most common carnivore, at about 7200 - 7700 individuals living in the Tanzania part of the ecosystem (Hofer & East; 1995) .

Table 1. Carnivores living in the Serengeti ecosystem (Adapted from Frame, 1986).

Common name	**Scientific name**
Aardwolf	*Proteles cristatus* Sparrman, 1783
African wild dog	*Lycaon pictus* Temminck, 1820
African civet	*Viverra civetta* Schreber, 1778
Banded mongoose	*Mungus mungo* Gmelin, 1788
Black-backed jackal	*Canis mesomelas* Schreber, 1775
Cape clawless otter	*Aonyx capensis* Schinz, 1821
Caracal	*Felis caracal* Schreber, 1776
Cheetah	*Acinonyx jubatus* Schreber, 1776
Dwarf mongoose	*Helogale parvula* Sundevall, 1846
Egyptian mongoose	*Herpestes ichneumon* Linnaeus, 1758
Golden jackal/common jackal	*Canis aureus* Linnaeus, 1758
Large-spotted genet	*Genetta tigrina* Schreber, 1778
Leopard	*Panthera pardus* Linnaeus, 1758
Lion	*Panthera leo* Linnaeus, 1758
Marsh mongoose	*Atilax paludinosus* Cuvier, 1777
Ratel	*Mellivora capensis* Schreber, 1776
Serval	*Felis serval* Schreber, 1776
Small-spotted genet	*Genetta genetta* Linnaeus, 1758
Side-striped jackal	*Canis adustus* Sundevall, 1846
Spotted hyena	*Crocuta crocuta* Erxleben, 1777
Spotted-necked otter	*Lutra maculicollis* Lichtenstein, 1835
Striped hyena	*Hyaena hyaena* Linnaeus, 1758
Striped polecat	*Ictonyx striatus* Perry, 1810
Two-spotted palm civet	*Nandinia binotata* Gray, 1830
White-tailed mongoose	*Ichneumia albicauda* Cuvier, 1829
Wild cat	*Felis libyca* Forster, 1780

2.6.3 Seasonality and prey abundance

The migratory herds move along a rainfall gradient (from the dry southeaster plains to the wet northwest Kenya), in an approximately circular pattern. In the dry season, migratory herds linger in the northern Serengeti National Park and Masai Mara National Reserve and then move to southeast in the wet season (December to May) to give birth in the short-grass plains. In May or June, when the dry season begins, they move northwest and aggregate in the northern woodlands of the Serengeti National Park and Masai Mara National Reserve, until the rain begins in December, when they return to the south (Frame, 1986; Sinclair, 1995; Thirgood et al., 2004; Boone, Thirgood & Hopcraft, 2006).

Rainfall in Serengeti National Park influence prey abundance with the territories of spotted hyena clans owing to migratory prey movements. There are periods when clan territories contain a high (238.5 animals/km^2), medium (31.0 animals/km^2) or low (7.2 animals/km^2) abundance of prey (Hofer & East, 1993a). Besides seasonality, prey abundance also varies within years (Hofer & East, 1993a) leading to years which can be defined as either good or poor in terms of food abundance within a clan territory (Hofer & East 2008). During high prey abundance (large migratory herds inside a clan territory), all hyenas feed inside their clan's territory, but when prey abundance is low (presence of just residence prey) or medium (presence of small, dispersed migratory herds), there are not sufficient food resources to sustain all clan members and individuals leave their clan territory on regular long-distance, relatively short-term foraging trips to feed on large migratory herds. These long-distance foraging trips have been termed commuting trips (Hofer & East, 1993b). The maximum commuting distances registered in the Serengeti are <75km/night. The mean commuting distance is <61km/night during dry season and <47km/night during wet season (Hofer & East, 1993b).

2.7. Ribosomal ribonucleic acid genes

Ribosomal ribonucleic acid (rRNA) is the RNA component of the ribosome, critical for the protein synthesis across species. The differences in the rRNA genes across species preserve the structure and function of the rRNA. Thus, rRNA genes and the associated spacer regions (figure 7) evolve at different rates and include highly conserved regions (regions that have little or no variability across the rRNA gene amongst species), which reflects the evolutionary relationships between different species (Hillis & Dixon, 1991; Smit, Widmann & Knight; 2007). However, highly conserved regions may constitute a problem in molecular phylogenetic inferences because there may be insufficient differences to infer a phylogenetic relationships. Moreover, selecting regions which have high variability can also give non robust phylogenetic

results due to an increasing level of homoplasy (similarities not attributable to coancestry) resulting in an alignment that provides little information about coancestry.To overcome these problems it has been suggested that DNA regions with a similarity greater than 70% but less than 100% should be used (Hillis & Dixon, 1991).

Ribosomes are formed with two subunits, the large subunit (LSU), containing the 28S, 5,8S and 5S rRNAs, and the small subunit (SSU) , containing the 18S rRNA. The 5S RNA gene is not usually linked to the main transcription unit which has the 18S, 28S and 5,8S rRNA coding regions, illustrated in figure 7 (Long & Dawin, 1980; Torres-Machorro, Hernández, Cevallos & López-Villaseñor, 2010). The 18S rRNA genes has been widely used in phylogenetic analysis of coccidian parasites (Carreno et al. 1998; Morrison et al., 2004; Dahlgren & Gjerde, 2009; Zhijun, Mingwei, Hongliang & Yuping, 2011; Matsubayashi et al., 2011).

Figure 7. rRNA main transcription unit coding region of a eukaryotic. Coding regions (18S, 5,8S and 28S) are separated by intergenetic regions, the internal transcribed spacers (ITS) and external transcribed spacers (ETS). S represents Svedberg units for for sedimentation rates. (Adapted from Hillis & Dixon, 1991).

2.8. Phylogenetic status of the coccidian parasites

The coccidian belong to the phylum Apicomplexa, and are obligate intracellular parasites which have either a monoxenous (i.e. having a direct life cycle) or heteroxenous (using intermediate hosts) life cycle. Coccidian organisms typically have been classified based on the phenotypic characteristics, such as morphology of the oocysts and/or their life-cycle. The taxonomic classification of the coccidian, especially within the genus *Cystoisospora/Isospora* requires clarification (Carreno et al., 1998; Tenter et al., 2002; Morrison et al., 2004; Matsubayashi et al., 2011). The problem with phenotypic classification in the eimeriid coccidian is that only one morphologic stage of the parasite is known, the oocyst and the host from which it was recovered (Tenter et al., 2002). Incomplete information on the parasite biology and the possibility of misunderstanding its natural host, because it is possible for animals to present oocysts in their faeces without being the host of the parasite, might lead to misunderstandings (Tenter et al., 2002). Thus, molecular information on the parasite is useful, and as said above, the SSU 18S rRNA gene has been widely used for inference of evolutionary hypothesis (Carreno et al. 1998; Morrison et al., 2004; Dahlgren & Gjerde, 2009; Zhijun, Mingwei, Hongliang & Yuping, 2011;

Matsubayashi et al., 2011). However, only roughly 4% of the eimeriid coccidia from vertebrates are known, so phylogeny inference on the species-level must be taken cautiously as a result of a poor statistical representative sample (Tenter et al., 2002; Morrison et al., 2004). Molecular phylogeny inference has generally supported the existence of the major Apicomplexan groups. Within the eimeriid coccidian group, there is support for differentiation into a cyst-forming coccidian group, i.e. the Sarcocystidae family and an intestinal oocyst-forming coccidian group, i.e. the Eimeriidae family (Tenter et al., 2002; Morrison et al. 2004; ; Zhijun, Mingwei, Hongliang & Yuping, 2011; Matsubayashi et al., 2011).

Nevertheless, the former genus *Isospora* had inconsistencies with the taxonomic relationships proposed by both phenotypic characteristics and molecular data (Tenter et al., 2002). The genus *Isospora* contained both a bird-host species clade, related to Eimeriidae and a mammal-host species clade, related to Toxoplasmatinae. Thus, the genus *Cystoisospora* was proposed for the mammal-hosts parasites, to differentiate it from the bird-host species, termed Isospora (Carreno et al., 1998; Morrison et al., 2004; Matsubayashi et al., 2011). For further inference on the relationships between closely related species, the SSU 18S rRNA gene is not sufficient, and further molecular analysis should be performed, using nuclear and organelles genes (Tenter et al., 2002).

Chapter 3. MATERIAL AND METHODS

3.1. Study population

The study population included three closely monitored clans of spotted hyenas ("Isiaka", "Mamba" and "Pool") that are part of an ongoing long-term research program in the centre of Serengeti National Park in Tanzania. Sample collection was carried out during the period of March 2010 to August 2011. Individuals were recognized by their spot patterns, ear notches, scars and bald patches (Frank, 1986a; Hofer & East, 1993a; Golla et al, 1999) and sexed using dimorphic shape of the phallic gland (Frank et al., 1990). Age was determined from date of birth or based on the observation of pelage, size, level of coordination when walking and position of the ears, with an accuracy of ± 7days (East et al., 1989). The social status within litters was determined by observing juvenile's behaviour in agonistic encounters when competing for milk access, such as biting, lunging, chasing the sibling and retreating (Golla et al., 1999).

3.2. Sampling

Collection of 164 faecal samples belonging to 104 known spotted hyena juveniles, of age between 36 and 726 days, was performed immediately after defecation, between March, 2010 and August, 2011. Samples were mixed, subsampled, stored in formalin 4 % for parasite screening and preserved in RNAlater (Sigma–Aldrich Inc., St Louis, MO, USA), initially stored frozen at -10 ºC, transported frozen and stored at -80 ºC for the faecal glucocorticoid metabolites (fGCM) measurement or genetic analysis (East et al., 2013). As a consequence of the amount of debris in 3 samples, it was not possible to perform faecal egg counts (FEC), although they were positive for *Ancylostoma* sp. in qualitative floatation tests. For fGCM measurement, 132 faecal subsamples belonging to 84 spotted hyena individuals were used.
For the genetic analysis 17 samples from 7 carnivore species were used including 2 from spotted hyenas, 2 side-striped jackals (*Canis adustus*), 4 lions (*Panthera leo*), 2 aardwolfs (*Proteles cristatus*), 3 black-backed jackals (*Canis mesomelas*), 1 golden jackal (*Canis aureus*) and 3 bat-eared foxes (*Otocyon megalotis*) collected by the same procedures.

3.3. Parasite screening

A modification of the McMaster flotation technique (Gordon & Whitlock 1939) was applied to access gastrointestinal parasite burden in the spotted hyena juveniles. To enhance the detectability of eggs, a solution of potassium iodide (KI) was used with a specific weight of

1.5 g/mL (Meyer-Lucht & Sommer, 2005; Schwensow et al., 2007). Four McMaster chambers were counted for each sample with a dilution factor of 1:15 (2 g of faeces with 28 mL of KI solution). After combining the faeces with the KI solution, it was mixed 3 minutes in the vortex and then sieved in order to remove bigger debris. After 1 h of preparing the samples, the suspension was introduced into the McMaster chambers and 5 minutes later, parasite eggs/oocysts were counted and identified accordingly with their morphology using a light microscope, with the magnification of 100x (10x eyepiece lens x 10x objective lens). The photographs were taken with the software ProgRes CapturePro 2.5, 2007. The results are expressed in EPG/OPG.

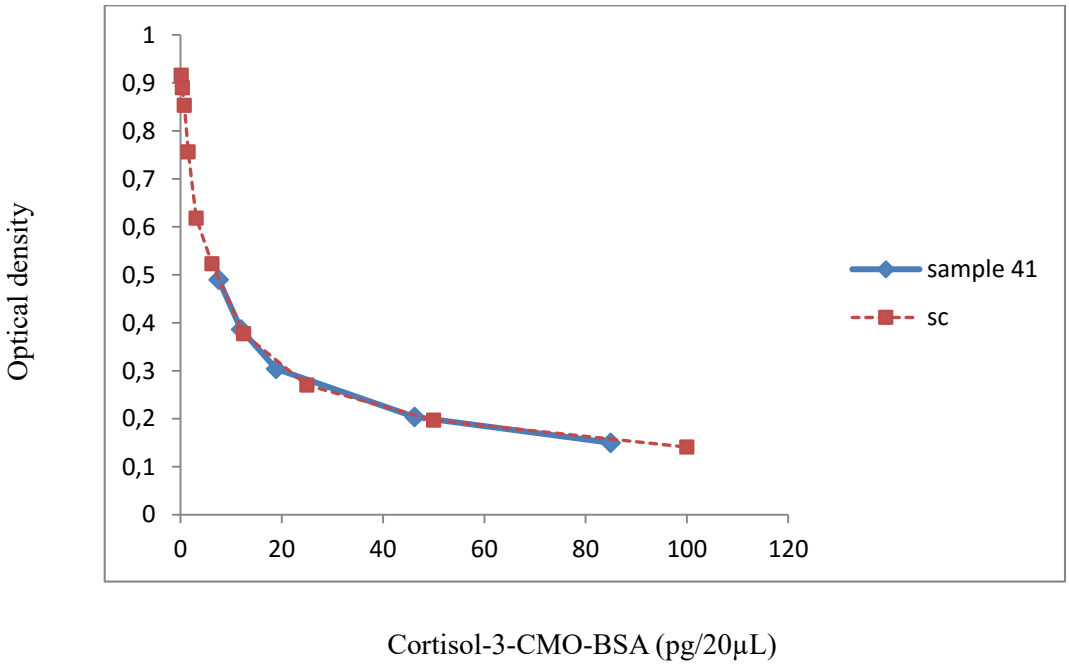
3.4. Extraction and measurement of faecal glucocorticoid metabolites

The extraction of fGCM was performed as previously described (Benhaiem et al., 2012a), after lyophilizing the samples for 22 h using a freeze drier (EPSILON 1-4 LSCplus, Martin Christ GmbH, Osterode, Germany), and homogenising them into a powder using a mortar and pestle. Excessive debris was removed. For the extraction, an aliquot of 0.20 g of faecal powder was mixed in a 2 mL Eppendorf tube with 1.8 mL of 90% methanol (MeOH) and shaken for 30 min using an universal shaker (SM-30, Edmund Buhler GmbH, Hechingen, Germany), then centrifuged for 15 min at 1200G. An aliquot of 0.5 mL was transferred to another Eppendorf tube and a dilution of 1:1 was made with distilled water.

3.5. Enzyme Immunoassay

To measure fGCM, several EIA previously validated in the spotted hyenas were performed (Benhaiem et al., 2012a). For the procedure, an antibody polyclonal anti-IgG, raised in rabbits directed against cortisol-3-CMO-BSA and the label cortisol-3-CMO-peroxidase were used.
The faecal extracts were diluted 1:5 and some, due to high concentration were repeated with the dilution 1:50. The measurements were performed in duplicate and the conditions for acceptance were 1) concentration <25 pg/20 μL and 2) Variation coefficient ≤5 %.
The assay was validated by demonstrating parallelism of faecal samples to the cortisol-3-CMO-BSA standard curve (Figure 8). The intra-assay and inter-assay coefficient of variation were determined by using faecal samples and buffer pools containing known concentrations of cortisol-3-CMO-BSA. The inter-assay coefficients were 14.7 % (n = 6) for buffer pools containing low and 16.3% (n =6) for high concentrations of cortisol-3-CMO-BSA. The intra-assay coefficients were 6.5 % (n = 9) for faecal samples containing high and 6.5% (n = 9) for samples containing low concentrations of cortisol-3-CMO-BSA.

Figure 8. Parallelism test for cortisol-3-CMO-BSA performed using serial dilutions of faecal extracts from one individual (sample 41) and compared displacement curve with the displacement curve produced by the steroid standard (sc). The identities of curves indicate a high degree of parallelism in case of faecal extracts.



3.6. EIA procedure

The faecal glucocorticoid metabolites analysis procedure was described previously (Pribbenow et al., 2014). The assay was performed using in-house microtiter plates, which were previously coated with goat anti-rabbit IgG (1 µg IgG/100 µL coating buffer per well) overnight at 4° C. The plates were blocked with BSA in assay buffer (7.12 g/L Na2HPO4 * 2H2O, 8.5 g/L NaCl, 1 g/L BSA, pH 7.2) for 45 min at room temperature and then decanted and stored at 25° C. Previously to use, the plates were washed one time using 300 µL washing buffer (0.05 % Tween80) in a plate washer (SLT 96PW, Tecan, Crailsheim, Germany). Standard solutions of cortisol-3-CMO, ranging from 0.2 to 100 pg/20 µL were previously prepared in 40 % MeOH. With the aid of a diluter dispenser, 20 µL duplicates of standard solutions, quality controls (P1 and P2), samples and blanks were pipetted together with 100 µL of the Cortisol HRP conjugate (4-Pregnene-11b,17a,21-triol-3,20-dione-3-CMO-POD) in assay buffer solution into respective wells. Then 100 µL of the antibody (4-Pregnene-11b,17a,21-triol-3,20-dione-3-CMO) in assay buffer solution was pipetted into each well (expect blanks) using a multipipette. After overnight incubation at 4° C, the plate was washed 4 times with 300 µL washing buffer and 150 µL

substrate (solution A: 1 g/L CH4N2O * H2O2, 18 g/L Na2- HPO4 *2H2O, 10.3 g/L C6H8O7 * H2O, 100 µL Kathon, pH 5.0; solution B: 0.3 g/L C16H2ON2, 40mL C2H6OS, 10.3 g/L C6H8O7 * H2O, pH 2.4) was added to each well, following incubation for 40 min in 4° C in the dark. The colour reaction was then stopped adding 50 µL 4N H_2SO_4 to each well and the optical density was measured at 450 nm, using a spectrophotometer plate reader (Infinite M200, Tecan). The hormone concentrations were calculated using Magellan software V 7.0 (Tecan, 2008) and the results expressed in ngg^{-1} faecal matter.

3.7. Genetic analysis

For the extraction of parasite DNA from spotted hyena faeces I used a DNA extraction kit NucleoSpin®Soil (Macherey-Nagel, Düren, Germany) and applied the manufacturer's instructions, including a homogenisation step (Precellys 24, Bertin Technologies, Montigny-le-Bretonneux, France) to release the DNA from eggs and oocytes. The extracts were stored at -20 °C.

Two different primer pairs (*Isosp_spec* forward 5'TAGGGGTGTGTACGTGGTGA 3', reverse 5'CTGGACCTGGTGAGTTTCCC3' and *Isosp_felis* forward 5'CCTTAGGGGTGTGTACGTGG3', and reverse 5'CGTGCAGCCCAGAACATCTA3') were designed to obtain a fragment of the 18S rRNA gene of 556 base pair (bp) and 803 bp minus primers, respectively, using the template *Cystoisospora felis* (Carreno et al, 1998, Gen bank accession number: L76471.1) through NCBI Primer- Basic local alignment search tool (BLAST) (Altschul, Gish, Miller, Myers & Lipman, 1990). Their properties were calculated using Oligo Calculator (Kibbe, 2007) and checked for specificity in Arb-Silva (Quast et al., 2013).

The Polymerase Chain Reactions (PCRs) were performed in a volume of 25 µL, using 2.5 µL of template, with approximately 10–100 ng per 20 µL reaction volume, and containing 0.5 µM of each primer, 1 unit of Thermo Scientific Phire Hot Start II DNA Polymerase (Thermo Fisher Scientific Biosciences GmbH, St. Leon-Rot, Germany), 200 µM each dNTPs, 1.5mM $MgCL_2$ (buffer) and nuclease free water. The PCR cycling conditions are as follows: initial denaturation at 98° C for 30 seconds, denaturation at 98° C for 5 seconds, primer annealing at 55° C for *Isosp_spec* and 53° C for *Isosp_felis* for 5 seconds and extension at 72° C for 10 seconds, followed by a final extension at 72° C for 1 minute, using 35 cycles in total.

Purification of the PCR products was conducted using FastAP ™ Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific Inc., Waltham, MA, USA). Sequencing was bidirectional, except for the three sequences and BrightDye® Terminator Cycle Sequencing Kit

(NimaGen B.V., Nijmegen, The Netherlands) and D-Pure™ Dye Terminator Removal kit (NimaGen B.V., Nijmegen, The Netherlands) were used. The PCR products were analysed by gel electrophoresis and visualised by GelRed staining (Biotium Inc, Hayward, CA, USA). Sequences were visualised on a Hitachi 3130 Genetic Analyzer (ABI) and then, manually visualised and edited using BioEdit 7.2.5. A BLAST search was performed in GenBank database (Altschul et al., 1990).

3.8. Phylogenetic analysis

The sequences obtained of a partial 18SrRNA coding region and 18S rRNA coding region sequences from apicomplexan parasites available in GenBank were aligned using Clustal W in Mega 6 (Tamura, Stecher, Peterson, Filipski & Kumar, 2013). The allignement had 555 base pairs (bp) including gaps. Various coccidian 18S RNAs were included in the analyses (Figure 11). The alignment was checked manually and used for phylogenetic analysis with MEGA 6 (Tamura et al., 2013). The method Maximum Likelihood with 1000 bootstraps, using the model "General Time Reversible model" was used which allows all six possible substitution rates to vary, having invariant sites because it is a conserved region in the genome.

3.9. Statistical analysis

All statistical analyses were performed using R program Version 3.1.2 (2014-10-31). Data set included 164 samples from 104 individually known juveniles. For the present statistical analysis, in order to prevent pseudo replication, mean values were calculated for multiple samples belonging to the same individuals. Prevalence describes the presence-absence of a parasite in host's sample. Mean intensity of infection describes the average value of intensity of a particular parasite species among infected host in a sample and mean abundance describes the average abundance value of a particular parasite species among all host examined (Margolis et al., 1982; Bush, Lafferty, Lotz & Shostak, 1997). A Spearman's rank correlation test was used to investigate the correlation between fGCM concentration and FEC/FOC of *Ancylostoma* spp., *Spirometra* sp. and *Cystoisospora* spp. To investigate the factors influencing *Ancylostoma* spp. infection intensity (high, above or low, below the abundant mean of infection), a binary logistic regression model was used. A univariate analysis of each variable was performed and parameters with a p-value below of 0.2 based on the Wald test from logistic regression were selected to the multivariate analysis (Bursac, Gauss, Williams & Hosmer, 2008). The selected parameters were then combined and the final model was chosen based on the Akaike Information Criterion (AIC) value. The final model included (1) fGCM concentration as a

measure of allostatic load of each juvenile (Benhaiem et al., 2013); (2) Juvenile age as younger as or older as 180 days, reflecting the dependence on maternal milk, as juveniles are totally dependent on milk until around 4-6 months, however they are not weaned until 12-18 months of age; and (3) presence of *Spirometra* sp., as a possible parasite interaction factor. Other factors considered that did not participate in the model were within-litter dominance status combined with size, clan identity, presence of *Cystoisospora* spp. and sex. Co-infection (no other parasite species present besides *Ancylostoma* spp., one other parasite species present, two or more parasite species present) was excluded in favour of *Spirometra* sp. and *Cystoisospora* spp. infection.

Chapter 4. RESULTS

4.1 Prevalence and intensity of parasite species infecting spotted hyena juveniles

Most parasites were identified at genus level based on their morphology and size. Seven parasite taxa were identified in the samples, additionally, five egg types remain unknown. Prevalence and means are shown in table 2. On average, each individual was infected with 3.1 ± (SD) 1.6 different parasite species (range 0 - 9). Distribution of individual parasites was overdispersed (variance > mean) and the dispersion of *Ancylostoma* spp., *Cystoisospora* spp. and *Spirometra* sp. parasites are shown in figure 9.

The overall prevalence was very high, since 98.1 % of the sampled animals were shedding at least one egg and/or oocyst.

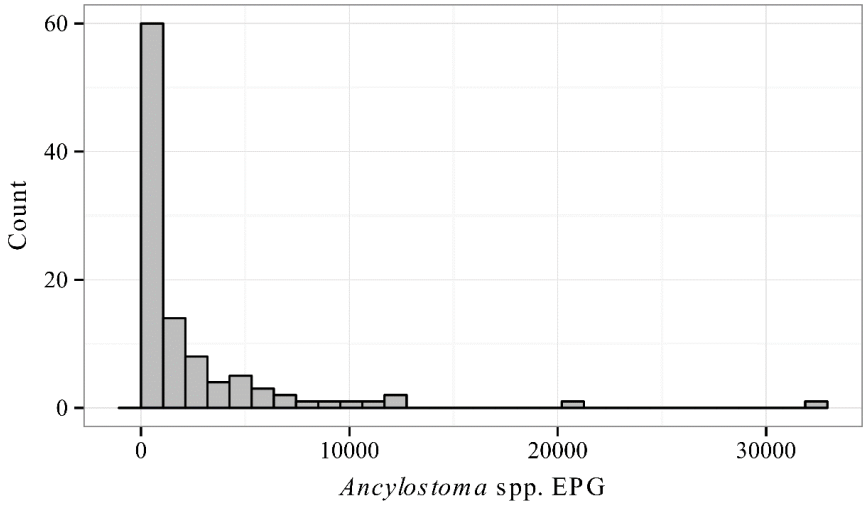
The most prevalent parasite was a hookworm, from the phylum Nematoda, with two morphological distinguished egg types (Type 1: 67 x 42 µm and type 2: 70 x 40 µm) found (figure 10). Genetic analysis demonstrated that they were *Ancylostoma* spp. (unpublished data). *Trichuris* sp. (egg size 61 x 37 µm, see appendix I) and a parasite from the order Spirurida (egg size 34 x 16 µm, see appendix I), with relatively low prevalence (7.69 %) were also present.

Spirometra sp. (egg size 67 x 36 µm, figure 11) was the most common cestode (53.85 %) and had the highest mean intensity of infection. Egg packages from *Dipylidium* sp. (117 x 95 µm, figure 5) and eggs from family Taeniidae (44 µm, see appendix I) were also observed.

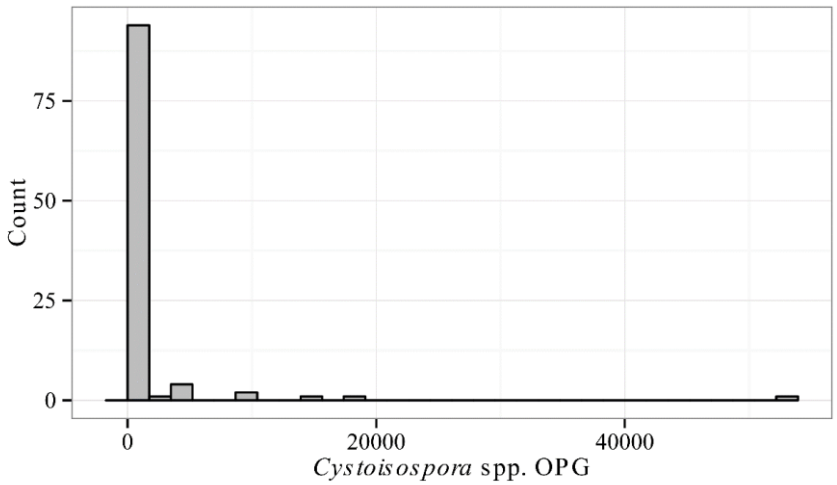
One protozoan genus was identified, corresponding to *Cystoisospora* spp. oocysts (figure 12), with differentiation in two types based on its morphology and size (type 1: 14 µm and type 2: 31 µm).

Figure 9. Dispersion of a) Ancylostoma spp., b) Cystoisospora spp. and c) Spirometra spp. FEC from juvenile spotted hyena.

a)



b)



c)

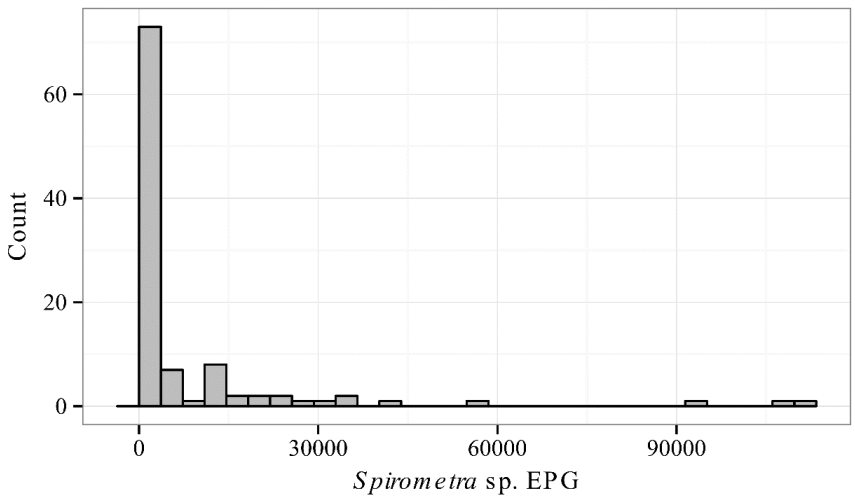


Table 2. Parasite screening results from faecal samples (N=104) with prevalence (%), mean abundance of parasites (eggs per gram, EPG), mean intensity of infection and respective confidence interval, maximum intensity range (eggs per gram, EPG), mean of egg size (µm) and variance to mean ratio of abundance of the study population.

Parasite	Prevalence (%)	Mean abundance (EPG)	Mean intensity (EPG)	Confidence Interval (EPG)		Maximum intensity (EPG)	Variance / mean ratio of abundance
				Low	High		
Ancylostoma spp.	95.2	2389	2509.6	1633.2	3386.1	31880	8211.3
Ancylostoma type 1	94.2	2348	2492.1	1611.1	3373.2	31880	8377.1
Ancylostoma type 2	16.4	40.6	248.5	163.6	333.5	1850	939.2
Spirometra sp.	53.9	8053	14954.7	10146.2	19763.2	109700	47506.4
Cystoisospora spp.	55.8	1319	2364.4	904.0	3825.0	52180	24719.0
Cystoisospora type 1	31.7	837.7	2639.9	819.5	4460.4	52100	34318.4
Cystoisospora type 2	42.3	481	1136.8	594.2	1679.5	14700	7420.6
Spirurida	7.7	6.5	84.4	69.7	99.1	225	138.4
Trichuris sp.	35.6	3.9	37.2	30.5	43.9	100	62.7
Taeniidae	11.5	3.2	27.9	24.5	31.3	63	34.9
Dipylidium sp.	27.9	10.6	38.1	32.5	43.6	225	48.6

Note: In the *Cystoisospora* spp. rows, unit counts are in oocysts per grams (OPG).

Figure 10. *Ancylostoma* spp. egg found in juvenile spotted hyena faeces. The orange arrow point to Ancylostoma type 1 eggs, and the green arrow points to type 2 (original).
spotted hyena (original).

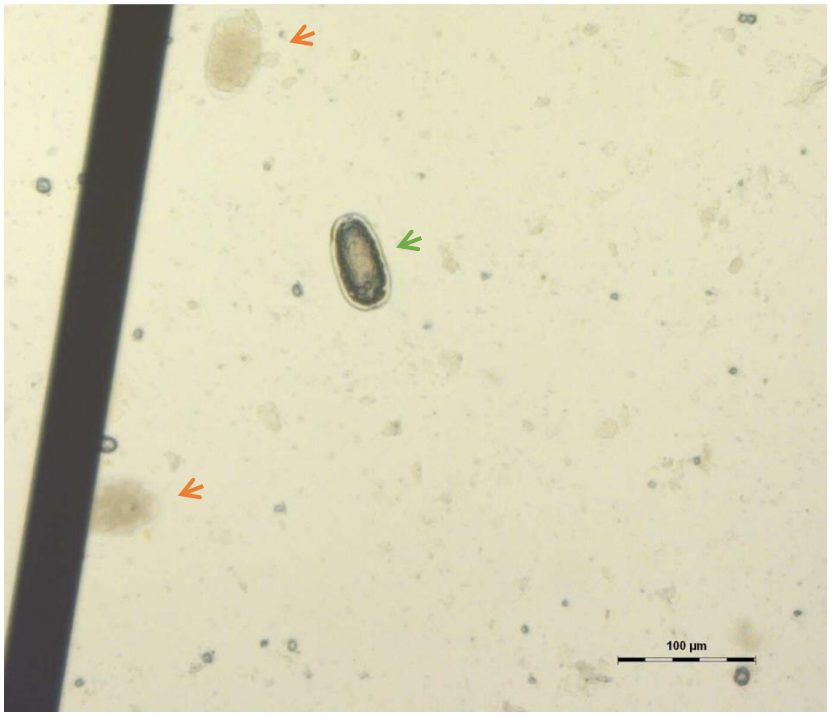


Figure 11. *Spirometra* sp. (blue arrow) and *Ancylostoma* sp. egg (orange arrow), found in faeces from a juvenile spotted hyena (original).

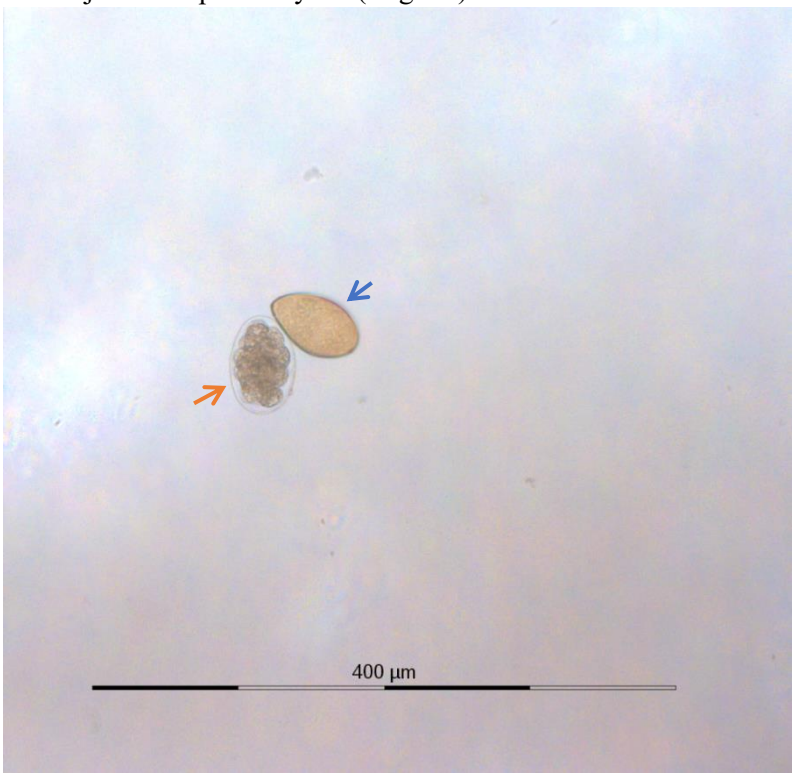
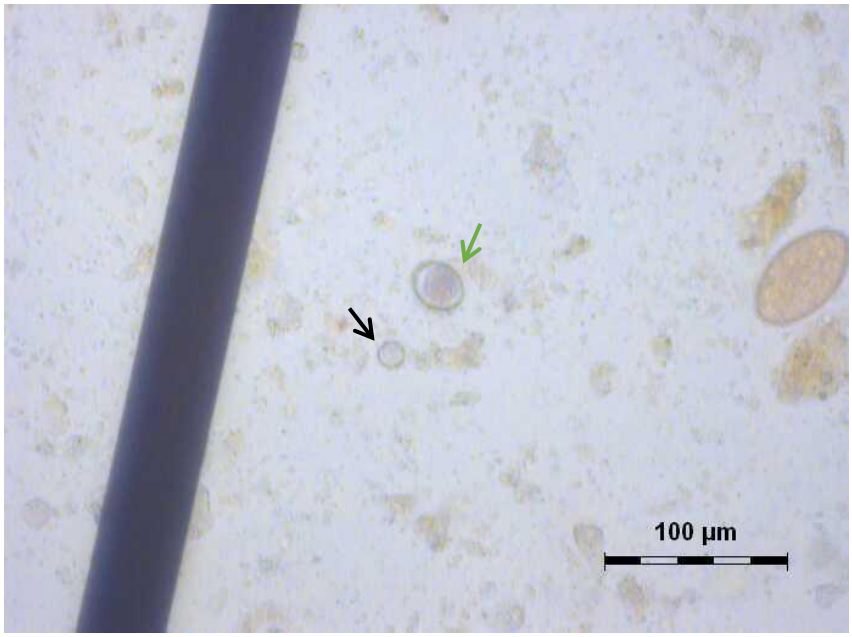


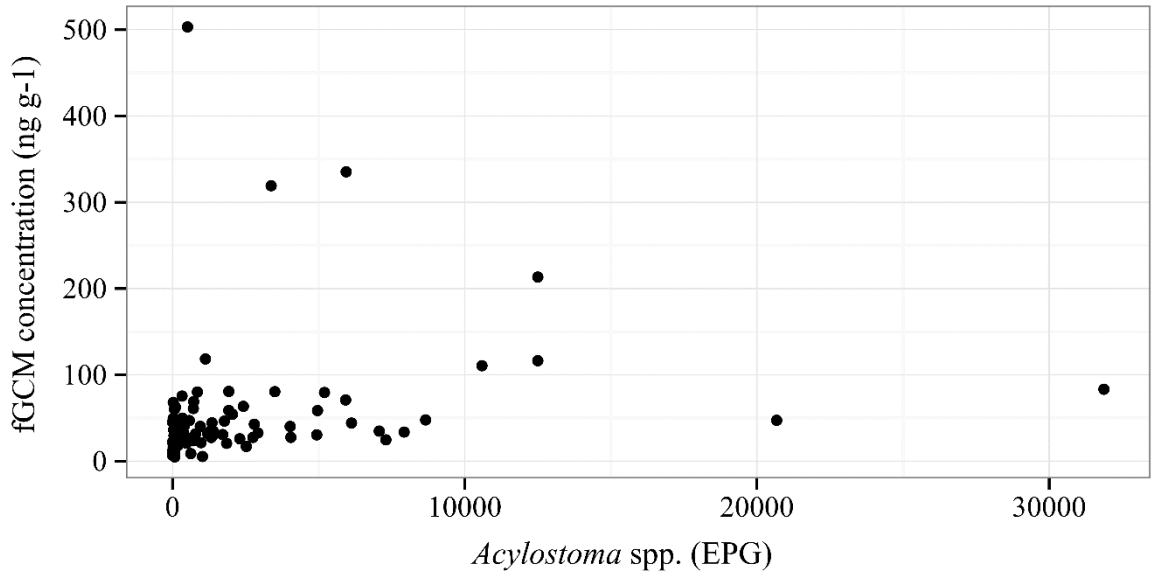
Figure 12. *Cystoisospora* spp. oocyst found in spotted hyena juvenile faecal sample. Oocysts type 1 pointed out by the black arrow and type 2 by the green arrow (original).



4.2. Faecal glucocorticoid metabolites concentration

Juvenile fGCM concentration ranged between 4.9 and 503.2 ng g^{-1} (mean 55.8 ± (SD) 72.4 ng g^{-1}).There is a significant correlation between FEC from *Ancylostoma* spp., *Spirometra* sp. and *Cystoisospora* spp. in relation to fGCM (Spearman's rank correlation test, rho=0.371, p<0.001, rho=0.272, p<0.05, rho=0.287, p<0.01 respectively), with *Ancylostoma* spp. correlation being the strongest (figure 13).

Figure 13. Faecal egg counts of *Ancylostoma* spp., in eggs per gram (EPG) in relation to faecal glucocorticoids metabolites (fGCM) concentration, in ng g-1.



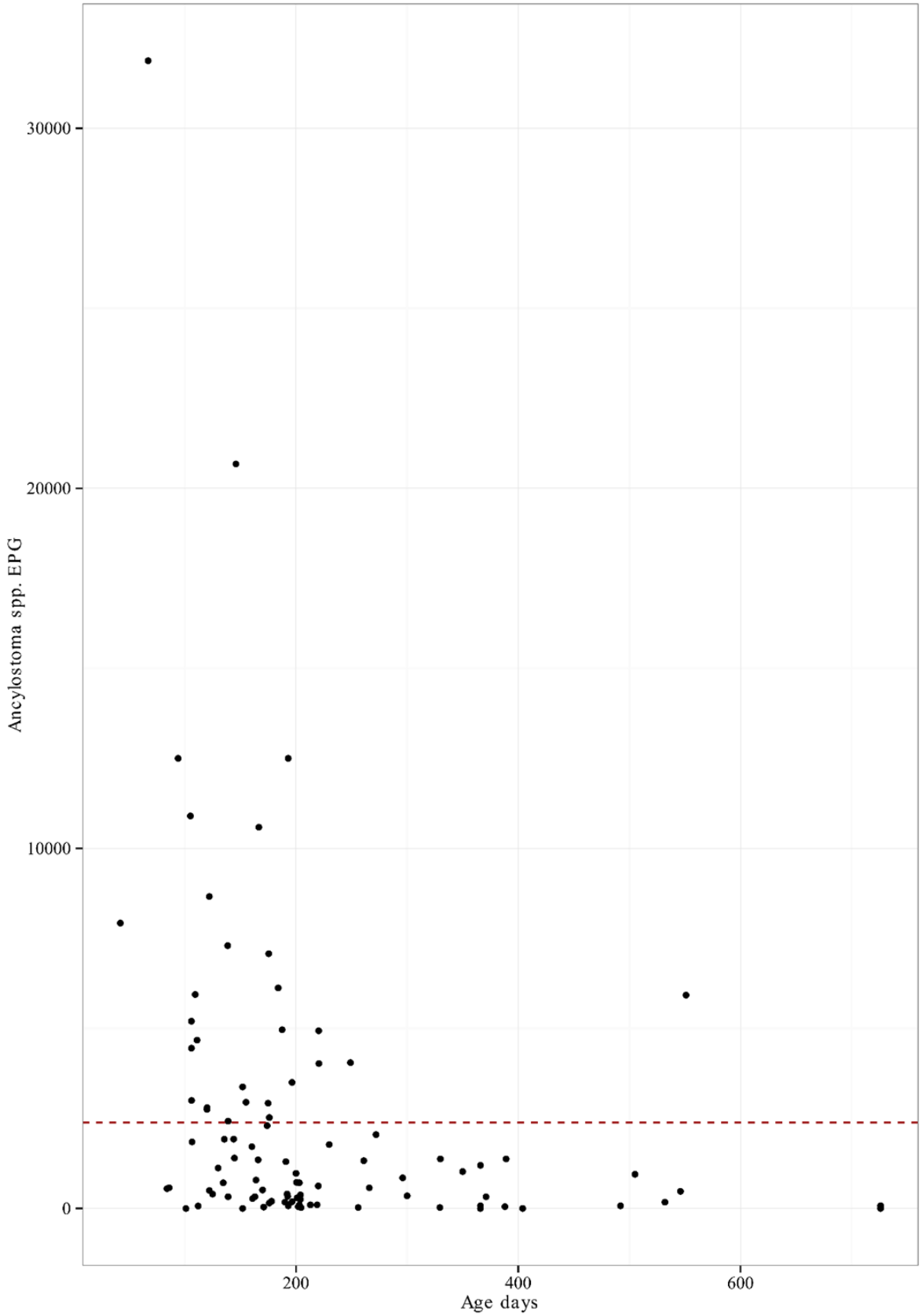
4.3 Factors modulating *Ancylostoma* scale.

The binary logistic regression (Likelihood ratio test, G=12.04, df=3, p=0.007, table 3) demonstrated that juveniles older than 180 days were significantly less likely (p=0.05) to be infected with *Ancylostoma* spp. than younger juveniles. Furthermore, the majority of juveniles with high *Ancylostoma* egg loads (eggs per gm faeces) were young animals (figure 14). Also, individuals infected with *Ancylostoma* spp were significantly more likely to have a concurrent infection of *Spirometra* sp. (p=0.03) than individuals not infected with *Ancylostoma spp*. The likelihood of *Ancylostoma* infection was not significantly affected by fGCM concentration , Inclusion of this factor in the model decrease the AIC value.

Table 3. Effects of biological and ecological factors on the likelihood of infection intensity (high or low) with *Ancylostoma* spp. in juvenile spotted hyenas. Parameters estimates, their standard errors with respective confidence intervals, Z value and its associated p-values are shown below, resulting from a logistic regression. Negative parameter estimates inform that the likelihood of the intensity of infection decreases when there is an increase in the value of the parameter. Positive parameter estimates informs of an increase of the likelihood of intensity of infection when the value of the parameter increases. This model was selected using Akaike Information Criterion (AIC).

Parameter	Estimate	Standard error	Z	p-Value	95% confidence intervals Lower	Upper
Constant	-1.293	0.537	-2.406	0.016	-2.346	-0.239
Older juveniles	-1.065	0.541	-1.968	**0.049**	-2.125	-0.005
fGCM concentration	0.004	0.004	0.945	0.345	-0.004	0.011
Infection with *Spirometra* sp.	1.203	0.565	2.128	**0.033**	0.095	2.310

Figure 14. Relation of age (days) with *Ancylostoma* spp. faecal egg counts in eggs per gram (EPG). The red dashed line marks the mean abundance of intensity, the cutoff value for considering low or high infection.

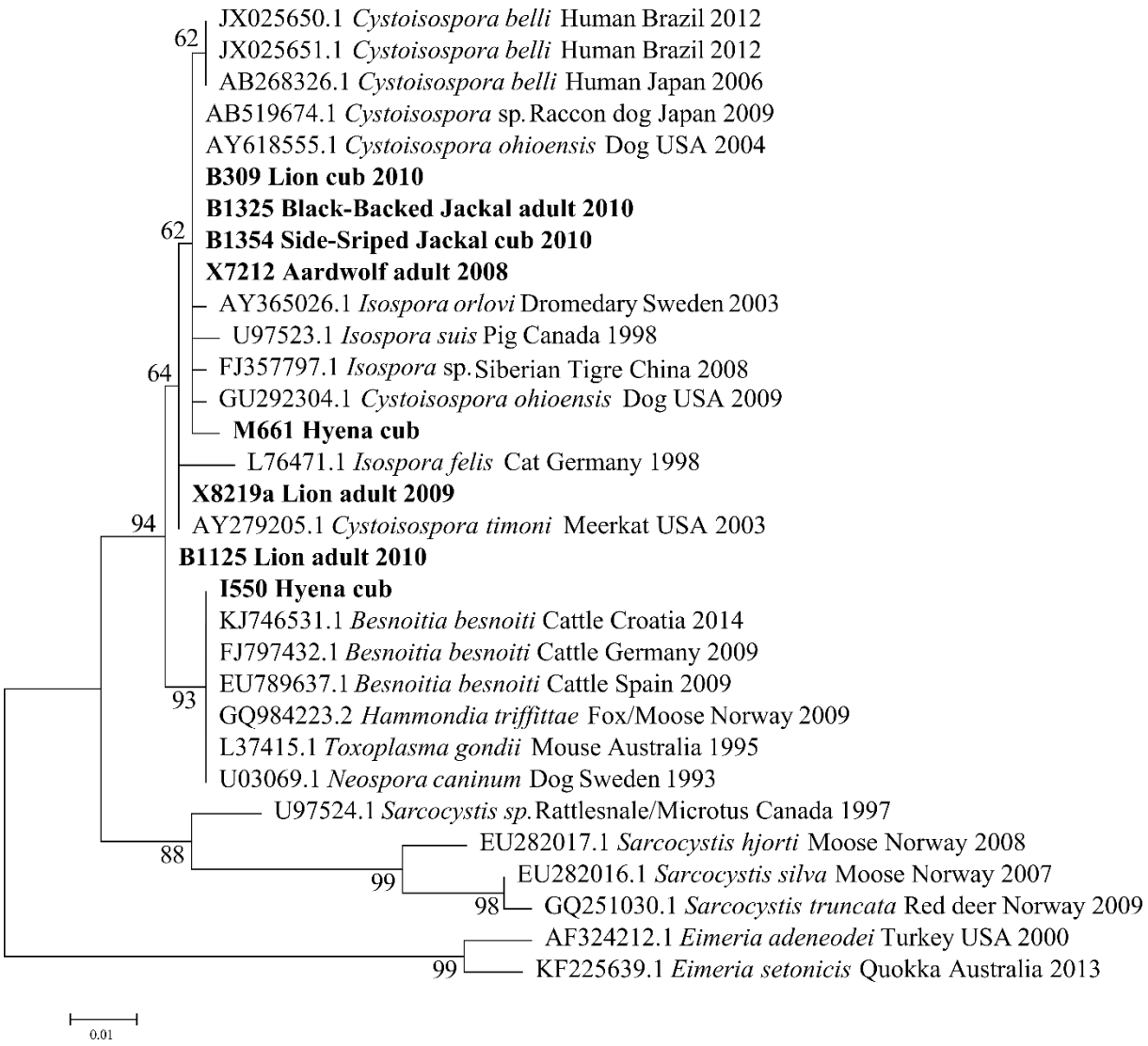


4.4 Phylogenetic analysis

Genetic screening for coccidian parasites produced positive results in 8 out of 17 samples tested (2 spotted hyenas, 2 side-striped jackals, 4 lions, 2 aardwolf, 3 black-backed jackals, 1 golden jackal and 3 bat-eared foxes species). Positive results were found in 3 lions, 1 black backed jackal, 1 side striped jackal and 2 spotted hyena samples. Amplification with the primer pair *Isosp_spec* produced DNA fragments of 501 base pairs (bp) using the primer pair *Isosp_felis*, fragments of 716, 706, 761, 754, 721, 753 and 761 bp, excluding primers.

The sequences were cut to achieve an allignment of the obtained sequences and the sequences obtained in the GenBank, having a final length of 555 bp including gaps. The phylogenetic relationships are represented in a tree and are based on Maximum Likelihood inference, shown in figure 15. All obtained sequences belong to the family Sarcocystidae, and the two sequences from the GenBank that belong in the family Eimeriidae were included as the outgroup. Five of eight sequences clustered within other isolates of *Cystoisospora* from several localities worldwide, one isolate from a spotted hyena was placed within the same cluster but closer to *Isospora*, one isolate from a spotted hyena was place is a different cluster of strains including *Toxoplasma*, *Neospora*, *Besnoitia*. Using NCBI nucleotide megablast (Altschul et al., 1990) the highest alignment scores for this isolate had a similarity of 99% with *Besnoitia besnoitia*. Finally one isolate from a lion was place between this cluster and the *Isospora* isolates (Figure 15).

Figure 15. Phylogenetic trees of the coccidia 18S small subunit ribosomal RNA gene locus, including isolates in the present study and publically available sequence data from elsewhere. GenBank accession numbers of parasites are shown next to species names, host where it was isolated from, location and date. Bootstrap percentages from 1000 replicates per analysis are shown for clades that were supported in the analysis.



Chapter 5. DISCUSSION

5.1. Coprological survey of parasites of free-ranging spotted hyena juveniles

Faecal flotations are classic, simple methods which can be applied to free-ranging animals without direct intervention, to identify and quantify parasites (Anderson & Schad, 1985; Campo, Manga-González & González-Lanza, 2000; Engh et al., 2003; Berentsen et al., 2012; Borecka et al., 2013; Stringer, Smith, Kerley & Linklater, 2014).

Overall prevalence of gastrointestinal parasites is high in this free-ranging spotted hyena juvenile population, since 98.1% were positive for at least one parasite genus. All parasites identified were previously described in the spotted hyena (Engh et al. 2003; Berentsen et al. 2012; East et al., 2013), however, there is no record of gastrointestinal parasites infecting juveniles. The prevalence as well as the intensity of infection, measured in terms of mean intensity is expected to be different during this life history stage, when compared with the entire population, because of differences in behaviour and physiology. Thus, the difference of prevalence and mean intensity between the population analysed in Engh et al., (2003) and the one from this study can be explained due to sampling methodology that included animals in different life history stages in the first and the different location.

Hookworms (*Ancylostoma* spp.) were the most prevalent parasite in spotted hyenas juveniles in Serengeti National Park (table 2), similar to the population living in the Masai Mara National Reserve, Kenya (Engh et al. 2003).

Parasite abundance is linked to host population density (Arneberg, Skorping, Grenfell, & Read, 1998; Jaenike & Perlman, 2002; Bajer, Bednarska & Rodo, 2011), therefore because all the offspring of female clan members under the age of 12 months rest in underground burrows at the clan communal den (Hofer & East, 1993c) and mothers and other clan members frequently visit the den, the communal den is likely to enhance transmission of parasites among clan members. Naive young cubs are likely to be particularly susceptible to parasite infection especially to those parasites with direct life cycles, such as *Ancylostoma* sp. The reason for this is that infection with these parasites depends on the faecal soil contamination with the infective stage and the level of exposure in the environment, not needing vectors or intermediate hosts, like *Spirometra* sp. or *Spirurida* (Schmidt & Roberts, 1996; Urqhhart et al. 1996; Bowman, 1999; Bajer et al., 2011). Moreover, environmental faecal contamination is likely to be higher at communal dens than at most other locations in a clan territory. The ecological conditions

such as temperature, moisture and substrate should also be suitable for the development and survival of the infective larvae.

Juveniles younger than two months old stay in the communal dens, and rarely go outside the entrance, but mothers typically ingest the young juvenile faeces (East et al., 2013). Older juveniles defecate in communal latrines within the vicinity of the communal den (East et al., 2004). Infection of susceptible individuals through ingestion is likely to be enhanced for parasites monoxenous, which infective form is present in the faeces or rapidly goes through external sporulation such as *Cystoisospora* sp. (Lindsay et al., 1997), but the same does not apply to hookworms, because to be infective they need to mature outside the host to L3 stage, nor for *Trichuris* sp., which requires longer periods (28 days at 25° C, 15 days at 30° C and 13 days at 34° C) for external embryonation until the infective stage (Bundy & Cooper, 1989). Therefore, transmammary infection, ingestion of paratenic hosts and skin penetration of the L3 are likely to be more important infection routes for *Ancylostoma* spp. in juvenile animals than ingestion of the L3 larva stage.

My results reveal that *Cystoisospora* spp. was the parasite with the second highest prevalence in juvenile spotted hyenas. Several oocyst sizes were found in the faeces (figure 12), leading to the possibility that different *Cystoisospora* species were present. However, morphological identification is not sufficient to determine species, and other approaches, such as molecular phylogenetic analyses need to be employed (Tenter et al., 2002; Matsubayashi et al. 2011).

Several Cestoda parasites were identified in the juvenile spotted hyenas examined. *Spirometra* sp. was the third most prevalent parasite in the sampled animals and had the highest mean intensity of infection, in terms of eggs per gram faeces. The infection with cestodes depend on the presence of the infected intermediate or paratenic host in the environment, and in the case of genus *Spirometra*, any vertebrate which have ingested an infected copepode (the first intermediate host) can be the second intermediate host and infect the juveniles (Bowman, 1999).

Dipylidium sp. infection prevalence revealed by the egg floatation method applied in this study (27.9%) was lower than the prevalence found using genetic screening and the presence of proglottids (55.1%) by a previous study of juveniles in the same study clans (East et al, 2013). It is possible that FEC are less sensitive to identify egg packages of *Dipylidium* sp. because 1) they are stored in formalin 4%. It is anticipated that there may be some damage in the package wall, thus not all of them will float to be identified. Also, due to the fact that *Dipylidium* eggs are agglomerated in egg packages, when broken they spread in the faeces, making them hard to be identified. 2) Like other cestodes (Taeniidae), they are released in proglottids, and the presence of egg packages depends on the presence of gravid proglottids in the analysed faecal

aliquot. So, this method may not be the best to assess parasite burden of cestode other than *Spirometra* sp. which releases its eggs through the uterine pore of the attached proglottids (Bowman, 1999). Finally, I cannot exclude the possibility that the prevalence of *Dipylidium* infection in juvenile spotted hyenas may have changed between my study period and that covered by the study of East et al. 2013 even though I suspect the differences in prevalence are most likely due to the different methods applied.

As expected, my results revealed a significant, positive correlation between parasite burden, measured in terms of EPG/OPG and allostatic load, measurement in terms of fGCM concentration. Three parasites were considered individually i.e. *Ancylostoma* spp., *Spirometra* sp. and *Cystoisospora* spp. Hookworm (*Ancylostoma* spp.) FEC had the strongest correlation with fGCM concentration. For this reason and for the high prevalence and mean intensity, the factors modulating the intensity of infection in juvenile's spotted hyena were investigated in *Ancylostoma*.

5.2. Factors influencing hookworm parasite intensity in spotted hyena juveniles

Human hookworm (*Ancylostoma duodenale*) FEC are useful to discriminate between individuals with low or high intensity of infection even though fecundity is density dependent (Anderson & Schad, 1985; Romeo et al., 2014).

The period of growth in juvenile spotted hyenas is an important life history stage because juveniles with higher growth rates are more likely to survive to the age of adulthood (2 year old) and females that high juvenile growth rates have their first litter at a younger age (Hofer & East, 1993c; Hofer & East, 2003). Consequently growing is an important life history stage with clear fitness consequences, therefore trade-offs are expected to occur between life history traits to prioritize growth in juveniles with insufficient food intake to sustain growth and other life processes.

Because, the immune system is energetically demanding, when there is a sustained increase in allostatic load, such as intense sibling competition and hunger both of which are associated with periods of low food intake in juvenile spotted hyenas (Golla et al. 1999; Hofer and East 2008; Benhaiem et al. 2012), allocation of resources may be reduced and certain branches of the immune system are expected to be down regulated, which leads to a higher susceptibility to infections (Sheldon & Verhulst, 1996; Lochmiller & Deerenberg, 2000, Martin, 2009). Additionally, parasite infections have shown to be costly to the hosts (French et al., 2007; Colditz, 2008; Reed et al, 2008; Patterson et al, 2013), consequently increasing the host's allostatic load. The correlation between FEC of *Ancylostoma*, *Cystoisospora* and *Spirometra*

species corroborates this hypothesis, however it was fairly weak, especially with *Cystoisospora* and *Spirometra* species, indicating that other factors than allostatic load are important and influence parasite infections, such as age of the host and interactions between co-infecting parasites. Nevertheless, immune responses reflect the environmental context and the host's life history (Schmid-Hempel, 2003; Ardia et al., 2011).

A previous study on the factors influencing *Dipylidium* sp. prevalence found no effect of sex in spotted hyena juveniles (East el al., 2013). Likewise, in this study sex was not identified as a factor influencing *Ancylostoma* spp. infection intensity.

5.2.1. Allostatic load

Allostatic load in the spotted hyena juveniles is influenced by sibling competition and resource availability (Benhaiem et al., 2013). Hence, it was expected that subordinate siblings should have higher parasite load than dominants. The model did not showed a significant influence of the concentration of fGCM on the scale of *Ancylostoma* sp. infection in juvenile's spotted hyena. However, it only considers the effect on high or low infection of *Ancylostoma* spp. as a binary predictor (explanatory variable), thus further statistical analysis should be performed in order to access the biological effect of allostatic load on parasite infections, as it is suggested by the correlation of *Ancylostoma* spp. FEC and fGCM concentration.

5.2.2. Host age

As expected, hookworm intensity of infection in the spotted hyena was higher in animals less than 200 days of age than in older juveniles (figure 14). It is possible that the variation in susceptibility to *Ancylostoma* spp. is caused by younger juveniles being naïve to hookworms. Once infected, immunity towards parasites can be acquired by the host. Acquired immunity usually increases with age, to a peak until stabilization or decline (Hayward, 2013). Moreover, the development of immunity depends on exposure, the intensity of exposure, whether juveniles acquire protection from their mother and to what extent body resources can be allocated to immunity (Hayward, 2013). Also, younger juveniles have a higher allostatic load than older ones, very likely as a consequence of their environment being less predictable and a decline in within-litter sibling aggression with age (Benhaiem et al, 2012a). Hence, these two reasons can explain this variability in parasite load between young and old juveniles. Mother's may transfer a degree of immunity against intestinal parasites via milk. It is unlikely that maternal immunity explains the data presented in figure 14 given that young juveniles of less than 200 days of age are more dependent on milk than older animals (Hofer & East, 1993c). My results suggest that

immune responses by juveniles to parasite infection are probably more important than maternally derived immunity.

5.2.3. Co-infection interaction

There is a link between co-infection with *Spirometra* sp. and intensity of hookworm *Ancylostoma* spp. infection in juvenile spotted hyenas. Parasite densities and host health are influenced by interactions between different co-existing parasites and parasite-host interactions, such as suppression of inflammatory immune responses or resource competition (Maizels et al., 2004; Riet, Hartgers & Yazdanbakhsh, 2007; Graham, 2008; Helmby, 2009; Råberg et al., 2009). Helminths modulate immune responses from the host, generally down-regulating it, in order to create a favourable environment in which to feed and reproduce (Maizels et al., 2004), thus altering the host tolerance to parasites. This helminth immune regulation can explain why the presence of *Spirometra* sp., a parasite that needs exposure of intermediate hosts to infect the definitive hosts (Bowman, 1999), the spotted hyena, is positively linked to parasite intensity infection of *Ancylostoma* spp., which infection depends on the environmental contamination by eggs present in the faeces and suitable conditions to survival of the L3 in the environment. Other reason it may be that once intensity of infection is high, tolerance may be adaptive so that pathogenicity provoked by inflammatory responses is avoided, resulting in higher parasite load.

5.3. Phylogenetic analysis of *Cystoisospora* spp. infecting several carnivores in the Serengeti National Park in Tanzania

The 18S rRNA gene is often used in molecular phylogenetic inference in coccidian species (Carreno et al. 1998; Morrison et al., 2004; Dahlgren & Gjerde, 2009; Zhijun et al., 2011; Matsubayashi et al., 2011). In this study, a partial sequence of the coccidian 18S rRNA gene was obtained from faeces from six carnivore species living in the Serengeti National Park. Although the primers were designed to obtain sequences specific to *Cystoisospora* spp., they targeted a very conservative region and hence we obtained sequence data from other genuses (figure 15).

Even though the genetic sequence data obtained in this study was from a relatively short fragment, it was sufficient to infer the phylogenetic relationship between parasite isolates from different species (figure 15). Future research will need to target longer sequences and regions less conserved to robustly determine the relationships between the different coccidians present in different carnivore host species.

One isolate from a spotted hyena juvenile (I550) is closely related to *Besnoitia besnoiti*, *Hammondia triffittae*, *Toxoplasma gondii* and *Neospora caninum*, indicating that it does not belong to the *Cystoisospora* genus. Moreover it revealed high similarity with *Besnoitia besnoitia. Besnoitia* species are heteroxenous, classified in the family Sarcocystidae. The definitive host of *Besnoitia* infecting large mammals is not yet known. Moreover, bovine besnoitiosis seems to be spreading from Southern Europe throughout other countries, and caprine besnoitiosis apparently is enzootic in Kenya and Iran (Olias, Schade & Mehlhorn, 2011; Oryan, Silver, & Sadoughifar, 2014). The spotted hyena may be a definitive host for *Besnoitia* infecting large mammals in this part of Africa, since further south *Besnoitia besnoiti* was already identified as a source of cattle infection in South Africa (Dubey, Wilpe, Blignaut, Schares & Williams, 2013).

Chapter 6. CONCLUSION

Parasite infections are a constant threat in free-ranging animals and the factors driving individual and population susceptibility to infections are not yet completely elucidated.

This study revealed insights into the parasites infecting the spotted hyena juveniles, a highly social carnivore living in the Serengeti National Park, and contributed to the elucidation of factors driving infections in the wildlife. Furthermore, this study also compared parasites (coccidian) within different carnivores that may share the same parasite species.

Spotted hyena juveniles were found to harbour a high overall infection prevalence of gastrointestinal parasites, with a mean of 3.1 different parasite species per individual. The parasites identified in this study were, by prevalence order, *Ancylostoma* spp., *Cystoisospora* spp., *Spirometra* sp., *Trichuris* sp., *Dipylidium* spp., Taeniidae and Spirurida.

Allostatic load correlated positively with EPG for the three most prevalent parasites in this study, however it is not a strong correlation, indicating that there are other factors influencing parasite infections.

The hookworm *Ancylostoma* spp. was an important species in the intestinal parasite community of juvenile spotted hyenas because of its high prevalence (95.2). This study identified key factors modulating *Ancylostoma* spp. infection intensity. Host age and co-infection with *Spirometra* sp. are shown here to influence infection intensity of *Ancylostoma* spp. Younger juveniles and individuals infected with *Spirometra* sp. are more likely to have higher *Ancylostoma* spp. infection intensity. This is consistent with the hypothesis that young individuals have not yet acquired effective immunity and have higher allostatic load (Benhaiem et al., 2013), and modulation of immune responses by parasites has potential impacts in other co-infecting parasites, respectively.

The large community in the Serengeti Mara ecosystem is a big carnivore guild and it is likely that they share the same type of parasites. This study also provided a preliminary phylogenetic analysis on the coccidian parasites infecting different carnivores from the Serengeti National Park. Phylogenetic inference may provide information on the relationship amongst taxa and elucidate on the coccidian life-cycles and host-parasite relations. This might help to elucidate the taxonomic classification within the coccidian group, which is a current question.

Chapter 7. FUTURE PERSPECTIVES

Additional research on the physiological effects of allostatic load on parasite infections and vice-versa may provide insights into the evolutionary costs of parasites in this host species. A further analysis considering the effect of egg burden rather than the binary outcome of the high or low infection intensity with *Ancylostoma* spp. may provide better understanding of these host-parasite relation.

A deeper research approach on the possibility of cross transmission of *Cystoisospora* spp. between different hosts, and the possibility of the spotted hyena being one definitive host of *Besnoitia* parasites which is currently unknown, together with a further phylogenetic analyses with additional genetic data, should be performed. Longer fragments of the rRNA genes, including regions with more variability, should be obtained. Additionally, a more intensive analysis, comparing different models to support the results, with more isolates in the analysis and other gene sequences will help explore the taxonomic relationships within the coccidian group. This analysis, combining phenotypic and molecular data and extended data from other carnivores sharing the same environment and likely the same parasites, could provide information on parasite transmission and dynamics of the parasite life-cycles infecting this community.

Chapter 8. BIBLIOGRAPHY

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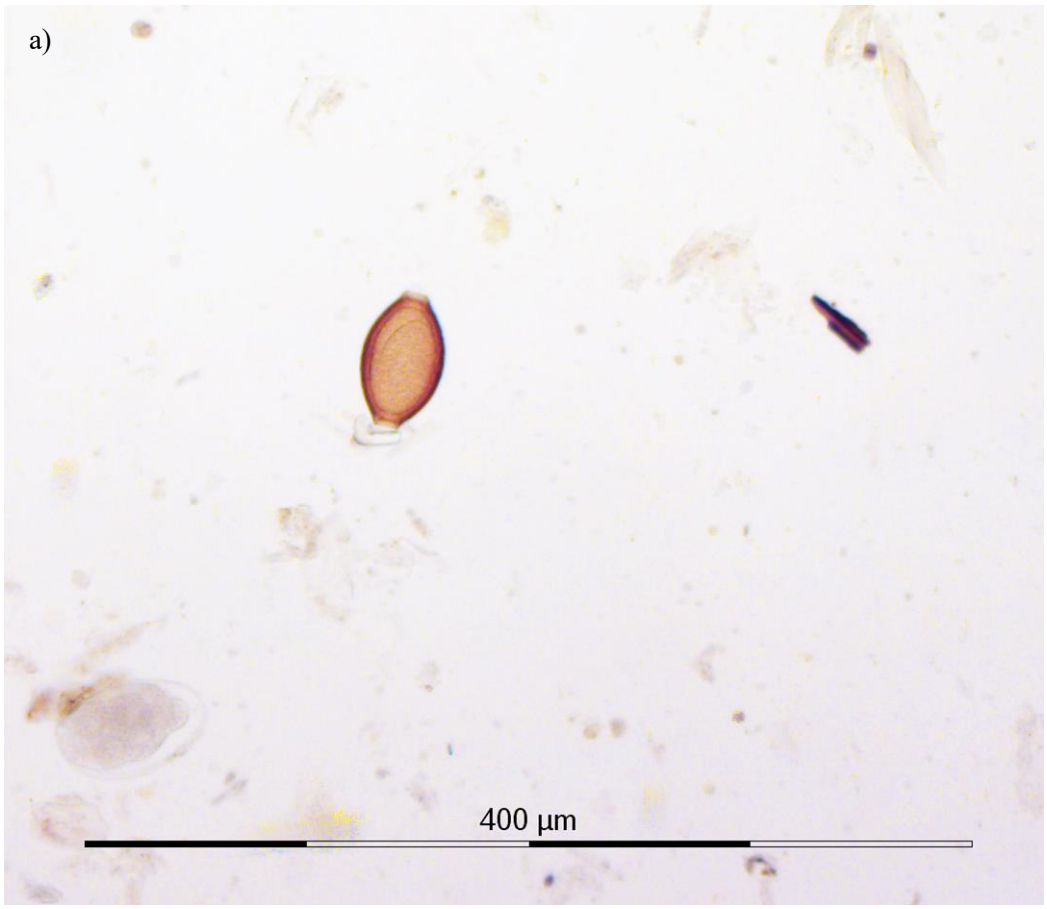
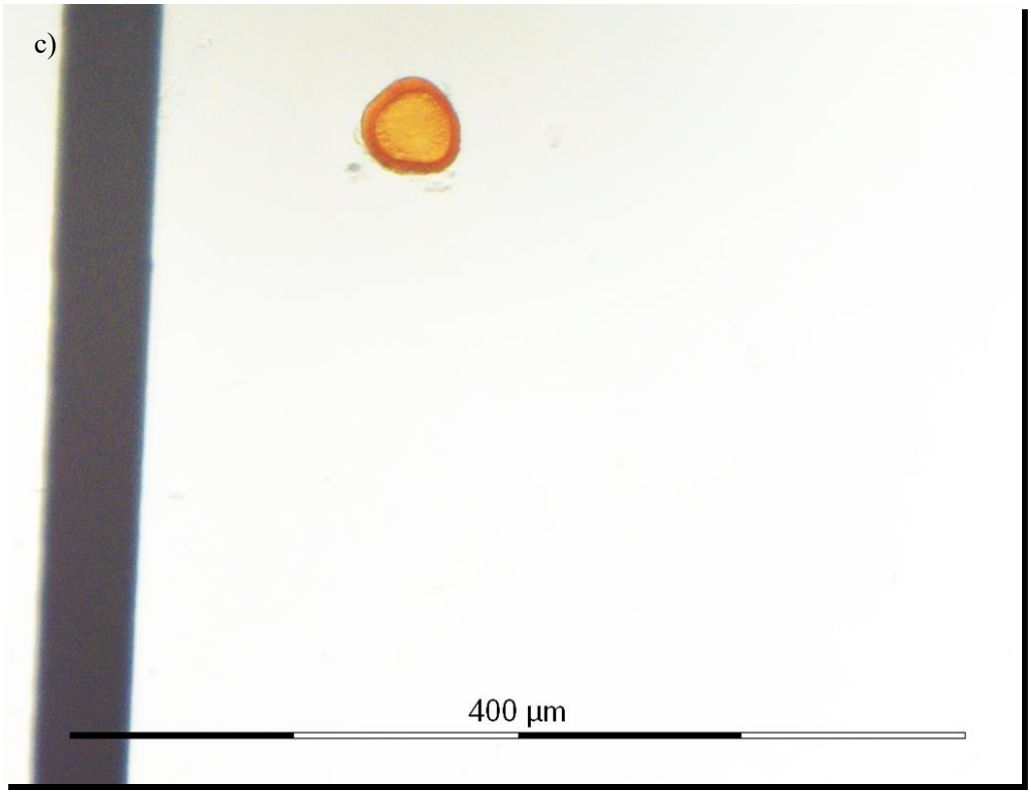
APPENDIX I

Eggs recovered from the faeces of a spotted hyena juvenile under light microscope (original). a) egg from a *Trichuris* sp.; b) egg from a Spiruridae family c) egg from a Taeniidae family.




c)
400 µm