Aus der Abteilung für Ecological Dynamics
im Leibniz-Institut für Zoo- und Wildtierforschung
des Fachbereichs Veterinärmedizin der Freien Universität Berlin

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Inaugural-Dissertation
zur Erlangung des Grades eines
PhD of Biomedical Sciences an der Freien Universität Berlin

vorgelegt von
Susana Carolina Martins Ferreira
Tierärztin aus Coimbra, Portugal

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Gedruckt mit Genehmigung
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Dekan: Univ.- Prof. Dr. Jürgen Zentek
Erster Gutachter: Univ.-Prof. Dr. Heribert Hofer
Zweiter Gutachter: Univ.-Prof. Dr. Georg von Samson-Himmelstjerna
Dritter Gutachter: Univ.-Prof. Dr. Justyna Wolinska

Deskriptoren (nach CAB-Thesaurus): *Crocuta crocuta*; *hyaena hyaena*; *Otocyon megalotis*; *Panthera leo*; fitness; populations; digestive tract; biodiversity; *Toxoplasma gondii*; *Cystoisospora*; *Ancylostoma*; epidemiology

Tag der Promotion: 13.08.2019

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List of abbreviations

Abbreviation	**full term**
CDV	canine distemper virus
CI	confident interval
CITES	convention on international trade in endangered species of wild fauna and flora
COSTECH	Tanzania commission for science and technology
CPV	canine parvovirus
DNA	Deoxyribonucleic acid
GRK 2046	Research training group 2046
ELISA	enzyme-linked immunosorbent assay
FCV	feline calicivirus
FEC	faecal egg counts
FHV1	feline herpesvirus 1
FIV	feline immunodeficiency virus
FPLV	feline panleukopenia virus
fGCM	faecal glucocorticoid metabolite
FOC	faecal oocyst count
GI	gastrointestinal
GLM	generalized linear models
IgG	immunoglobulin G
IgM	immunoglobulin M
IHA ELISA	indirect haemagglutination
KI	potassium iodide
MDS	multidimensional scaling
MHC	major histocompatibility complex
NP	national park
PLS	partial least squares
QAIC	quasi Akaike information criterion
RNA	ribonucleic acid
RSV	ribosomal sequence variants
SSU	small ribosomal subunits
T. gondii	*Toxoplasma gondii*
Th2	T helper cell type 2
Zoo	zoological garden

Chapter 1 - General introduction

Symbiosis refers to close associations between dissimilar organisms, either permanent or temporary and was first introduced by de Bary (1879) as "*Betrachtung der Erscheinungen des Zusammenlebens ungleichnamiger Organismen*", i.e., co-habitation of unlike organisms. When referring to the organisms living within or on a host, the outcome of the interaction of symbionts with their host can range from positive to negative, i.e. organisms can be mutualists (positive), commensals (neutral) or parasites (negative) of the host, and this is context dependent (Van Baalen and Jansen, 2003; Leung and Poulin, 2008). In the context of symbiosis, a parasite is therefore broadly defined as an organism that lives at the expense of another organism.

In this thesis I define "infection" as the entry of an organism into the host. Following infection, the infecting agent is either eliminated from the host or successfully colonises the host, i.e. establishes itself for a variable amount of time. The resulting damage to the host varies from none to severe. The clinical manifestation of infection is here defined as disease (Casadevall and Pirofski, 2000). When referring to disease causing parasites, the term usually used is pathogen (Begon, 2009). Unfortunately, the terms "parasites" and "pathogens" are often used interchangeably in the literature. Historically, the field of Parasitology deals with four major phyla of eukaryotes: Apicomplexa, Plathyhelminthes, Nematoda and Arthropoda, whereas Fungi, Bacteria and Viruses are studied in the field of Microbiology. Throughout this thesis I will use the narrow definition of parasites: A diverse multiphyletic group included in the domain Eukaryota studied by the scientific discipline of Parasitology (Urquhart et al., 1996), unless specified otherwise. The examples I provide from the literature review in this chapter emphasise gastrointestinal (GI) parasites because these were the focus of my thesis. When referring to pathogens, I use the broad definition proposed by Begon (2009) which includes viruses, bacteria and parasites as disease causing agents.

Parasitic relationships, in the broad sense, occur at all taxonomic levels, from viruses (La Scola et al., 2008) to mammals (Stephens et al., 2017). Parasitism, in the broad sense, has been proposed as the most common lifestyle on Earth (Poulin and Morand, 2000), with parasitic organisms contributing a considerable biomass within ecosystems. Lambden and Johnson (2013) estimated an average of 25% total parasite biomass within a snail host from an aquatic community. Parasite biodiversity and its role in ecological and evolutionary processes and ultimately human health have been increasingly recognized and appreciated (Hudson et al., 2006; Gómez and Nichols, 2013).

All mammalian species are infected by a range of both external and internal parasites. For parasites to infect a host, they need to overcome its defences and adapt to its physiological and microbiological environment. Consequently, hosts defend themselves using preventive and controlling measures such as mounting immune responses (Moreau and Chauvin, 2010; Reynolds et al., 2012) and reducing the chance of being infected by parasites by modifying their behaviour (Hart and Hart, 2018). Parasites are a significant component of ecological systems (Tompkins and Begon, 1999) and exert an important evolutionary selective force on their host. Similarly, the host's defences exert a selective force on parasites as well as shaping the communities of parasites that inhabit hosts (Schmid-Hempel, 2011; Leung et al., 2018).

The structure of ecological communities in terms of the abundance, distribution and/or diversity of organisms and species they contain is influenced by top-down and bottom-up mechanisms. In top-down processes, the lower trophic levels depend on the activity of higher levels, whereas in bottom-up processes, community structure is influenced by the effects at the lower trophic levels and/or in the availability of resources (Hunter and Price, 1992). Importantly, the relative importance of these mechanisms varies among species and ecosystems. Parasites can influence host community structure by decreasing survival, curtailing longevity or reproductive output and

ultimately influencing host abundance (top-down effect), as, for instance seen for the gastrointestinal (GI) nematode *Teladorsagia circumcincta* in Soay sheep (*Ovis aries*) in St. Kilda, UK (Gulland, 1992) and the GI nematode *Trichostrongylus tenuis* in red grouse (*Lagopus lagopus scoticus*) in northern England, UK (Newborn et al., 1998). Furthermore, parasites can change the host phenotype indirectly, thereby affecting host communities and possibly entire ecosystems (Mouritsen and Poulin, 2005; Hudson et al., 2006; Dunn et al., 2012). As a result, parasites have been proposed as ecosystem engineers (Thomas et al., 1999), i.e., as organisms that have a significant impact on an ecosystem structure and function.

Wild mammalian hosts are typically infected by a taxonomically diverse community of parasites. GI parasites live, feed and multiply in the host's gut and shed eggs, oocysts or larvae into the environment; typically through faeces. In some parasite species, infection is directly transmitted from one individual to another within the same host species. In parasites with indirect life-cycles, the parasite is transmitted to another species (intermediate host) before being re-transmitted to the final host (definitive host) in which the parasite sexually reproduces. Some parasites also infect "paratenic hosts", where development of the parasite does not occur until the paratenic host is ingested by the definitive host (Urquhart et al., 1996). It is likely that new parasite species will be identified from wild mammalian species because their identification through classical parasitological techniques make it difficult to unambiguously identify them down to the level of species. This is because the morphology of eggs and oocysts produced by parasites within a given parasite genus are similar (and typically undistinguishable). The genus is often the lowest taxonomic resolution at which the presence of parasites in host faeces can be distinguished and used to screen wildlife for infection with GI parasites. Hence, the morphological identification and counts of the number of faecal eggs or oocysts (faecal egg count FEC, faecal oocyst count FOC) is a common non-invasive method to

measure the infection load among individuals in populations when lethal sampling is not possible or counter-productive, when the research aim is to study the long-term impact of infection on individuals. Infection loads are typically heterogeneous within a given population (Wilson et al., 2002; Poulin, 2013). This variation is influenced by differences in the host's risk of exposure to infective parasite stages and the susceptibility of hosts to infections (Wilson et al., 2002). Knowledge of the processes and patterns of the distribution is key to understand parasite dynamics and host-parasite interactions, as a small proportion of individuals might suffer the highest costs of infection and contribute to the spread (transmission) of parasites to other hosts and strongly alter the potential for parasite transmission (Paull et al., 2002).

Parasites cause direct costs to their host by several mechanisms, by usurping or re-directing resources of the host that it might otherwise use for production, including the removal of nutrients from their host, the activation of host immune cells, damage and inflammation of host tissue and the repair of damaged tissue (Colditz, 2008). The accumulative effect of parasites might reduce host survival (Nussey et al., 2014) and reproductive success (Marzal et al., 2007). The outcome of a given parasite infection varies considerably among individuals, partially because of individual differences in host defences (Watson et al., 2016; Budischak et al., 2017). Once infected, hosts can limit the parasite burden by various mechanisms, a process termed host "resistance", and minimize the damage caused by the parasite, a process termed host "tolerance". Both processes are non-exclusive. Studies of host defences have been focusing on the mechanisms and determinants of resistance to infections whereas less is known about the determinants of tolerance (Råberg et al., 2009). Laboratory studies suggest that host tolerance to infections varies with host genotype and the resources available. Laboratory mice fed with low-protein diet had higher internal damage when infected with the worm *Heligmosomoides polygyrus*, than mice fed with normal food (Clough et al., 2016) and this effect varied between

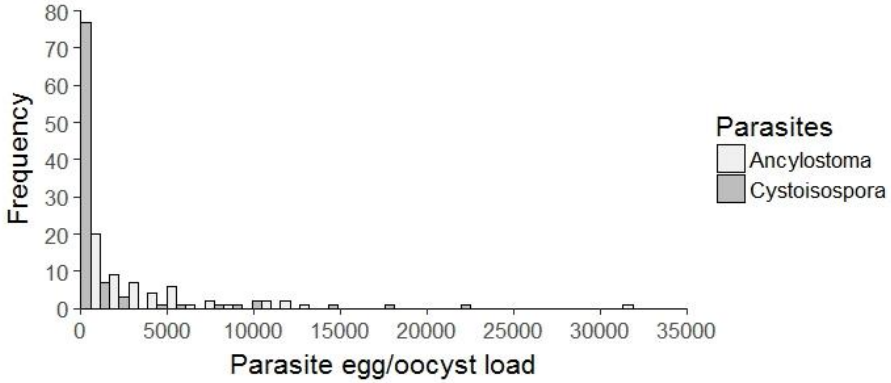
mice strains. In line with the idea that individuals exhibit genetic variation for tolerance, Raberg et al. (2007) found that different strains of mice infected with the protozoan *Plasmodium chabaudi* varied in tolerance as seen by different rates of decline in terms of anaemia and weight loss with parasite density. In studies of natural host-parasite relationships, accumulating evidence suggests that there is a genetic basis of tolerance (Aidoo et al., 2002; Hayward et al., 2014; Wanelik et al., 2018).

1.1 Determinants contributing to parasite heterogeneity in natural systems

Typically hosts within a population have different infection loads (Woolhouse et al., 1997), with most individuals having low parasite loads and a few individuals having high parasite loads and a few having none (see Figure 1, chosen as an example from my own work, see Chapter IV). Several factors relating to the host as well as factors relating to characteristics of the parasite are likely to contribute to the variation in parasite loads observed in natural populations (Wilson et al., 2002). Factors associated with hosts that are known to affect parasite loads in mammals are generally those that determine host immunity, such as host immune genotype (Meyer-Lucht and Sommer, 2005; Schad et al., 2012; Hayward et al., 2014), resource allocation trade-offs during energetically costly life stages such as lactation (Turner et al., 2012; East et al., 2015), the development of immune process in juveniles with increasing age (Dowling and Levy, 2014) and in social species, rank related access to food which in turn determines resource allocation to immune processes (East et al., 2015; Foerster et al., 2015). Tables 1 summarises a literature review of studies on biological aspects of spotted hyenas (*Crocuta crocuta*), my host study species, likely to contribute to the variation of parasite infections. Table 2 summarises a literature review on the pathogens of spotted hyenas and the host and pathogen factors analysed in relation to the pathogen infection presence or infection load. Both tables exclusively present results from the

Serengeti ecosystem in Tanzania and Kenya, East Africa – my study area.

Figure 1. Frequency distributions of *Ancylostoma* faecal egg load and *Cystoisospora* oocyst load (eggs or oocysts/g faeces) in juvenile spotted hyenas.



Juvenile mammals tend to have the highest infection loads (Cattadori et al., 2005) and suffer differential costs of infection (Jackson et al., 2014; Marescot et al., 2018) although this age class is often overlooked by studies on parasite infections in wild mammals. High infection loads in young mammals are likely a consequence of poorly developed immunocompetence following birth and the gradual improvement of immune responses and acquired immunity during juvenile development (Ramsburg et al., 2003; Adkins et al., 2004; Watson et al., 2016), and increased exposure to parasites with increasing age and life time range (Cattadori et al., 2005).

It is well known that social contacts between infected and susceptible hosts will facilitate parasite transmission. In comparison to solitary or monogamous species, social mammals are likely to be particularly exposed to parasites as a consequence of social interactions between members of a group. Hence, the number of animals within a group and factors, such as social status, age and sex, which determine contacts between animals in a group are likely to affect the probability of a susceptible animal contacting an infected one

and may in part explain parasite load heterogeneities between individuals within a group (Anderson and May, 1992; Altizer et al., 2003; Patterson and Ruckstuhl, 2013). For parasites that are directly transmitted between individuals in a host population, density-dependent effects (Anderson and May, 1992) should increase transmission as population densities increase. The rate of direct parasite transmission may also be expected to increase with group size in social mammals, as has been shown for symbionts in social mammals (Moeller et al., 2016). Intestinal parasites that have to pass through a number of development stages once they are shed by an infected host are expected to increase with group size if exposure to infective stages occurs in locations regularly frequented by members of a group (Patterson and Ruckstuhl, 2013). Positive relationships between group size and parasite prevalence and intensity have been found for several taxa (Côté and Poulin, 1995; Patterson and Ruckstuhl, 2013).

In social mammals, high-ranking animals have a higher social value than low-ranking animals (Seyfarth, 1977) and for this reason generally are involved in more social interactions (East et al. 1993) resulting in increased exposure to pathogens spread by direct or close contact (East et al., 2001; Habig et al., 2018; Marescot et al., 2018). In species with well-defined linear hierarchies, where access to resources depend on social status, high-ranking animals have priority to resources (Clutton-Brock and Huchard, 2013). Thus life-history theory predicts that animals holding high social status should less often resort to resource allocation trade-offs between important life processes, such as maintenance, immune processes and reproduction, than animals holding low social status, in order to optimize fitness. Immune responses are costly (Sheldon and Verhulst, 1996; Lochmiller and Deerenberg, 2000; Colditz, 2008). An accumulating body of research has shown evidence of trade-offs between immunity and reproduction (Verhulst et al., 2005) and lactation (Cattadori et al., 2005; Turner et al., 2012; East et al., 2015). In line with this idea, high-ranking spotted hyenas

have higher immunoglobulin M (IgM) serum concentration than low-ranking individuals (Flies et al., 2016).

Table 1. A literature review of aspects of spotted hyena biology likely to affect the heterogeneity of parasite infections in individual spotted hyenas. Studies cited are restricted to those from the Serengeti ecosystem, including the Serengeti National Park and Ngorongoro Conservation Area in the Tanzanian sector and the Maasai Mara National Reserve in Kenya. genotype - partial sequencing of genes of interest.

Host factors	**Reference**
Resource availability and social environment: Prey availability Social organization Maternal effects Sibling rivalry Siblicide	Kruuk, 1972 Frank, 1986b, 1986a Hofer and East, 1993, 1993a, 1993b, 1993c, 1995, 1997, 2003, 2008 East and Hofer, 1991, 2001 East et al., 1993, 2001, 2009 Smale et al.,1993 Holekamp and Smale, 1993 Holekamp et al., 1996 Cooper et al., 1999 Golla et al., 1999 Wachter et al., 2002 Höner et al., 2005, 2007, 2010 Benhaiem et al., 2012b Vullioud et al., 2018
Host state: Nutritional Physiology Age Sex Allostatic load Reproductive stage Hormonal levels	Holekamp et al., 1996 Cooper et al., 1999 Goymann et al., 1999, 2001, 2003a, 2003b Benhaiem et al., 2012a, 2013, 2018 Flies et al., 2012, 2015, 2016 East et al., 2013, 2015 Pribbenow et al., 2015 Funk et al., 2018
Trade-offs and constraints: Lactation	East et al., 2015 Hofer et al., 2016
Genotype: Toll-Like receptors Major histocompatibility class	Califf et al., 2013 Flies et al., 2014

Table 2. A literature review of the studies analysing pathogen prevalence and/or host and pathogen factors of pathogen infections. Studies cited are restricted to those from the Serengeti ecosystem, including the Serengeti National Park and Ngorongoro Conservation Area in the Tanzanian sector and the Maasai Mara National Reserve in Kenya. Parasite is defined here as eukaryotic parasites. In blue are the contributions of the current thesis. CDV- canine distemper virus; FIV - feline immunodeficiency virus; FPLV/CPV - feline panleukopenia virus/canine parvovirus; FCV - feline calicivirus; FHV1 - feline herpesvirus 1. co-infection – studies that considered the effect of the presence or infection load of more than one parasite taxon; genotypes - partial or complete sequencing of pathogen of interest; Intestinal biome – eukaryome and bacterial microbiome; GI parasites – coprological surveys of gastrointestinal parasites

		Host factors								Pathogen factors			
Pathogen		**Prevalence**	**Prey abundance**	**Social status**	**Sex**	**Clan composition**	**Clan size**	**Age**	**Lactation**	**Co-infection**	**Clan ID**	**Genotype**	**Reference**
virus	Rabies	X		X	X			X			X	X	East et al., 2001
	CDV	X		X	X			X				X	Harrison et al., 2004; Nikolin et al., 2017 Marescot et al., 2018
	FIV	X		X	X			X					Harrison et al., 2004
	FPLV/CPV	X		X	X			X					Harrison et al., 2004
	FCV	X		X	X			X					Harrison et al., 2004
	FHV1	X		X	X			X					Harrison et al., 2004

	Sapovirus	X			X	X			X	X				X	Olarte-castillo et al., 2016
	Coronavirus	X												X	East et al., 2004; Goller et al., 2013; Harrison et al., 2004
	Kobovirus	X												X	Olarte-Castillo et al., 2015
bacteria	*Streptococcus equi ruminatorum*	X	X		X	X	X		X	X				X	Höner et al., 2006, 2012
	Bacillus anthracis	X													Lembo et al., 2011
parasite	GI parasites	X										X			**Chapter II** **Chapter IV** Engh et al., 2003
	Cystoisospora	X			X	X	X			X		X	X		**Chapter IV**
	Ancylostoma				X	X	X			X	X	X	X		**Chapter IV** East et al., 2015
	Toxoplasma	X								X					**Chapter III**
	Dipylidium	X	X				X			X			X		East et al., 2013
	Hepatozoon													X	East et al., 2008
	Trichinella	X													Pozio et al., 1997
	Trypanosoma													X	Auty et al., 2012

symbiont	intestinal biome	X	X		X	X	X	**Chapter II**

1.2 The community of the gastrointestinal tract - Intestinal biome

The gastrointestinal tract of mammals is progressively colonized by a collection of viruses and eukaryotic and prokaryotic organisms - the intestinal biome (Palmer et al., 2007; Funkhouser and Bordenstein, 2013). The intestinal biome and their host have a long, joint evolutionary history (Ley et al., 2008; Sharpton, 2018). The contribution of the intestinal biome to host phenotype include the development of local and systemic immunity (Hooper et al., 2012), the facilitation of host metabolism by producing essential vitamins, and the provision of nutrients otherwise indigestible to the host (Ramakrishna, 2013). Interaction between species of the intestinal community can affect immune responses towards helminths (Brosschot and Reynolds, 2018) and host fitness as reported for invertebrate hosts (Koch and Schmid-Hempel, 2011; Gould et al., 2018).

Other factors may account for the observed individual variation in parasite load, such as the presence of other co-existing symbionts (Kreisinger et al., 2015), including co-infecting parasites (Graham, 2008; Knowles et al., 2013). Co-infecting taxa can interact through bottom-up processes by resource competition or indirectly through top-down processes, for instance via the immune system of the host (van Riet et al., 2007; Graham, 2008; Råberg et al., 2009; Budischak et al., 2018; Maizels et al., 2018) and have positive, negative or neutral effects on the interacting taxa (Christensen et al., 1987; Zele et al., 2018).

Currently the dynamics of parasite presence, abundance and diversity within intestinal parasite communities are still poorly understood. Most studies on this topic have focused on the bacterial microbiome and less so on the eukaryotic biome termed the eukaryome. Research on both the mammalian bacterial microbiome and eukaryome has mostly been restricted to primates, including humans, captive wild mammals and laboratory model species. As a result, relatively little is known about the non-bacterial components of the microbiome and particularly the eukaryome in large wild mammalians. To gain a

better understanding of the composition, diversity, stability and function of the mammalian intestinal biome (Hird, 2017) research is required on the intestinal biomes of known individuals in wild mammal population in ecosystems where 'natural' ecological processes prevail.

1.3 Darwinian fitness consequences of parasite infections

Variation in fitness among individuals in a population arises from differences in components of fitness such as survival to adulthood, longevity and lifetime reproductive success (Stearns, 1992). The measurement of relevant components of fitness throughout the lifespan of long-lived mammals requires the collection of long-term data from individually recognisable animals throughout their lives (Clutton-Brock and Sheldon, 2010).

The relationship between parasite infection load and measured components of fitness may not be straight-forward (Graham et al., 2011; Sparks et al., 2018). Besides the direct impact of infection, parasites can also have indirect effects via the increased energetic cost to the host of establishing or maintaining host defences and mounting responses to infection, resulting in a reduction of host resources available for life processes relevant for fitness such as survival and reproduction (Sheldon and Verhulst, 1996; Lochmiller and Deerenberg, 2000). The outcome of parasite infection for the host, in terms of fitness components such as survival, can depend on host related determinants such as host social status (Habig et al., 2018), age (Lynsdale et al., 2017) and body condition (Gulland, 1992) as well as the parasite taxon (Budischak et al., 2017).

In addition, parasites can impose other costs, including increased susceptibility to predation (Bakker et al., 1997; Östlund-Nilsson et al., 2005). Pathogens are proposed as the evolutionary reason for the maintenance of males among eukaryotes, i.e. the existence of sexual reproduction. This is a direct consequence of the theory known as the "Red Queen" hypothesis (van Valen, 1977; Bell, 1982), coined after a character from a novel by Lewis Carroll. The character, the "red queen" tells the main character from the novel, "Alice", she must

keep running to stay in the same place. The metaphor here being that one evolutionary change that gives a pathogen an advantage is then reduced by a counter evolutionary change evolved by the host in response to the change in the parasite. Sexually reproduction is advantageous over asexual reproduction by facilitating genetic variability and the potential production of offspring with rare genotypes that tend to be more resistant to co-evolved parasites (Jaenike 1978; Hamilton 1980). This hypothesis has been expanded and intensively studied in diverse systems (Hamilton et al. 1990; Wolinska and Spaak, 2009; Gibson et al., 2018). Consistent with the idea that parasites are an importance selective force on the evolution of the host, (Hamilton and Zuk, 1982) proposed that the choosier sex, typically females, prefer to mate with males that display traits associated with parasite resistance and good condition, implying that parasites can have a negative impact on the reproductive success, an important fitness component. Based on this hypothesis, the role for immune genes, in particular the major histocompatibility complex (MHC) loci in mate choice has been extensively studied (Wedekind et al., 1995; von Schantz et al., 1996; Ober et al., 2006). Viewed together, the total costs of parasites are difficult to measure.

1.4 Study population and ecological setting

The Serengeti-Mara ecosystem, hereafter the Serengeti ecosystem, includes the Serengeti National Park, Ngorongoro Conservation Area, Maswa Game Reserve, Grumeti Game Reserve, Ikorongo Game Reserve and Loliondo Controlled Area in Tanzania and the Maasai Mara National Reserve in Kenya. It was created to protect the migratory route of large herds of wildebeest (*Connochaetes taurinus*), Thomson gazelle (*Eudorcas thomsoni*) and zebra (*Equus quagga*) (Norton-Griffiths and Sinclair, 1979). The ecosystem remains a relatively intact natural ecosystem with most of the area used by migratory herbivores being located within some form of protected area. Several large predators occur in the Serengeti ecosystem, with the spotted hyena (*Crocuta crocuta*) being the most numerous large carnivore (Hofer and East, 1995).

Spotted hyenas are social carnivores that live in large, stable, territorial groups termed clans (Kruuk, 1972) with separate linear hierarchies among natal females and immigrant males (East and Hofer, 1991; Hofer and East, 1993b). All adult females in a clan and their offspring are socially dominant over males (East et al., 1993; East and Hofer, 2001). Female spotted hyenas remain in their natal clan throughout their life whereas most males leave their natal clan to join other clans after they reach adulthood at 2 - 2.5 years (Frank, 1986a; Hofer and East, 1995; Höner et al., 2007).

Spotted hyenas are efficient hunters and scavengers (Kruuk, 1972). In the Serengeti National Park they mostly prey on migratory herbivores (Hofer and East, 1993b; Kruuk, 1972). Due to the seasonal migratory movements, prey availability fluctuates substantially within a clan's territory throughout the year. When there is not enough prey within a clan's territory, clan members travel long distances in a non-synchronized pattern up to 75km one way to feed outside their territories in areas containing a high abundance of migratory herbivores (Hofer and East, 1993a, 1993b, 1993c).

All juveniles from a clan are based in a communal den located within a clan's territory (Figure 2). The communal den is a social centre for the clan (Hofer and East, 1993a, 1993b). During the heat of the day adults and older juveniles, i.e. animals between 12 and 24 months of age, generally rest away from the communal den. In the evening adult females return to the den to nurse their offspring (Golla et al., 1999; Hofer et al., 2016), forage within their clan's territory or leave the clan territory on a commuting trip (Hofer and East, 1993b, 1993c). Lactating females suckle their young typically at dawn and dusk at the vicinity of the den. The lactation period is circa 12 – 18 months long and the milk is highly nutritious (Hofer and East, 1995; Hofer et al., 2016). Prey availability influences maternal nursing patterns (Hofer and East, 1993a). When prey abundance declines, lactating females go on commuting trips and the frequency of den visits to suckle their offspring declines, resulting in lower growth rates of the dependent juvenile (Hofer and East, 1993a). High-ranking females have priority of access

to food on their territory (Frank, 1986b; Hofer and East, 1993b) and hence need to commute for a far shorter period of the year than low-ranking females. When prey abundance is high, all females feed within the territory and when prey abundance is low, all females go on costly commuting trips to feed on migratory herds outside their territory. When prey abundance is medium, high-ranking females less often commute long distances than low-ranking females. As a consequence, high-ranking females suckle their offspring more often, their offspring grow faster and these females have a higher lifetime reproductive success (Hofer and East, 2003).

Figure 2. Spotted hyenas from one of the study clans at the communal den, in the Serengeti National Park, Tanzania.

1.5 Aims of my research

Currently little is known about the factors determining individual heterogeneity in parasite infections in unmanaged wild mammal populations, including the gastrointestinal community of most wildlife species. In particular, the eukaryotic proportion of the biome, the eukaryome, remains largely unknown, and little is known about the fitness consequences which parasite infections impose on their hosts.

The research outlined in this thesis was embedded in an on-going, long running research programme initiated in 1987 on a population of several hundred individually known spotted hyenas in the Serengeti National Park in Tanzania. The study population consists of animals belonging to three large clans. Detailed data on the demography of the study population, social status, occurrence of pathogen infections and faecal samples from animals in these clans were available for my study. One key aim of this long-term project is to increase knowledge on the evolutionary history and epidemiology of pathogens in wild carnivores from a natural environment. Primary to my study, there have been several studies on viral infections with massive consequences in the ecosystem such as canine distemper virus (CDV). I expand this research by improving knowledge on the less conspicuous gastrointestinal parasites, which may also play critical roles in ecological and evolutionary processes.

My research was primarily focused on individually known spotted hyenas in three large clans in the Serengeti NP. Fortunately, information of each animal's age, sex and social status was known as well as information on the size and composition of the clan. This information permitted a more detailed examination of my data than would have been otherwise possible. As my study population was in a National Park, interventions in terms of capturing animals to obtain blood samples to assess parasite infection and host immune responses were kept to a minimum and non-invasive measures were mostly applied. In general, the use of non-invasive methods is preferable because there is growing evidence that interventions used during capture and sampling can have a

negative impact on a broad range of animals and hence affect the results of studies (Hofer and East, 2012).

My thesis aims to investigate determinants of gastrointestinal parasite infections of a wild social mammal and to assess the fitness consequences of infection. I hypothesise that individual variation of parasite infections is determined by 1) life-history traits; 2) social, ecological and abiotic environmental factors; 3) host immune-competence and 3) gastrointestinal community.

In **chapter 2**, I describe my investigation of the intestinal biome of spotted hyenas. In this study I applied the relatively new method of multi-amplicon sequencing (metabarcoding) using faecal samples from spotted hyenas. The aim of this research was to determine the effect of social status in adult females (high vs low) and life-history stage (juveniles vs adults) on the intestinal biome of spotted hyenas in the Serengeti ecosystem. We hypothesised that the intestinal biome differs in terms of composition, richness and diversity with life history traits between age classes as a result of physiological and social factors, and social status as a consequence of differences in their behaviour and access to resources.

In **chapter 3**, I detail my investigation of exposure to *Toxoplasma gondii* in spotted hyenas and other sympatric carnivores in the Serengeti ecosystem. For this purpose, I used sera from carnivore species in the Serengeti ecosystem, including sera collected during a period of 28 years from spotted hyenas. My aim was to determine whether exposure to this parasite in spotted hyenas changed during this long monitoring period and how life-history stage (juvenile vs adults) affected seroprevalence in terms of seropositivity to *T. gondii* in the spotted hyena population. We hypothesised that infection with *T. gondii* is influenced by diet.

In **chapter 4**, I present my investigation of factors likely to determine the infection load for two directly transmitted, energetically costly and highly prevalent parasites – *Cystoisospora* and *Ancylostoma* – and test predictions derived from known mechanisms of parasite-host and parasite-parasite interactions, and life-history theory. This research focused on the juvenile life stage, which in spotted hyenas includes animals

less than 24 months of age. I also investigated the Darwinian fitness consequences of the infection loads of these two parasites in terms of juvenile survival to adulthood (at 24 months of age). This research combined non-invasive faecal sampling, classical parasitological methods, and statistically modelling. We hypothesised that 1) nutritional status influences the allocation of resources to immune processes, predicting that low-ranking juveniles, that are generally less nourished have higher parasite loads than better nourished high-ranking juveniles; 2) the immune system develops with age, as a result of which parasite loads are predicted to decrease with age; 3) social status enhances the transmission of parasites with oral-faecal infection routes, predicting that high-ranking juveniles have higher *Cystoisospora* infection loads than low-ranking juveniles, as they engage more frequently in greeting ceremonies (East et al. 1993); 4) energetically costly parasites such as *Cystoisospora* and *Ancylostoma* should negatively affect survival to adulthood, a key component of fitness.

Chapter 2 - The intestinal eukaryotic and bacterial biome of spotted hyenas: the impact of social status and age on diversity and composition

(Published article)

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The study design of the work, in terms of applying an appropriate assay was developed
by Emanuel Heitlinger (EH). The concept of the study on spotted hyenas was provided by Susana C. M. Ferreira (SF), Heribert Hofer (HH), and Marion L. East (ME). Faecal samples were collected by ME and HH. Laboratory analysis was performed by EH, SF, and Dagmar Thierer (DT). Statistical analysis was done by EH and SF. The manuscript was written by EH, SF, HH, and ME. All authors approved the final version of the manuscript.

Front Cell Infect Microbiol. 2017; 7: 262.
doi: 10.3389/fcimb.2017.00262

2.1 Abstract

In mammals, two factors likely to affect the diversity and composition of intestinal bacteria (bacterial microbiome) and eukaryotes (eukaryome) are social status and age. In species in which social status determines access to resources, socially dominant animals maintain better immune processes and health status than subordinates. As high species diversity is an index of ecosystem health, the intestinal biome of healthier, socially dominant animals should be more diverse than those of subordinates. Gradual colonization of the juvenile intestine after birth predicts lower intestinal biome diversity in juveniles than adults. We tested these predictions on the effect of: (1) age (juvenile/adult) and (2) social status (low/high) on bacterial microbiome and eukaryome diversity and composition in the spotted hyena (Crocuta crocuta), a highly social, female-dominated carnivore in which social status determines access to resources. We comprehensively screened feces from 35 individually known adult females and 7 juveniles in the Serengeti ecosystem for bacteria and eukaryotes, using a set of 48 different amplicons (4 for bacterial 16S, 44 for eukaryote 18S) in a multi-amplicon sequencing approach. We compared sequence abundances to classical coprological egg or oocyst counts. For all parasite taxa detected in more than six samples, the number of sequence reads significantly predicted the number of eggs or oocysts counted, underscoring the value of an amplicon sequencing approach for quantitative measurements of parasite load. In line with our predictions, our results revealed a significantly less diverse microbiome in juveniles than adults and a significantly higher diversity of eukaryotes in high-ranking than low-ranking animals. We propose that free-ranging wildlife can provide an intriguing model system to assess the adaptive value of intestinal biome diversity for both bacteria and eukaryotes.

Keywords: eukaryotome, eukaryome, parasites, amplicon sequencing, spotted hyena, social status, bacterial microbiome, age classes

2.2 Introduction

Mammalian hosts have a long evolutionary history with the diverse communities of prokaryotes (Dethlefsen et al., 2007; Ley et al., 2008; Douglas and Werren, 2016) and eukaryotes (Hafner and Nadler, 1988; Glendinning et al., 2014), here designated as the intestinal biome, present in their gastrointestinal tract. Although the intestinal biome was traditionally viewed as a rather inert species assembly, recent insights have revealed that its composition is influenced by many host traits and that it in turn can have a considerable impact on its host (Turnbaugh et al., 2007; Graham, 2008; Costello et al., 2012; Wegner Parfrey et al., 2014; Kreisinger et al., 2015). Recently, interest in the community of bacteria in the intestine (often called the microbiome, even though bacteria do not comprise all microscopic organisms in the intestines) has grown (Round and Mazmanian, 2009; Turnbaugh et al., 2009; Lozupone et al., 2012), whereas the communities of unicellular (Wegner Parfrey et al., 2014) and especially multicellular eukaryotes in the gastrointestinal tract (eukaryome or eukaryotome) have received less attention.

Following birth, the mammalian gastrointestinal tract is gradually colonized by prokaryotes and eukaryotes (Palmer et al., 2007; Koenig et al., 2011; Lozupone et al., 2012; Yatsunenko et al., 2012). Throughout an individual's lifespan, the composition of the intestinal biome can be altered by various factors, including diet (Turnbaugh et al., 2009; Amato et al., 2013), as exemplified by the transition from the consumption of milk during infancy (Pond, 1977) to an “adult” diet when weaned. Composition and diversity is also influenced by interactions between species within the intestinal biome (Ezenwa, 2004; Benson et al., 2010; Glendinning et al., 2014; Lee et al., 2014; Kreisinger et al., 2015), developmental changes in immune function (Dowling and Levy, 2014) and age related changes in exposure to prokaryotes and eukaryotes in the environment, food and conspecifics (Palmer et al., 2007; Koenig et al., 2011; Lozupone et al., 2012). Despite these factors that induce variation in the intestinal biome among individuals within a species, there is evidence of species-

specific bacterial microbiome signatures in mammals (Ley et al., 2008; Ochman et al., 2010; Yildirim et al., 2010; Degnan et al., 2012; Menke et al., 2014).
While the majority of studies on the diversity and composition of bacterial microbiomes are based on partial sequencing of 16S ribosomal RNA genes, the application of metagenomics (the sequencing of the complete genetic repertoire of the host's intestinal microbiome) has revealed links between the bacterial microbiome of the intestine and the host's metabolism (Gill et al., 2006; The Human Microbiome Consortium, 2012). The bacterial microbiome influences host nutrition, fat storage and the metabolism of vitamins and minerals (Turnbaugh et al., 2009; Tremaroli and Bäckhed, 2012; Leone et al., 2015). Experimental studies with germ-free mice also show that the bacterial microbiome of the intestine can affect postnatal development of the hypothalamic-pituitary-adrenal axis (Sudo et al., 2004). Furthermore, bacterially derived fermentation products from the intestinal microbiome help regulate the maturation of microglia which contribute to an active immune defense of the central nervous system (Erny et al., 2015) and there is now general agreement that the bacterial microbiome influences the host immune system (Round and Mazmanian, 2009; Hooper et al., 2012; Sivan et al., 2015).
The impact of intestinal eukaryotes on their hosts has mostly been studied in relation to the pathologies caused by specific helminths and protozoans (Wegener Parfrey et al., 2011; Andersen et al., 2013; Rajilić-Stojanović and de Vos, 2014). We are not aware of any studies on intestinal biomes comprehensively screening for eukaryotes, including multicellular species. This is surprising, as it has been argued that eukaryotes may have an important ecological function in intestinal ecosystems similar to that of keystone species in terrestrial or aquatic ecosystems (Lukeš et al., 2015). By extension this should particularly apply to large multicellular eukaryotes. Currently, most research on the intestinal biomes of mammals has focused on the bacterial microbiome of humans, wild and captive non-human primates, laboratory mice and zoo

animals; hence knowledge on the intestinal biomes of free-ranging wild mammals is limited.

In social mammals, social status often determines access to resources (Clutton-Brock and Huchard, 2013) thereby affecting health status (Sapolsky, 2005), wound healing (Archie et al., 2012), immune gene expression (Tung et al., 2012), immune defenses (Flies et al., 2016; Snyder-Mackler et al., 2016) and the likelihood and impact of pathogen infection (East et al., 2001, 2015; Höner et al., 2012). Social status is also likely to affect the species composition and abundance of bacteria and eukaryotes in the gastrointestinal tract.

We present, to our knowledge, the first study to simultaneously investigate both the bacterial microbiome and eukaryome of a wild mammalian species. We applied a multi-amplicon sequencing approach for bacteria and eukaryotes (metabarcoding) to fresh feces to investigate the effect of social status and age on the (distal) intestinal bacterial microbiome and eukaryome of individually known spotted hyenas (Crocuta crocuta) in the Serengeti National Park (NP) in northwestern Tanzania. This highly social carnivore lives in fission-fusion groups termed clans, in which natal females and their offspring are socially dominant over immigrant males (Kruuk, 1972). Migratory movements of ungulates cause large fluctuations in the abundance of food resources in clan territories (Hofer and East, 1993a,b), which profoundly affect the foraging behavior of clan members (Hofer and East, 1993a,b). When migratory ungulates are absent, clan members undertake regular long-distance (approximately 80–140 km) foraging trips (termed commuting trips) to areas containing abundant migratory prey (Hofer and East, 1993b). As access to food within a clan's territory is determined by social status, high-ranking females commute far less often than low-ranking females (Hofer and East, 2003) and their reduced foraging effort is reflected in their lower fecal glucocorticoid metabolite (fGCM) concentrations (Goymann et al., 2001b).

High-ranking females are more often exposed to infectious pathogens than low-ranking females because of their more frequent social interactions with clan members (East et al.,

2001). Even so, high-ranking females have lower intestinal parasite burdens than low-ranking females, probably because they can allocate more resources to immune processes than low-ranking females (East et al., 2015). Hence, we expect the bacterial microbiome and the eukaryome of high-ranking animals to be more diverse than those of low-ranking animals, because high species diversity is generally considered an index of ecosystem health (Cardinale et al., 2006; Costello et al., 2012; Reich et al., 2012). In contrast, we expect the intestinal biome of low-ranking animals to be less diverse than those of high-ranking animals.

As in all mammals, the intestinal bacterial microbiome and the eukaryome of juvenile spotted hyenas develops after birth. In our study population, juveniles are more often infected with specific pathogens than adults (Goller et al., 2013; Nikolin et al., 2017), including the eukaryote Dipylidium sp. (East et al., 2013). Moreover, juveniles have lower protection from antibodies (East et al., 2001) and their diet includes maternal milk until 12–18 months of age (Hofer and East, 1995). For these reasons we expect the bacterial microbiome and eukaryome of juveniles to be less diverse than that of adults.

We tested our predictions concerning the effects of age and social status by assessing sequence read counts of taxonomically annotated ribosomal sequence variants (RSV) and hence the composition and abundance of genera in the bacterial microbiome and the eukaryome of individually known hosts. We also compared these results with those generated by the classical coprological method of parasite egg or oocyst counts as applied, for instance, by East et al. (2015) to assess whether the results of these methods were strongly correlated.

2.3 Methods

2.3.1 Study population

The spotted hyena (hereafter hyena) study population included three closely monitored clans that are part of an ongoing long-term research program in the center of Serengeti NP, northwest Tanzania. Individuals were recognized by their spot patterns, ear notches, scars and bald patches (Frank, 1986; Hofer and

East, 1993a) and sexed using the dimorphic shape of the phallic gland (Frank et al., 1990). Age was determined from the observed date of birth or based on observations of pelage, position of the ears, level of coordination when walking and body size, with an accuracy of ±7 days as previously described (East et al., 2003). Animals were categorized as juveniles when <24 months of age, and adult when ≥24 months of age. Females were allocated a social rank within the dominance hierarchy using submissive responses in dyadic interactions (East et al., 2003). To compare rank positions across clans, individuals were assigned a standardized rank within a dominance hierarchy by distributing ranks evenly between the highest rank (standardized rank +1) and the lowest rank (standardized rank −1), with the median rank being scored as 0 (Goymann et al., 2001a). Females holding standardized ranks with 0 or above 0 were categorized as high-ranking, those holding a standardized rank of less than 0 as low ranking.

2.3.2 Sampling

Forty-two fecal samples (35 adult females and 7 juveniles) were collected immediately after defecation from individually known animals between 2009 and 2012. Samples were thoroughly mixed and aliquots were stored in formalin (4%) for parasite egg counts and preserved in RNAlater (Sigma–Aldrich, St Louis, MO, USA) for molecular genetic analyses. Samples in RNAlater were initially stored frozen at −10°C, transported frozen and then stored at −80°C for genetic analysis (East et al., 2013). DNA was extracted using the Macherey-Nagel Nucleo-spin soil DNA extraction kit (Macherey-Nagel, Düren, Germany) following the manufacturer's recommendations and using the Peqlab Precellys 24 homogenisator (VWR International Group, Erlangen, Germany). We assume that fecal biomes are representative of intestinal biomes, since a strong relationship between the two was demonstrated for freshly collected (Menke et al., 2015) and properly stored samples (Menke et al., 2017).

2.3.3 Parasite egg counts

Parasite egg or oocyst counts were conducted on aliquots of a subset of 32 fecal samples (27 adult females and 5 juveniles, 20 high-ranking and 12 low-ranking individuals) that were suitable for this procedure using a modification of the McMaster flotation technique (Gordon and Whitlock, 1939). To enhance the detectability of eggs, a solution of potassium iodide (KI) was used with a specific weight of 1.5 g ml−1 (Meyer-Lucht and Sommer, 2005; Schwensow et al., 2007). Four McMaster chambers were counted for each sample with a dilution factor of 1:15. After combining the feces with the KI solution, it was vortexed for 3 min and then sieved in order to remove bigger debris. Parasite eggs or oocysts were identified according to their morphology and counted using a light microscope, 1 h after preparing the fecal suspension, with a magnification of 100x (10x eyepiece lens × 10x objective lens). Pictures were captured using the software ProgRes CapturePro version 2.5, 2007 (Jenoptik, Jena, Germany). During fecal egg or oocyst counts, eukaryote parasites were identified at the genus or family level on the basis of their morphology and size, with the exception of oocysts from the order Coccidia because they are very similar in terms of their morphological appearance. For Ancylostoma, the Taeniidae and the Coccidia two morphological types based on two size classes of eggs or oocysts were distinguished. For the nematode Ancylostoma, the two identified size classes for eggs were <80 µm and ≥80 µm, for the cestodes from Taeniidae, the two size classes were <45 µm and ≥45 µm, and for the Coccidia oocysts, the two size classes were <20 µm and ≥20 µm, respectively. The results are expressed as fecal egg counts per g feces (FEC) or fecal oocyst counts per g feces (FOC). All egg and oocyst counts were done blind with respect to the life history stages and characteristics (age, social status) of the individual hyenas from which the fecal sample was taken and analyzed.

2.3.4 Multi-amplicon PCRs and sequencing

The Fluidigm Access Array integrated fluidic circuit (Fluidigm, San Francisco, California, USA) was used to run 48 × 48 PCR

reactions in 2,306 compartments of a microfluidics device. Target specific PCR primers (Supplementary File 1) for 18S and 16S small ribosomal subunits (SSU) were used in a “four primer” PCR approach, following the manual provided by Fluidigm. Briefly, target specific primer pairs were combined with “CS1” and “CS2” adapters at their 5′ and 3′ ends, respectively, on the microfluidics device. These target specific primer pairs were used to prime 48 target specific reactions for each of 48 samples, using default cycling parameters. After harvesting all products from the separate samples into a 96 well microliter plate, a second PCR was performed on a 10-fold dilution, by introducing Illumina sequencing oligonucleotides “PE5” at the “CS1” adapter and “PE7” at the “CS2” adapter as well as a sample identifier sequence between “CS2” and “PE7” based on the Access Array Barcode Library (Fluidigm, San Francisco, California, USA) for Illumina Sequencers (384 single direction). Samples were pooled and selected by size using Agencourt AMPure XP Reagent beads (Beckman Coulter Life Sciences, Krefeld, Germany). For further cleanup, PCR fragments between 400 and 1,000 bp were purified by PippinPrep using the 1.5% agarose DNA gel cassettes (Sage Science Inc., Beverly, Massachusetts, USA). Suitability of PCR products for sequencing (e.g., by checking for an absence of primer multi-meres) was confirmed using the Agilent 2200 Tape Station with D1000 ScreenTapes and D1000 Reagents (Agilent Technologies, Santa Clara, California, USA). Sequences were generated at the Berlin Center for Genomics in Biodiversity Research (BeGenDiv) on the Illumina MiSeq machine (Illumina, San Diego, California, USA) using version 3 chemistry and 600 cycles of (paired-end) sequencing. The sequencing data can be accessed through the accession number PRJNA386767 at NCBI Short Read Archive (SRA).

2.3.5 Bioinformatic analyses

Sorting of sequencing reads in different samples was performed using the bcl2fastq utility version 2.17.1.14 (Illumina, San Diego, California, USA) based on the sample identifier oligos. All subsequent bioinformatic, taxonomic and statistical analyses

were performed in R version 3.3.2 (R Development Core Team, 2016); below we cite the R packages used for specific steps in the analysis.
Sequences were quality trimmed and screened for erroneous reads using the fastqPairedFilter function of package dada2 version 1.2.1 (Callahan et al., 2016) with parameter settings of truncLen = c(170,170), maxN = 0, maxEE = 2, truncQ = 2. Further stratification of the full “samples by amplicon” matrix was performed using package MultiAmplicon version 0.1 (Heitlinger, 2017): primer sequences were trimmed in read pairs matching with zero mismatches starting at position one in both forward and reverse reads. Sequencing reads were sorted into an amplicon when they contained the sequences of a specific pair of primers. The MultiAmplicon package was also used as a wrapper to process identified amplicons with the dada2 workflow. Briefly, sequences were dereplicated, RSVs were inferred using the function dadaMulti (with options err = NULL, selfConsist = TRUE), forward and reverse reads were concatenated (using function mergeMulti, option justConcatenate = TRUE), a table of RSV occurrence was collated for each sample and sequences likely to be chimeric and introduced during PCR were screened and discarded.
Technical replicates for which PCRs failed were identified by hierarchical clustering of primer-stratified read numbers, with water as negative controls, and marked for exclusion. For each sample, RSV counts were obtained as the sum of the remaining technical replicates and normalized for sequencing depth using a simple scaling by the median of the per sample RSV counts. All taxonomic assignments were done blind with respect to the life history stages and characteristics (age, social status) of the individual hyenas from which the fecal sample was taken and analyzed.

2.3.6 Taxonomic and statistical analyses

Taxonomy was inferred for RSVs using a ribosomal database project naïve Bayesian classifier (Wang et al., 2007) through dada2's “assignTaxonomy” function. As a training data set for the classifier, the SILVA_123 database (Quast et al., 2013) was

expanded with highest scoring unique BLAST hits (blastn; Altschul et al., 1997) of our RSVs in the NCBI nt database. This training dataset was thus curated with a focus on eukaryotic 18S sequences in our study system and then used in an assignment of the taxonomy levels “phylum,” “class,” “order,” “family,” and “genus” to each of our RSVs. Problems arose for annotation of putative Eimeria reads (among others) because of an incongruence between the taxonomic systems of SILVA and NCBI, so two separate sets of results will be described for this genus.

RSV counts, annotation with taxonomy information and hyena specific sample data were combined into a single R object for all amplicons using the package phyloseq version 1.18.0 (McMurdie and Holmes, 2013). Data were merged across different amplicons via their taxonomic annotation at the genus level using the function “tax_glom” with the parameter “NArm = TRUE.” This excludes genera not annotated at the genus level (or annotated with uninformative terms such as “undefined”). We also excluded genera annotated as the only genus in their respective phylum and genera with an “undefined” phylum annotation. We report estimates of RSV diversity derived from different amplicons with caution, as we recognize that many raw RSVs prior to taxonomic annotation represent different parts of the same marker genes and hence our diversity estimates are inflated for this measure. Even so, they can be used to compare the diversity between individuals, because all samples were processed using the same amplification and bioinformatics workflow.

We used the strength of association between “sequence abundance” (number of ribosomal sequence reads annotated for specific genera or higher level taxa) and FEC or FOC to screen for the most likely sequenced taxon in samples for which we had morphological evidence from the egg or oocyst counts. Because the larger size class of Ancylostoma occurred in only two samples, FEC for both size classes were added together for the correlation of egg counts with sequence abundance data. Spearman rank correlation coefficients were calculated in R base and recorded not only for “target taxa” within the

taxonomic scope of morphological discrimination but in a "blinded" approach for the comprehensive set of all sequence counts at all levels of taxonomy. The strongest correlations were then screened for taxonomic agreement with FEC and FOC. The four highest positive correlations always contained the target taxa as defined by the observed morphology. In the results (below) we report p-values to test for significant association between the number of ribosomal sequence reads and FEC or FOC for the highest or second highest Spearman correlation coefficients. We also constructed linear models using (1+log10) transformed data to visualize the linearity of the association and report the regression equations as a predictive tool.

Richness—the number of taxa (RSVs or genera) present —, evenness—the evenness in the distribution of sequencing read numbers for different taxa—and diversity—an index that takes into account both richness and evenness—(see Legendre and Legendre, 2012) were calculated after the random selection of a data subset (rarefaction) from all sample counts at the sequencing depth of the library sequenced to the lowest depth. We used rarefaction instead of normalization for diversity estimates to avoid problems of overestimating presence in more deeply sequenced samples. We used the package phyloseq with its function estimate_diversity to estimate measures of diversity and species richness with the help of package vegan version 2.4-1. We calculated the number of genera as "observed richness," the Chao1 index of diversity (Chao, 1984) as a measure of species diversity and Pielou's J as a measure of evenness (Pielou, 1975). Number of genera, Chao1 index and Pielou's J were compared between juveniles and adults or high-ranking and low-ranking hyenas using the exact version of the Mann-Whitney U test in the package coin version 1.1-2 (Hothorn et al., 2006) to obtain appropriate p-values for small sample sizes and in the presence of ties.

In order to compare the composition of the bacterial microbiome and the eukaryome between individuals, life history stages and social status categories, the diversity of the bacterial microbiomes and eukaryomes and its underlying variation

across individuals was efficiently summarized by multidimensional scaling (MDS), a non-parametric ordination technique. This technique has previously been highly successful in summarizing similarly complex data sets (e.g., Burgener et al., 2009), along new dimensions called here MDS axes. MDS uses pairwise Bray-Curtis dissimilarities (Bray and Curtis, 1957) on (1+log10) transformed data of sequence abundances per genus. On the same transformed data partial least squares (PLS) models (Hastie et al., 2013), as calculated by package caret version 6.0-73, were used as a supervised machine learning technique (Wold et al., 1984) to predict age and rank category for the individual from which the sample was collected. PLS models also produce a set of axes or "directions" (Hastie et al., 2013) called here PLS axes. The optimal number of PLS axes retained in the final model was determined using leave-one-out cross-validation. Each sample received a PLS score on each PLS axis, which documents how well sample categories can be differentiated on that axis, and each taxonomic unit as a "predictor" received a PLS "loading" on each axis which documents to what extent the taxonomic unit contributed to the PLS axis. We subsequently used Fisher's exact test from R base to test for overrepresentation or underrepresentation of phyla visually identified to be abundant at the extremes of the distribution of loadings. We tested highest and lowest quartile of loadings on the single PLS axis of the model addressing rank category differences in the eukaryome. As the PLS model for differences of the microbiome in age categories contained more than one axis, the PLS loadings of the first two axes separating the samples were combined by multiplication before this procedure.

Package DESeq2 version 1.14.0 (Love et al., 2014) (function "DESeq") was used to test for differences in the abundance of individual genera between age and rank categories. In contrast to normalization or rarefaction used in other analyses the function estimated "size factors" to address differences in sequencing depth between different samples. These factors were then used as offset when a mean abundance was fitted for each taxon in generalized linear models (glm) using a

negative binomial distribution and a dispersion parameter specific to that taxon. Maximum likelihood estimates for glm coefficients were obtained and likelihood ratio tests were conducted by subtracting the log-likelihood of the full model including different estimates for a focal contrast (age or rank category) from a reduced model without this difference. The resulting likelihood-ratio was compared then to a $\chi 2$-distribution. Resulting p-values were corrected for multiple testing using the Benjamini and Hochberg method (Benjamini and Hochberg, 1995) and expressed as false discovery rates, with the significance threshold set to 0.05.

2.4 Results

High throughput sequencing of multiple amplicons provided a comprehensive survey of the intestinal biome of hyenas, comprising 986 genera, most of which belonged to the eukaryotic biome, as described in detail below.

2.4.1 Sequencing based assessment

We obtained a total of 3,195,831 sequencing read-pairs from amplicon sequencing for 18S small ribosomal subunits of eukaryotes and 16S ribosomal subunits of bacteria. Variant inference on a single base resolution level for these sequences revealed a total of 24,604 RSVs, which were summarized via shared taxonomic assignments at the genus level.

2.4.2 Taxonomic diversity of hyena intestinal biomes

We identified 201 genera (3,725 RSVs) of bacteria as constituents of the bacterial microbiome of the hyena. We also identified 656 genera (20,879 RSVs) of eukaryotes in the same fecal samples. The number of genera annotated varied greatly between phyla of both bacteria and eukaryotes (Figure 1A, Table1). High genus level diversity was observed in the bacterial phylum Firmicutes and in the eukaryote phyla Ascomycota, Chlorophyta and Basidiomycota. The classic phyla of intestinal parasites—Nematoda, Apicomplexa and Platyhelminthes—showed an intermediate number of genera but a high number of sequencing reads. Phylum Chordata was

represented with few genera and a large number of sequencing reads (Figure 1A, Table Table 1).
The eukaryote sequences derived from 18S amplification did not solely originate from organisms in the eukaryome but also included organisms that were part of the hyena's diet or those that were accidentally ingested. Taxonomic annotation of eukaryote derived DNA can help to assess these potential sources. For some taxa a role as intestinal inhabitants or food items can safely be assigned. We therefore categorized eukaryote phyla in our taxonomic annotation in categories of likely (1) eukaryome (including parasites, commensals and mutualists), (2) food items, (3) passing material, and (4) undetermined role (Table 1). We consider for example Nematoda, Platyhelminthes, Apicomplexa and Microsporidia as classical eukaryotic parasites and hence members of the eukaryome. Other organisms not conventionally considered to be parasites but rather commensals or with an unknown effect on their host comprised most phyla of fungi. DNA sequences from the phylum Chordata were most likely originating from hyena food items (Table 1).

2.4.3 Sequence based abundance counts correlate with fecal egg or oocyst counts but are more sensitive

Eukaryote parasites were identified at a genus level for the genus Ancylostoma (detected in 25 of 32 samples), more conservatively at the family level for cestodes in families Diphyllobothriidae (detected in 26 of 32 samples) and Taeniidae (detected in 6 of 32 samples) and protists only at the level of order for Coccidia (detected in 17 of 32 samples). We also detected in one or two samples species from Spirurida, Trichuris spp. and Dipylidium spp. (Table 2).

Figure 1. Taxonomic diversity of the intestinal bacterial microbiome and eukaryome of the spotted hyena in terms of (A) number of genera and (B) number of sequence reads inferred for different phyla of bacteria and eukaryotes from taxonomically annotated multi-amplicon sequencing reads. Bars are colored according to the likely predominant interaction with their host conventionally assumed for these taxa (see main text).

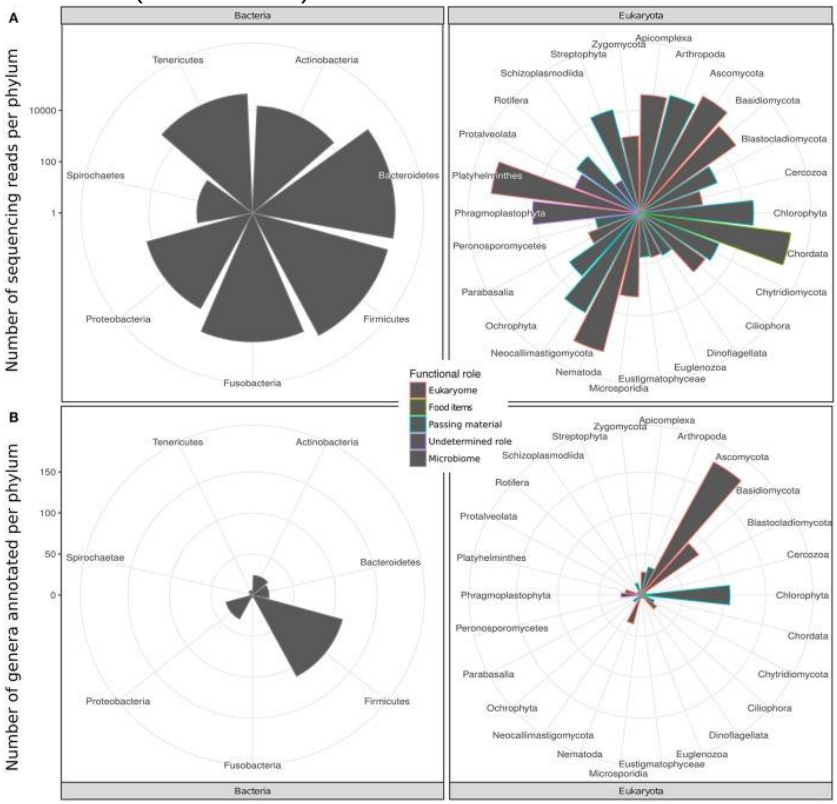


Table 1. The diversity of genera and phyla of bacteria and eukaryotes recorded from the intestinal biome of the spotted hyena as extracted by amplicon sequencing of fecal samples. *The first number is for the annotated dataset analyzed for genera; the second number is the full dataset analyzed for RSVs. **Number of genera not considered correctly annotated based on phylum level abundance (see methods) or annotated as "undefined" at the phylum level.

Taxon	Functional role	Ribosomal sequencing variants (RSV)	Identified genera	Number of sequencing reads
Bacteria	**Microbiome**	**3,559/*3,725**	**201/*202**	**768,581/*807,654**
Actinobacteria	Microbiome	184	24	14,690
Bacteroidetes	Microbiome	787	20	328,848
Firmicutes	Microbiome	2,073	112	259,513
Fusobacteria	Microbiome	183	3	105,138
Proteobacteria	Microbiome	248	33	16,881
Spirochaetes	Microbiome	13	3	140
Tenericutes	Microbiome	71	6	43,371
Undetermined**	Microbiome	166	1**	38,928
Eukaryotes	**Multiple**	**18,202/*20,879**	**656/*784**	**2,129,418/*2,388,177**
Apicomplexa	Eukaryome (parasites)	1,074	28	39,899
Arthropoda	Food items	600	35	53,075
Ascomycota	Eukaryome	4,841	184	146,864
Basidiomycota	Eukaryome	1,675	85	36,241
Blastocladiomycota	Passing material	126	7	1,676
Cercozoa	Eukaryome	47	4	288
Chlorophyta	Passing material	1,131	107	25,680
Chordata	Food items	1,743	8	782,156
Chytridiomycota	Passing material	144	17	1,944
Ciliophora	Eukaryome	254	32	1,264

Dinoflagellata	Passing material	30	4	74
Euglenozoa	Eukaryome	13	4	61
Eustigmatophyceae	Passing material	6	3	53
Microsporidia	Eukaryome (parasites)	39	4	1,869
Nematoda	Eukaryome (parasites)	1,431	36	380,347
Neocallimastigomycota	Passing material	337	6	25,520
Ochrophyta	Passing material	184	11	2,008
Parabasalia	Eukaryome	29	3	131
Peronosporomycetes	Passing material	11	3	52
Phragmoplastophyta	Undetermined role	420	24	13,602
Platyhelminthes	Eukaryome (parasites)	1,195	19	600,119
Protalveolata	Undetermined role	85	3	478
Rotifera	Passing material	100	5	930
Schizoplasmodiida	Undetermined role	11	2	28
Streptophyta	Passing material	107	15	14,066
Zygomycota	Eukaryome	98	7	993
Undetermined**	Undetermined role	2,471	128**	216,753

The amplicon sequencing based abundance estimates correlated significantly and positively with egg or oocyst counts, as detailed below, for those species for which we had more than six positive FEC or FOC samples. These correlations also helped to assign plausible taxonomic status to taxa with morphologically indistinguishable eggs or oocysts. Ancylostoma FEC correlated best with the sequence counts for the genus Ancylostoma among all reported genera (Spearman's rho, $\rho = 0.54$, $n = 32$, $p = 0.002$). There was a slightly better positive correlation with counts summarized for the order Rhabditida, to which Ancylostoma belongs ($\rho = 0.58$, $n = 32$, $p < 0.001$): seven samples with relatively high sequencing counts (range 84–4,035) and zero FECs were recovered and all samples reporting FEC in Rhabditida had at least 407 sequences counted for Ancylostoma. Within this order, annotations for 23 other genera were reported. The genus Ancylostoma contributed most (59%) sequencing reads annotated within the order. The genera Ostertagia (20%) and Haemonchus (12%) also contributed substantial numbers of sequencing reads annotated as Rhabditida. Adding together reads annotated as Ancylostoma and Haemonchus resulted in a correlation slightly stronger ($\rho = 0.59$, $n = 32$, $p < 0.001$) than that observed for the whole order Rhabditida. When reads classified as Ostertagia were added to the reads annotated as Ancylostoma, the correlation with Ancylostoma spp. in the FEC became slightly weaker ($\rho = 0.54$, $n = 32$, $p = 0.002$) (Figure 2A).

Similarly, FEC for taxa identified as originating from one or several species in the family Diphyllobothriidae correlated best with annotated RSV counts for the family Diphyllobothriidae ($\rho = 0.69$, $n = 32$, $p < 0.001$). The genus Diphyllobothrium provided the vast majority (98%) of counts annotated in the family Diphyllobothriidae and reads for the genus Spirometra provided the remaining (2%) of counts in this family. The correlation of FEC with annotated RSV counts for Spirometra alone was slightly weaker ($\rho = 0.67$, $n = 32$, $p < 0.001$) than for the entire family. The FEC results for four samples contained no Diphyllobothrium eggs but produced positive Diphyllobothrium sequence results, with annotated RSV counts ranging from 9 to

39,699; all samples with FEC above zero produced annotated RSV counts in the range of 79–44,993 for Diphyllobothriidae. Figure2B visualizes this relationship.
When annotated RSV counts for the genera Besnoitia, Toxoplasma, Isospora, and Eimeria were added together, their total number correlated best ($\rho = 0.79$, $n = 32$, $p < 0.001$) with FOC for the small size class (<20 µm) of Coccidia oocysts. This comparison produced a linear model on log transformed data with an excellent fit ($r2 = 0.92$; Figure 2C). FOC were zero for three samples with sequencing counts ranging from four to five for these genera. The number of RSV counts annotated as Eimeria correlated best ($\rho = 0.60$, $n = 32$, $p < 0.001$) with the large size class (≥20 µm) of Coccidia oocyst counts (Figure 2D). This relationship did not follow the pattern reported for other correlations, since substantial numbers of FOC were reported for samples with zero abundance in terms of annotated RSV reads.

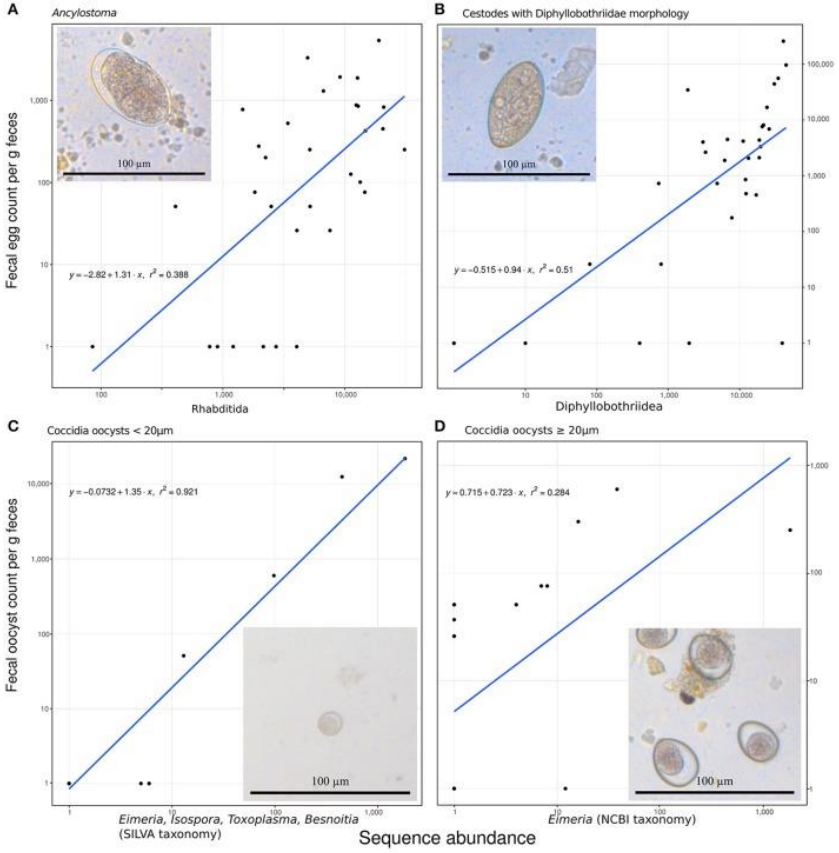
2.4.4 Adult female hyenas have a bacterial microbiome which is more diverse than and differs in composition from that of juveniles

Consistent with our prediction, the bacterial microbiome of juvenile hyenas contained a significantly lower number of genera (lower richness; Mann-Whitney U-test, $U = 184.5$, $n = 42$, $p = 0.012$), had a lower diversity ($U = 174.5$, $n = 42$, $p = 0.034$) and showed a trend toward lower evenness ($U = 168$, $n = 42$, $p = 0.063$) than that of adults (Figure.3A). The bacterial microbiome of adult hyenas contained a median of 49 bacterial genera whereas those of juveniles hosted a median of 41 genera.

Table 2. The intensity of infection in terms of parasite egg or oocyst counts per g feces from fecal samples (n = 32) of spotted hyenas. Indicated are identified taxa, their prevalence in %, the mean and median with respective 95% confidence intervals. Table was sorted by prevalence. "spp.": an unknown number of species which may or may not belong to more than one genus.

Taxon	Taxon level	Phylum	Prevalence	Intensity of infection					
			%	Mean	Confidence intervals		Median	Confidence intervals	
					Low	High		Low	High
Diphyllobothriidae	family	Platyhelminthes	81.2	21,461	1,137	41,784	3,650	850	6,850
Ancylostoma total	genus	Nematoda	78.1	802	320	1,284	275	100	773
Ancylostoma < 80 µm	spp.	Nematoda	78.1	786	308	1,264	275	100	773
Ancylostoma ≥ 80 µm	spp.	Nematoda	6.2	200	0 [−94]	494	200	NA	NA
Coccidia total	order	Apicomplexa	53.2	2,171	0 [−664]	5,007	50	25	75
Coccidia < 20 µm	spp.	Apicomplexa	12.5	8,800	0 [−1504]	19,104	6,588	NA	NA
Coccidia ≥ 20 µm	spp.	Apicomplexa	53.1	101	29	173	50	25	50
Taeniidae total	family	Platyhelminthes	18.8	89	15	164	62	25	75
Taeniidae < 45 µm	spp.	Platyhelminthes	3.1	85	10	161	50	25	75
Taeniidae ≥ 45 µm	spp.	Platyhelminthes	18.8	25	NA	NA	25	NA	NA
Spirurida	order	Nematoda	6.2	412	0 [−249]	1,074	412	NA	NA
Trichuris	genus	Nematoda	3.1	50	NA	NA	50	NA	NA
Dipylidium	genus	Platyhelminthes	3.1	50	NA	NA	50	NA	NA

Figure 2. Predicting fecal egg or oocyst counts per g feces from the number of annotated ribosomal sequence variants reads. (A) Ancylostoma FEC vs. sequence counts for the order Rhabditida. (B) Diphyllobothriidae FEC vs. sequence counts for the same family. (C) A small size class of oocyst counts vs. added sequence counts for Eimeria, Isospora, Besnoitia, and Toxoplasma. (D) A large size class of coccidian oocysts vs. Eimeria sequences. All panels contain the formula for the specific linear model on (1+log10) transformed data, R2 as a measure of goodness of fit, and a line representing the predicted relationship. The panels additionally include a representative micrograph depicting the egg or oocyst counted.



A more detailed analysis for each bacterial phylum revealed that the higher richness and diversity of genera in adults than juveniles predominantly occurred in the phyla Tenericutes ($U = 203$, $n = 42$, $p < 0.001$) and Bacteroidetes ($U = 173$, $n = 42$, $p = 0.037$). Microbiomes of adults had a significantly lower diversity for Actinobacteria than those of juveniles ($U = 53$, $n = 42$, $p = 0.021$). The differences in composition between the bacterial microbiomes of juveniles and adults were confirmed using multidimensional scaling ordination, which showed that juvenile microbiomes are less uniform in composition between individuals than those of adults (Figure 3B).

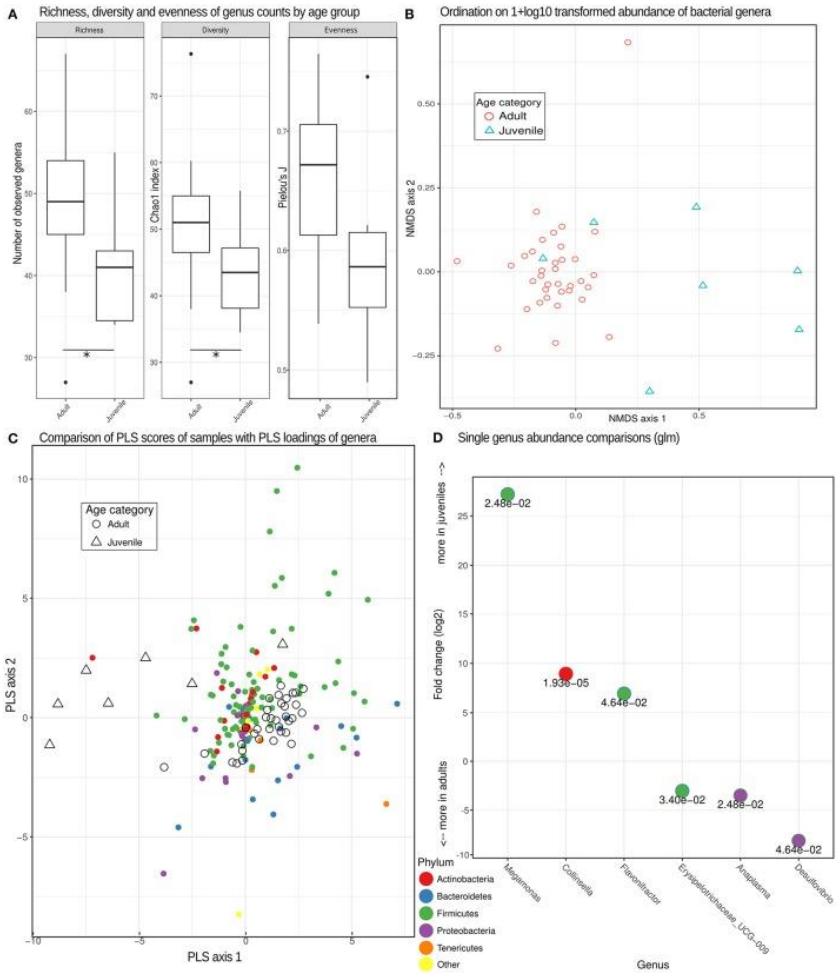
The distinct composition of adult and juvenile bacterial microbiomes was underlined by the results of a PLS regression which correctly assigned samples to age categories with an accuracy of 93% in leave-one-out cross evaluations. The optimal model retained three PLS axes, the first two of which are plotted with PLS scores of samples and loadings of bacteria phyla in Figure 3C, illustrating how well age categories were separated by their PLS scores. A more detailed analysis demonstrated that genera in the phyla Tenericutes tended to be (odds ratio = 4.83, Fisher test, $p = 0.069$) and Bacteroidetes were (odds ratio = 5.42, Fisher test, $p < 0.001$) characteristic for adult microbiomes, Actinobacteria for juvenile microbiomes (odds ratio = 2.93, Fisher test, $p = 0.009$). Testing individual genera of bacteria for differences in abundance between age categories resulted in six genera with significant false discovery rates of <0.05, with three more abundant in juveniles and three more abundant in adults (Figure 3D).

2.4.5 A more diverse eukaryome in high-ranking than low-ranking hyenas

Estimates of observed richness ($U = 293$, $n = 42$, $p = 0.004$) and diversity ($U = 288$, $n = 42$, $p = 0.007$) in terms of RSVs in the eukaryome were significantly higher in high-ranking than low-ranking hyenas (Figure 4A). The same was true for inferred genera (richness, $U = 299$, $n = 42$, $p = 0.002$; diversity, $U = 299.5$, $n = 42$, $p = 0.002$; Figure 4B). Social status had no significant effect on eukaryome evenness. A significantly higher

number of genera (richness) occurred in high-ranking than low-ranking individuals for the phyla Basidiomycota ($U = 275$, $n = 42$, $p = 0.021$), Ascomycota ($U = 272.5$, $n = 42$, $p = 0.025$) and Blastocladiomycota ($U = 250$, $n = 42$, $p = 0.049$). There was a trend toward higher diversity in high-ranking than low-ranking hyenas for Apicomplexa ($U = 254.5$, $n = 42$, $p = 0.079$).

Figure 3. Bacterial genera richness, diversity and microbiome composition in different age categories. (A) Box plots depicting distributions of richness (observed counts of genera richness per phylum), diversity (Chao1 index) and evenness (Pielou's J) estimates on rarefied (see methods) genera counts for juveniles and adults. *Significant differences ($p < 0.05$) based on exact Mann-Whitney U tests. (B) Non-metric multidimensional scaling (MDS) ordination based on pairwise Bray-Curtis dissimilarities partially separated juvenile from adult samples based on different compositions of taxonomic units. (C) A comparison of PLS scores (for samples) and PLS loadings (for genera) from the first two PLS axes of an optimized partial least squares model, demonstrating a clear separation of adult and juvenile samples. Genera colored by phylum can be used to assess the taxa contributing (PLS loading) to the differences underlying this distinction. (D) Log2-fold change inferred by generalized linear models testing for differences between adults and juveniles for each genus with a false discovery rate (adjusted p-value) of < 0.05. The numerical value of the false discovery rate is given below the dot for each genus color-coded for its respective phylum.



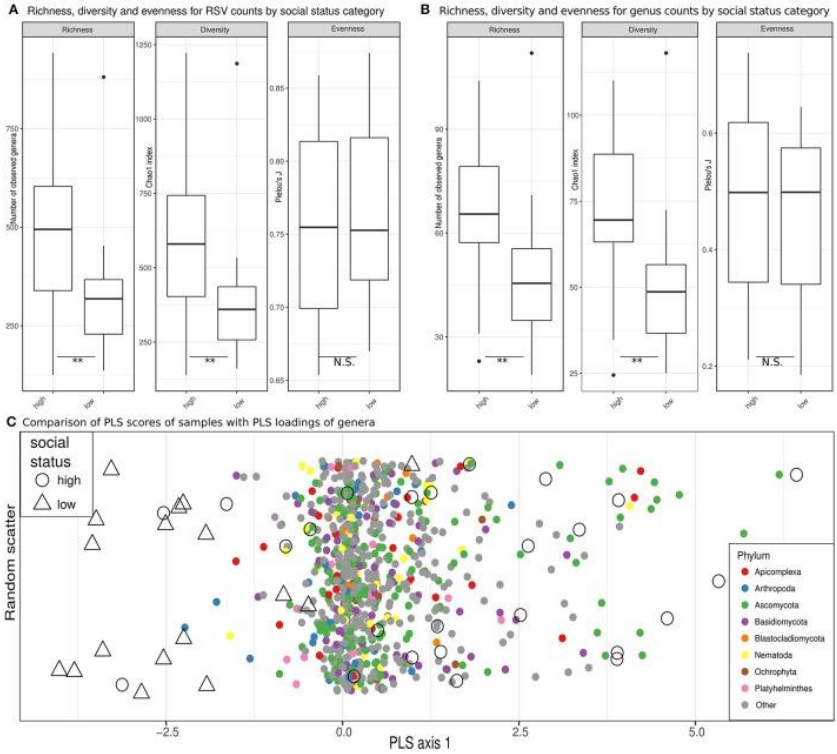
In contrast to the bacterial microbiome, we detected no significant differences in eukaryome richness (U = 106, n = 42, p = 0.75), diversity (U = 96, n = 42, p = 0.50), evenness (U = 128, n = 42, p = 0.68) or genus abundance between hyena age categories (false discovery rate for all single genera glms >0.05).

It was not possible to determine compositional differences between eukaryomes of high-ranking and low-ranking animals in our dataset using unsupervised ordination techniques. PLS regression models, however, were able to assign animals to social status categories with 71% accuracy in leave-one-out cross evaluations. Most high-ranking individuals were clearly separated from low-ranking individuals by the single PLS axis retained in the model, the overlap with low-ranking individuals being confined to a minority of high-ranking individuals (Figure 4C). The loading of several genera belonging to the phylum Basidiomycota tended to increase the PLS scores for high-ranking individuals (odds ratio = 1.55, Fisher test, $p = 0.074$). There was also a trend that genera in the Apicomplexa through their loadings increased the PLS scores for low-ranking individuals (odds ratio = 2.01, Fisher test, $p = 0.057$). Both trends contributed to the separation of high-ranking and low-ranking individuals. Interestingly, different genera in the Apicomplexa were drivers in terms of their PLS loadings on the single PLS axis for the classification of samples as belonging to both high-ranking and low-ranking individuals (odds ratio = 3.17, $p = 0.011$; testing the combined upper and lower quartiles of loadings).

In contrast to the eukaryome, we detected no significant differences in bacterial microbiome richness ($U = 184.5$ $n = 42$, $p = 0.84$), diversity ($U = 182.5$, $n = 42$, $p = 0.80$), evenness ($U = 184$, $n = 42$, $p = 0.84$) or genus abundance between high-ranking and low-ranking animals (false discovery rate for all individual genera glms >0.05).

Figure 4. Eukaryome diversity and composition in high ranking vs. low ranking hyenas. (A) Estimates of richness (observed counts of genera richness per phylum), diversity (Chao1 index) and evenness (Pielou's J) on rarefied (see methods) ribosomal sequence variant (RSV) counts for high-ranking and low-ranking individuals. (B) Counts for annotated RSV per genus are compared in box plots for high and low ranking hyenas. **Significant differences ($p < 0.01$) based on exact Mann-Whitney U tests. (C) A comparison of PLS scores (for samples) and PLS loadings (for genera) are visualized on the single PLS axis of an optimized partial least squares model, demonstrating a

separation of the majority of samples from high-ranking individuals from samples from low-ranking animals. On the y-axis random scatter is introduced for visualization. The underlying genera are color-coded for their respective phylum.



2.5 Discussion

Amplicon sequencing (metabarcoding) revealed that spotted hyenas in the Serengeti NP had a diverse intestinal biome, with at least 201 identified genera of bacteria and 656 identified genera of eukaryotes (Table1, Figure1A. The bacterial phylum with the highest diversity of identified genera was the Firmicutes, as were the Ascomycota, Chlorophyta and Basidiomycota among the eukaryotes (Figure 1B). Positive, significant correlations between amplicon sequence abundance estimates and FEC or FOC (Figure 2) suggest that results from amplicon sequencing are quantitatively valid and highly

sensitive measures, and also can aid the differentiation of taxa with similar egg and oocyst morphologies. Consistent with our predictions, adult females had a significantly more diverse intestinal bacterial microbiome than juveniles (Figure 3) and the diversity of eukaryotes was significantly greater in high-ranking than low-ranking animals (Figure 4). However, contrary to our prediction, there was no effect of social status on the diversity of the bacterial microbiome and also no difference between the diversity of eukaryotes in adult and juvenile intestinal biomes.

Studies of the intestinal bacterial microbiome in humans report an increase in the number of taxa and taxonomic diversity of bacteria with age (Palmer et al., 2007; Tannock, 2007; Koenig et al., 2011). Our results were similar in that the distal intestinal bacterial microbiome of adult hyenas was significantly more diverse and differed in composition from that in juvenile hyenas. Probably both social and physiological factors contributed to these age related differences. During approximately the first 12 months of life, juveniles remain at the clan's communal den (Hofer and East, 1993c) and for most daylight hours they rest together in underground burrows where conditions are probably conducive to the spread of bacteria among resting juveniles (Höner et al., 2012). Dens also function as important social centers where clan members meet and interact, thereby enhancing the transmission of hyena-associated pathogens between clan members (East et al., 2013; Olarte-Castillo et al., 2016). Greeting ceremonies (East et al., 1993), in which participants stand head-to-tail and sniff and lick each other's anogenital area, are probably important for the spread of bacteria and their inclusion in the intestinal biome by the fecal-oral route. Since juveniles frequently participate in greeting ceremonies (East et al., 1993), greetings probably aid the colonization of the juvenile intestinal biome by both bacteria and eukaryotes. In line with this idea there is growing evidence that socially mediated transmission contributes to the maintenance of a diverse bacterial microbiome in a taxonomically broad range of species, including chimpanzees (Pan troglodytes), in which social processes help maintain a diversity rich in commensals and mutualists (Moeller et al., 2016), and social

insects where social transmission of bacteria provides protection against virulent pathogens (Koch and Schmid-Hempel, 2011). Throughout the long period in which they are nursed, juvenile hyenas receive bacteria from their mother, and the nutritional and immunological components of milk (Hofer et al., 2016) may also affect the composition of the intestinal bacterial microbiome of juvenile hyenas.

As high-ranking individuals are highly sought after social partners, we predicted a socially mediated spread of a more diverse bacterial microbiome in high-ranking than low-ranking individuals but our results did not show this. One possible explanation is that our resolution, to the level of RSVs and genera, was insufficient. Our approach might have failed to distinguish functional differences in the relationship (e.g., mutualistic vs. pathogenic) of variants of the same species with their hosts, as in the case of distinct variants of Escherichia coli (von Mentzer et al., 2014). Hence our taxonomic resolution—even at the RSV level—was possibly insufficient for a fine-grained analysis of the diversity of the bacterial microbiome and the functional relationships of variants to their host. An alternative interpretation of our results is that high rates of contact between adult female clan members homogenize their intestinal bacterial biomes, as proposed by ecological network theory (Wilson, 1992).

In contrast to the bacterial microbiome, intestinal eukaryotes are—with the exception of some fungi—traditionally considered parasites and thus detrimental to their host. However, extending the findings made on bacteria in the last decades it seems likely that the mammalian intestinal biome also includes diverse commensals and mutualists (Wegner Parfrey et al., 2014; Lukeš et al., 2015). In microbial communities at least, an increase in species richness increases the potential for metabolic interactions and dependencies between community members, resulting in more stable communities because they become more independent from the environment (Zelezniak et al., 2015). More stable communities are less likely to suffer perturbations in their composition (dysbiosis) as reported for many diseases. To what extent this also applies to intestinal

eukaryotic communities is unclear at present. We found that high-ranking animals had a significantly more diverse eukaryome than low-ranking animals and we interpret this result to indicate a healthier intestinal ecosystem in high-ranking animals. Previously we have shown that female social status determines access to food resources within the clan territory and foraging effort (Hofer and East, 1993b). As a result, low-ranking females have higher foraging costs and higher fGCM concentrations (Goymann et al., 2001b) indicative of an elevated allostatic load. Low-ranking adult females more often resort to resource allocation trade-offs that reduce allocation of resources to immune processes, which therefore resulted in higher burdens of the intestinal helminth Ancylostoma spp., especially during lactation (East et al., 2015). A greater allocation of resources by high-ranking females to immune processes might contribute to maintaining mutualistic microorganisms whilst keeping pathogens in check (Hooper et al., 2012). It seems likely that the immune system of high-ranking females better limits parasitic infections than those of low-ranking females. Their higher contact rate with other clan members (East et al., 2001) and monopolization of parasite-infected social resting sites and parts of carcasses (on which they feed) can then enable high-ranking females to absorb, establish and maintain a more diverse eukaryome.

To alleviate primer bias and handling problems arising from primers targeting mainly food items we used a multi-amplicon sequencing approach (Heitlinger, 2017). To assess to what extent these methods provide a quantitative estimate of the abundance at a particular level of resolution achieved between taxa, we correlated FEC or FOC with sequence reads. Our results indicate that sequencing based estimates were more sensitive both in the detection and the identification of parasite taxa than morphologically based FEC or FOC. We also found that the number of sequence reads was moderately to strongly positively correlated with FEC or FOC counts (Figure2), which generally indicates that a degree of quantification was possible using amplicon sequencing, as reported by previous studies

(Kartzinel et al., 2015; Pornon et al., 2016). For analyses of this kind, it is important to take into account the fact that taxa vary in the amount of DNA present in traditionally counted entities such as eggs, oocysts or whole individuals as this will influence the number of sequencing reads obtained (Blanckenhorn et al., 2016). The stronger correlations between the number of annotated sequence reads and coccidian FOC counts than between annotated sequence reads and helminth FEC may be due to the different life cycles of these parasites. Coccidian parasites live in the cells of the epithelial lining, thus contribute DNA via oocytes shed into the lumen of the intestines whereas adult helminths reside in the lumen of the intestines, thus potentially can contributed DNA both in the eggs they shed and from adult worms. The extent of discrepancies introduced by adult worm DNA would then depend on the relationship between egg numbers and helminth tissue in the DNA preparation. For some helminth parasites, this relationship may also depend on adult female/male ratios and other biological processes such as density-dependent effects on worm fecundity, which introduce biases in the estimate of hookworm burdens (Anderson and Schad, 1985).

Cestodes in the family Diphyllobothriidae produce eggs with a very similar morphology, thus reliable identification of Spirometra spp. and Diphyllobothrium spp. based on egg morphology is challenging (Thanchomnang et al., 2016). Our sequencing based taxonomic assignments suggest that eggs from both Spirometra spp. and Diphyllobothrium spp. were included in these egg counts. Similarly, Ancylostoma spp. have a typical strongyle egg type, hence differentiation between Ancylostoma spp. eggs and those of species such as Haemonchus spp. is difficult. Although Haemonchus spp. are parasites of ungulates, they might be detected in hyena feces by both amplicon sequencing and FEC after ingestion if hyenas fed on the viscera of an infected ungulate prey. The detection of Haemonchus spp. eggs in the feces of hyena does not indicate to us at present that these parasite species are members of the hyena's eukaryome. Our sequencing results revealed that our FOC counts for the order Coccidia (phylum Apicomplexa) likely

comprised oocysts from species in three genera. We thus conclude that our amplicon sequencing approach offered an improved resolution and sensitivity over traditional egg or oocyst identification techniques and that combining the results from both approaches can provide complementary information.

Currently, little is known about the gastrointestinal biome of most wildlife species in “natural” ecosystems. Nevertheless, components of Darwinian fitness correlated with intestinal biome features can provide insight into the function of associated organisms for their host. At a more basic level, research on a broad range of wild mammals is required to permit the correct functional role (parasite, commensal or mutualist) to be assigned to individual taxa present in intestinal biomes, and in the case of predatory species to correctly differentiate passaging material such as parasites of prey from true host gastrointestinal biome constituents. For the latter, high resolution amplicon markers (e.g., COI; Hebert et al., 2003) and analysis of correlations of the composition of the apparent intestinal biome and ingested food items might offer solutions. Annotation of taxa with functional roles additionally requires databases collating such information (Poelen et al., 2014). Whereas studies on humans and laboratory animals have revealed the importance of the bacterial microbiome in host nutrition, physiology and immune processes, little is known about the impact of intestinal eukaryote diversity and composition on any host. We propose that the assessment of intestinal biomes in free-ranging wildlife in the context of host fitness in terms of survival or reproductive success can help to identify beneficial and adverse community compositions for different demographic or social categories of host populations and distinguish those from dysbiosis. The hologenome concept of evolution proposes that evolution in complex organisms should, in addition to considering interactions between an individual's genome and its environment, also consider its interactions with the products and physiological processes arising from the combined genomes of the microorganisms it hosts (Zilber-Rosenberg and Rosenberg, 2008; but see Douglas and Werren, 2016). This has led some to suggest that,

during the current period of rapid environmental change, the plasticity of the gastrointestinal microbiome may help some vertebrate populations adjust in an appropriate manner (Alberdi et al., 2016). We suggest that research on the roles of intestinal biomes for humans and wildlife should in addition encompass both unicellular and multicellular eukaryotes, including those traditionally thought of as parasites and the vast majority of organism so far unknown for their impact on hosts, before we can arrive at a balanced view of the benefits and costs of different community compositions of intestinal biomes.

2.6 Data deposition

Raw data has been deposited under accession number PRJNA386767 at NCBI Short Read Archive (SRA).

2.7 Ethics statement

All protocols were non-invasive and adhered to the laws and guidelines of Tanzania. Permission to conduct research in Tanzania was granted to HH, ME, and SF by the Tanzania Commission for Science and Technology. Permission to undertake research within the Serengeti National Park was granted by the Tanzanian National Parks Authority, and the research was approved by the Tanzanian Wildlife Research Institute. The research was also approved by the Committee for Ethics and Animal Welfare of the Leibniz Institute for Zoo and Wildlife Research under the approval number 2008-11-02.

2.8 Author contributions

EH, SF, HH, and ME designed the study, EH, SF, DT, HH, and ME collected the data, EH and SF analyzed the data, EH, SF, HH, and ME wrote the manuscript. All authors approved the final version of the manuscript.

2.9 Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

2.10 Acknowledgments

We thank Sarah Benhaiem, Nelly Boyer, Annie Francis, Katja Goller, Janine Helms, Thomas Shabani for their assistance, Luis Santos for his contribution to PCR primer selection, Ines Lesniak for help with preparing the Fluidigm Access Array PCR chips and two reviewers for their constructive comments on the manuscript.

2.11 Footnotes

Funding. The study was funded by the Leibniz Institute for Zoo and Wildlife Research and the DFG Research Training Group 2046 “Parasite Infections: From Experimental Models to Natural Systems” (SF).

2.12 Supplementary material

The Supplementary Material for this article can be found online at:
http://journal.frontiersin.org/article/10.3389/fcimb.2017.00262/full#supplementary-material

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Chapter 3 – Evidence of high exposure to *Toxoplasma gondii* in free-ranging and captive African carnivores

(Published article)

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The study design of the work, in terms of applying an appropriate assay was developed by Susana C. M. Ferreira (SF), Francesca Torelli (FT), Frank Seeber (FS), Marion L. East (ME). The concept of the study on spotted hyenas was provided by Susana C. M. Ferreira (SF), Heribert Hofer (HH), and Marion L. East (ME). Blood samples from the Serengeti National Park were collected by Robert Fyumagwa (RF), ME, HH and SF. Laboratory analysis was performed by SF, FT, Sandra Klein (SK) and William B. Karesh (WK). Statistical analysis was done by SF. The manuscript was written by SF, FT, FS and ME. All authors approved the final version of the manuscript.

Int. J. Parasitol. Parasites Wildl. 2019; 8, 111-117
doi: 10.1016/j.ijppaw.2018.12.007

3.1 Abstract

Toxoplasma gondii is an ubiquitous intracellular protozoan parasite. Mammals and birds are intermediate hosts and felid species are definitive hosts. In most human altered habitats the domestic cat is the predominant definitive host. Current knowledge of *T. gondii* infection in African ecosystems is limited. This study aimed to assess exposure to *T. gondii* in wild carnivores in the Serengeti ecosystem in East Africa. Carnivores can be infected by the consumption of tissue cysts when feeding on infected animals and by incidental ingestion of oocysts from environmental contamination. Incidental ingestion should occur regardless of a species' diet whereas the consumption of cysts should increase the chance of infection in carnivorous species. This predicts higher seropositivity in carnivorous than in insectivorous carnivores and lower seropositivity in juvenile carnivores with a long dependency on milk than in adults. We found high seropositivity in carnivorous species: 100 % (15 of 15 samples) in adult African lions, 93 % (38 of 41 samples) in adult spotted hyenas and one striped hyena sample was positive, whereas all four samples from the insectivorous bat-eared fox were negative. Juvenile hyenas (11 of 19 sera) had significantly lower seropositivity than adults (38 of 41 sera). Long-term monitoring of spotted hyenas revealed no significant difference in seropositivity between two periods (1988 to 1992 and 2000 to 2016). Identical results were produced in lion and hyena samples by a commercial multi-species ELISA (at serum dilution 1:10) and an in-house ELISA based on a recombinant *T. gondii* protein (at serum dilution 1:100), making the latter a useful alternative for small amounts of serum. We suggest that diet, age and lifetime range are factors determining seropositivity in carnivores in the Serengeti ecosystem and suggest that the role of small wild felids in the spread of *T. gondii* in the African ecosystem warrants investigation.

Keywords: African lion; bat-eared fox; *Toxoplasma gondii*; parasite; Serengeti ecosystem; spotted hyena

3.2 Introduction

Toxoplasma gondii is an intracellular apicomplexan parasite with a worldwide distribution. All mammal and bird species are thought to be potential intermediate hosts and all known definitive host species belong to the family Felidae (Tenter et al., 2000). Humans can also be infected and although the outcome is typically asymptomatic, infection can cause serious health problems, and therefore *T. gondii* is considered to be an important zoonotic pathogen (Dubey, 2010; Robert-Gangneux and Darde, 2012).

Infection with *T. gondii* occurs orally either via ingestion of oocysts shed by infected felids or via the consumption of raw meat containing viable tissue cysts (Dubey, 2010). In the majority of urban landscapes worldwide, the domestic cat, *Felis catus*, is the most abundant definitive host species and primarily responsible for the contamination of urban areas with *T. gondii* oocysts (Dabritz et al., 2007; Torrey and Yolken, 2013). In landscapes where domestic cats are scarce or absent, environmental contamination by *T. gondii* oocysts stems mostly from wild felid species (Afonso et al., 2013; Bevins et al., 2012; Lewis et al., 2017). After an initial acute infection, domestic cats shed oocysts for a limited period of days or weeks (Afonso et al., 2006; Zulpo et al., 2018). Antibodies induced by infection are presumed to persist throughout life (Dubey et al., 1995; Afonso et al., 2006; Zarnke et al., 2001) but may decline without further antigenic restimulation, as reported in humans (Rougier et al. 2017).

Toxoplasma gondii oocysts are resilient, particularly in moist environments, thus inadvertent ingestion of oocysts in water or on food may frequently occur in highly contaminated environments (Tenter et al., 2000; VanWormer et al., 2016). This transmission route most likely explains the variable levels of *T. gondii* seropositivity reported in several wild herbivore species worldwide (Bartova et al., 2007; Gauss et al., 2006; Hove and Mukaratirwa, 2005; Jardine and Dubey, 1996; Kutz et al., 2001; Riemann et al., 1975). The consumption of *T. gondii*-infected carcasses is also an important transmission route to carnivorous mammalian and avian species, as is the

consumption by humans of insufficiently cooked infected meat (Jones and Dubey, 2012). Vertical transmission of *T. gondii* (in utero) has also been reported from several mammals, including cats and humans (Calero-Bernal et al., 2013; Parameswaran et al., 2009; Powell and Lappin, 2001; Robert-Gangneux and Darde, 2012; Sato et al., 1993; Vargas-Villavicencio et al., 2016; Verma et al., 2016).

Currently little is known about *T. gondii* infection in large carnivores in protected landscapes in Africa, where domestic cats are absent or scarce. One of the largest protected landscapes in East Africa is the 25,000 km^2 Serengeti-Mara ecosystem and adjacent Ngorongoro Conservation Area (Sinclair and Arcese, 1995), hereafter termed the Serengeti ecosystem. This landscape holds 26 wild carnivore species, including six felid species. The largest felid is the African lion, *Panthera leo* (hereafter lion), which mostly preys on wildebeest (*Connochaetes taurinus*), plains zebra (*Equus quagga*), Thomson's gazelle (*Eudorcas thomsoni*), African buffalo (*Syncerus caffer*), topi (*Damaliscus korrigum*), and kongoni (*Alcelaphus buselaphus*) and warthog (*Phacochoerus aethiopicus*) (Scheel and Packer, 1995). The spotted hyena (*Crocuta crocuta*, family Hyaenidae), is the most numerous large carnivore in the Serengeti ecosystem (Hofer and East, 1995). It is an efficient predator and scavenger that consumes a wide range of species (East and Hofer, 2013). In the Serengeti ecosystem it predominantly consumes wildebeest, plains zebra and Thomson's gazelle (Hofer and East, 1993a), and in comparison to other large carnivores, maternal input in terms of lactation is high and the period of lactation long (Hofer et al., 2016). The striped hyena (*Hyaena hyaena,* family Hyaenidae), is predominantly a scavenger that occurs at low densities in the Serengeti ecosystem (Kruuk, 1976). The bat-eared fox (*Otocyon megalotis*, family Canidae), is a small insectivorous species, that occasionally consumes small mammals and birds (Lamprecht, 1978). Currently little is known about *T. gondii* infections in these four species in the Serengeti ecosystem, beyond reports that none of 112 faeces from lions contained *T. gondii*-like oocysts (Müller-Graf, 1995) or that 12 % of 33 faeces

from lions contained *T. gondii*-like oocysts (Bjork et al., 2000), and that a single serum sample from a lion was positive for *T. gondii* neutralizing antibodies (Riemann et al., 1975). Measures of seropositivity in carnivores are considered a useful general index of the combined infection caused by both the presence of *T. gondii* in the environment (oocysts) and the consumption of tissue cysts by carnivorous species (Burrells et al., 2013; Millán et al., 2013; Zarnke et al. 2001). Inadvertent infection as a result of the ingestion of oocysts from the environment is expected in all four carnivore species, but infection from the consumption of infected prey is less likely in the insectivorous bat-eared fox than the carnivorous species and in juvenile carnivores than in adults.

Our study aims to (1) determine the proportion of wild carnivores in the Serengeti ecosystem with anti-*T. gondii* antibodies (2) determine whether the occurrence of anti-*T. gondii* antibodies was lower in juvenile spotted hyenas than adults; (3) contribute new but limited data on the number of African carnivores in European zoological gardens (zoos) with anti-*T. gondii* antibodies, and (4) compare results from a commercial multispecies ELISA for detecting anti-*T. gondii* antibodies with those from an in-house ELISA with a recombinant antigen from *T. gondii,* using commercially available anti-cat antibodies, with the aim of assessing whether or not the in-house ELISA with defined properties might require less serum, and hence be an attractive proposition when only limited serum is available.

3.3 Material and Methods

3.3.1 Sample collection

In the Serengeti ecosystem, serum and plasma samples were mostly collected by veterinarians from animals that were anaesthetized for other purposes, such as the removal of wire snares set by bushmeat hunters (Hofer et al., 1993) between 1988 and 2016. Serum and plasma samples were also obtained opportunistically from fresh carcasses of animals killed by predators or vehicles between 1988 and 2016. All samples from species other than the spotted hyena were obtained from adult

animals. In spotted hyenas, juveniles were aged at first sighting to an accuracy of ± 7 days and were categorized as juvenile when less than 24 months old and as adult when 24 months of age or older (Golla et al., 1999). Juvenile spotted hyenas are dependent on milk for at least the first six months of life (Hofer et al., 2016). Infection with *T. gondii* via milk has been reported (but see Costa and Langoni, 2010; Powell et al. 2001) but we do not know if this occurs hyenas.
Samples were stored in liquid nitrogen (at -196°C) or a freezer (-15°C) in the Serengeti until transported frozen to Germany where they were stored (at -80°C) until analysed. Sera from 7 captive carnivores from six zoos from Germany and the Netherlands (including Tierpark Berlin, Hodenhagen Zoo, Schwerin Zoo, Opel Zoo and Leipzig Zoo in Germany and Amersfoort Zoo in the Netherlands) were collected by zoo veterinarians primarily during health examinations of animals, including 3 spotted hyenas, 3 lions and one brown hyena, *Hyaena brunnea*.

3.3.2 Commercial immunological assays

A total of 35 sera (27 adults, 8 juveniles) collected from spotted hyenas in the Serengeti ecosystem between 1988 and 1992 were tested for anti-*T. gondii* antibodies in 1992 at the Animal Health Diagnostic Center at Cornell University (Ithaca, New York), using protocols established by J.P. Dubey and the application of a commercial indirect haemagglutination (IHA ELISA) test (Toxoplasmosis TPM-Test indirect haemagglutination kit, Wampole Laboratories, Princeton, NJ, USA.) Subsequent comparison of this indirect haemagglutination test with a modified commercial multi-species IgG ELISA (Toxo IgG II ELISA kit, Wampole Laboratories, Princeton, NJ, USA) showed very good agreement between the results produced by the two assays using sera from 6 domestic species, including cats and dogs (Schaefer et al., 2011).
Furthermore, we screened 45 samples of serum or plasma collected from wild carnivores in the Tanzanian section of the Serengeti ecosystem between 1997 and 2016, including 25

samples (14 adults and 11 juveniles) from spotted hyenas, 15 samples from adult lions, 4 samples from adult bat-eared foxes, and one from an adult striped hyena. To these samples and to the samples from European zoos we applied the ID Screen® Toxoplasmosis Indirect Multi-species ELISA (IDVet, Grabels, France). The ID Screen® Toxoplasmosis Indirect Multi-species ELISA was used per supplier's instructions. Serum and plasma samples were diluted 1:10. Samples were considered positive if the Sample/Positive control ratio (S/P %) was higher than 50 %, doubtful if the S/P % was between 40 and 50 % and negative if the S/P % was below 40 %. Controls were provided in the kit. We conducted blind screening with regard to species and age.

3.3.3 In-house ELISA

We applied an in-house ELISA to all lion, spotted hyena and stripped hyena samples, including the samples from European zoos. Expression and purification of recombinant SAG1 (rSAG1-6H) was done as follows. Briefly, C-terminally 6His-tagged SAG1 (amino acids 31-289) from pSAG1-GPI (Seeber et al., 1998) was cloned into plasmid pASG-IBA33 (IBA, Göttingen, Germany) according to the manufacturer's instructions. For expression the resulting plasmid pASG33-SAG1 was transformed into *Escherichia coli* SHuffle® T7 Express cells (New England Biolabs, Frankfurt am Main, Germany) together with plasmid pMJS9 (Nguyen et al., 2011) to aid in proper disulphide bonding of rSAG1-6H. After induction of expression with 0.5 % arabinose and 200 ng/ml anhydrotetracycline for 18 h at 30°C, rSAG1-6H protein was purified using a HisTrap FF 1 ml column on an ÄktaPurifier FPLC system essentially as described by the manufacturer (GE Healthcare, Chicago, USA). Finally, buffer was exchanged to PBS on a PD10 column (GE Healthcare) before the protein was stored at -20°C until further use. Protein concentration was determined using the BCA assay (Thermo Fisher, Darmstadt, Germany). Protein purity was assessed by SDS-polyacrylamide gel electrophoresis, silver staining and immunoblot using anti-His tag antibodies and found to be ~95 % pure.

For the ELISA MaxiSorp® plates (Thermo Fisher) were coated overnight at 4°C with 100 ng of rSAG1-6H per well or PBS as control. All further steps were performed at room temperature. Unspecific binding of serum to the plate was blocked by incubation with 5 % soluble milk powder in PBS for 1 h. Then sera from lions and hyenas were serially diluted 1:100, 1:200 and 1:400, added in duplicates to wells and incubated for 90 min. As positive and negative controls we used seropositive plasma and seronegative serum from domestic cats (kindly provided by G. Schares; Friedrich-Loeffler-Institut, Riems, Germany). Controls were used at a dilution of 1:2000. As secondary antibody we used a peroxidase-conjugated goat anti-cat IgG (H+L) at a dilution 1:4000 (KPL, Gaithersburg, MD, USA). SureBlue® TMB Peroxidase substrate (KPL, Gaithersburg, MD, USA) was added and the reaction stopped after 10 minutes by adding sulphuric acid. The resulting colour signal measured at 450 nm (650 nm reference) at a Tecan Infinite M200 PRO reader. Samples were considered positive when the value was higher than the mean from two independent experiments + 3 standard deviations of negative cat or hyena sera of the same dilution.

3.3.4 Data analysis

Statistical analyses were performed in R, version 3.3.0 (R core team, 2016). Proportions were calculated with the Clopper-Pearson confident intervals (CI) at confidence level of 0.95 using the package DescTools version 0.99.24 (Signorell et al, 2018). We tested for differences between groups using the Pearson's chi-squared test (R core team, 2018). In all statistical tests, the significance threshold was fixed at 5 % and all tests were two-tailed.

3.4 Results

3.4.1 Anti-*T. gondii* antibodies in carnivores in the Serengeti ecosystem

Results from a commercial IHA ELISA revealed that most serum/plasma samples from spotted hyenas between 1988 and 1992 had anti-*T. gondii* antibodies, including 6 of 8 samples (75

%, CI: 35 - 97 %) from juveniles and 24 of 27 samples (89 %, CI: 71 - 98 %) from adults. Results from a commercial ID Screen® indirect multi-species ELISA for samples from spotted hyenas obtained between 2000 and 2016 revealed that 5 of 11 samples (45 %, CI: 17 - 77 %) from juveniles and 14 of 14 samples (100 %, CI: 77 - 100 %) from adults had anti-*T. gondii* antibodies. As we found no significant difference in the results from adults sampled in these two periods (Chi-squared test, $\chi^2 = 0.44$, df = 1, p = 0.51) or for those from juveniles in these two periods ($\chi^2 = 0.67$, df = 1, p = 0.41) we combined the results for adults from the two periods, and the results from juveniles in the two periods. Overall, these combined results revealed that 11 of 19 juvenile (58 %, CI: 33 - 80% %) and 38 of 41 adult (93 %, CI: 80 - 98 %) spotted hyenas had anti-*T. gondii* antibodies. Juveniles were less likely to have anti-*T. gondii* antibodies than adults ($\chi^2 = 8.30$, df = 1, p = 0.004).

In total we investigated 20 serum samples from adults in three additional carnivore species in the Serengeti ecosystem between 1997 and 2005, using the ID Screen® indirect multi-species ELISA. The results revealed that all 15 sera (100 %, CI: 78 - 100 %) from adult lions and one sample from a striped hyena had anti-*T. gondii* antibodies whereas sera from four bat-eared foxes were all negative (0 % CI: 0 - 60 %). There was no difference in the occurrence of anti-*T. gondii* antibodies in samples from adult spotted hyenas and adult lions ($\chi^2 = 0.17$, df = 1, p = 0.68).

Results from the ID Screen® indirect multi-species ELISA for seven samples from captive carnivores (3 spotted hyenas, 3 lions and one brown hyena) in European zoos were all positive (Tables 1, 2).

3.4.2 Comparison of an in-house ELISA assay with a commercial multi-species ELISA

We assessed a total of 48 samples obtained from lions, spotted hyenas and a striped hyena between 2000 and 2016 in the Serengeti ecosystem and from captive lions, spotted hyenas and a brown hyena, for the presence or absence of anti-*T. gondii* antibodies using the commercial ID Screen® indirect

multi-species ELISA kit and our in-house ELISA. The results in terms of the antibody being detected or not for each of these 48 samples were identical, even though for the commercial ID Screen® indirect multi-species ELISA we applied a serum dilution of 1:10 whereas for the in-house ELISA we used a serum dilution of 1:100.

3.5 Discussion

Our study revealed that a high proportion of large carnivores in the Serengeti ecosystem had anti-*T. gondii* antibodies but none of the four samples from the insectivorous bat-eared fox had positive titres. Juvenile spotted hyenas had positive titres less often than adults. The proportion of adult spotted hyenas with anti-*T. gondii* antibodies in the study population was similar during two sampling periods: from 1988 to 1992 and from 2000 to 2016. In line with previous studies (Tables 1, 2) we found high *T. gondii* seropositivity in large African carnivores in zoos.

Our long-term survey of anti-*T. gondii* antibodies in spotted hyenas in the Serengeti ecosystem revealed that 93 % of 41 sera from adults were positive. The only previous report we are aware of found that all of six sera from wild spotted hyenas in Kenya were positive (Bakal et al., 1980). We found that juvenile spotted hyenas in the Serengeti ecosystem were less often seropositive (58 % of 19 sera from juveniles) than adults. This in part may be because juveniles in our study population are dependent on milk for the first six months of life and are weaned between 12 and 18 months of age (Hofer et al., 2016; Hofer and East, 1995). As a result, juveniles are less likely to consume *T. gondii* infected tissue than adults. Also, the likelihood of exposure to a pathogen can increase with age and life-time range, as illustrated by the increase with age in seropositivity to rabies specific virus-neutralizing antibodies in our study population (East et al., 2001). Juveniles remain at communal dens until approximately 12 months of age (Hofer and East, 1993b) and hence their life-time range during their first year of life is small. Incidental contamination of communal den areas with oocysts is probably lower than in other areas in the ecosystem where felids more often deposit faeces, thus

chance encounters with contaminated areas should increase with life-time range. In other mammalian (humans, sheep) intermediate hosts and definitive hosts (domestic cats and wild felids), age-dependent increases in seropositivity has been reported (Afonso et al., 2010; Dubey, 2009; Wilking et al., 2016; Zarnke et al., 2001). An age-dependent increase in seropositivity has also been reported in European avian species (Cabezón et al. 2011). We speculate that the consumption of infected tissue probably induces anti-*T. gondii* antibodies in spotted hyenas more often than the incidental ingestion of oocysts.

Toxoplasma gondii seropositivity in wild populations of the two other carnivorous species in the family Hyaenidae, the striped hyena and brown hyena, is unknown. We found that serum from one adult striped hyena in the Serengeti ecosystem was positive and that most spotted, striped and brown hyenas held in zoos (Table 2) have anti-*T. gondii* antibodies. Sera from all four bat-eared foxes, an insectivorous canid, in the Serengeti ecosystem were seronegative. To our knowledge, *T. gondii* seropositivity in the insectivorous member of the family Hyaenidae, the aardwolf (*Proteles cristatus*) is unknown, but it may be that seropositivity in aardwolves is lower than in carnivorous hyena species. Consistent with the idea of the importance of diet, carnivorous birds have higher *T. gondii* seropositivity than other bird species (Cabezón et al. 2011), and carnivorous mammals in zoos and circuses in Italy have higher seropositivity than herbivorous mammals (Marková et al., 2018).

In line with studies on lion sera from other locations in Africa (Table 1) we found that all of 15 sera from adult lions in the Serengeti ecosystem contained anti-*T. gondii* antibodies. Whether lions shed oocysts during a relatively short period after an initial acute infection, as in the domestic cat (Afonso et al., 2006; Zulpo et al., 2018) is not known. Standard coprological examination of samples from lions in the Serengeti ecosystem has revealed either the presence of *T. gondii*-like oocysts in 12 % of fecal samples (Bjork et al., 2000) or the absence of oocysts in feces (Müller-Graf, 1995). Studies on both the

Eurasian lynx (*Lynx lynx*) and Canadian lynx (*Lynx canadensis*), also report an absence of *T. gondii* oocysts in faeces (Ryser-Degiorgis et al., 2006; Simon et al., 2013). Little if anything is known on *T. gondii* infection and oocyst shedding by the three smaller felid species in the Serengeti ecosystem: the serval (*Felis serval*), the caracal (*Felis caracal*) and the African wild cat (*Felis sylvestris*) or in the rodent and bird prey species of these three species (Hunter and Bowland, 2013; Stuart and Stuart, 2013; Stuart et al., 2013).

Information on *T. gondii* seropositivity in African wild ungulates provides information on environmental contamination, as herbivores are probably infected by the incidental consumption of oocysts in the environment while grazing or drinking water. In the Serengeti ecosystem, plains zebras are consumed by large predators (Grange et al., 2004) and 3.6 % of 29 sera from plains zebra have anti-*T. gondii* antibodies (Riemann et al., 1975). In Zimbabwe, a wide range of seropositivity (between 10 % and 90 %) has been reported in wild ungulates (Hove and Dubey, 1999; Hove and Mukaratirwa, 2005). Research is required to investigate the factors determining the occurrence of both anti-*T. gondii* antibodies and tissue cysts in wild ungulate species, to better understand the relative importance of environmental contamination and the consumption of tissue cysts as transmission routes for *T. gondii* to definitive felid hosts and intermediate carnivorous mammals and bird hosts. As none of four sera from the insectivorous bat-eared fox were seropositive we cautiously interpret the results from this small samples to suggest that environmental contamination with *T. gondii* oocysts in the habitats occupied by these bat-eared foxes was low and the chance of infection through the consumption of prey (mostly insects and occasional small birds and mammals) was also low.

Our current knowledge of *T. gondii* epidemiology in ecosystems, such as the Serengeti, that contain many wild felid species and few domestic cats is limited. If we assume that all six wild felid species in the Serengeti ecosystem are infected with *T. gondii* and that oocyst shedding is mostly restricted to a limited period following a primary infection, as in the domestic

cat, then it could be argued that because of their shorter lifespans, smaller ranges and generally higher densities (Hunter and Bowland, 2013; Stuart and Stuart, 2013; Stuart et al., 2013), small felid species (the African wild cat, serval and caracal) might be expected to contribute more to environmental contamination than the larger felid species (lion, leopard and cheetah) (Caro 2013; Hunter et al., 2013; West and Packer 2013).

The current worldwide genetic diversity of *T. gondii* has been classified into three clonal lineages (named type I, II, or III), that are three of 16 described haplogroups belonging to six major clades (Su et al., 2012). Currently little is known about the genetic diversity of *T. gondii* strains in Africa, particularly from East Africa (Galal et al., 2017). This lack of information on *T. gondii* in this region of Africa is also apparent in the Global Mammal Parasite Database (Stephens et al., 2017). Of 568 entries specifying *T. gondii* as a parasite species, only 28 are from Africa, and none are from East Africa.

Even though experimental infection of laboratory mice with two *T. gondii* strains of different genotypes has been reported (Brandão et al., 2009; Burrells et al., 2013), it is currently unclear whether this occurs in wild mammalian hosts. Infection with more than one *T. gondii* genotype challenges the view that *T. gondii* induces life-long immunity (Zulpo et al., 2018). Analysing tissues known to harbour *T. gondii* cysts (brain, heart, muscles) from lions and hyena carcasses that potentially sampled a collection of genotypes from infected prey could give insights into the genetic diversity of *T. gondii* in the Serengeti ecosystem.

A commercial multi-species Toxoplasmosis ELISA kit that is based on the major *T. gondii* antigen SAG1 was previously shown to work with sera from domestic cats and dogs (Roqueplo et al., 2011) as well as lions (Dărăbuş et al., 2014; Kamga-Waladjo et al., 2009). We showed that this kit and our in-house ELISA, using recombinant SAG1 and commercially available anti-cat antibodies, provided identical results with respect to seropositivity. The in-house ELISA has the advantage of demonstrating seropositivity at dilutions of one or

more orders of magnitude higher than the commercial kit. Our ELISA is both cost-effective since it only requires cheap reagents, i.e. antigen and antibody, and is resource-saving as much less serum is required to obtain identical qualitative results. The latter is of importance when serum from wild animals (which can be time consuming and costly to obtain) need to be screened for evidence of exposure to multiple pathogens. Given the recent advances in the use of smartphones as colorimetric readers for ELISA-based assays (Vashist et al., 2015; Vashist and Luong, 2018) together with smartphone apps that can subsequently analyse the data we envisage that cheap in-house ELISAs like ours could be used to provide useful results on the relationship between *T. gondii* and wildlife in the field.

3.6 Permits

The research was approved by The Ethics Committee of Leibnitz Institute for Zoo and Wildlife Research (permit number 1997-02-01). Samples from lions in Tanzania were transported with CITES permits issued by the relevant Tanzanian and German Authorities. Research in Tanzania was conducted under clearance granted by the Tanzania Commission for Science and Technology (COSTECH) to HH, SCMF and MLE.

3.7 Acknowledgements

We thank the Tanzania Wildlife Research Institute, Tanzanian National Park Authority and COSTECH for their support of our research. SCMF and FT are supported by and SCMF, FT, HH, FS and MLE are members of the Research Training Group 2046 “Parasite Infections: From Experimental Models to Natural Systems” funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) – Projektnummer 251133687 / GRK 2046. We thank the DFG (grant EA 5/3-1), the Leibniz-Institute for Zoo and Wildlife Research, the Robert Koch-Institute, the Georg und Agnes Blumenthal-Stiftung and the Max-Planck-Institute for Behavioral Physiology for financial assistance. We thank Klaus Eulenberger, Frank Göritz, Robert Hermes, Thomas

Hildebrandt, Gisela von Hegel, Richard Hoare, Mark Jago, Titus Mlengeya, Henning Wiesner, Raymond van der Meer and Harald Wiik for veterinary advice, Mauridi Mdaki and Justine Shamanche at the Serengeti Wildlife Research Centre, and also Annie Francis, Stephan Karl, Sonja Metzger, Thomas Shabani and Dagmar Thierer for assistance. We are grateful to Gereon Schares for cat sera, Lloyd W. Ruddock for plasmids and Timo Schippers for his valuable comments on the manuscript. We thank the editor and two anonymous reviewers for their helpful and constructive comments.

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Table 1. Anti-*Toxoplasma gondii* antibodies in African lions in zoos and in natural populations worldwide. N, number of samples tested. IFAT, indirect fluorescent antibody test; LAT, latex agglutination test; MTA, modified agglutination test; ELISA, Enzyme-Linked ImmunoSorbent Assay; * ID-Screen *T. gondii* ELISA also used in this study; N, number of samples tested.

Location	**Positive/N (%)**	**Test used**	**Reference**	**Captive/Wild**
Germany	3/3 (100)	ELISA*	Current study	Captive
Brazil	5/9 (56)	IFAT	André et al., 2010	Captive
Brazil	14/27 (52)	MAT	Silva et al., 2001	Captive
China	6/6 (100)	MAT	Yang et al., 2017	Captive
Czech Republic	2/2 (100)	IFAT	Sedlak and Bártová, 2006	Captive
Italy	13/14 (93)	IFAT	Marková et al. 2018	Captive
Mexico	7/7 (100)	MAT	Alvarado-Esquivel et al., 2013	Captive
Romania	3/3 (100)	ELISA*	Dărăbuş et al., 2014	Captive
Senegal	3/7 (43)	ELISA*	Kamga-Waladjo et al., 2009	Captive
South Africa	10/14 (71)	IFAT	Cheadle et al., 1999	Captive
Thailand	1/7 (14)	LAT	Thiangtum et al., 2006	Captive
USA	12/22 (55)	MAT	de Camps et al., 2008	Captive
USA	8/10 (80)	IFAT	Spencer et al., 2003	Captive
Tanzania (Serengeti NP)	15/15 (100)	ELISA*	Current study	Wild
South Africa (Kruger NP)	12/12 (100)	IFAT	Penzhorn et al., 2002	Wild
Zimbabwe	21/21 (100)	IFAT	Penzhorn et al., 2002	Wild
Botswana	49/53 (92)	IFAT	Penzhorn et al., 2002	Wild
South Africa (Hluhluwe-Umfolozi NP)	30/30 (100)	IFAT	Penzhorn et al., 2002	Wild
Namibia	65/66 (98)	ELISA	Spencer and Morkel, 1993	Wild

Table 2. Anti-*Toxoplasma gondii* antibodies in Hyaenidae previously reported in the literature. N, number of samples tested; SFDT, Sabin-Feldman dye test; IFA, immunofluorescence assay; MAT, modified agglutination test; ELISA, Enzyme-Linked ImmunoSorbent Assay; * ID-Screen *T. gondii* ELISA. Spotted hyena (*Crocuta crocuta*), Brown hyena *(Parahyaena brunnea*), Striped hyena (*Hyaena hyaena*)

Hyena species	**Location**	**Positive/N (%)**	**Test**	**Reference**
Spotted hyena	Zoos in Germany and Netherlands	3/3 (100)	ELISA*	Current study
Brown hyena	Zoo in Germany	1/1 (100)	ELISA*	Current study
Spotted hyena (juveniles)	free ranging, Serengeti NP (Tanzania)	11/19 (58)	ELISA*	Current study
Spotted hyena (adults)	free ranging, Serengeti NP (Tanzania)	34/41 (93)	ELISA*	Current study
Spotted hyena	free ranging, Kenya	6/6 (100)	SFDT	Bakal et al., 1980
Brown hyena	Zoos in Czech Republic	3/3 (100)	IFA	Sedlak and Bártová, 2006
Striped hyena	Zoo in France	1/2 (50)	MAT	Alerte, 2008
Spotted hyena	Zoo in France	1/1 (100)	MAT	Alerte, 2008
Striped hyena	Breeding Centre for Endangered Arabian Wildlife, UAE	3/6 (50)	MAT	Dubey et al., 2010
Spotted hyena	Zoos in Australia	5/10 (50)	IFA	Wait et al., 2015

Chapter 4 - Parasite infections in a social carnivore: evidence of their fitness consequences and factors modulating infection load

(Published article)

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Author contributions:
The study design of the work was developed by Susana C. M. Ferreira (SF), Marion L. East (ME) and Heribert Hofer (HH).Faecal samples were collected by ME and HH. Laboratory analysis was performed by SF under the supervision of Luís Madeira de Carvalho (LMC). Statistical analysis was done by S. The manuscript was written by SF ME and HH. All authors approved the final version of the manuscript.

Ecol Evol. 2019; 9, 8783-8799
doi: 10.1002/ece3.5431

4.1 Abstract

There are substantial individual differences in parasite composition and infection load in wildlife populations. Few studies have investigated the factors shaping this heterogeneity in large wild mammals or the impact of parasite infections on Darwinian fitness, particularly in juveniles. A host's parasite composition and infection load can be shaped by factors that determine contact with infective parasite stages and those that determine the host's resistance to infection, such as abiotic and social environmental factors, and age. Host-parasite interactions and synergies between co-infecting parasites may also be important. We test predictions derived from these different processes to investigate factors shaping infection loads (faecal egg/oocyte load) of two energetically costly gastrointestinal parasites: the hookworm *Ancylostoma* and the intracellular *Cystoisospora*, in juvenile spotted hyenas (*Crocuta crocuta*) in the Serengeti National Park, in Tanzania. We also assess whether parasite infections curtail survival to adulthood and longevity. *Ancylostoma and Cystoisospora* infection loads declined as the number of adult clan members increased, a result consistent with an encounter-reduction effect whereby adults reduced encounters between juveniles and infective larvae, but were not affected by the number of juveniles in a clan. Infection loads decreased with age, possibly because active immune responses to infection improved with age. Differences in parasite load between clans possibly indicate variation in abiotic environmental factors between clan den sites. The survival of juveniles (< 365 days old) to adulthood decreased with *Ancylostoma* load, increased with age and was modulated by maternal social status. High-ranking individuals with low *Ancylostoma* loads had a higher survivorship during the first four years of life than high-ranking individuals with high *Ancylostoma* loads. These findings suggest that high infection loads with energetically costly parasites such as hookworms during early life can have negative fitness consequences.

Keywords: gastrointestinal parasites; fitness; juvenile survival; resistance; Serengeti ecosystem; spotted hyena; tolerance

4.2 Introduction

Parasites and their mammalian hosts have complex and dynamic relationships (Bush et al., 2001; Lello et al., 2004; Irvine, 2006; Knowles et al., 2013) with long joint evolutionary histories (Hafner and Nadler, 1988). Wild mammals are typically infected with a taxonomically diverse range of gastrointestinal (GI) macroparasites and microparasites and individuals vary considerably in their infection loads and diversity of co-infecting parasite taxa (e.g., Irvine et al., 2000; MacIntosh et al., 2010; Heitlinger et al., 2017; Seltmann et al. 2019). This heterogeneity is thought to be shaped by interactions between numerous factors associated with the host, the infecting parasite(s) and the environment. Disentangling the contribution of these various factors to GI parasite infection is difficult, particularly in unmanaged, wild mammalian hosts (Pedersen and Fenton, 2007; Rynkiewicz et al., 2015; VanderWaal and Ezenwa, 2016).

The ability of an individual to prevent, control and clear infection is termed resistance, whereas tolerance is defined as the ability to limit the damage caused to an individual's health status and the associated Darwinian fitness cost of a given infection load (Råberg et al., 2009). There is growing interest in the effect of GI parasite infections on health status and components of fitness such as survival, longevity and reproductive success in mammals (Kutz et al., 2004; Hayward et al., 2014; Lynsdale et al., 2017) and how phenotypic expression of both resistance and tolerance change during an individual's life-span. Most studies focus on adults, even though juveniles may be more susceptible to infection and may suffer more severe outcomes of infection than adults (East et al., 2008; Chilvers et al., 2009; Marcus et al., 2014; Shrestha et al., 2015).

Resistance to parasite infection depends on both intrinsic and extrinsic factors (Ardia et al., 2011; Hayward et al., 2011, Jackson et al., 2011). In mammals, age is one intrinsic factor that can alter resistance to infection, because immune processes generally improve from early life to adulthood and then decline in old age (Simon et al., 2015). When young, the cellular immune responses of juveniles typically differ both qualitatively and quantitatively from those of adults (Ramsburg et al., 2003; Watson et al., 2016) and juveniles are less likely to have the acquired immunity that develops following exposure to pathogen antigen than adults, (Cattadori et al. 2005; Ferreira et al. 2019a), and thus juveniles are generally more vulnerable to infection than adults.

The period of rapid growth and development during the juvenile life-stage is energetically costly and when food intake is insufficient, juveniles may resort to resource allocation trade-offs to support growth and development at the expense of immune responses (Roff, 1992; Stearns, 1992; Sheldon and Verhulst 1996). When this occurs, resistance to GI parasite infection should decline and infection loads increase. Furthermore, high infection loads of energetic costly parasites might compromise a host's nutritional status, thereby curtailing resource allocation to immune processes. When this leads to an increase in host susceptibility to infection, the outcome may be increased infection loads and an increase in the number of co-infecting taxa. This negative cycle might have detrimental fitness consequences such as reduced survival (Beldomenico et al., 2008; Beldomenico and Begon, 2010). Reduced survival in individuals with high infection loads indicates a lower tolerance of infection than that in animals that survive these levels of infection.

An individual's behaviour may determine its chance of pathogen infection and the outcome of infection. For example, the aggregation of animals at breeding sites and water sources is thought to promote both the direct transmission of pathogens between hosts and the level of

environmental contamination of such sites with infective stages (Cattadori et al., 2005; East et al. 2013; Stommel et al., 2016). In social mammals, interactions between members of a group can affect the chance and outcome of infection for an individual (Kappeler et al., 2015; Nunn et al., 2015; Duboscq et al., 2016). Within-group social status (social rank) emerges from the outcome of behavioural interactions between animals in a group and typically, high-ranking animals are more attractive social partners than low-ranking animals (Seyfarth, 1977). This suggests that more frequent social interactions by high-ranking individuals enhance the likelihood of contact with pathogens and hence they may require greater resistance against infection and/or a higher tolerance of higher infection loads than low-ranking individuals (Marescot et al., 2018). High-ranking individuals in mammalian groups typically have greater access to food resources and a higher nutritional status than low-ranking individuals, and as a result offspring reared by high-ranking mothers are often better nourished than those reared by low ranking-mothers (Clutton-Brock and Huchard, 2013; Hofer et al., 2016). This suggests that juveniles reared by high-ranking mothers (high-ranking juveniles) should find it easier to allocate the energy and protein (Jones et al., 2011) required for the maintenance of effective immune processes, and the repair of parasite damaged tissue than those reared by low-ranking mother (low-ranking juveniles). As immune processes and tissue repair are key components of resistance to infection, high-ranking juveniles should experience a less severe impact of infection (for any given infection load) on their health and on fitness components such as survival than low-ranking juveniles. Thus high-ranking juveniles are expected to exhibit greater tolerance of infection than low-ranking juveniles. Several studies provide evidence that adult high-ranking individuals do allocate more resources to immune processes and the repair of parasite damaged tissue than low ranking animals (Ardia et al., 2011; East et al., 2015; Flies et. al., 2016; Marescot et al., 2018), but studies of this kind on juveniles are rare.

A range of intestinal parasites infect wild spotted hyenas (*Crocuta crocuta*) (Baylis, 1937; Graber and Blanc, 1979; Engh et al., 2003; East et al., 2013; Berentsen et al., 2012), and recently a metabarcoding study extended knowledge on the composition and diversity of the bacterial microbiome and eukaryome in this species (Heitlinger et al., 2017). Spotted hyenas (hereafter termed hyenas) are social carnivores that live in multi-female, multi-male fission-fusion societies termed clans (Kruuk, 1972) that defend territories (Frank 1986). In the Serengeti National Park (NP), the main prey species are migratory ungulates, and as a result of their movements there are large fluctuations in food abundance in clan territories (Hofer and East, 1993a, 1993b). High-ranking females have priority of access to food resources in their clan's territory and thus forage for a substantial proportion of each year in their territory. In contrast, for a substantial proportion of each year low-ranking females travel long distances (from 80 to140 km per foraging trip) to forage outside their territory in areas containing large herds of migratory ungulates (Hofer and East, 1993b). The greater the distance females travel the larger the number of days between nursing visits to their dependent offspring stationed at communal dens in clan territories (Hofer and East, 1993c). Low-ranking mothers have longer inter-nursing intervals (Hofer et al., 2016) their offspring have lower growth rates and are less likely to survive to adulthood than offspring of high-ranking mothers (Hofer and East, 2003). Females reproduce throughout the year, their offspring are dependent on milk for the first six months of life and are weaned between 12-18 months of age (Hofer and East 1993c; Hofer et al., 2016).

Our study aims to investigate determinants of both *Ancylostoma* (nematode) and *Cystoisospora* (coccidian) infection loads in high-ranking and low-ranking juvenile spotted hyenas and to assess the resistance and tolerance of juveniles in these two social categories to parasite infection. We focus on *Ancylostoma* and *Cystoisospora* because both have direct life cycles that need no

intermediary host, both cause damage to the epithelial lining of the small intestine and are considered energetically costly parasites (Seguel and Gottdenker, 2017; East et al., 2015; Shrestha et al., 2015). We test predictions derived from six hypotheses: (1) The resource allocation hypothesis of life-history theory expects high-ranking juveniles to have lower infection loads than low-ranking juveniles, because they should have more resources to allocate to immune processes. (2) An improvement of juvenile immune responses with age should result in a decline in infection loads with age. Higher resistance is thus expected in high-ranking than low-ranking juveniles and in older than younger juveniles. (3) The transmission of *Cystoisospora* by the faecal-oral route during social contacts predicts higher infection loads in high-ranking than low-ranking juveniles, if resistance to infection is similar in these rank categories. (4) Environmental contamination with both *Ancylostoma* and *Cystoisospora* infective stages is expected to increase as the number of clan members increases, thereby elevating infection loads. (5) The energetic cost of high infection loads of either *Ancylostoma* or *Cystoisospora* should compromise immune responses, thereby increasing the number of co-infecting taxa. (6) Finally, we expect the survival of young juvenile hyenas to adulthood and their longevity to decrease with increasing *Ancylostoma* and/or *Cystoisospora* infection loads, and the fitness cost of high infection loads to be less in high-ranking juveniles than low-ranking juveniles, i.e., we expect high-ranking juveniles to be more tolerant of high infection loads.

4.3 Material and methods

4.3.1 Study host population

The study was conducted between March 2010 and August 2011 in three large clans that held territories at the junction between the woodland and plains in the centre of the Serengeti NP. As this area is not within either the wet or dry season ranges of the migratory ungulates, females in all three clans conducted regular long distance foraging trips to

locations outside their clan territory (Hofer and East, 1993a; East et al., 2003). The mean number of animals (± SEM, plus minimum and maximum values) in these clans during the study period were 79.4 ± 0.6 adults (range 67 - 86 adults); 22.5 ± 0.6 juveniles, (range 11 - 32 juveniles) in the Isiaka clan, 66.0 ± 0.6 adults (range 54 - 69 adults), 28.8 ± 0.4 juveniles (range 22 - 32 juveniles) in the Pool clan, and 81.5 ± 0.9 adults (range 67 - 86 adults), 20.0 ± 0.7 juveniles (range 12 - 28 juveniles) in the Mamba clan, respectively. These clans have been studied since May 1987, November 1989 and August 1990 respectively (East et al., 2003). Information on individual longevity used in this study was collected until the end of July 2018. Individuals were recognized by their unique spot patterns and other features (Frank, 1986; Hofer and East, 1993a). Juveniles were aged using a range of characteristics (pelage, size, locomotion) when first detected, typically within their first few weeks of life, as previously described (Golla et al., 1999; East et al., 2003). Sex was assessed at approximately 3 months of age using the dimorphic glans morphology of the erect clitoris or penis (Frank et al., 1991). Weaning typically occurs at 12-18 months of age (Hofer and East, 1995). Juveniles remain at the clan's communal den until approximately 12 months old where they shelter in underground burrows small enough to prevent entry of adults (Hofer and East, 1993c; Golla et al., 1999).

Juveniles obtain their social position as a result of the behavioural support they receive from their mother during interactions with other members of the clan (Smale et al., 1993; East et al., 2009). As a result, juveniles were allocated the social rank held by their mother on the day the juvenile was sampled (Hofer and East, 2008). To construct linear dominance hierarchies for females in each clan, we recorded ad libitum submissive behaviours during dyadic interactions between adult females during frequent observation periods of approximately three hours at dawn and dusk, mostly at the clan's communal den, as previously detailed (East et al., 2003; Hofer and East, 2003).

Dominance hierarchies were adjusted after each loss or recruitment of adults and when dyadic interaction data revealed that an individual had increased or fallen in rank. The social ranks held by the mothers of juveniles in this study changed little between March 2010 and August 2011. To permit the comparison of ranks held by individuals in different clans, we computed a standardized rank. This measure places the ranks within a given hierarchy evenly between the highest (standardized rank: +1) and the lowest (standardized rank: −1) rank (Goymann et al., 2001; East et al., 2003). Juveniles were then categorized as high-ranking if their mother's standardized rank was equal to or above 0, and low-ranking if their mother's standardized rank was below 0 (as detailed by East et al., 2015; Marescot et al., 2018). We term juveniles reared by high-ranking mothers as high-ranking juveniles and juveniles reared by low-ranking mothers as low-ranking juveniles.

4.3.2 Parasites

The GI parasite community in juveniles found by this study included helminths (*Ancylostoma*, Spirurida and *Trichuris, Dipylidium*, *Diphyllobothrium*, Taeniidae) and the apicomplexan *Cystoisospora* (Table 1). Infection with adult *Ancylostoma* hookworms is energetically costly (Seguel and Gottdenker, 2017) because adults live attached to the mucosal layer of the small intestine and feed on intestinal mucosa and blood. Adults shed eggs into the intestinal lumen which are voided in host faeces (Urquhart et al., 1996; Sowemimo and Asaolu, 2008). Eggs hatch when environmental conditions (substrate, temperature and moisture) are suitable and larvae moult twice before they are infective. Infection occurs by ingestion or when larvae penetrate the skin of a host and then migrate through host tissue to the small intestines (Urquhart et al., 1996). Larvae can be transmitted to the offspring through the trans-mammary route during lactation in some host species (Burke and Roberson, 1985; Urquhart et al., 1996) or by ingestion of paratenic hosts with larvae infected tissue (Lee et al.,

1975). The pathogenic effects result from the blood loss instigated by adult worms and the damage adult worms cause to the intestinal epithelium (Urquhart et al., 1996).
Cystoisospora (formerly termed *Isospora*) infects epithelial cells lining the host's small intestine and is predominantly transmitted by the faecal-oral route (Lindsay et al. 1997; Shrestha et al. 2015). Oocysts in faeces can remain infective for weeks under favourable environmental conditions and sporulation may take less than 16 h at 30°C (Lindsay et al., 1997). Following the infection of intestinal epithelial cells, asexual reproduction occurs, thereby enhancing the infection load in these cells in relation to the infection dose. During this phase, histological changes in the small intestine occur (Lindsay et al. 1997; Shrestha et al. 2015) resulting in clinical signs such as diarrhoea and weight loss (Lindsay et al. 1997; Mengel et al. 2012). Sexual reproduction occurs after a period of several to many days, depending on the species of *Cystoisospora*. Oocysts are then shed in the host's faeces (Lindsay et al., 1997; Mengel et al. 2012). For details on life-cycles of spotted hyena GI parasites other than *Ancylostoma* and *Cystoisospora* please see Table S1.

4.3.3 Parasite screening

Fresh faeces (*N* = 154) were immediately collected after deposition from 96 known juveniles (*N* = 50 males; *N* = 46 females) between 36 and 726 days of age (median age 188 day, mean age 214 days) between. Each faecal sample was mechanically mixed, and one aliquot was stored in a 4% formalin solution at room temperature until processed.
We determined faecal egg counts (FEC, eggs/g faeces) and faecal oocyst counts (FOC, oocysts/g faeces) as previously described (East et al., 2015; Heitlinger et al., 2017) using the McMaster egg flotation technique following Foreyt (2001). We screened 2 g of faeces per sample and used a potassium iodide (KI) solution with a specific weight of 1.5 g ml^{-1} and with a dilution ratio of 1:15 (Meyer-Lucht and Sommer, 2005). The McMaster slide counting chambers were loaded and left for 5 min before parasite eggs/oocysts

were identified, measured and counted in four chambers per sample using a compound microscope at 100x magnification (Jenaval, Carl Zeiss, Jena, Germany). Taxa were identified by conventional morphological criteria using veterinary parasitology reference manuals (Foreyt, 2001; Bowman et al., 2002; Eckert et al., 2008; Zajac and Conboy, 2012). Taxonomic identification to the resolution of order, family or genus level varied between taxa. Results are presented as eggs or oocysts per gram of faeces (EPG or OPG). These values were calculated by dividing the total number of eggs counted by the mass of faeces in the counted chambers, which corresponds to the mass of faeces measured per sample (2g) divided by the volume of KI solution used (28 mL) and multiplied by the total volume counted (0.6 mL). To investigate the reliability of detecting the presence or absence of infection at low parasite loads, we repeated the analysis using three samples with low parasite egg loads. This revealed that the chance of detecting *Dipylidium* was 33 % (2 of 6 counts), *Trichuris* 50 % (7/14), Taeniidae 83 % (5/6), Spirurida 100 % (4/4) and *Cystoisospora* 100 % (4/4). In three of five taxa there was a chance of a false negative result, hence results from these three taxa should be interpreted with caution.

For *Ancylostoma duodenale,* fecundity (the number of eggs/g faeces per worm) is density-dependent, declining from 287 eggs to approximately 100 eggs for the first 100 worms, then remains largely independent thereafter (Anderson and Schad, 1985), hence FEC are a reasonable index of adult worm infection load. Also, Heitlinger et al. (2017) found a significant positive correlation between egg or oocyst counts and the amount of parasite DNA in the faeces for the taxa identified as *Ancylostoma*, *Diphyllobothrium* and Coccidia in this study population. In pigs, *Trichuris suis* egg counts are deemed a reliable approximate estimate of the number of adult worms per host, i.e., parasite load (Gassó et al. 2015). FECs are not a reliable method to quantify parasite loads in cestodes of the order Cyclophillidea (e.g., Taeniidae) which release eggs

from gravid proglottids. *Dipylidium* eggs are released inside egg packages or as gravid proglottids which creates a clumped distribution of eggs whereas when egg packages are broken, eggs are spread in the faeces (Bowman et al., 2002). To estimate infection prevalence with *Dipylidium* we combined the presence of eggs in faeces and the occurrence of *Dipylidium* proglottids on the surface of faeces when collected (for details see East et al., 2013).

4.3.4 Data analysis

Prevalence of infection is the proportion of individuals infected by a particular parasite taxon. Infection load corresponds to the FEC or FOC of a particular parasite taxon per faeces. Mean intensity of infection describes the mean value of infection load of a particular parasite taxon among infected hosts. Mean abundance describes the mean of infection load of a particular parasite taxon among all host examined (Margolis et al., 1982; Bush et al., 1997). The ratio of the variance to the mean abundance was calculated to assess the degree of overdispersion (Table 1).

We used infection loads for *Ancylostoma* and *Cystoisospora* and presence or absence of co-infection with the other parasite taxa identified in our study (Table 1) as recommended by Fenton et al. (2010). We applied generalized mixed-effect linear models (GLMM) using the package "lme4" (Bates et al. 2015) in which *Ancylostoma* load (Table 2) and transformed $\log_{10}(1+)$ *Cystoisospora* load (Table 3) were the response variables. Because the raw data were overdispersed, we used a negative binomial distribution with a log link function (Hilbe, 2011) and confirmed that negative binomial regressions adequately accounted for overdispersion. We used individual ID as random effect to prevent pseudo-replication of 38 individuals contributing more than one faecal sample.

Fixed effect predictors of parasite load were (1) clan membership - an index of environmental variability in terms of abiotic conditions in the vicinity of clan communal den sites, which may affect the survival of parasite eggs/oocysts

and infective stages (Altizer et al., 2006), (2) absolute number of juvenile clan members - an index of social contact between juveniles at the clan communal den and the deposition of juvenile faeces in the vicinity of den sites, (3) absolute number of adult clan members - an index of social contact between adults and juveniles, the deposition of adult faeces in the vicinity of dens, and the potential for these factors to influence parasite transmission to juveniles (see, East et al., 2013; Olarte-Castillo et al., 2016), (4) age at sampling for individuals with several measurements, (5) the number of GI co-infecting parasite taxa (between 0 and 6 taxa), and (6) maternal social rank (high or low) - an indicator of juvenile body condition (see Hofer et al., 2016) and contact with pathogens mediated by within-clan social interactions (Marescot et al., 2018).

We used log-likelihood ratio tests and the Akaike information criterion (AIC) in R (R Core Team, 2015) to check whether the complete models were superior to reduced models. Models were considered similar if differences in AIC were less than 2.5 and preferable if the difference exceeded 6.0 (Hilbe, 2009). The significance of each predictor variable was assessed as the marginal contribution of each parameter to the full model by subtracting from the full model the log-likelihood ratio of a second model with each specific parameter removed and testing the difference against a chi-square distribution with appropriate degrees of freedom (see discussions in Hilbe, 2011; Hosmer et al., 2013). To assess the global goodness-of-fit we used log-likelihood ratio tests to compare full models with intercept-only models. In preliminary models we included season in terms of wet and dry season and sex as fixed effects but neither had a significant effect nor improved the models as described above.

To investigate factors influencing survival to adulthood at 2 years of age, we selected samples from juveniles younger than 12 months (364 days). This resulted in a dataset with 135 samples from 84 individuals. We used a binomial logistic regression with a logit link function calculated with

the R package MASS version 7.3-45 (Venables and Ripley, 2002) and included as predictors (1) age at sampling, (2) maternal social rank (high or low), (3) *Ancylostoma* infection load*,* (4) *Cystoisospora* infection load, (5) the number of GI co-infecting parasite taxa (between 0 and 4 taxa), and (6) an interaction between age at sampling and maternal social status. To avoid pseudo-replication we randomly chose one sample per juvenile younger than 12 months (N = 84).

To investigate the tolerance of juveniles to high *Ancylostoma* infection load and to infection with *Cystoisospora* we determined the longevity (in days) of those juveniles sampled before the age of 6 months (180 days). We applied this cut-off because juveniles less than 180 days old had the highest *Ancylostoma and Cystoisospora* infection loads (see results), were entirely dependent on milk and hence their intake of milk was likely to be sensitive to maternal rank-related access to food and the period in days between maternal nursing visits (Hofer and East, 2003, 2016). Longevity was scored as an exact value if the death date was known, or as a right-censored value (1) at the age of dispersal for males that dispersed (and whose fate could not be subsequently followed), (2) at their age on the day of the road accident for individuals killed in road accidents, and (3) the age reached by females on the last day of observation for this study (31 July 2018) if they were still alive on that day. We used Kaplan-Meier survivorship functions to construct survivorship curves for maternal rank (high and low) and infection intensity (high considered above and low below mean values of *Ancylostoma* load, (Figure 7a). Similarly, for *Cystoisospora,* we constructed survivorship curves for maternal rank (high and low) but chose infection status (positive and negative) rather than infection load to improve sample size, as only 9 out of 50 individuals had infections loads above the mean whereas 25 out of 50 individuals were scored as infected. We excluded duplicates resulting from repeated samples from the same individual that were placed in the same categories. The dataset for the *Ancylostoma* and maternal rank survivorship functions had 5

individuals sampled twice with different *Ancylostoma* load categories. The dataset for the *Cystoisospora* infection status and maternal rank survivorship function had 8 individuals sampled twice with different *Cystoisospora* infection status categories. To avoid pseudoreplication we chose one sample per individual randomly. This data set contained 50 juveniles.

This survival analysis was conducted using the R package survival v.2.38 (Therneau, 2015).

In all statistical models, the significance threshold was fixed at 5 % and all tests were two-tailed. All statistical analyses were performed using R version 3.2.2 (R Core Team, 2015). Unless otherwise indicated, results are presented as means ± S.D.

4.4 Results

We quantified eggs or oocysts from seven parasite taxa in the faeces of juvenile hyenas (Table 1). Infection prevalence was 94.2 % for *Ancylostoma*, and 53.9 % for *Cystoisospora.* Infection loads were overdispersed (variance to mean abundance ratios >1, Table 1) for all seven parasite taxa, and are illustrated for *Ancylostoma*, *Diphyllobothrium* and *Cystoisospora* in Figure 2. In 4 of 154 (2.6%) samples no parasite eggs or oocysts were found. Prevalence of *Dipylidium* based on egg counts alone was 20.8%, and increased to 59.7 % when data on the presence of *Dipylidium* proglottids on faeces at the time of collection were combined with egg count data. Changes of infection loads with age are illustrated for *Ancylostoma, Cystoisospora* and *Diphyllobothrium* in Figure 3a, b and c, respectively, for all individuals sampled on at least three dates (*N* = 46 samples from 13 individuals). In general, infection load declined with age for these three most prevalent parasite genera.

Concurrent infection with more than one parasite taxon was frequent. Individuals were infected with 0 to 5 parasite taxa, the mean number of parasite taxa per individual was 2.8 ± 1.2 (*N* = 154). The frequency distribution of the number of

different parasite taxa found in individual hyenas is illustrated in Figure 4.

Several factors influenced *Ancylostoma* egg load (log likelihood ratio test $G = 52.11$, $df = 7$, $p < 0.001$, Table 2) and *Cystoisospora* oocyst load ($G = 33.96$, $df = 7$, $p < 0.001$, Table 3). *Ancylostoma* and *Cystoisospora* loads significantly decreased with host age and significantly increased with the number of co-infecting parasite taxa (Tables 2, 3, Figure 5). *Cystoisospora* load and *Ancylostoma* load significantly decreased as the number of adults in a clan increased. Both *Ancylostoma* and *Cystoisospora* loads significantly differed between clans (Tables 2, 3).

Survival to adulthood among juveniles sampled in the first 12 months of life (log likelihood ratio test, $G = 33.38$, $df = 6$, $p < 0.001$) decreased as *Ancylostoma* load increased but was independent of *Cystoisospora* load (Table 4). The effect of maternal social status was modulated by age. In low-ranking juveniles, survival early in life was substantially lower than that of high-ranking juveniles. As age increased, survival to adulthood of low-ranking juveniles increased steeply whereas survival to adulthood of high-ranking juveniles increased modestly with age (Figure 6). Kaplan-Meier survivorship curves indicated that during approximately the first four years of life, high-ranking juveniles with low *Ancylostoma* loads (Figure 7a) had an overall better survivorship than those with high *Ancylostoma* loads. The survivorship of low-ranking juveniles with high *Ancylostoma* loads was mostly below that of low-ranking juveniles with low *Ancylostoma* loads during approximately the first five years of life (Figure 7a). In contrast, high-ranking juveniles infected with *Cystoisospora* had a higher survivorship until early adulthood than those that were not infected with this parasite, whereas low-ranking juveniles that were infected with *Cystoisospora* had a lower survivorship than those not infected with *Cystoisospora* during approximately their first 5 years of life (Figure 7b).

4.5 Discussion

In line with other studies on parasite infections in free-ranging wild mammals, our study on spotted hyenas revealed substantial heterogeneity between juveniles in both their *Ancylostoma* and *Cystoisospora* infection loads (Figure 2) and their number of co-infecting parasite taxa (Table 1, Figure 4). Consistent with evidence that immune processes in juvenile mammals improve with age, both *Ancylostoma* (Table 2) and *Cystoisospora* (Table 3) loads decreased as juveniles increased in age. We interpret differences in infection loads between juveniles in different clans as possible evidence that abiotic conditions at communal dens sites that influence the survival of parasite infective stages differed between clans. In contrast to our prediction, *Ancylostoma* and *Cystoisospora* loads decreased with the number of adults in a clan (Table 2). One plausible explanation could be an encounter-reduction effect whereby adult hyenas reduce the chance of juveniles encountering *Ancylostoma* infective larvae. Also contrary to expectation, we found no evidence that *Cystoisospora* loads were affected by social interactions among juveniles (Table 3), possibly because asexual reproduction by *Cystoisospora* in infected intestinal epithelial cells contributes more to elevating infection loads in individuals than faecal – oral transmission during social interactions. We found evidence that the survival of young juveniles to adulthood (Table 4) decreased as *Ancylostoma* infection loads increased but was not affected by *Cystoisospora* infection loads (Table 4). Furthermore, the longevity of young juveniles with high *Ancylostoma* infection loads was poor compared to those with low *Ancylostoma* infection load, and this was particularly so in juveniles reared by low-ranking mothers (Figure 7). These findings indicate that energetically costly parasites such as *Ancylostoma* can have negative fitness consequences for juveniles and that high-ranking juveniles may be more tolerant of infection (i.e., have lower fitness costs) than low-ranking juveniles.

Juvenile hyenas in our study population are entirely dependent on maternal milk for their growth during their first six months of life (Hofer and East, 2003; Hofer et al., 2016). During this early period of life they often have their peak infection loads of *Ancylostoma*, *Cystoisospora* and *Diphyllobothrium* (Figure 3) and hence their peak energetic costs of infection with these parasites. The survival of juveniles to adulthood and their longevity were the two components of Darwinian fitness we used to investigate the fitness consequences of *Ancylostoma* infection loads in juvenile hyenas. Our analyses revealed evidence that survival to adulthood decreased as *Ancylostoma* infection loads increased (Table 4) and that high-ranking juveniles had a higher probability of reaching adulthood than low-ranking juveniles (Table 4, Figure 6). Furthermore, survival to adulthood increased with age and there was an interaction between juvenile age and maternal rank (Table 4). During the first six months of life, the probability of survival for low-ranking juveniles was very low and for the few low-ranking juveniles that did survive this initial period, their probability of survival was then similar to the higher survival of high-ranking juveniles (Figure 6). We also found evidence that in both high-ranking and low-ranking juveniles, survivorship (longevity) appears to be less in animals with high than those with low *Ancylostoma* loads. High-ranking juveniles with high infection loads lived longer than low-ranking juveniles with high loads (Figure 7a). This result is based on a relatively small number of juveniles and thus the effect of *Ancylostoma* loads on longevity should be further investigated with a larger sample of juveniles. Our results indicate that most animals in our study were infected with *Ancylostoma.* Given this high prevalence of *Ancylostoma* infection (Table 1) and the absence of a maternal rank effect on *Ancylostoma* loads (Table 2), we interpret these results to indicate that high *Ancylostoma* loads decrease longevity. Furthermore, we interpret the lack of a maternal rank effect on *Ancylostoma* loads (Table 2) to suggest that higher milk intake and higher growth rates in high-ranking than low-

ranking juveniles (Hofer and East 2003; Hofer et al. 2016) does not provide evidence that high-ranking juveniles are more resistant to infection than low-ranking juveniles (Figure 7a), but rather that better nourished high-ranking juveniles have a higher 'tolerance' phenotype to *Ancylostoma* load than low-ranking juveniles. This idea needs to be more rigorously tested with a larger sample of juveniles, as it is possible that, in combination with other factors, even low *Ancylostoma* loads may reduce longevity.

Previously we have shown that offspring reared by low-ranking mothers have lower growth rates than those reared by high-ranking females (Hofer and East, 2003). This is because low-ranking mothers in our study population more often travel long distances (round trips of up to 140 km) to forage outside the clan territory than high-ranking mothers (Hofer and East, 1993c, 2003). As a result, during the first six months of life, when growth is fuelled by milk, low-ranking juveniles are nursed less often and overall receive less milk than high-ranking juveniles (Hofer et al., 2016). Furthermore, juveniles in our study population with low growth rates in the first six months of life have a lower probability of survival to adulthood than those with high growth rates (Hofer & East, 2003). High *Ancylostoma* infection loads are energetically costly because of substantial blood loss and damage to the intestinal wall (Urquhart et al., 1996), and have been reported to retard juvenile growth, and cause anaemia and malnutrition in other mammals (Sakti et al., 1999; Stoltzfus et al. 2004; Seguel and Gottdenker, 2017). This suggests that the energetic cost of high *Ancylostoma* infection loads in rapidly growing, milk-dependent juvenile hyenas may compromise growth and thus curtail survival to adulthood. This idea is supported by evidence that pup growth in New Zealand sea lion (*Phocarctos hookeri*) is reduced by infection with the hookworm *Uncinaria* sp (Chilvers et al., 2009), and body mass, fat deposits and fecundity in reindeer (*Rangifer tarandus platyrhynchus*) are reduced by the nematode *Ostertagia gruehneri* (Stien et al., 2002). Using all these

strands of evidence we interpret our current finding to suggest that high-ranking juveniles are more tolerant of high *Ancylostoma* infection loads than low-ranking juveniles, because they are generally better nourished and thus can allocate more resources to replace the blood lost to feeding adult *Ancylostoma* and to repair the damage to the intestinal wall than low-ranking juveniles. Similarly, a study of nematode infections in female Soay sheep on the island of St Kilda (Hayward et al., 2014) reported considerable variation in weight loss in relation to a given strongyle egg load and that animals that lost weight more slowly had a higher lifetime breeding success, indicative of a greater tolerance of infection and higher fitness. Tolerance is a trait that is thought to lead to a higher infection prevalence of a parasite in a host population and this should promote the persistence of infection (Roy and Kirchner 2000). Consistent with this idea, our results from juvenile hyenas and those of a previous study (East et al., 2015) indicate both a high prevalence and persistence of *Ancylostoma* infection in our study clans.

Although we found no evidence that high *Cystoisospora* infection loads decreased the survival of juvenile hyenas to adulthood (Table 4), the survival probability for low-ranking individuals that were infected with *Cystoisospora* when they were juveniles was lower throughout the juvenile period and into early adulthood than that of low-ranking juveniles not infected by *Cystoisospora* (figure 7b). Our study does not assess infection loads during the first few weeks after birth when we are unable to obtain faeces from cubs, hence we cannot rule out the possibility that infection during this period might decrease survival. Infection with *C. suis* within days of birth of domestic piglets can cause severe pathologies, increased mortality and poor weight gain (Lindsay et al. 1997; Koudela and Kučerová, 1999; Shrestha et al., 2015), and an enhanced chance of co-infection (Mengel et al., 2012). Furthermore, our analysis does not distinguish between juvenile hyenas with chronic *Cystoisospora* infections that may curtail juvenile survival to adulthood from

those with relatively short-term, high infection loads that may have a limited effect on juvenile survival (Figure 3b). A preponderance of such short-term infections in our sample may contribute to the lack of a significant effect of high oocyst infection load on juvenile survival to adulthood.

Our analysis found no evidence for the expected positive relationship between *Cystoisospora* loads in juveniles and the number of juveniles in a clan (Table 3). This relationship was expected because *Cystoisospora* is transmitted by the faecal-oral route and social interactions, particularly greeting ceremonies between young juveniles, in which participants sniff and lick their partner's anal genital region (East et al., 1993), might be expected to increase with the number juveniles in a clan (Olarte-Castillo et al., 2016). Environmental contamination of communal den sites might also be expected to increase with the number of juveniles, thereby raising the chance of inadvertent ingestion of oocysts by juveniles. We studied three clans that contained roughly a similar mean number of juveniles (between 20 and 29 juveniles). It is possible that the inclusion of a larger number of clans that differed substantially in the number of juveniles they contained might yield results that differ from ours.

Our results revealed that heterogeneity in *Cystoisospora* oocyst loads shed by juveniles was considerable (Figure 2, 3b) and that high infection loads occurred mostly early in life (Figure 3b) and declined as juvenile age increases (Table 3). These findings suggest that at any given time, high infection loads are probably mostly apparent in a few younger juveniles in a clan. It is possible that high infection loads in these individuals may be linked to rapid asexual reproduction of the parasite within the intestinal epithelial cells of some individuals, followed by sexual reproduction and the shedding of high oocyst loads – rather than high infection loads resulting from faecal-oral transmission during social interactions or from environmental contamination. Juveniles that shed high *Cystoisospora* oocyst loads may act as 'super-shedders' for infection. 'Super-shedders' are

thought to be important for disease transmission in several pathogens (Lloyd-Smith et al., 2005; Paull et al., 2011; Courtenay et al., 2017). Because adult females in our study clans give birth throughout the year (Hofer and East, 1995; East et al., 2003), for a substantial proportion of each year, communal dens are likely to contain the young juveniles (Figure 3b) that typically shed high oocyst loads. Communal dens are likely to be hotspots for *Cystoisospora* transmission to susceptible individuals because both juveniles (this study) and lactating females at dens (East et al., 2015) shed oocysts in faeces. Hyena communal den sites are locations of high use by both adults and juveniles (Figure 1) and thus can act as hotspots for the spread of pathogens, similar to bird feeders (Adelman et al., 2015) and savannah waterholes (Stommel et al., 2016). Both super-spreaders and hotspots of infection are important for the spread and maintenance of pathogens in host populations (Lloyd-Smith et al., 2005; Paull et al., 2011; Adelman et al., 2015; Courtenay et al., 2017). Juvenile hyenas with high infection loads at communal dens may be important for the spread and maintenance of not only *Cystoisospora* and *Ancylostoma* (this study) but other parasite such as *Dipylidium* (East et al., 2013). For approximately two months after birth, defecation by young juveniles is stimulated by maternal grooming of their offspring's anus and mothers typically consume the faeces produced (ML East and H Hofer, personal observations). This maternal behaviour may help limit oocyst contamination of dens.

Ancylostoma larvae emerge from eggs shed in faeces, then moult twice before becoming infective filariform larvae that remain viable for weeks in moist and cool environmental conditions (Urquhart et al., 1996). Hyenas are infected when *Ancylostoma* infective larvae either burrow into their feet or pass from infected mothers to their offspring during nursing. We previously found that *Ancylostoma* infection loads were higher in low-ranking than high-ranking lactating females (East et al., 2015). These rank related differences in maternal infection loads might be expected to result in the

passage of more infective larvae from low-ranking than high-ranking mothers to their offspring during nursing. However, even though *Ancylostoma* egg load is a good indicator of the infection load of adult worms in a host (Anderson and Schad, 1985), the number of adult *Ancylostoma* worms in the intestine may not reflect the number of migrating larvae in the female's tissue or the number of larvae shed in her milk, and this may explain why there was no significant effect of maternal rank on *Ancylostoma* infection load in juvenile hyenas (Table 2). In several species of sea lions, transmission of the hookworm *Uncinaria* by the transmammary route is thought to be important (Marcus et al., 2014).

The three study clans varied in the mean number of adults they contained (between 66 and 82 adults) and although the number of clans studied was small, our results indicate that infection loads of *Ancylostoma* (Table 2) and *Cystoisospora* (Table 3) decreased as the number of adult clan members increased. This suggests an encounter-reduction effect (Mooring and Hart, 1992, Côté and Poulin, 1995; Keesing et al., 2006), which in multi-species host assemblages is termed a 'dilution' effect. Evidence of an encounter-reduction effect contradicts the expected positive relationship between the number of adults in a clan and infection loads in juveniles. How might the presence of adult clan members reduce *Ancylostoma* infection loads in juveniles? Firstly, adults exposed to the infective stages of both parasites at den sites become infected and then rapidly clear these infections, thereby decreasing the population of infective stages at dens. When the number of visits by adults to den areas is high, the removal of infective stages by adults may be sufficient to reduce encounters between juveniles and infective stages. Secondly, since adults deposit faeces in communal latrines near dens, this might reduce the chance of juveniles encountering infective stages because the *Ancylostoma* infection load in the faeces of adult females (East et al., 2015) is typically less than that in faeces produced by juveniles (this study), probably because of a

more effective immune response to infection by adults than juveniles. Unless encountered in a fresh condition, the faeces of adult hyenas are often extremely hard and dry (Kruuk, 1972) whereas the faeces of juveniles are more often moist and less hard (ML East, SCM Ferreira, and H Hofer, personal observations). This suggests that *Ancylostoma* larvae and *Cystoisospora* oocysts may develop and survive less well in adult than juvenile faeces. These three possible encounter-reduction effects may all contribute to the negative relationship between *Ancylostoma* and *Cystoisospora* infection loads in juveniles and the number of adults in the clan. Encounter-reduction effects have been reported for a range of pathogens, including the bacterium *Mycobacterium bovis* (Vicente et al., 2007; Huang et al., 2013), and both the cestode *Dipylidium* (East et al., 2013) and the enteric *Sapovirus* (Olarte-Castillo et al., 2016) in our study population.

In line with the findings of previous studies that provide evidence that immunocompetence in juvenile mammals improves with age (e.g., Koudela and Kučerová, 1999; Ramsburg et al., 2003; Simon et al., 2015), both *Ancylostoma* and *Cystoisospora* infection loads in juvenile hyenas decreased with age (Tables 2, 3). This age effect was also apparent for infection with *Ancylostoma, Cystoisospora* and *Diphyllobothrium* in juvenile hyenas sampled several times during their development (Figure 3a, b, c). The less developed immune responses of neonates develop during the juvenile period into the fully mature immune system of adults. For example, during early life, juveniles are generally more susceptible to infection by pathogens than adults, partly because of their limited capacity to mount an effective immune responses due to qualitative differences in the T cell populations of juveniles and adults (Ramsburg et al., 2003), and naïve, mostly young animals that lack exposure to pathogen antigens have not developed the acquired immune responses that develop as a result of exposure (Koudela and Kučerová, 1999; Cattadori et al. 2005). The decrease in *Ancylostoma* and

Cystoisospora infection loads in juvenile hyenas with age may be the combined outcome of increased immunocompetence due to active immune responses induced by parasite exposure, which is likely to increase with age (e.g., East et al., 2001; Ferreira et al., 2019a) and the general maturation of the mammalian immune system in juvenile hyenas with increasing age. Currently, little is known about factors that affect immunocompetence in juvenile hyenas, so in addition to age, factors such as genotype and gene expression (Gulland et al., 1993, Jackson et al., 2011), allostatic load, physiological processes, behaviour and diet may be relevant (Ardia et al., 2011; VanderWaal and Ezenwa, 2016).

Our results revealed a significant effect of clan membership on both *Ancylostoma* (Table 2) and *Cystoisospora* (Table 3) infection loads, with juveniles in the Isiaka and Mamba clans having higher infection loads than those in the Pool clan. This finding suggests that the survival of eggs, oocysts and infective larvae at den sites in the Pool clan territory was lower than those in the Mamba and Isiaka clan territories, possibly because of abiotic conditions that reduce survival, such as high ambient temperatures and low soil moisture (Urquhart et al., 1996; Turner et al., 2012). Clan differences in infection loads may also result from behavioural differences between juveniles in the Pool and other two clans. For example, juveniles in the Pool clan may have more often changed the location of the latrines they used than those in the other two clans, thereby avoiding latrines highly contaminated with infective stages. Similarly, the use of latrines in shorter vegetation where peak daytime temperatures were higher and soil moisture was lower, might also reduce the survival of infective stages and hence infection loads.

Most wild mammal populations are concurrently infected with more than one parasite (Behnke et al., 2005; Telfer et al., 2010; Seltmann et al. 2019) and this is also true for juvenile hyenas (Table 1, Figure 4). Our results revealed that as either *Ancylostoma* (Table 2, Figure 5a) or *Cystoisospora*

(Table 3, Figure 5b) loads increased, the number of co-infecting GI parasite taxa hosted per juvenile also increased. The explanation for the positive relationship between infection loads and co-infecting taxa is unknown and requires further research. As maternal rank category did not have the predicted effect on either *Ancylostoma* (Table 2) or *Cystoisospora* infection loads (Table 3), we lack evidence that the immune processes of low-ranking juveniles are more often compromised by resource allocation trade-offs than those of high-ranking juveniles, and thus the role of immune processes in determining the number of co-infecting parasite taxa is also unclear. Even though potential interactions between taxa in the GI parasite community of juvenile hyenas are unknown, there was a tendency for an increase in juvenile survival to adulthood with the number of co-infecting GI parasite taxa per host (Table 4). This suggests the intriguing idea that the fitness cost of a more diverse GI parasite community may be lower than one dominated by a limited number of taxa. Previously we found that the eukaryome in high-ranking hyenas is more diverse than that of low-ranking animals (Heitlinger et al., 2017) which may indicate that high-ranking animals have a more diverse, stable and ecologically 'healthy' GI community than low-ranking animals.

Our study identifies several factors that shape heterogeneity of parasite infections among juveniles in a wild social mammal. Disentangling key environmental factors, parasite-host mechanisms and interactions between co-infecting parasites is difficult, particularly when non-invasive methods are required, as is the case in many protected areas or in endangered species. The benefit of non-invasive methods is that they allow repeated measures of both infection loads and components of fitness throughout an individual's lifespan. Further research is needed to examine in detail the effect of the variation of clan size and composition on parasite infections.

4.6 Ethics statement

All protocols were non-invasive and adhered to the laws and guidelines of Tanzania. Permission to conduct research in Tanzania was granted to HH, ME and SF by the Tanzania Commission for Science and Technology through the Tanzanian Wildlife Research Institute. Permission to undertake research within the Serengeti National Park was granted by the Tanzanian National Parks Authority. The research was also approved by the Committee for Ethics and Animal Welfare of the Leibniz Institute for Zoo and Wildlife Research under the approval number 2010-05-02.

4.7 Data accessibility

The dataset analysed for this study is available from the Dryad Digital Repository, doi:10.5061/dryad.5qv7v47 (Ferreira et al. 2019b).

4.8 Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

4.9 Acknowledgments

We are grateful to the Commission for Science and Technology of Tanzania, the Tanzania Wildlife Research Institute and Tanzania National Parks for their support of our research. We thank the Deutsche Forschungsgemeinschaft (grants EA 5/3-1, DFG-Grako 2046 Parasitic Infections) and the Leibniz-Institute for Zoo and Wildlife Research for financial assistance. We thank Malvina Andris, Nelly Boyer, Annie Francis, Robert Fyumagwa, Janine Helms, Stephan Karl, Sonja Metzger, Thomas Shabani and Dagmar Thierer for assistance.

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Table 1. Gastrointestinal parasite taxa in 154 samples from juvenile spotted hyenas (*N* = 96) identified in faecal samples. Indicated are the percentage of infected host individuals (prevalence, %), mean and median infection loads of infected hosts (mean and median intensity, number of eggs or oocysts/g faeces), mean infection load across all hosts, including the non-infected hosts with a load of 0 (mean abundance, number of eggs or oocysts/g faeces) and the ratio of the variance to the mean of abundance.

Parasite	Phylum	Prevalence	Mean intensity	Median intensity	Mean abundance	Ratio variance / mean abundance
Ancylostoma	Nematoda	94.2	1784	503	1680	13530
Dipylidium	Platyhelminthes	59.7	-	-	-	-
Cystoisospora	Apicomplexa	53.0	1811	114	976	40783
Diphyllobothrium	Platyhelminthes	50.0	9732	2451	4866	73146
Taeniidae	Platyhelminthes	8.4	24	16	2	83
Trichuris	Nematoda	8.4	29	16	2	105
Spirurida	Nematoda	6.5	60	16	4	296

Table 2. Predictors of *Ancylostoma* load (eggs/g faeces) in juvenile spotted hyenas (*N* = 154) as estimated by a mixed-effect negative binomial regression. Potential predictors included age, co-infection (scored as the number of other gastrointestinal parasite taxa), clan membership and maternal social status. Shown are the model parameter estimates, their standard errors (SE) in natural log units, and z values with associated P-value. Positive (negative) estimates indicate that an increase in the value of the parameter increased (reduced) egg load. Also shown are the tests for the significance of each parameter using log-likelihood ratio tests (G), with associated p-values and the values for the Akaike Information Criterion (AIC) for each alternative model when the specific predictor was removed. AIC for the full model was 2564.5. GI: gastrointestinal.

Fixed effect	Estimate	SE	CI		Z	P	df	G	p	AIC	ΔAIC
			Low	Low							
(Intercept)	10.45	1.83	NA	NA	5.70	<0.001					
Age at sampling	-0.01	0.001	NA	NA	-5.48	<0.001	1	22.14	<0.001	2584.6	20.1
No. of co-infecting GI parasite taxa *	0.41	0.13	NA	NA	3.22	0.001	1	10.59	0.001	2573.1	8.6
Clan Isiaka **	1.76	0.65	NA	NA	2.73	0.01	2	8.59	0.01	2569.1	4.6
Clan Mamba **	1.68	0.73	NA	NA	2.29	0.02					
Maternal rank (high>low)	-0.39	0.25	NA	NA	-1.55	0.12	1	2.60	0.11	2565.1	0.6
Number of adult clan members	-0.06	0.03	NA	NA	-2.09	0.04	1	15.17	<0.001	2577.7	13.2
Number of juvenile clan members	0.05	0.03	NA	NA	1.37	0.17	1	1.89	0.17	2564.4	-0.1

Table 3. Predictors of *Cystoisospora* load (oocysts/g faeces) in juvenile spotted hyenas log10 (1+) transformed (N = 154) as estimated by a mixed-effect negative binomial regression. Potential predictors included age, co-infection (scored as the number of other gastrointestinal parasite taxa), clan membership and maternal social status. Shown are the model parameter estimates, their standard errors (SE) in natural log units, 95 % confidence limits (CI) and z values with associated P-value. Positive (negative) estimates indicate that an increase in the value of the parameter increased (reduced) egg load. Also shown are the tests for the significance of each parameter using log-likelihood ratio tests (G), with associated p-values and the values for the Akaike Information Criterion (AIC) for each alternative model when the specific predictor was removed. QAIC for the full model was 479.88. GI: gastrointestinal.

	Estimate	SE	CI		Z	P	df	G	P	AIC	ΔAIC
			Low	High							
(Intercept)	1.63	0.87	-0.12	3.39	1.86	0.06	-				
Age at sampling	-0.001	0.001	-0.003	-0.00002	-1.96	0.05	1	4.95	0.05	481.83	1.95
No. of co-infecting GI parasite taxa *	0.22	0.08	0.05	0.39	2.63	0.01	1	6.79	0.01	484.68	4.8
Clan Isiaka **	1.48	0.33	0.82	2.14	4.44	<0.001	2	18.24	<0.001	494.12	14.24
Clan Mamba **	1.34	0.37	0.61	2.10	3.61	<0.001					
Maternal rank (high>low)	0.06	0.15	-0.24	0.35	0.42	0.68	1	0.17	0.68	478.06	-1.82
Number of adult clan members	-0.04	0.01	-0.07	-0.01	-2.95	0.003	1	8.49	0.004	486.37	6.49
Number of juvenile clan members	0.02	0.02	-0.01	0.06	1.33	0.18	1	1.66	0.20	479.54	-0.34

Table 4. The effects of *Ancylostoma* and *Cystoisospora* load (eggs/oocysts/g faeces), age and maternal social status on survival to adulthood (at 2 years of age) for less than one year old juvenile spotted hyenas (*N* = 84). Shown are the logistic regression coefficient estimates with their standard errors (SE) and 95 % confidence limits in natural log units as well as their conversion into odds ratios with their respective 95 % confidence limits (CI), the z-values and associated P-values for each parameter and the results of log-likelihood ratio tests (G) with associated p-values. Positive (negative) estimates indicate that an increase in the value of the parameter increased (reduced) the survival to adulthood. Survival for juveniles with low maternal rank was lower than for juveniles with high maternal rank. * Presence of one or more of the following taxa: *Diphyllobothrium*, *Dipylidium*, Taeniidae, Spirurida, *Trichuris*

Parameter	**Regression coefficients**									**Odds ratios**		
	Estimate	**SE**	**z**	**P**	**df**	**G**	**p**	**CI**			**CI**	
								lower	**upper**	**Estimate**	**lower**	**upper**
(Intercept)	-0.32	1.47	-0.22	0.83				-3.36	2.50	0.72	0.03	12.16
Ancylostoma egg load	-0.0001	0.0001	-2.07	0.04	1	4.78	0.03	-0.0003	-0.00001	1.00	1.00	1.00
Cystoisospora oocyst load	0.0001	0.0001	1.00	0.32	1	1.70	0.19	-0.00003	0.0003	1.00	1.00	1.00
Age at sampling	0.01	0.01	0.77	0.44	2	20.34	<0.001	-0.01	0.02	1.01	0.99	1.02
No. of co-infecting parasite taxa *	0.38	0.35	1.07	0.28	1	1.16	0.28	-0.31	1.09	1.46	0.74	2.97
Maternal rank: high>low	-6.86	2.83	-2.43	0.02	2	8.03	0.02	-13.20	-1.86	0.001	0.000002	0.16
Interaction age and maternal rank	0.04	0.02	2.23	0.03	1	6.38	0.01	0.01	0.07	1.04	1.01	1.08

FIGURE LEGENDS

Figure 1. Spotted hyenas at the communal den.

Figure 2. Frequency distributions of *Ancylostoma*, *Cystoisospora* and *Diphyllobothrium* faecal egg or oocyst load (eggs or oocysts/g faeces) in juvenile spotted hyenas (n=154).

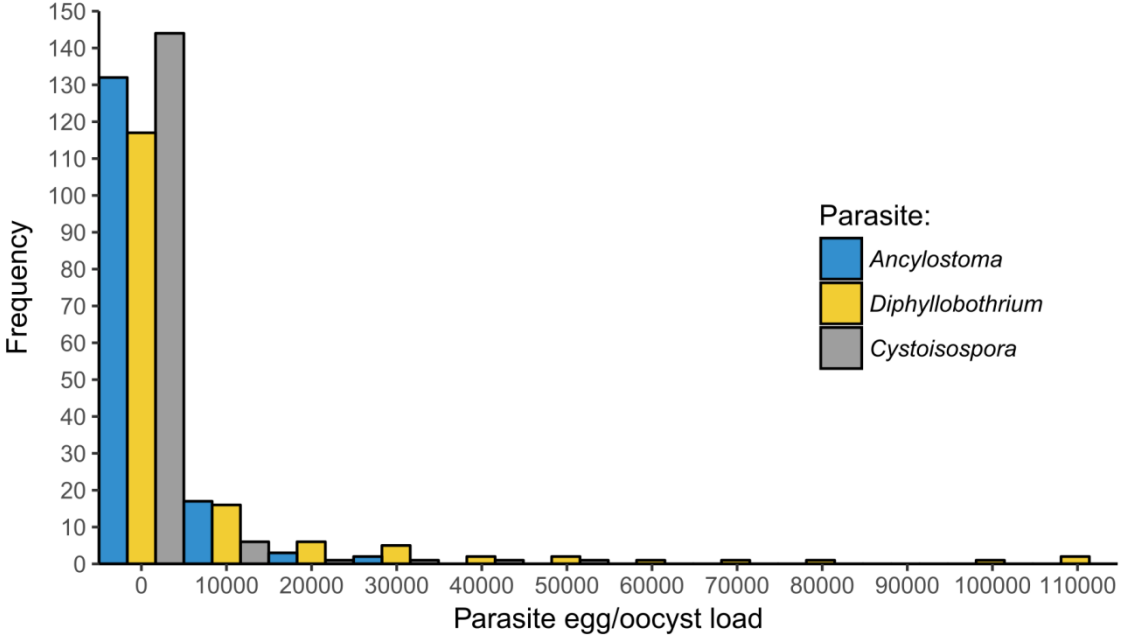


Figure 3. Changes in parasite infection load (eggs or oocysts/g faeces) in individual juvenile spotted hyenas with (a) *Ancylostoma*, (b) *Cystoisospora*, (c) *Diphyllobothrium.*

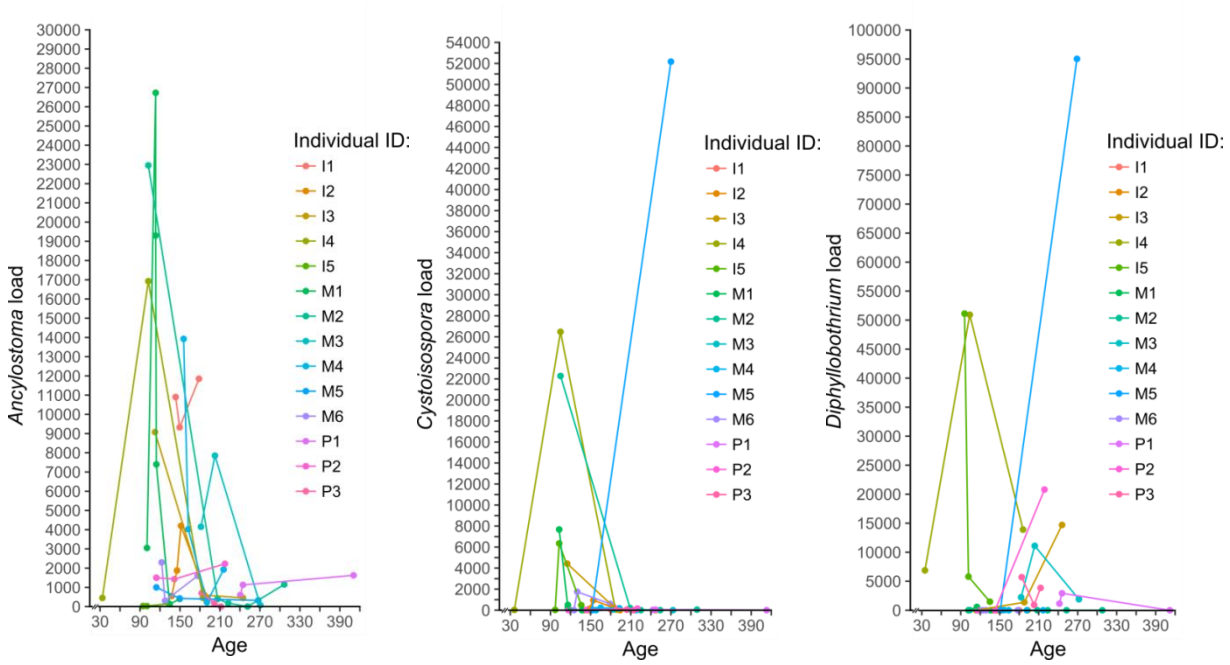


Figure 4. Frequency distribution of the number of gastrointestinal parasite taxa in juvenile spotted hyenas (n=154).

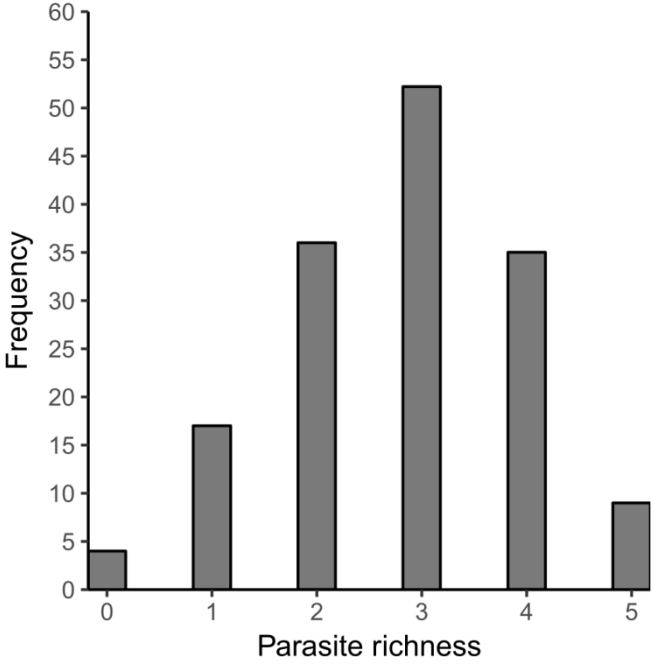


Figure 5. Association between the presence of different co-infecting gastrointestinal parasite taxa and (a) infection load (eggs/g faeces) by *Ancylostoma*; (b) infection load (oocysts/g faeces) by *Cystoisospora* (n=154).

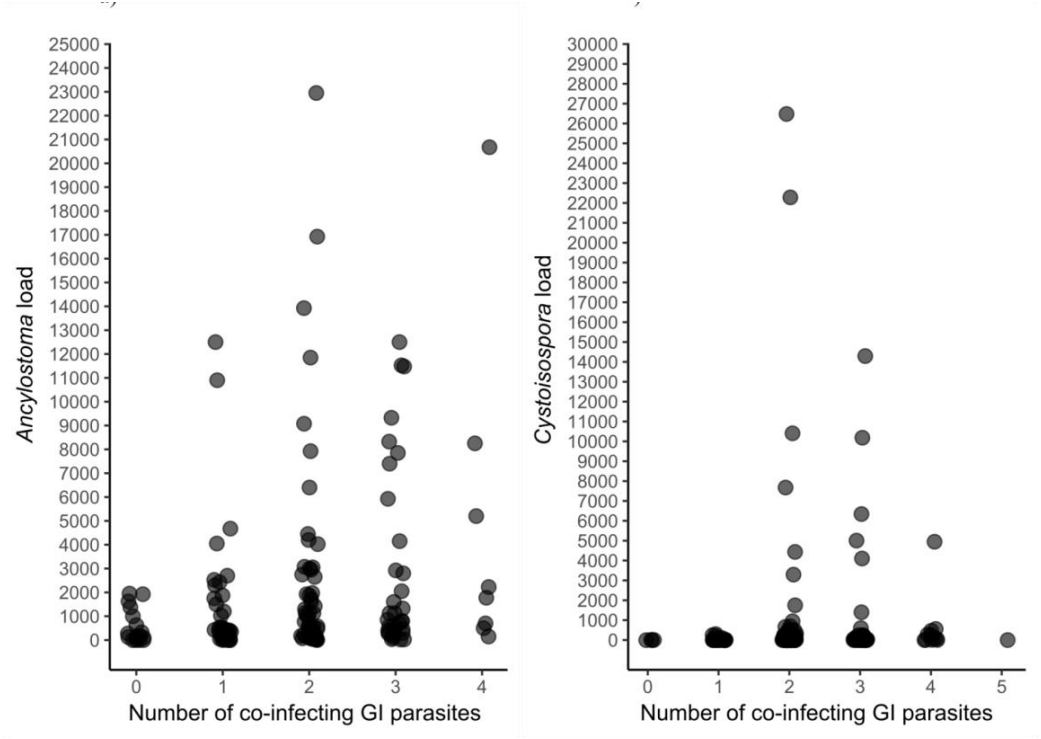


Figure 6. Predicted effect of juvenile age and maternal social status on the probability of juvenile survival to adulthood for spotted hyenas younger than 12 months of age with 95% confidence intervals (n=84). Alongside as “dots” are the raw data of juveniles included in the model that survived (probability of survival to adulthood=1) and did not survive (probability of survival to adulthood=0) to adulthood.

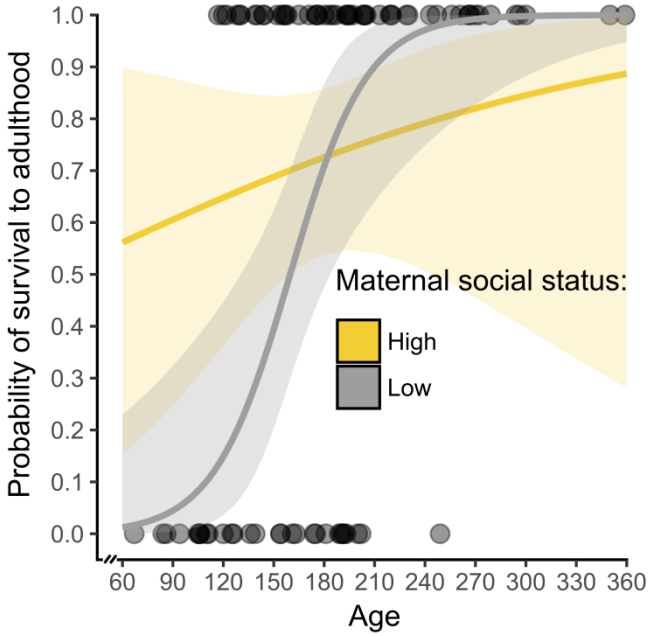
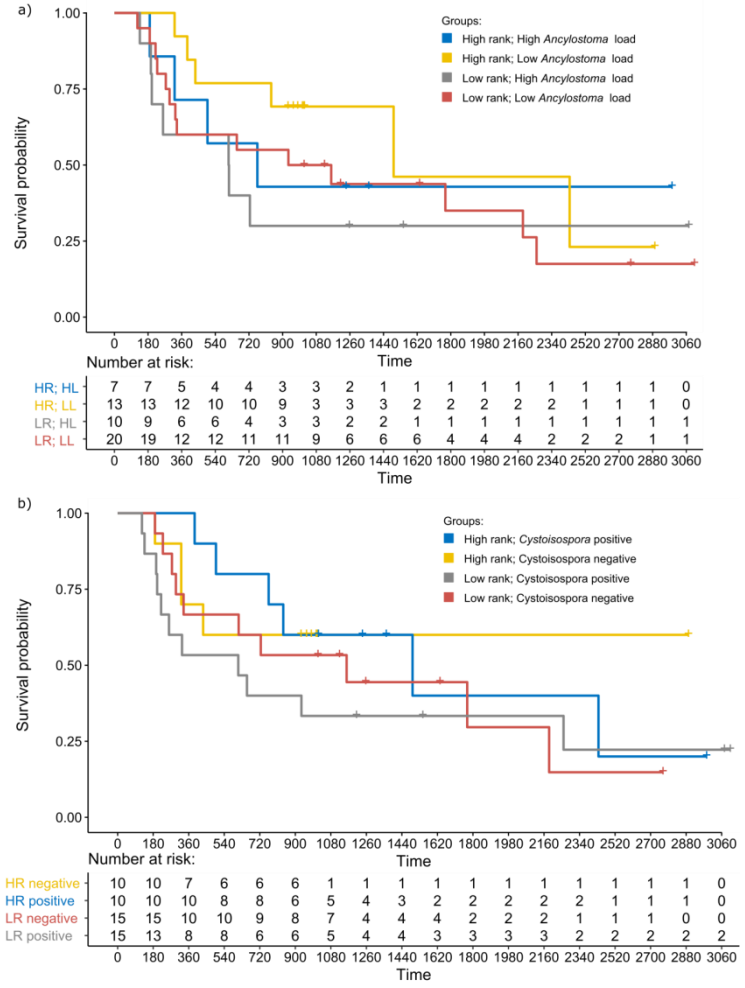


Figure 7. Survivorship of juvenile spotted hyenas sampled before 6 months (180 days) of age (n= 50). a) Juveniles with either high (HL) or low (LL) *Ancylostoma* egg load and either a high-ranking (HR) or low-ranking (LR) mother; b) Juveniles either infected (positive) or not infected (negative) with Cystoisospora and either a high-ranking (HR) or low-ranking (LR) mother. Right-censored measures of survival are marked as tick marks and include males that dispersed (and whose fate could not be subsequently followed), animals killed by road accidents and females that survived until the last day of observation.



Chapter 5 - General discussion

The full set of factors which determine the heterogeneity of parasite infections in individual hosts are not fully understood. Some factors are likely to be essential (key determinants) which are therefore likely to influence exposure and susceptibility to parasite infections in many circumstances (Tables 1 and 2) and contribute to the observed variation. In the introduction to this thesis I reviewed some of the main processes and mechanisms that have been uncovered to contribute to the considerable differences between host individuals in terms of the infection load of the different parasite species they harbour. The aims of this thesis were to investigate key determinants of gastrointestinal parasite infection and its impact on Darwinian fitness. Spotted hyenas in the Serengeti ecosystem are highly interesting to study host-parasite interactions, as they are a long-lived, key carnivore in the ecosystem with a social organisation of similar complexity to that of many old world primate species. This is because the social position a female obtains at adulthood is determined by the social support received from its mother (Smale et al., 1993; East et al., 2009). Furthermore, the exceptionally high maternal input in young in terms of lactation is unique amongst African terrestrial carnivores and this long dependence on milk is likely to have a profound effect on the intestinal biome. The spotted hyena project provides a stimulating opportunity to analyse patterns of infection and its long-term consequences for the host population due to the opportunity to follow individuals across generations.

In the preceding chapters I expanded on the knowledge of the symbiont and particularly parasite community of spotted hyenas in the Serengeti NP, and explored several ecological, demographic, social and physiological determinants of intestinal biome composition, richness and diversity (chapter 2), infection prevalence of the ubiquitous and human relevant parasite *Toxoplasma gondii* (chapter 3) and infection load of energetically costly parasites and its fitness consequences (chapter 4).

5.1 The effect of physiology, diet and the communal den on the intestinal biome and parasites

Juvenile mammals tend to be more susceptible to infections and suffer higher costs associated with infection than adults (East et al., 2008; Chilvers et al., 2009; Marescot et al., 2018). In chapter 4 I show that *Ancylostoma* and *Cystoisospora* infection loads decrease with age, probably as a consequence of the development of competent immune responses (Ramsburg et al., 2003; Hayward, 2013; Shrestha et al., 2015; Simon et al., 2015; Watson et al., 2016). Additionally, glucocorticoid concentrations decrease with age in juveniles of spotted hyenas (Benhaiem et al., 2012a), and therefore a reduction in the negative impact on immune responses by prolonged exposure to circulating glucocorticoids (Sapolsky et al., 2000; Martin, 2009; Hofer and East, 2012) might have an effect on the decrease of parasite loads observed.

Spotted hyenas have a long lactation period of about 12 to18 months (Hofer and East, 1995) and juveniles are stationary at communal dens until they are approximately 12 months old (Hofer and East, 1993a; Golla et al., 1999; Hofer et al., 2016). In chapter 2, I find that the composition of the bacterial microbiome in juveniles differed from that in adult females and overall the intestinal microbiome of juveniles had a low richness and diversity than that of adult females, probably because of the milk-based diet of juveniles. Additionally, the more diverse diet of adults may contribute to the differences in composition, richness and diversity seen. Particularly the consumption of carcasses infected with various parasites, whose intermediate hosts include ungulates, as *Toxoplasma gondii*, may contribute to differences in parasite prevalence between juveniles and adults. This is consistent with the results in chapter 3, where I showed that the seropositivity of antibodies against *T. gondii* was significantly lower in juvenile spotted hyenas than in adults. The effect of carnivorous diet on the increased exposure to *T. gondii* is consistent with our findings that adult spotted hyenas and lions (*Panthera leo*) had a higher proportion of seropositivity than sera from insectivorous bat-eared foxes (*Otocyon megalotis*). In the particular case *of T. gondii*, felids

are the only known species that shed oocysts into the environment (Tenter et al., 2000). Being stationary at the communal den may decrease the exposure of juvenile hyenas to areas contaminated by lions.

On the other hand, communal dens, not only house juveniles but are also social centres for adult clan members and are therefore likely to play an important role as 'pathogen transmission hotspots' for directly transmitted and vector-borne parasites (Woolhouse et al., 1997). Communal dens harbour juveniles that are the most susceptible individuals of the populations, typically with highest pathogen loads, as observed elsewhere (Adelman et al., 2015). Additionally, communal dens are regularly visited by lactating females, which are known to have increased infection loads, as seen for *Ancylostoma* egg loads, in their faeces (East et al., 2015), but also in other mammal species (Cattadori et al. 2005). In line with the idea that communal dens are important hotspots of pathogen transmission, juveniles maintain *Dipylidium* infection in the flea population at communal dens (East et al., 2013). In chapter 3 we found that an increase of juveniles in the den is associated with an increase of *Cystoisospora* loads, suggesting that oocysts shed in the faeces of juveniles increase contamination in the vicinity of the communal den.

Interestingly we found a significant negative association between *Ancylostoma* load and the number of adults in a clan. One plausible explanation could be an encounter-reduction effect whereby adult hyenas reduce the chance of juveniles encountering *Ancylostoma* infective larvae. The role of communal den in the parasite dynamics in spotted hyenas need to be explored further. From observing the three monitored clans, we noticed that the underground burrows used by spotted hyenas as communal dens are regularly changed and so is the location of the den. The relocation of juveniles by their mothers increases the risk of predation by lions. We can speculate that parasite infections, including ectoparasites play a crucial role when making the decision to change dens, although the role of parasites in the relocation of communal dens remains an open question.

5.2 The effect of social environment on the intestinal biome and parasites

Like other species where access to resources depends on social status, high-ranking spotted hyenas are better fed than low-ranking females. This may allow them to allocate more resources to immune responses and thereby achieve a lower infection probability and pathogen infection loads while keeping beneficial symbionts (Hooper et al., 2012). Consistent with this idea, high-ranking hyenas were less infected with the bacterium *Streptococcus equi ruminatorum* (Höner et al., 2012), and the nematode *Ancylostoma* (East et al., 2015) than low-ranking females. Also, offspring of high-ranking females which are suckled more often than those of low-ranking mothers (Hofer et al., 2016) are less likely to become infected with the virus CDV (Marescot et al., 2018). This is in line with the idea that a richer and more diverse eukaryome indicates a more stable community (Zelezniak et al., 2015) that is less prone to disturbance (dysbiosis). Also consistent with this idea are the results presented in chapter 2 indicating that high-ranking females have a richer and more diverse eukaryome than low-ranking females, with distinct composition differences. In contrast, the results presented in chapter 4 indicated that the social status of a mother had no significant effect on *Ancylostoma* and *Cystoisospora* infection loads in their offspring. This finding might indicate that the significant key determinant of immunocompetence in juvenile hyenas is the development of immune responses with age, including acquired immunity rather than the allocation of resources to immune processes.

In social mammals, high-ranking animals are more valuable social partners than low-ranking ones (Seyfarth, 1977) and in spotted hyenas they engage more frequently in greeting ceremonies and are more exposed to viral pathogens with fast transmission such as rabies and CDV (East et al., 1993, 2001; Marescot et al., 2018). Contrary to our prediction, we found no evidence that *Cystoisospora* transmission is influenced by contact rates mediated by social status. It may be that the direct transmission of *Cystoisospora* during greeting ceremonies is

unlikely to result in infection because *Cystoisospora* oocysts have to mature outside the host before they become infective.

5.3 Measuring fitness in wild populations

Parasites, by definition, are costly to their hosts but the outcome of parasite infection varies between hosts (Budischak et al., 2012, 2017). Measuring components of fitness in wild populations is difficult, particularly in relation to parasites with direct and indirect costs of sub-lethal infections, typical for gastrointestinal helminths, in contrast to acute lethal infections typically caused by viral and bacterial organisms (Höner et al., 2006; Benhaiem et al., 2018). In such sub-lethal infections, cross-sectional studies or short-term studies pose serious challenges to measure the cost of the reduction of reproductive success and survival. Using measures of physiological processes can successfully quantify the costs of infection (Budischak et al., 2012; Garrido et al., 2016), although they largely rely on invasive methods. In chapter 4, the effect of *Ancylostoma* and *Cystoisospora* infection loads during early life (in young less than 12 months of age) on the survival to adulthood was investigated. *Ancylostoma* infection can have fitness consequences in juvenile spotted hyenas as juvenile survival to adulthood declined as *Ancylostoma* infection loads increased. *Ancylostoma* is associated with considerable blood loss, malnutrition and growth retardation in humans (Hotez et al., 2005) but also some wild mammal populations (Seguel and Gottdenker, 2017). As *Ancylostoma* is found in most juveniles, it is plausible that this parasite plays an important role in determining population structure of spotted hyenas in the Serengeti NP.

5.4 Conclusions

Mammals and their intestinal biomes have a long co-evolutionary history. The intestinal biome can be considered to be an ecosystem composed of an assemblage of organisms. Direct and indirect interactions between the organisms and their host determine this particular ecosystem structure, in terms of abundance and density of each organism, with fitness consequences to the host. So far, the composition, diversity and

functional role of the intestinal biome, including parasites, and its consequences to the host, at the individual and population level, remain largely open questions. In my thesis, I set out to answer questions on parasite and intestinal biome determinants in spotted hyenas living in a natural ecosystem, and the long-term consequences of energetically costly parasites. I hope my work will serve as foundation for further studies on this exciting field. To better understand host-parasite interactions and host defences, including the mechanisms of resistance and tolerance, it is important to quantify immune responses of the host. The field of immunology traditionally concentrates on a small number of model species, humans and domestic animals. Most current methods to measure immune responses are not suitable to apply in wild populations because they are invasive. Currently there are few non-invasive immunological assays that have been validated for use with faeces from wild mammals, so there is an urgent need to develop new non-invasive methods. Applying non-invasive methods to measure host immune responses to longitudinal studies, particularly when repeated measures of the same individual are available, will be essential to the progress of the understanding of the mechanisms behind host-parasite interactions and the patterns of infection in natural populations.

Intrinsische und extrinsische Determinaten der Infektion mit Parasiten in Tüpfelhyänen des Serengeti Nationalparks

Zusammenfassung:

Obwohl parasitische Infektionen bisher nur in einer sehr begrenzten Anzahl von Wildtierarten untersucht wurden, zeigten diese Studien eine große Vielfalt an Infektionen in Individuen einer Population. Zurzeit ist wenig über die Faktoren bekannt, die für die beobachtete Heterogenität verantwortlich sind. Ebenso wenig Information exisitert über den Einfluss parasitischer Infektionen auf die individuelle Fitness. Zu den Schlüsselfaktoren, die bisher in der Literatur für individuelle Unterschiede in der Infektionslast verschiedener Parasitentaxa verantwortlich gemacht wurden, gehören Umweltfaktoren des Wirts, einschließlich der sozialen Umgebung. Diese beeinflussen, inwiefern der Wirt Parasiten ausgesetzt ist und könnten auch die Entwicklung seines Phänotyps beeinflussen. Ein weiterer Schlüsselfaktor ist die Phase des Lebenszyklus des Wirts, die dessen Anfälligkeit und Immunkompetenz gegenüber parasitischen Infektionen beeinflussen könnte. Das gemeinsame Vorkommen von gastrointestinalen Parasiten, d.h. die Zusammensetzung von Parasitenarten im gastrointestinalen Trakt des Wirts, das intestinale Biom, ist vermutlich ebenfalls ein Schlüsselfaktor für die Höhe der Infektionslast. Über die Zusammensetzung, Diversität und Reichtum des intestinalen Bioms von Wildtieren ist nur wenig bekannt.

In meiner Arbeit untersuchte ich, welche Faktoren gastrointestinale Parasiteninfektionen beeinflussen und ob Parasiteninfektionen einen Einfluss auf die Fitness in einem hochsozialen Säugetier, der Tüpfelhyäne (*Crocuta crocuta*), haben, die in drei als Klans bezeichneten sozialen Gruppen innerhalb des Serengeti-Nationalparks in Tanzania leben. Ich stellte die Hypothese auf, dass individuelle Unterschiede parasitischer Infektionen durch 1) Merkmale des Lebenszyklus, 2) soziale, ökologische und abiotische Umweltfaktoren, 3) die Immunkompetenz des Wirts und 4) das intestinale Biom bestimmt werden.

In Kapitel 2 führte ich die erste Metabarcoding-Studie (*Amplicon Sequencing*) des intestinalen Bioms von weiblichen

Tüpfelhyänen durch, sowohl des Eukaryoms als auch des bakteriellen Mikrobioms. Wie erwartet unterschieden sich juvenile in der Zusammensetzung des bakteriellen Mikrobioms von adulten Tieren und zeigten insgesamt einen geringeren Parasitenreichtum und eine geringe Parasitendiversität. Die Zusammensetzung, der Reichtum und die Diversität des Eukaryoms von Weibchen variierte mit deren sozialem Rang: hochrangige Weibchen wiesen einen höheren Reichtum und Diversität auf als niedrig-rangige Weibchen.

In Kapitel 3 untersuchte ich einen ubiquitären intrazellulären Parasiten warmblütiger Tiere: *Toxoplasma gondii*. Ich untersuchte Seren von Tüpfelhyänen und sympatrisch lebenden Raubtieren. Davon stammten die meisten Proben von adulten Löwen (*Pathera leo*), der häufigsten Großkatzenart in der Serengeti. Außerdem gingen Proben von einer Streifenhyäne (*Hyaena hyaena)* und vier insektivoren Löffelhunden (*Otocyon megalotis*) in die Studie ein. Ich konnte zeigen, dass sowohl bei adulten Tüpfelhyänen als auch bei Löwen eine hohe Seroprävelenz vorliegt. Juvenile Tüpfelhyänen wiesen eine geringere Seroprävalenz als adulte Tiere auf, was auf eine Infektion durch den Verzehr von befallenen Kadavern hinweist, so dass eine mögliche Aufnahme von Oozysten aus der Umwelt keine große Rolle zu spielen scheint. Vor dem Hintergrund, dass *T. gondii* hauptsächlich durch das Verzehren von infiziertem Fleisch übertragen wird, war es keine Überraschung, dass bei keinem der vier untersuchten Löffelhunde Antikörper gegen *T. gondii* festgestellt wurden.

In Kapitel 4 untersuchte ich eine Vielzahl von Faktoren, die wahrscheinlich mit gastrointestinalen Parasiteninfektionen bei juvenilen Tüpfelhyänen in Verbindung stehen. In dieser Studie konzentrierte ich mich auf zwei energetisch kostspielige und weit verbreitete Parasiten: die intrazellulären *Cystoisospora* und der Blut konsumierende, extrazelluläre Hakenwurm *Ancylostoma*. Ziel der Studie war es, Faktoren zu identifizieren, die die Infektionslast beeinflussen. Außerdem untersuchte ich die Auswirkung der Infektionslast auf die Überlebenschance

juveniler Tüpfelhyänen bis zum Erwachsenenalter als Maß für die Darwinsche Fitness.
Meine Ergebnisse zeigen, dass (1) eine hohe Infektionslast mit Hakenwürmern Fitness reduziert (mit zunehmender Infektionslast sank die Überlebensrate juveniler signifikant), (2) die Infektionslast beider Parasiten mit zunehmendem Alter abnahm, was vermutlich an einer mit dem Alter wachsenden Immunkompetenz bei juvenilen Säugetieren liegt, (3) eine hohe Infektionslast in Zusammenhang mit einer hohen Anzahl von ko-infizierenden Taxa steht, was mit einer Herunterregulierung der Immunantwort und einer höheren Anfälligkeit gegenüber anderen ko-infizierenden gastrointestinalen Parasiten zusammenhängen könnte, (4) die Hakenwurm-Infektionslast mit sinkender Anzahl von adulten Hyänen pro Klan abnimmt, was einen „*Encounter-Reduction*"-Effekt vermuten lässt und (5) *Cystoisospora*-Infektionslasten mit zunehmender Anzahl von juvenilen Tieren im Klan steigen, was wahrscheinlich mit einer Übertragung während sozialer Interaktionen zwischen Juvenilen bei den gemeinschaftliche genutzten Bauen und der Verunreinigung der Umgebung des Baus mit Ooozysten in Verbindung steht.
Die Ergebnisse, die ich in meiner Arbeit präsentiere, unterstreichen die Komplexität der Faktoren, welche die Infektion mit Parasiten in Wildtierpopulationen beeinflussen und zu der beträchtlichen Heterogenität zwischen Individuen bezüglich parasitärer Last, Zusammensetzung der Parasitengemeinschaft und ihrer Exposition, führen. Ich konnte zeigen, dass Lebensalter (und sein Einfluss auf die Entwicklung des Immunsystems und der Ernährung), sozialer Status, die Anzahl der erwachsenen Tiere wie die Anzahl der Jungtiere in Gemeinschaftsbauen eine Schlüsselrolle für die infektion mit Parasiten von Tüpfelhyänen spielen. Meine Forschungsarbeit enthüllte zudem, dass eine hohe Infektionslast mit energetisch kostspieligen Parasiten einen negativen Effekt auf wichtige Komponenten der Darwinschen Fitness wie dem Erreichen des Erwachsenenalters hat. Diese Ergebnisse unterstreicht die ökologische und evolutionäre Bedeutung der energieintensiven Parasiten in Wildtsäugetierpopulationen. Zusammenfassend

tragen meine Erkenntnisse zu einem verbesserten Wissensstand über Parasiten-Wirt-Interaktionen und parasitärer Gesellschaften in Wildsäugetierpopulationen bei.

Intrinsic and extrinsic determinants of parasite infections in spotted hyenas in the Serengeti National Park

Summary:
Parasite infections have been examined in a limited number of wild mammalian species. These studies have revealed substantial heterogeneity between individuals within a population. Currently little is known about the Darwinian fitness consequences of parasite infections and the factors determining the observed heterogeneity. Key determinants expected to affect individual differences in the infection load of different parasite taxa are host environmental factors, including the social environment, that influence exposure to parasites, and phenotypic characteristics and life-history stages of individual hosts that may be linked to susceptibility to parasite infections. The biotic environment of gastrointestinal parasites, i.e., the assemblage of organisms found in the gastrointestinal tract of the host, collectively called intestinal biome, is also expected to be a key determinant of infection load. Knowledge is limited on the composition, diversity and richness of the intestinal biomes of wild mammals.
In this thesis I aimed to investigate determinants of gastrointestinal parasite infections and their fitness consequences in a free-ranging population of a highly social large mammal, the spotted hyena (*Crocuta crocuta*), in three social groups termed clans in the Serengeti National Park, Tanzania. I hypothesised that individual variation in parasite infections is determined by 1) life-history traits; 2) social, ecological and abiotic environmental factors; 3) host immuno-competence and 4) the gastrointestinal biome community.
In chapter 2, I conducted the first metabarcoding (amplicon sequencing) study of the intestinal biome of female spotted hyenas, including both the eukaryome and bacterial microbiome. Consistent with my predictions, juveniles differed in the composition of their bacterial microbiomes from adults, and overall showed a low richness and diversity. The composition, richness and diversity of the eukaryome of females varied with their social status, with high-ranking individuals having a higher richness and diversity than low-ranking individuals.

In chapter 3, I investigated the ubiquitous intracellular parasite of warm blooded animals *Toxoplasma gondii*. I analysed sera from spotted hyenas and sympatric carnivores, including mostly adult lions (*Pathera leo*), the most abundant large felid in the Serengeti, and also a striped hyena (*Hyaena hyaena*) and four bat-eared foxes (*Otocyon megalotis*), an insectivore. This study revealed high seropositivity of adult spotted hyenas and lions. Juvenile spotted hyenas had significantly lower seropositivity than adults, suggesting exposure is primarily caused by the consumption of infected carcasses rather than environmental contamination with *T. gondii* oocysts. In line with the likely importance of a carnivorous diet for exposure to *T.gondii* in the Serengeti ecosystem, none of the four analysed bat-eared foxes were positive.

In chapter 4, I explored a range of factors likely to determine gastrointestinal parasite infection in individually known juvenile spotted hyenas. In this study I focused on two energetically costly and common parasites, to investigate determinants of infection loads: the intracellular apicomplexan *Cystoisospora* and the blood feeding, extracellular hookworm (nematode) *Ancylostoma*. I also assessed the fitness consequences of individual infection load in terms of juvenile survival to adulthood. The results indicate that (1) high hookworm infection load decreased fitness, as the chance of juvenile survival significantly declined as *Ancylostoma* infection load increased; (2) infection loads of both parasites decreased with age, most likely because immunocompetence in juvenile mammals increases with age; (3) high infection loads were associated with an increase in the number of co-infecting taxa, suggesting a downregulation of immune responses and increased susceptibility to other co-infecting gastrointestinal parasites; (4) hookworm infection load decreased as the number of adult hyenas per clan increased, suggesting an encounter-reduction effect, and (5) *Cystoisospora* infection load increased as the number of juveniles in the clan increased, suggesting that both transmission during social interactions between juveniles at communal dens and environmental contamination with oocysts at den sites contributed to infection.

Overall, the findings presented in my thesis highlight the complex range of factors that affect parasite infections in wild mammal populations and contribute to the considerable heterogeneity between individuals in terms of parasite infection load, parasite community composition and parasite exposure. I found that age (and its effect on both the development of immune processes and diet), social status, the number of juveniles and the number of adults at clan communal dens were key drivers of parasite infection in spotted hyenas. My research revealed that high infection loads with an energetically costly parasite had a negative effect on an important fitness component in juveniles, i.e., survival to adulthood. This finding highlights the ecological and evolutionary importance of such energetically costly parasites in wild mammal populations. Together, the findings in my thesis contribute to the body of knowledge on parasite – host interactions and parasite communities in wild mammalian populations.

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Publication 2:
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Publication 3:
Ferreira, S.C.M., Hofer, H., Madeira de Carvalho, L., East, M.L., 2019a. Parasite infections in a social carnivore: Evidence of their fitness consequences and factors modulating infection load. Ecol. Evol. 9, 8783–8799. DOI: 10.1002/ece3.5431

Publication 4:
Seltmann, A., Webster, F., Ferreira, S.C.M., Czirják, G., and Wachter, B. (2019). Age-specific gastrointestinal parasite shedding in free-ranging cheetahs (*Acinonyx jubatus*) on Namibian farmland. Parasitol Res. 118(3), 851-859. DOI: 10.1007/s00436-018-6190-2

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Acknowledgments
I would like to first acknowledge the support I received from my supervisors, Marion East and Heribert Hofer. Thank you for the scientific discussions, for discussions on philosophy and feminism, and for the encouragement to keep my interests broad. Above all, for giving me the opportunity to be part of the hyena research project and fort the amazing opportunity of doing field work in the Serengeti National Park.
I would like to thank my advisors, Emanuel Heitlinger, Gábor Czirják, Sarah Benhaiem and Svenja Steinfelder for their valuable guidance and expert advice. Thank you Emanuel for encouraging me to learn bioinformatics. Thank you Gábor and Sarah for the moral support and relaxing words when needed.
An important part of my project was organising the field and laboratory work requiring me to dig into foreign bureaucracy. I must thank Dagmar Thierer for taking over so many times, Dagmar Boris for the patience, and Stephan Karl and Katja Pohle for their help and for sharing their experience with me.
I would like to thank my graduate schools, GRK2046, ZIBI and DRS, for funding and scientific support.
During the seven months of field work in Serengeti I had truly a special time. During my stay I met incredible people that help me overcome the hurdles of field work and with whom I shared many sunsets. Thank you Sonja Metzger for introducing me to the hyenas and teaching me about African animals. Asante sana squash banana Stan Mwampeta, kwa kua rafiki yangu wa kwanza Tanzania, kwa kunishibisha baada yankazi nyingi za porini, kwa vipindi vya kutambua ndege, kwa kunifanya nifurahi hata pale mambo yanapoenda mrama na kwa kunikaribisha nyumbani kwako. Asante sana Thomas Shabani for always finding a solution in the research station. I would also like to thank Dr. Robert Fyumagwa for his support and Annie Francis and Gideon Orio for the support in Arusha. Thank you Louis for the many games you made me loose at badminton.
In the past 3 years I had incredible colleagues that quickly became friends. I thank every single member of the “Parasite group”. Most notably, I want to acknowledge Alice, Francesca and Totta for the informal discussions on parasites, even when

all I wanted was a quiet evening with my friends. I would also like to thank my family: obrigada mãe, pai e irmão.
Finally, a heartfelt thank you to Timo, for keeping me fed, sane and happy.

Finanzierungsquellen
Funded by the Research Training Group 2046 “Parasite Infections: From Experimental Models to Natural Systems” funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) and the Leibniz-Institute for Zoo and Wildlife Research.

Interessenskonflikte
Es besteht kein Interessenskonflikt durch finanzielle Unterstützung der Arbeiten.

Selbständigkeitserklärung
Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin 13.08.2019 Susana Ferreira