AN ABSTRACT OF THE DISSERTATION OF

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Title: INFECTIOUS DISEASES OF THE FELIDAE: PARASITE COMMUNITIES FROM MINIATURE TO MASSIVE

Abstract approved: _____

Anna Jolles

Direct anthropogenic stressors have caused drastic declines in wildlife populations over the past two centuries. In the face of these threats, spillover of infectious disease from domestic animals and livestock into wildlife, and novel interactions between parasites and pathogens within wildlife communities, have further suppressed already vulnerable populations. As management officials and conservationists fight to counteract these influences, a sound understanding of how pathogens and parasites interact to shape host health, and the level of disease threat posed from outside species, has become paramount to ensuring long term population viability. Here I use two model systems to examine these interactions.

The first study system (Chapters 1, 2, and 3) examines the potential implications of an immunosuppressive lentivirus, feline immunodeficiency virus (FIV), for structuring host health, immunity, and coinfection dynamics in a population of 219 free-ranging African lions (*Panthera leo)* living in Kruger National Park, South Africa. Similar to HIV in humans, FIV has been linked to an AIDS-like syndrome in domestic cats characterized by decreased functional immunity; alterations to biochemical, histological, and serological markers; and increased susceptibility to other parasites and pathogens. While recent evidence suggests similar mechanisms may be at play in FIV-infected lion hosts, less is known about the health implications of FIV for this species or what role coinfections may play in structuring disease outcome. In Chapter 1 I set the stage for this investigation by expanding the available toolbox for lion health research and creating a set of normal reference intervals against which health metrics for free-ranging lions can be compared. Using metrics and tools established in Chapter 1, I then use Chapters 2 and 3 to show that FIV exhibits wide scale immunosuppressive and

negative health effects within lion hosts, but that parasite and pathogen communities facilitated by the virus may be of equal or greater importance for determining both health outcome and susceptibility to other coinfections when compared to FIV alone.

In the second study system, I examine disease prevalence and incidence in a common, highly adaptable species, the feral cat (*Felis catus)*, to determine its potential as a disease reservoir for sympatrically dwelling human and animal populations. Using a healthy population of 129 feral cats presented at a local trap-neuter-release program in Portland, Oregon, I show that prevalence and incidence of viral and bacterial pathogens, as well as endo- and ectoparasites, is high among this untreated population. Together, findings of these respective studies show potential points for disease intervention on both the parasite and host level.

INFECTIOUS DISEASES OF THE FELIDAE: PARASITE COMMUNITIES FROM MINIATURE TO MASSIVE

by
Heather Broughton

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APPROVED:

Major Professor representing Integrative Biology

Chair of the Department of Integrative Biology

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Heather Broughton, Author

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CONTRIBUTION OF AUTHORS

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Chapter 1: Dr. Patrick Chappell was directly involved in the development of RIA methods used to measure endocrine markers for leptin and ghrelin against which clinical normals could be established and malnutrition evaluated for FIV-positive and negative populations.

Chapter 2: Dr. Emmanuel Serrano served as our resident statistical genius for this chapter through his adaptation of a partial least squares structural equation model to our data in order to evaluate causal relationships between FIV, coinfections, immunity, and impaired clinical health.

Chapter 3: Dr. Erin Gorsich is a postdoc in our lab and was the brain behind the monopartite network and cluster analysis used in this chapter to evaluate parasite community assemblages and host-parasite connectivity between FIV-positive and FIV-negative lion networks.

Chapter 4: Technician Leah Kennon is the primary overseer of all Feral Cat Coalition operations in Portland, Oregon and was the sole reason we were allowed to collect cat samples alongside their spay-and-neuter efforts. Drs. Ling Jin, Kathy O'Reiley, Janelle Bishop-Stewart, and Jana Gordon helped to collect data for this study through their supervision of diagnostic laboratory assays used to detect the various pathogens identified in the study.

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INTRODUCTION

Human population expansion, leading to the encroachment of humans into wildlife areas and overlap with previously disparate species, has heralded challenges for disease control and prevention both within human and animal communities (Daszak et al., 2000). As overlap between species continues to grow, other anthropogenically derived ecological changes to host and parasite systems continue to increase the potential for emergence of novel or previously innocuous disease threats (Schrag & Wiener, 1995; Daszak et al., 2000). In the face of these threats, extant species have entered a race for survival, where they can either adapt to new exposures or face negative consequences for fitness and survival.

While traditional concepts of adaptive evolution rely on genotypic selection for favorable traits over long expanses of time, rapidly changing disease threats in response to human-derived shifts may dictate adaptation through phenotypically determined behavioral plasticity rather than genetic mechanisms, with certain species less able to keep up (Sih et al., 2011; Cain et al., 2011). As changes occur, behavioral modifications may predict species survival in the face of anthropogenic stressors, while others may foretell of eventual extinction. Primarily, these favorable adaptations include the following: 1) ability to avoid or coexist with natural or novel enemies or competitors; 2) ability to utilize new temporal, spatial, or trophic niches in response to increasing competitive demand; 3) development of coping mechanisms to mitigate stressors; and 4) adaptation to ecosystem level changes such as population fragmentation, biodiversity loss, and changing biotic and abiotic threats including disease emergence (Sih et al., 2011; Rymer et al., 2013).

For less adaptable species, such as those with physiologically dictated home ranges, high or specific resource requirements, and foraging/predation habits that put them in direct conflict with humans, restriction to smaller, geographically isolated patches of habitat places them at substantial risk for population declines (Kissui & Packer, 2004; Daszak et al., 2000; Ferreira & Funston, 2010; Cain et al., 2011). As a result, changes to even one of the aforementioned stressors, such as disease emergence, may tip small populations into an extinction vortex (Cain et al., 2011). Meanwhile, more adaptable species with behavioral traits that confer resilience or even favorability towards human population expansion may be less impacted and provide a potential reservoir for disease to already vulnerable populations (Begon & Bowers, 1995; McCallum & Dobson, 1995; Murray et al., 1999; Cleaveland et al., 2007).

As these threats grow, understanding the community dynamics of infectious parasites and pathogens that may shape health and population resilience within imperiled wildlife systems, as well as the potential for some species to pose as reservoirs for disease emergence, has become critical in buffering already declining populations against anthropogenic change. In response to these challenges, the work presented here investigates parasite communities in two separate model systems, African lions (*Panthera leo)* and feral cats (*Felis catus),* on either side of the ecological continuum. Primarily we ask:

1. What impacts do coinfection and parasite community assemblages carry for health, immunity, and disease susceptibility in a species threatened by anthropogenic change? (Chapters 1, 2, 3)
2. What potential exists for other, more adaptable species to serve as reservoir hosts for disease in humans, domestic animals, and imperiled wildlife? (Chapter 4)

To achieve these goals, the first study utilizes a model system, Feline Immunodeficiency Virus (FIV) in African lions, to examine the role of parasite assemblages for shaping host health, immunity, and coinfection patterns in a study population of 219 free-ranging lions residing in Kruger National Park (KNP), South Africa. Over the past two decades lion populations have declined by 43%, leading to extirpation over much of their historical range (Riggio, 2013; Bauer et al., 2016). These changes have been largely mediated by anthropogenic stressors, including overharvesting due to improperly imposed hunting quotas or illegal poaching (Lindsey et al., 2012; Packer et al., 2009; Packer et al., 2011; Riggio et al., 2013); epizootics introduced from surrounding domestic animal and livestock populations (Roelke-Parker et al., 1996; Renwick et al., 2007); and direct human conflict stemming from reductions to naturally large home range and reduced prey access, which place lions and humans at odds where land and livestock are concerned (Ikanda & Packer, 2008; Lehmann et al., 2008; Dolrenry et al., 2014).

As a stronghold for wildlife conservation, KNP supports a viable lion population of approximately 1700 individuals bolstered largely by the park's bountiful supply of natural prey species, large expanses of undeveloped, uncontested space, and abundance of riparian habitat along natural water sources, which serves as a favorite hunting ground for this threatened large predator (Lehmann et al., 2008; Ferreira & Funston, 2010; Loarie et al., 2013). While the KNP lion population has remained relatively stable despite drastic declines in other populations throughout much of Africa, the emergence of a novel pathogen, *Mycobacterium bovis* (bTB),

from domestic livestock into Cape buffalo (*Syncerus caffer)* in the 1960s, with subsequent transmission to lions via infected buffalo meat, has prompted new concerns about this species' ability to cope with new pathogenic exposures (Michel et al., 2009).

Recent studies have shown that parasite and pathogen infracommunities may play a stronger or equal role in shaping disease progression and severity within coinfected hosts (Petney & Andrews, 1998; Pedersen & Fenton, 2007; Graham, 2008; Telfer et al., 2010; Ezenwa & Jolles, 2011). Through top-down and bottom-up mechanisms, pathogen and parasite assemblages may shape transmission success, disease persistence, and pathogenicity of parasites in a host population. These mechanisms may be either facilitative or inhibitory, with characteristics like destruction of mechanical barriers, cross-regulation of the host immune response, or immunosuppression carrying a positive association with pathogen invasion (Petney & Andrews, 1998; Pedersen & Fenton, 2007; Ezenwa & Jolles, 2011); while mechanisms like cross-immunity and resource competition limit pathogen success (Petney & Andrews, 1998; Graham, 2008; Telfer et al., 2010).

Feline Immunodeficiency Virus (FIV), a pathogenic lentivirus related to human and simian immunodeficiency viruses (HIV and SIV, respectively), has been identified in up to 40% of lions within the park boundary (Alexander et al., 2008). As in humans, FIV is known to cause an acquired immunodeficiency syndrome (AIDS) in domestic cats (Pedersen et al., 1987, Gardner, 1991) with immunosuppression and disease symptoms closely resembling AIDS in humans (Pedersen et al., 2001; VandeWoude & Apetrei, 2006; Hartmann, 2011; Tejerizo et al., 2012). While previous studies in wild species have identified this virus as an innocuous, endemic pathogen (Hofmann-Lehmann et al., 1996; Brown et al., 1994, Packer et al., 1999; Troyer et al., 2004), recent findings reveal that FIV-Ple (the strain of FIV known to infect African lions) may carry immune and health consequences for lions through CD4+ cell depletion along with clinical, biochemical, histological, and serological abnormalities consistent with feline AIDS (Roelke et al., 2006; Roelke et al., 2009).

As with other immunosuppressive pathogens, FIV in cats has been shown to exacerbate the occurrence and pathogenicity of other pathogens within its host system (Hartmann, 2011). Despite these trends, little research has been done to investigate the role of FIV as a mediator of parasite community assemblages and health outcomes in African lions. As such, this three-part

study sought to investigate these dynamics using a suite of analytical tools to compare health and coinfections in FIV-infected and –uninfected lions at KNP.

Chapter 1 sets the baseline for this comparison by using validated laboratory methods to expand the available tools used to measure health and immune outcomes in African lions. Previously, health studies in free-ranging lions have been confined by a lack of validated laboratory assays and published reference ranges against which to compare wildlife health values. Here, clinical health and demographic data were compared against biochemical analytes, immune and hematologic parameters, and endocrine markers using parametric bootstrapping techniques in order to establish a set of normal reference values that may be utilized for future lion health studies.

Next, utilizing the findings and methods from Chapter 1, Chapter 2 investigates the role of FIV and associated coinfections for determining immune and health outcomes in infected lion hosts. Specifically, we use mixed models and a path analysis to compare 30 health and immune metrics against FIV and 21 other parasitic coinfections in order to determine causal associations between metrics for FIV, coinfection, impaired immunity, and decreased clinical health.

Extrapolating from the second chapter, Chapter 3 uses mixed models, cluster, and network analyses to investigate parasite community groupings, as well as FIV's role in determining connectivity of host and pathogen networks. Through these analyses we pinpoint what parasites tend to affiliate, which ones tend to drive a higher prevalence of coinfection, and ultimately which parasites may be therapeutically targeted to reduce disease burdens in the face of novel disease threats.

The second study system investigates the potential of a highly abundant and adaptable host, the feral cat (*Felis catus)*, to serve as a disease reservoir for surrounding human and animal communities. High fecundity, small size, behavioral compatibility, and the general perception of cats as a pet and rodent control species have favored the growth of cat populations alongside human population expansion (Wodzicki, 1973). Within the United States alone, between 60 and 73 million feral cats live within proximity to wildlife refuges and human dwellings (Levy et al., 2003; Luria et al., 2004). While feral cats play an active role in the suppression of rodent borne diseases by serving as a biological control species, high population densities and low access to preventative health care may put these animals at higher risk for the establishment of disease reservoir populations.

While this concept is problematic in and of itself due to the implications for animal welfare, establishment of reservoir populations within feral cats may also carry a disproportionately high cost for surrounding wildlife species. Disease spillover and the emergence of novel pathogens from domestic reservoir hosts places added stress on already impacted wildlife populations by serving as continual source of new infection for threatened species (Hatcher & Dunn, 2011). Traditional epidemiology uses threshold density, N_T, which is defined as the host population size necessary to maintain transmission of a pathogen or parasite, to determine disease maintenance and risk within a population (Hatcher & Dunn, 2011). Following this principle, untreated domestic reservoir species can present a new disease threat for already threatened species by providing a mechanism for pathogen escape from the wild host population, and thereby preventing pathogen extinction despite small host population size. This in turn potentiates the risk of extinction in already small populations (Daszak et al., 2000).

To investigate the threat of disease within feral cat populations, Chapter 4 presents a study conducted alongside local non-profit trap-neuter-release programs in Portland, Oregon to collect disease prevalence data from otherwise healthy feral cat populations. Working collaboratively, data collected for this study provides a snapshot of the viral, bacterial, and endo-/ecto-parasitic and pathogenic infections that may be maintained in untreated feral populations, as well as potential areas that may be targeted alongside spay/neuter campaigns to promote health both within feral cats and surrounding animal and human communities.

CHAPTER 1: BRIDGING GAPS BETWEEN ZOO AND WILDLIFE MEDICINE: ESTABLISHING REFERENCE INTERVALS FOR FREE-RANGING AFRICAN LIONS (PANTHERA LEO)

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ABSTRACT

The International Species Information System (ISIS) has set forth an extensive database of reference intervals for zoological species, allowing veterinarians and game park officials to distinguish normal health parameters from underlying disease processes in captive wildlife. However, several recent studies comparing reference values from captive and free-ranging animals have found significant variation between populations, necessitating the development of separate reference intervals in free-ranging wildlife to aid in the interpretation of health data. Thus, this study characterizes reference intervals for six biochemical analytes, eleven hematologic or immune parameters, and three hormones using samples from 219 free-ranging African lions (*Panthera leo)* captured in Kruger National Park, South Africa. Using the original sample population, exclusion criteria based on physical examination were applied to yield a final reference population of 52 clinically normal lions. Reference intervals were then generated via 90% confidence intervals on log-transformed data using parametric bootstrapping techniques. In addition to the generation of reference intervals, linear mixed-effect models (LMEMs) and generalized linear mixed-effect models (GLMMs) were used to model associations of each focal parameter with the following independent variables: age, sex, and body condition score (BCS). Age and sex were statistically significant drivers for changes in hepatic enzymes, renal values, hematologic parameters, and leptin, a hormone related to body fat stores. Body condition was positively correlated with changes in monocyte counts. Given the large variation in reference values taken from captive versus free-ranging lions, it is our hope that this study will serve as a baseline for future clinical evaluations and biomedical research targeting free-ranging African lions.

1.1 INTRODUCTION

The establishment of reference intervals is standard practice in veterinary and human medicine, providing researchers and health care professionals with an estimated range of biological values for healthy populations against which they can interpret diagnostic data and make clinical decisions (Friedrichs et al., 2006). However, while reference intervals provide insight into underlying disease processes and can be used as markers to guide treatments or rule out disease conditions, reference intervals are prone to a wide range of error and variation.

Variations may result from differences in geographic location, individual laboratory, instrumentation, pre-analytical preparation, reference population, and species (Friedrichs et al., 2006; Gunn-Christie et al., 2012). Particularly, recent studies have shown substantial differences between reference intervals established in captive animals versus those determined for free-ranging wildlife (Brenner et al., 2002; Zuccarelli, 2004; Beechler et al., 2009; Garcia et al., 2010; Moen et al., 2010; Ruykys et al., 2012; Maas et al., 2013; Schook et al., 2015). These differences, which have been shown in multiple species including warru (*Petrogale lateralis),* bog turtles (*Clemmys muhlenbergii),* black rhinoceros (*Diceros bicornis),* Iberian and Canadian lynx (*Lynx pardinus and Lynx canadensis,* respectively*),* Nile crocodiles (*Crocodylus niloticus),* and giraffe (*Girrafa camelopardalis)* among others, have been attributed to differences in nutrition, stress, behavior, pathogenic exposures, and other biotic and abiotic variables between captive and free-ranging populations (Brenner et al., 2002; Zuccarelli, 2004; El Balaa & Blouin-Demers, 2010; Garcia et al., 2010; Osthoff et al., 2010; Schmidt et al., 2011; Ruykys et al., 2012; Schook et al., 2015). Variations in health parameters detected by these studies ranged widely from innocuous differences in body mass index and other morphometrics to more insidious changes in hepatic and renal biochemistry markers, glucose levels, insulin circulation, adiposity, hematological values, and systemic inflammation (Brennar et al., 2002; Zuccarelli, 2004; Beechler et al., 2009; Garcia et al., 2010; Moen et al., 2010; Osthoff et al., 2010; Ruykys et al., 2012; Maas et al., 2013). Thus, while current reference intervals may be sufficient for interpretation of data from captive animals, application of these ranges to free-ranging wildlife may be problematic.

To reduce variation and eliminate erroneous interpretation of results, the National Committee for Clinical Laboratory Standards (NCCLS) and the American Society of Veterinary

Clinical Pathology (ASVCP) have established guidelines for creating reference intervals among different populations (Friedrichs et al., 2006; Gunn-Christie et al., 2012; Paltrinieri et al., 2014). Thus far, these guidelines have been applied extensively by the International Species Information System (ISIS) and outside agencies to determine reference intervals for captive wildlife species. However, while these values have historically been used for reference in free-ranging wildlife species, recent efforts to establish separate intervals for these discrepant groups is an expanding area of interest due to previously mentioned differences between the two populations.

Given the lack of clinical data currently available, this study sought to determine reference intervals for a population of free-ranging African lions (*Panthera leo)* residing in Kruger National Park, South Africa, which serves as stronghold for lion populations. While there has been a recent upsurge in studies on lion health following their listing as "threatened" by the International Union for Conservation of Nature (IUCN), reference data for free-ranging lions are still unavailable for many important health parameters (Bauer et al., 2015). Thus, as part of a wider study on lion diseases and health, this study used guidelines set forth by the ASVCP to establish a set of reference intervals for six biochemical parameters; eleven hematologic or immune parameters; and three endocrine parameters commonly used to determine health and immunity in lion disease studies (Table 1.1).

1.2 METHODS

Preanalytical methods

Study system: Samples were obtained from a study on the impacts of bovine tuberculosis on the lion population in Kruger National Park, South Africa, which involved chemical immobilization of 219 free-ranging lions over a four-year period from March 2010 to September 2014. Throughout the study, all animals were captured at night during the dry season (March through November) to minimize circadian and seasonal variation in health parameters. Animals were baited using a mobile call-up station and freshly acquired bait carcass. Lions were tranquilized by South African National Park (SANParks) veterinarians via intramuscular injection using a CO_2 powered dart rifle (Dan-Inject International, Skukuza, South Africa). Each animal received a combination of either butorphanol (0.31 ± 0.034 mg/kg; Kyron Laboratories Ltd, South Africa), midazolam (0.21 ± 0.024 mg/kg; Kyron Laboratories Ltd, South Africa), and medetomidine (0.052 ± 0.006 mg/kg; Kyron Laboratories Ltd, South Africa); or tiletamine-

zolazepam (1.8 ± 0.5mg/kg; Kyron Laboratories Ltd, South Africa), and medetomidine (0.07 ± 0.01mg/kg; Kyron Laboratories Ltd, South Africa) as previously validated for use in lions (Jacquier et al., 2006; Wenger et al., 2010). Only animals over six months of age were darted due to risk of adverse reactions associated with small body size. After sample collection, animals received atipamezole (0.3 ± 0.1mg/kg; Orion Pharma, Finland) intramuscularly for reversal (Jacquier et al., 2006; Wenger et al., 2010). All capture and sampling protocols were approved by SANParks Animal Use and Care Committee (AUCC #FERSM5-767).

Sample collection and handling: Blood from each study animal was collected into redtop serum, EDTA, sodium heparin, erythrocyte sedimentation rate (ESR), and Cyto-Chex® tubes via jugular or femoral venipuncture. For the purposes of the various diagnostic assays, serum from red-top tubes was used for the detection of biochemical analytes, testosterone, and ghrelin; plasma and blood taken from sodium heparin tubes were used for the measurement of leptin, total solids, and packed cell volume; EDTA whole blood was used for total white blood cell counts and blood differentials; ESR whole blood was used for the ESR assay; and whole blood from Cyto-Chex® tubes was used for flow cytometric evaluation of specific T and B cell subsets.

In order to reduce analyte degradation and variation between sampling groups, blood to be used in the hormone assays was placed on ice for transport back to the lab and processed within eight hours to prevent analyte degradation. After separation, serum taken from red top tubes to be used for the ghrelin assay was further stabilized by adding hydrochloric acid prior to freezing. Blood for use in the other assays was transported at room temperature and constituents separated within eight hours. Though this time delay is likely to lead to the degradation of some analytes, the eight-hour cut-off was chosen in order to mimic standard field study conditions, where immediate processing of sample is often not feasible. All samples, excluding those used for flow cytometry, were aliquoted and stored at -60°C until use. Blood samples used for flow cytometry were stored in the original Cyto-Chex® tubes (VWR International, Radnor, Pennsylvania, USA) at 4°C for a maximum of two weeks before being processed in order to avoid the breakdown of cell markers.

Demographics and health

At the time of capture we recorded age, sex, and pride membership and conducted a physical exam, which included the following parameters: body condition, presence/absence of

suppurative lesions, lymph node enlargement, dehydration, ocular lesions, hygromas, periostitis, and gingivitis. These parameters were then used to determine eligibility for inclusion in the reference population.

Demographics: The sex of each captured individual was noted, and its age assessed using the presence/absence of deciduous vs permanent teeth, tooth wear patterns, pigmentation of the gingiva and nasal planum, and in males fullness and coloration of the mane (Smuts et al., 1978). In addition, pride membership was recorded from observed behavioral monitoring data provided by the larger parent study (Ferreira unpubl. data). This parameter was used as a random effect in place of capture location to avoid pseudoreplication among individuals from the same pride while accounting for potential drivers of our dependent variables including geologic substrate, prey density, and access to water (Fig. 1.1; Ferreira & Funston, 2010).

Health: Body condition score was determined from the degree of fat cover over the ribs, sacrum, lumbar vertebrae, stomach, and wings of the ilium. Animals were assigned a score from one to five, with animals showing a high degree of emaciation and easy palpation of boney prominences being assigned a score of one and healthy animals with normal to heavy fat cover a score of five (Ferreira & Funston, 2010). The presence of external lesions was also evaluated and the type of lesion was noted. Suppurative wounds were recorded separately and treated with antibiotic if severe.

Lymph node enlargement was recorded for the submandibular, axillary, prescapular, inguinal, popliteal, and other lymph nodes. Each lymph node was scored as either enlarged (one), normal (zero), or reduced (negative one). Total systemic lymph node enlargement was then scored on an additive scale from negative six to six by tallying each node's score together, with negative six being all lymph nodes reduced and six all nodes enlarged. Reduced nodes were classified as those nodes that could not be readily palpated or were significantly smaller and less firm than normal, while enlarged nodes were classified as those nodes that were at least twice the size and more firm when compared to a normal node. During evaluation, relative size and age were taken into consideration in order to account for anatomical variations.

Evidence of dehydration was evaluated from sunkeness of the eyes and skin turgor over the ribs. For the purpose of this study, animals with any sign of dehydration (skin tenting greater than two seconds or sunkeness of the eyes) were recorded as dehydrated. Ocular lesions were

also recorded during examination of the eyes and included any evidence of corneal ulceration or uveitis.

Hygromas, which most commonly appeared as a sterile fluid pocket palpable over the elbow joint, were recorded when observed. The diagnosis for periostitis was presumptive only and was based on physical examination showing any abnormal boney prominence that could be palpated. Possible causes for these two findings were not elucidated.

Gingivitis was ranked on a scale of zero to four, with zero representing no sign of redness or gingival erythema and four being severe erythema and ulcerations (Roelke et al., 2009). During the evaluation of the oral cavity papillomas, seen as raised growths under the tongue and in the buccal cavity, were also recorded.

In addition to the above parameters, female animals were evaluated for reproductive status via abdominal palpation for pregnancy, lactation via manual milking, and presence of cubs through behavioral monitoring.

Exclusion criteria

For the establishment of baseline reference intervals only clinically normal animals were included in the analysis, which included 52 of the original 219 animals captured. Animals were excluded from the reference group if they exhibited any of the following: Body condition score less than three, dehydration, hygromas, lymph node score less than negative two or greater than two, periostitis, pregnancy, lactation, severe suppurative wounds, or gingivitis score greater than two.

Analytical methods

Biochemical parameters: Serum was used for the evaluation of all biochemical parameters except for total solids, which was taken from heparinized plasma. A commercial Prep Profile II (Abaxis Inc.; PN 500-7124) was used on a Vet Scan VS2 chemistry analyzer (Abaxis Inc., Union City, California, USA) to evaluate the following serum enzymes: Alanine aminotransferase (ALT), alkaline phosphatase (ALP), blood urea nitrogen (BUN), creatinine (CRE), and glucose (GLU). Total solids (TS) were evaluated manually using a drop of plasma on a refractometer (Optika, Ponteranica, Italy; PN HR-160).

Hematologic and immune parameters: Total white blood cell counts were conducted from EDTA whole blood using an automated hematology analyzer (Vet ABC, Scil Animal Care Company, Gurnee, Illinois, USA). Blood differential counts for the evaluation of lymphocytes

and granulocytes were performed through microscopy on preserved blood smears prepared using EDTA whole blood and Hemacolor Diff-Kwik staining solutions (VWR International, Radnor, Pennsylvania, USA). Packed cell volume (PCV) was performed on heparinized blood using a microhematocrit tube spun at 15000XG for five minutes on a hematocrit centrifuge (Gemmy Industrial Corporation, Taipei, Taiwan; PN HKT-400).

CD4+ T helper cells, CD8+ cytotoxic T cells, CD21+ B cells, and general B and T cell levels were measured using flow cytometry. All cells were marked using a flow cytometric whole blood staining protocol and feline antibodies developed and obtained from Peter Moore (Leukocyte Antigen Biology Laboratory, University of California, Davis, CA, USA). Antibodies were conjugated to FITC labeled horse anti-mouse IgG (Vector Labs, Burlingame, California, USA; PN FI-2000) for radiolabelling as indicated by Moore's protocol. Control tubes without labeling were also included to allow proper gating of cells.

Non-specific inflammation was measured via ESR using equipment and protocols validated in humans (Greiner Bio-One, Lasec Laboratory and Scientific, Cape Town, South Africa; Vacuette ESR Systems; Ndakotsu et al., 2009). Blood for the test was collected directly into ESR tubes containing citrate solution. Samples were measured at 60 and 120-minute intervals.

Endocrinology: Three hormones were evaluated for each animal: Testosterone, leptin, and ghrelin. Testosterone was measured in serum using a commercially available EIA kit (Cayman Chemical, Ann Arbor, Michigan, USA; PN 582701) according to manufacturer's instructions. Initial assays were run in two dilutions (1:3 and 1:6) to test for the interference of contaminants. No interference was found, thus samples were not further altered for use in the assay and samples were run in duplicate at a single dilution (1:3).

Leptin and ghrelin were both run using commercially available RIA kits from Millipore Inc (Millipore Inc., Billerica, Massachusetts, USA; PN XL-85K and GHRA-88HK, respectively). Leptin was evaluated in plasma using the Multispecies Leptin RIA kit, which was previously validated in cheetahs and domestic cats (Depauw et al., 2012). Ghrelin was measured in serum using the Human Acylated Ghrelin kit, as feline ghrelin is analogous to the human form (Martin et. al., 2010).

Statistical analysis

All data used for the generation of reference intervals were evaluated and modeled using MedCalc Statistical Software and R (MedCalc Statistical Software bvba, 2014; R Core Team,

2014). Prior to evaluation, data were examined for violations of normality and constant spread using D'Agostino-Pearson tests and Tukey's Interquartile Ranges (IQR) included in the MedCalc program. Five outliers were identified in the ghrelin data set and excluded from the final analysis due to their strong influence on the data distribution and the high likelihood that they resulted from sample contamination. All other values were in line with model assumptions and were retained for final analysis.

The mean and reference intervals (90% confidence intervals), were generated using log-transformed data and MedCalc's Robust Method, which relies on built-in parametric bootstrapping techniques, as required by small sample size (Friedrichs et al., 2006). These methods were used for analytes for which greater than ten samples were available. Reference intervals were not generated for analytes with ten samples or less and instead raw values and 95% confidence intervals were reported for those analytes to account for increased variation due to a critically low sample size.

Following the generation of reference intervals, linear and generalized linear mixed-effect models (LMEMs and GLMMs, respectively) were modeled in R to test for associations of each focal parameter with the following independent variables: age, sex, and body condition score (BCS; R Core Team, 2014). All two-way interactions were considered, but only ones that were statistically significant were retained for the final model. In addition, a random effect for lion pride was included in each model to account for the fact that, in terms of environmental exposures and disease transmission, lions within a pride cannot be considered independent data points (Bolker et al., 2008; Bates et al., 2015).

Analyses in which data were normally distributed, or were log-transformable to normality as confirmed by a Shapiro-Wilk test (R Stats Package), were fit in R as LMEMs using the nlme Package and a maximum likelihood (ML) estimation (R Core Team, 2014; Pinheiro et al., 2015). Akaike information criterion (AIC) were then used for backwards selection of the final model using the stepAIC function (MASS Package; Venables & Ripley 2002). The final model was fit using a restricted maximum likelihood (REML) estimation and all log-transformed variables were back-transformed for ease of interpretation.

Data that violated assumptions of normality, namely blood urea nitrogen and all discrete count variables, were modeled as GLMMs. Blood urea nitrogen was fit in the lme4 Package using a Laplace Gaussian GLMM with a "log" link and the final model determined via

backwards selection using a combination of AIC values and likelihood ratio tests (Bates et al., 2014). Discrete counts, specifically all of the immune cell measures, were modeled in the MASS Package via a penalized quasi-likelihood Poisson GLMM and the final model was generated using backwards selection reliant on Wald x^2 statistics in the aod Package (Venables & Ripley, 2002; Lesnoff & Lancelot, 2012). Once again, all values were back-transformed prior to reporting.

All final models were confirmed visually using plots of residual versus fitted values and data trends examined using graphical representation (ggplot2 and Rmisc packages; Wickham, 2009; Hope, 2013). Use of LMEMs and GLMMs enabled us to evaluate both continuous (age and BCS) and categorical (pride and sex) variables in one model, allowing for improved statistical power while accounting for potentially confounding variables.

1.3 RESULTS

Reference intervals for all health parameters investigated in this study are presented in Table 1.2 alongside values previously established by ISIS. Due to restricted sample size, reference intervals could not be generated for all flow cytometric values and instead the raw values, mean, standard deviation, and 95% confidence interval are reported separately in Table 1.3.

Model outcomes for demographic associations are presented in Fig. 1.2 and Table 1.4 and include statistically significant interactions with age, sex, and body condition. Age was associated with decreases in overall leukocyte count and ALP, while it was associated with increases in ALT and BUN; PCV and leptin were higher in males than females, but males exhibited lower levels of ALT and BUN; and the only apparent association with body condition was a marginal increase in monocytes as body condition improved.

1.4 DISCUSSION

This study establishes baseline reference intervals for a select set of biochemical, hematologic, immune, and endocrine markers in the largest intact population of free-ranging African lions in southern Africa, providing a benchmark for future biomedical and conservation research targeting this species (Riggio et al., 2013). For comparison, reference values established for other free-ranging big cat species and domestic cats are provided in Table 1.5. The reference intervals determined by this study are similar to those observed in free-ranging Bengal tigers

(*Panthera tigris tigris*), with most values falling in or near currently published confidence intervals (ISIS, 2002; Shrivastav & Singh, 2012). Contrastingly, reference intervals for lions differ sharply from those seen in smaller species such as free-ranging jaguars (*Panthera onca)* and domestic cats (*Felis catus;* Widmer et al., 2012; Merck Veterinary Manual, 2013*).* Despite this, the later is often used as a baseline of comparison for wild felid species, a practice that should be avoided when interpreting data from larger free-ranging felids.

In addition to the above interspecific comparison, this study revealed striking differences between reference intervals for free-ranging and captive lions**.** Overall, reference intervals established in this study tended to be much narrower than those published in ISIS or in the limited previous studies on physiologic parameters in free-ranging lion populations (Erasmus unpubl. data; ISIS, 2002; AZA, 2012; Maas et al., 2013). Notably, ISIS data included very low values in many of its reference intervals, which would have been flagged as clinically significant in this study. This includes low values for all of the leukocyte subsets evaluated here, as well as some of the biochemical indicators of metabolic function – ALT, ALP, CRE and GLU. In addition, the upper boundary of the baseline range for CRE was higher in ISIS values, so much so that in this study it would have been attributed to renal pathology. Contrastingly, BUN was higher in the free-ranging lion populations when compared to captive lions.

There are several plausible explanations for these discrepant findings. Higher immune cell concentrations in free-ranging lions likely reflect persistent exposure to a diverse assemblage of pathogens and parasites that are controlled in captive settings (Maas et al., 2013). Moreover, the age range included in captive populations is likely to be much broader due to lions surviving longer in captivity than in the wild; and a less demanding environment with increased nutritional support in captive lions may mask poor clinical health, leading to the inclusion of lions that would have been excluded in this study (ISIS 2002; AZA 2012). Related to this, clinical data are often limited in studies conducted on free-ranging populations and as such, previous studies may have used more lenient exclusion criteria than what was stipulated in this study (Maas et al., 2013).

Higher BUN in free-ranging lions may be attributable to differences in behavioral ecology and diet compared to captive populations. Free-ranging lions drink as infrequently as once a week and consume a high protein, high blood diet punctuated by protracted periods of starvation, which may contribute to differences in renal physiology and muscle catabolism and lead to

increases of nitrogenous waste within the blood (Schaller, 1972; Thrall, 2004; Stockham & Scott 2008). However, while the slight increase in BUN may be attributed to natural variables, the increases in CRE captured by ISIS values would be pathologic if seen in a free-ranging population, a discrepancy that is most likely attributable to decreased renal function in captive animals of geriatric age (ISIS, 2002).

Age, sex, and body condition were found to have several statistically significant associations with the biochemical, hematologic, and endocrine parameters considered in this study. Increased age was associated with increases in ALT and BUN, while it was associated with decreases in ALP and total leukocytes. All trends were consistent with natural age-related findings in other species, though pathologic changes in immune cell parameters could not be ruled out. Decreases of ALP in older animals were likely attributable to diminishing bone development after the completion of significant growth phases; and increases of ALT probably linked to variations in muscle mass and the accumulation of age-related hepatocellular changes (Stockham & Scott, 2008). Additionally, increases of BUN in older animals are commonly seen in animals relying on a high protein, high blood diet, and in animals with prolonged periods of cachexia, both of which are characteristic of adult lions (Stockham & Scott, 2008; Backlund et al., 2011). Contrastingly, while hematologic changes (decreases in leukocytes) may reflect age or hormone related immunosuppression, they may also reflect increasing prevalence of subclinical infections in older animals. Several diseases are known to be prevalent throughout the KNP lion population (Alexander et al., 2008; Maas et al., 2012). In particular, Feline Immunodeficiency Virus (FIV), a pathogenic lentivirus that has been found with greater than 50% prevalence in Kruger, has been linked with similar hematologic derangements (Broughton unpubl. data; Alexander et al., 2008; Roelke et al., 2009). Thus, while most findings were as expected, the potential role for a subclinical disease or immune pathology should not be ruled out.

PCV and leptin were higher in males and mimicked what we would expect for sex related variation while lower ALT in males was unexpected, but unlikely to bear clinical significance (Thrall, 2004; Stockham & Scott, 2008). Increased PCV, resulting from the production of endogenous steroids, is a common finding in males of most species and increased leptin in males may be due to their larger size and better access to food items compared to females (Schaller et al., 1972; Thrall, 2004; Stockham & Scott, 2008).

Finally, a high body condition score was associated with increased levels of monocytes. While it is possible that this association stems from chronic inflammatory disease in animals of increased condition, it is more likely that this trend is associated with increased immunocompetence in animals with high nutrient availability, though neither explanation can be supported nor rejected based on current findings with the other immune cell parameters (Thrall, 2004). Given these findings, abnormal monocyte values should be interpreted with caution in animals of varying body condition.

This study does have several limitations. First, even though the initial sample size of 219 lions was sizeable for a field study, the sample size of lions that were able to be included in reference range calculations after exclusion criteria were applied was moderate, at 52 lions. This limited its ability to provide separate reference intervals for different age-sex groups of lions, even though some of the parameters examined do vary with age and sex. Additionally, animals were excluded from the study based solely on clinically evident health parameters rather than actual diagnostic measures of infections. It is therefore likely that many animals included for the generation of these reference intervals were harboring subclinical infections with endemic viruses, bacteria, and parasites. However, while inclusion of subclinical animals may affect reference intervals, it would be difficult to justify excluding animals based on subclinical infections given that infection by multiple parasites and pathogens is the norm for free-ranging animals (Jolles et al., 2008). Moreover, it would seem questionable to exclude animals based on some subclinical infections that were diagnosed, when there are doubtlessly many others that this study did not attempt to detect.

Despite the above limitations, we believe that the reference intervals set forth here are relevant owing to the use of robust analytical methods and the fact that subclinical infections, when present, should not cause enough systemic changes to drastically alter host physiology. Thus, this study provides a baseline against which future studies on the health of free-ranging African lions may be compared.

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1.5 TABLES AND FIGURES

Table 1.1 Analytes measured in this study, analyte category, and physiologic interpretation.

ANALYTE	CATEGORY	INTERPRETATION [a]
Alkaline phosphatase (ALP)	Biochemical	Increases associated with bone deposition, liver damage or biliary obstruction, hyperthyroidism, intestinal damage, abnormalities in glucocorticoids, and iatrogenic causes.
Alanine aminotransferase (ALT)	Biochemical	Increases due to hepatocellular damage or issues relating to muscle damage or hyperthyroidism.
Blood urea nitrogen (BUN)	Biochemical	Increases associated with alterations in metabolism, protein digestion, dehydration, and malfunction of the urinary or cardiovascular systems. Decreases associated with liver failure, protein deficiency, and portosystemic shunts.
Creatinine (CRE)	Biochemical	Increases associated with malfunctions of the urinary system. Changes associated with muscle mass and sample age.
Glucose (GLU)	Biochemical	Alterations associated with dietary intake, administration of certain medications or hormones, endocrine disease, pancreatic disease, fever, pregnancy, or stress.
Total solids (TS)	Biochemical	Alterations associated with nutritional disease, protein loss, hydrations levels, inflammation, infection, heart disease, and blood loss.
Packed cell volume	Immune/ hematologic	Measures relative levels of erythrocytes. Increases are associated with absolute or relative polycythemia; decreases associated with blood loss, hemolysis, or bone marrow disease.
Leukocytes	Immune/ hematologic	Measures relative levels of immune cells, including lymphocytes and granulocytes. Various causes.
Neutrophils	Immune/ hematologic	Non-specific immune cells that function for phagocytosis. Increases seen with inflammation, infection, iatrogenic drug administration, stress, and neoplasia. Decreases associated with sepsis, infections, bone marrow abnormalities, neoplasia, and cellular destruction.
Lymphocytes	Immune/ hematologic	Specific immune cells dedicated to antibody production and cell-mediated immunity. Increases associated with disease or neoplasia. Decreases seen due to neoplasia, iatrogenic drug administration, and viral infection.

[a] Information for this table was provided by peer-reviewed journal articles and veterinary textbooks (Shibata et al. 2003; Thrall, 2004; Stockham & Scott, 2008; Ndakotsu et al., 2009; Martin et al., 2010; Levinson 2012; Merck Veterinary Manual 2013).

Table 1.1 *Continued…*

ANALYTE	CATEGORY	INTERPRETATION [a]
Monocytes	Immune/ hematologic	Non-specific immune cells that function for phagocytosis. Increases associated with chronic disease, inflammation, or neoplasia.
Eosinophils	Immune/ hematologic	Non-specific immune cells that increase in association with hypersensitivity reactions, parasitism, injury, neoplasia, or hormonal fluctuations.
Basophils	Immune/ hematologic	Non-specific immune cells that increase in association with hypersensitivity reactions.
CD4+ T helper cells	Immune/ hematologic	Specific immune cells that help maturation of B cells into plasma cells for antibody production, activate cytotoxic T cells, and are involved with delayed hypersensitivity reactions.
CD8+ cytotoxic T cells	Immune/ hematologic	Specific immune cells that mediate the destruction of abnormal cells through initiation of apoptosis or direct destruction of cell membranes.
CD21+ B cells	Immune/ hematologic	Specific immune cells that function for antibody production and immune memory.
Erythrocyte sedimentation rate (ESR)	Immune/ hematologic	Increases associated with non-specific systemic inflammation.
Leptin	Endocrine	Produced by adipose tissue. Increases associated with higher body fat content.
Ghrelin	Endocrine	Secreted by gastric tissue. Increases in response to prolonged periods of fasting or starvation.
Testosterone	Endocrine	Increases associated with male sexual development, increased male sexual behavior, sperm production, aggression, and non-reproductive tissue development.

[a] Information for this table was provided by peer-reviewed journal articles and veterinary textbooks (Shibata et al. 2003; Thrall, 2004; Stockham & Scott, 2008; Ndakotsu et al., 2009; Martin et al., 2010; Levinson 2012; Merck Veterinary Manual 2013).

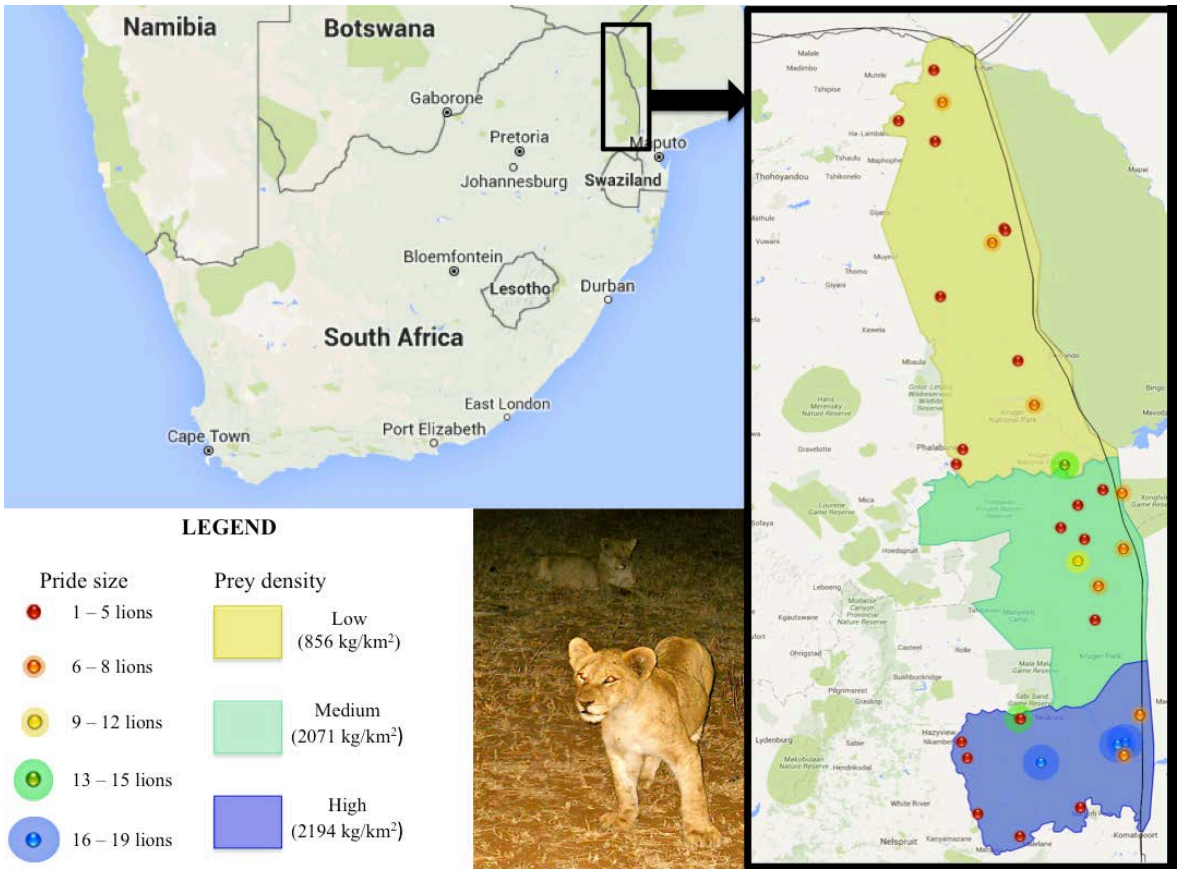

Fig. 1.1 Geographic distribution of sampled lion prides within Kruger National Park, South Africa. Markers illustrate regional prey density and pride size as indicated by the legend above. Data for prey density taken from Ferreira and Funston 2010. Photo courtesy of Heather Broughton (lion cub). Maps and data points generated in Maptive LLC (Maptive LLC, 2016).

Table 1.2 Select reference intervals for 16 commonly evaluated health parameters in free-ranging African lions.

PARAMETER	REFERENCE INTERVAL	MEAN	(n)	ISIS VALUES [a]
ALP (U/L)	6.8 - 86.0	24	52	0 - 168
ALT (U/L)	6.6 - 91.4	23.5	52	0 - 195
BUN (mg/dl)	8.3 - 78.5	45.7	52	13 - 70
CRE (mg/dl)	0.8 - 2.6	1.7	52	0 - 4.8
GLU (mg/dl)	42.6 - 151.0	80.2	52	0 - 245
TS (g/dl)	7.5 - 9.1	8.3	27	5.4 - 9.7
PCV (%)	33.7 - 46.7	39.8	45	24 - 54
Leukocytes (cells/mm3)	7139 - 31045	14630	38	5000 - 31000
Neutrophils (cells/mm3)	3893 - 18545	8540	27	38 - 26500
Lymphocytes (cells/mm3)	761 – 7220	2299	26	7.0 - 8340
Monocytes (cells/mm3)	153 – 3606	696	27	0 - 2900
Eosinophils (cells/mm3)	217 – 4015	868	27	0 - 2800
Basophils (cells/mm3)	0 – 1	0	27	0 - 3860
ESR (units at 120 minutes)	7.8-107.6	25.2	28	14
Leptin (ng/ml)	0.3 - 4.8	1.2	52	None
Ghrelin (pg/ml)	11.4 - 37.5	20.6	45	None
Testosterone (ng/ml)	0.06 - 10.5	8.4	15	10.4 - 11.5

[a] ISIS values provided by the International Species Information System and the Association of Zoos and Aquariums (ISIS, 2002; AZA, 2012).

Table 1.3 Flow cytometric values for lymphocyte subsets in free-ranging African lions.

	CD4+ T CELLS [a]	CD8+ T CELLS [a]	CD21+ B CELLS [a]	T/B GENERAL [a]
Low	267	34	120	1150
	299	159	292	1238
	343	204	336	1429
	413	268	339	1514
	422	293	524	1536
	465	317	538	1849
	476	323	550	2015
	511	374	580	2122
	558	397	1095	2274
High	672	551	1380	2922
Mean	427	243	471	1736
S.D.	1.3	2.2	2	1.3
95% CI	242 -745	51- 1140	119 -1874	969 – 3111

[a] All units are reported as the number of cells/10,000 counted.

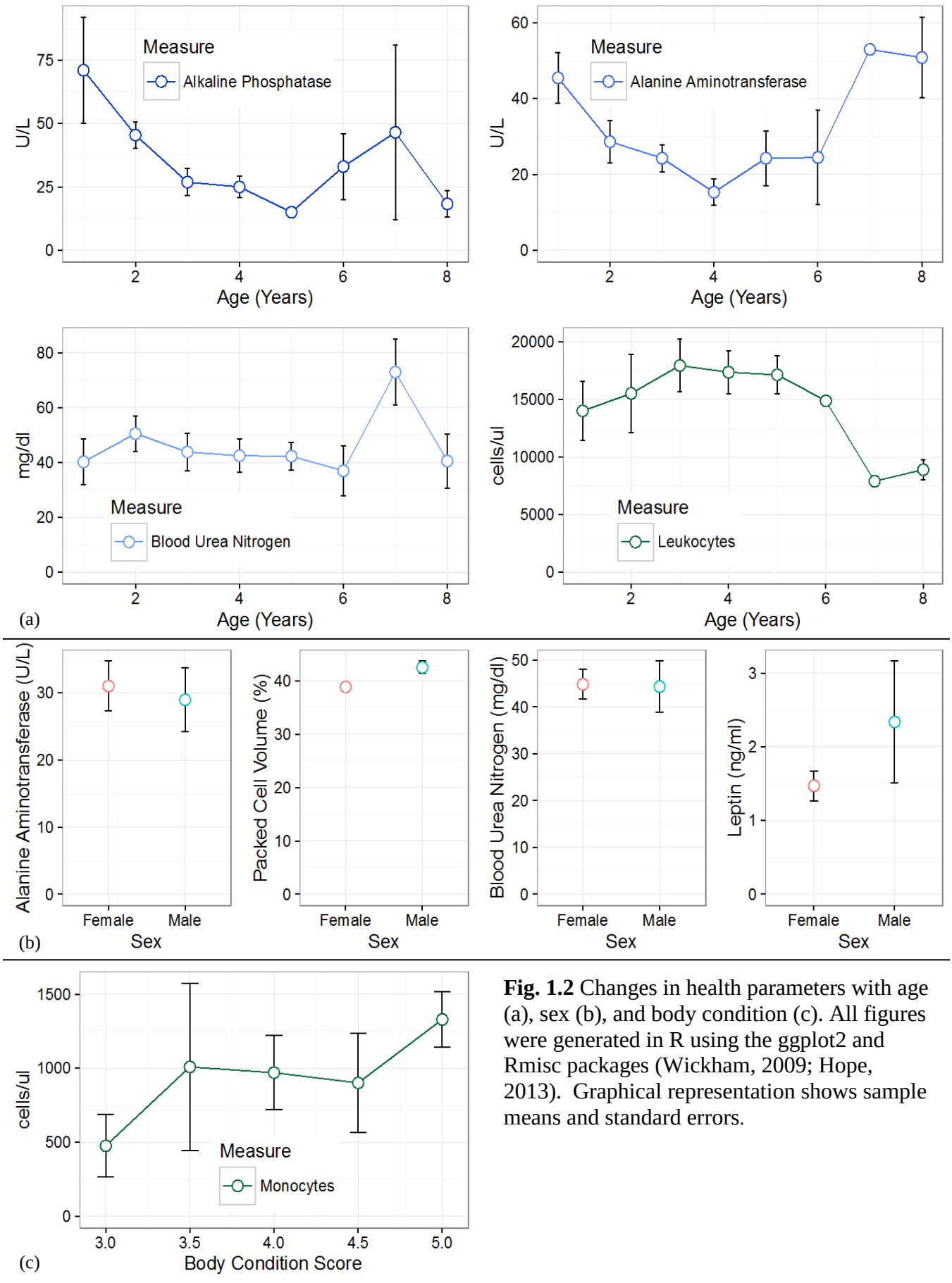

Fig. 1.2 Changes in health parameters with age (a), sex (b), and body condition (c). All figures were generated in R using the ggplot2 and Rmisc packages (Wickham, 2009; Hope, 2013). Graphical representation shows sample means and standard errors.

Table 1.4 Associations of physiologic and immune parameters with demographic variables and body condition score.

PARAMETER	*(n)*	*df*	age			sex			bcs			SIGNIFICANT
			β	*CI*	*P*	*β*	*CI*	*p*	*β*	*CI*	*p*	INTERACTIONS
ALP	51	27	0.85	0.78-0.93	**0.00**	-	-	-	0.79	0.59-1.05	0.11	none
ALT	51	24	1.53	1.04-2.25	**0.03**	0.17	0.03-1.08	**0.06**	1.07	0.61-1.89	0.80	age:bcs, sex:bcs
BUN	51	43	1.35	1.10-1.66	**0.00**	0.38	0.15-0.99	**0.05**	1.17	0.85-1.61	0.33	none
CRE	51	26	1.03	0.98-1.09	0.22	0.82	0.52-1.28	0.37	-	-	-	age:sex
GLU	51	26	26.35	0-59.52	0.11	-	-	-	34.35	0-76.58	0.11	age:bcs
TS	26	12	-	-	-	1.60	0-4.33	0.23	0.25	0-0.59	0.14	sex:bcs
PCV	44	24	-	-	-	3.80	1.43-6.18	**0.00**	-	-	-	none
Leukocytes	37	18	0.95	0.91-1.00	**0.09**	1.18	0.97-1.44	0.10	-	-	-	none
Neutrophils	27	14	1.02	0.95-1.08	0.66	-	-	-	-	-	-	none
Lymphocytes	27	14	0.97	0.84-1.12	0.63	1.39	0.50-3.84	0.51	-	-	-	age:sex
Monocytes	27	14	-	-	-	-	-	-	1.55	0.99-2.45	**0.06**	none
Eosinophils	27	14	0.90	0.79-1.02	0.10	-	-	-	-	-	-	none
Basophils	27	all values were zero and could not be modeled										
ESR	27	14	1.10	0.97-1.25	0.12	-	-	-	-	-	-	none
Leptin	50	25	0.99	0.88-1.12	0.91	3.54	1.22-10.34	**0.02**	-	-	-	age:sex
Ghrelin	50	21	0.43	0-1.99	0.59	8.09	0-20.65	0.21	-	-	-	age:sex
Testosterone	14	8	1.14	0.01-214.99	0.94	-	-	-	1.50	0.01-424.31	0.82	age:bcs

All estimates have been back-transformed for ease of interpretation. Fixed effects included in the final model, as well as significant two-way interactions, are reported above. Statistically significant associations with age, sex, and body condition are highlighted with bold-underline. Missing values denote that the fixed effect was dropped from the final model. Values for two-way interactions and random effects are not provided due to lack of interpretation.

Table 1.5 Select reference intervals for domestic and free-ranging felid species as provided by previous literature. Values from this study are provided on the right for comparison.

PARAMETER	DOMESTIC CAT (*Felis catus*)[c]	JAGUAR (*Panthera onca*) [a, c]	BENGAL TIGER (*Panthera tigris tigris*) [a, c]	AFRICAN LION (*Panthera leo*) [b]
ALP (U/L)	0 - 45	19 - 80	-	6.8 - 86.0
ALT (U/L)	25 - 97	20 - 68	21.2 – 109	6.6 - 91.4
BUN (mg/dl)	19 - 34	70 - 130	6.5 - 48.2	8.3 - 78.5
CRE (mg/dl)	0.9 - 2.2	0.7 - 1.5	1.6 - 4.6	0.8 - 2.6
GLU (mg/dl)	76 - 119	-	-	42.6 - 151.0
TS (g/dl)	5.4 - 7.5	7.1 - 8.2	3.7 - 8.7	7.5 - 9.1
PCV (%)	30 - 45	35.6 - 38.3	36 – 45	33.7 - 46.7
Leukocytes (cells/mm3)	5500 - 19500	15100 - 29000	6200 – 11050	7139 - 31045
Neutrophils (cells/mm3)	2500 - 12500	10500 - 19200	-	3893 - 18545
Lymphocytes (cells/mm3)	1500 - 7500	2100 - 7700	-	761 - 7220
Monocytes (cells/mm3)	0 - 900	300 - 1400	-	153 - 3606
Eosinophils (cells/mm3)	0 - 800	0 - 1000	-	217 - 4015
Basophils (cells/mm3)	0 - 200	0	-	0 - 1

[a] Indicates values taken from wild-caught populations.
[b] Indicates values taken from this study.
[c] Sources for this table included this study as well as outside peer-reviewed journal articles and veterinary texts (Shrivastav & Singh, 2012; Widmer et al. 2012; Merck Veterinary Manual, 2013).

# CHAPTER 2: EQUAL CONTRIBUTIONS OF FELINE IMMUNODEFICIENCY VIRUS AND ASSOCIATED COINFECTIONS TO MORBIDITY IN FREE-RANGING AFRICAN LIONS (*PANTHERA LEO)*

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ABSTRACT

Feline Immunodeficiency Virus (FIV) is a pathogenic lentivirus related to human and simian immunodeficiency viruses (HIV and SIV, respectively) that has been associated with AIDS-like pathologies in domestic cats (*Felis catus)*, as well as infection in several wild species of felidae and hyenidae. Progressive immunosuppression and wasting body condition are hallmarks of the disease caused by these lentiviruses, and secondary infections are common in humans and domestic cats. However, the role of secondary infections in disease progression is difficult to assess in these populations, as treatment for coinfections in domestic animals and humans is the norm. Here, we investigated the role of coinfections by gastrointestinal parasites and tick-borne hemoparasites for FIV disease progression in a structured survey of 195 wild African lions (*Panthera leo*) living in Kruger National Park (KNP), South Africa. Using a combination of generalized linear mixed models and a partial least squares structural equation model path analysis, we assessed the effects of FIV on a broad range of health indicators to explore how direct effects of FIV combine with indirect effects through immunity, behavior, and coinfections to determine lion health outcomes. We show that immunosuppression due to FIV is associated with increased richness and burden of hemoparasites and gastrointestinal parasites, and that these parasites in turn contribute to morbidity due to FIV disease. Specifically, FIV-infected lions experience progressive nutritional wasting and increases in hepatic biomarkers as both direct and indirect responses to prolonged anorexia and parasitism, findings that are both exacerbated by infection with gastrointestinal and hemoparasites. When taken together, the contribution of co-infecting parasites and pathogens to morbidity in lions is of similar magnitude as direct effects of FIV infection. These findings suggest that common coinfections are not merely symptoms of FIV infection, but play a central role as mediators of disease progression in natural lion populations. As such, variation in FIV disease outcome among populations must be understood in the context of differences in parasite and pathogen exposure profiles. This raises the question of whether interventions targeting key coinfections might help improve health outcomes in natural populations.

2.1 INTRODUCTION

The genus *Lentivirinae* comprises a group of pleomorphic, single-stranded, RNA viruses belonging to the family *Retroviridae* that includes well-known pathogens such as human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), and feline immunodeficiency virus (FIV) (Pedersen et al., 1987; Brown et al., 1994; Hartmann, 1998; Biek et al., 2003; Kanzaki & Looney, 2004). While the pathogenicity of these viruses varies widely based on factors such as strain, subtype, geographic location, and degree of host-pathogen coevolution, the general hallmark of infection is a protracted decrease in CD4+ T helper cell levels with resultant immunosuppression, decreased resistance to pathogenic and parasitic exposures, progressive weight loss, and increased mortality (Yamamoto et al., 1989; Barlough et al., 1991; Gardner, 1991; Yamamoto et al., 1991; Barr et al., 1995; Bendinelli et al., 1995; Willett et al., 1997; Hartmann, 1998; Dunham, 2006; VandeWoude & Apetrei, 2006; Pecon-Slattery et al., 2008; Troyer et al., 2011; Hartmann, 2011; Hartmann, 2012).

FIV infection is primarily transmitted via saliva to blood contact during aggressive encounters and has been documented in 19 wild felid species found throughout Africa; North, Central, and South America; and Asia, as well as in domestic cats and hyenas (Hartmann, 1998; Troyer et al., 2005; VandeWoude & Apetrei, 2006; Pecon-Slattery et al., 2008). While characterization of FIV infection in most wild felids is still on-going, evidence from natural and experimental infection in domestic cats supports widespread AIDS related pathology in infected hosts, which mimics HIV disease in humans (Pedersen et al., 1987; Gardner, 1991; Pedersen et al., 1993; English et al., 1994; Hofmann-Lehmann et al., 1997; Elder et al., 1998; Hartmann et al., 2011). Recent studies on FIV-ple (the lion-specific strain of the virus) in African lions (*Panthera leo)* suggest similar health effects. Findings from those studies show wide scale immunosuppression, increased liver pathologies, and declining clinical health among lions of Botswana infected with FIV-ple (Bull et al., 2003; Roelke et al., 2006; Roelke et al., 2009). Despite these advances, potential health interactions between FIV and other parasitic and pathogenic coinfections in this and other species with limited access to disease control remain difficult to characterize.

As an immunosuppressive pathogen, FIV is likely to cause significant health impairment by lowering the threshold for invasion and persistence of secondary pathogenic and parasitic infections through a combination of factors (Bendinelli et al., 1995; Dean et al., 1998; Hartmann,

2011; Hartmann, 2012). Similar to HIV in humans, destruction of immune cells and immune dysregulation may lead to increased coinfections and inflammation; disease related nutritional deficiency may limit the host's ability to compensate by increasing investment in immunity; and organ pathology may break down mechanical barriers to parasitic invasion (Bendinelli et al., 1995; Dean et al., 1998; Hartmann, 2011; Hartmann, 2012). In the face of these changes, alterations to sex steroids and glial cell populations of the brain may serve to upregulate aggressive behaviors, further increasing transmission potential (Hawley et al., 2011; McCaig et al., 2009; Fromont et al., 2003; Tejerizo et al. 2012). However, the role of secondary infections in disease progression of FIV and other pathogenic lentiviruses is difficult to assess, as management of immunodeficiency virus –associated disease in model species such as humans and domestic animals typically includes preventing or treating coinfections (Bentwich et al., 2000). For example, FIV-infected domestic cats are typically kept indoors, where they have limited exposure to pathogens and parasites, and any overt coinfections are treated. Yet, in natural animal populations, and arguably in human populations in developing countries, coinfection by common parasites and pathogens is the rule rather than the exception (Bentwich et al., 1999; Bentwich et al., 2000; Ivan et al., 2013). As such, understanding how immunodeficiency viruses affect coinfection profiles, and how coinfections contribute to disease progression, is of broad relevance to understanding variation in health outcomes of immunodeficiency virus infection in natural host populations. Given these characteristics we hypothesize that, in natural felid populations, coinfections by common pathogens and parasites play a central role in mediating health outcomes of FIV disease.

To investigate the role of coinfections by gastrointestinal parasites and tick-borne hemoparasites in FIV disease progression, we conducted a structured survey of 195 free-ranging lions in Kruger National Park, South Africa. Specifically, we asked: (1) what are the consequences of FIV for lion health in terms of immunity, maintenance of body condition, aggressive behaviors, biochemical homeostasis, and clinical signs of disease; (2) to what extent does FIV infection alter the richness (total number) of co-infecting parasite species; and (3) what role do coinfections play in causing morbidity in FIV disease? Key findings suggest that FIV plays a crucial role in structuring host health and susceptibility through direct pathologies, but that the contributions of coinfecting pathogens may be as important or even more critical when predicting disease progression and morbidity related to FIV.

2.2 METHODS

Study system, demographics, and sample collection

Data and samples were collected between March 2010 and September 2013 from 195 free-ranging lions as part of a study on lion health, behavior, and demographics. Captures were conducted by South African National Parks veterinary staff using chemical immobilization according to established protocols for lions (Jacquier et al., 2006; Wenger et al., 2010; AUCC #FERSM5-767). When possible, all pride members over six months of age were captured. Animals under six months of age were not immobilized due to risk of adverse reaction associated with the drug cocktails. During immobilization, blood was collected via venipuncture into EDTA, sodium heparin, Cyto-Chex® BCT, erythrocyte sedimentation rate (ESR) ®, and whole blood (red-topped) tubes. Feces were collected directly from the rectum. A full physical examination was performed and the following demographics recorded: approximate age, sex, and pride membership.

Age was estimated using established methods (Smuts et al., 1978). As all immobilized individuals were older than six months, sex was readily identifiable based on visualization of external genitalia. Pride membership was provided through known pride ranges and long-term behavioral monitoring data provided by the parent study.

Health, immunity, and behavior

In order to evaluate health and immune status in FIV-positive and negative individuals, this study used a combination of physical exam parameters, blood biochemistry and endocrinology, and functional and quantitative immune assays.

Physical examination: Each lion was evaluated for the presence of lesions and other abnormalities known to be associated with either FIV or other infectious diseases present in the population. The parameters evaluated included the following: body condition score (BCS), gingivitis, oral papillomas, ocular lesions, lymph node enlargement, dehydration, and hygromas, the later of which has been associated strongly with certain bacterial infections in other species (Roelke et al., 2009).

Body condition score was ranked on a scale from one (poor) to five (ideal) using fat coverage and palpation of bony prominences over the ribs, sacrum, hips, and stomach (Ferreira and Funston, 2010). Oral papillomas were identified via visualization of large raised plaques on the gingiva, tongue, or oral mucosa, while gingivitis was identified as inflammation or ulceration

of the gingiva seen along the gum line and ranked on a scale of zero (no inflammation) to four (marked inflammation and ulceration) (Roelke et al., 2009). Scores of two or above merited inclusion in the gingivitis-positive category. Ocular lesions were identified using light–facilitated examination of the cornea and anterior chamber of the eye for signs of corneal ulceration or infiltrate that may signify uveitis. A gross examination of each lion was used to determine the presence of dehydration (seen as sunken eyes and decreased skin turgor over the ribs), hygromas (soft fluctuant swellings over the elbow), and peripheral lymph node enlargement (palpable enlargement of one or more lymph nodes). For further evaluation, degree of systemic inflammation was determined from the number of enlarged lymph nodes ranked on a scale from negative six (all nodes reduced) to six (all nodes enlarged). This score was calculated by adding the number of enlarged lymph nodes and subtracting the number of reduced nodes detected in the submandibular, prescapular, axillary, inguinal, and popliteal regions, as well as a separate "other" category that included lymph nodes that were only rarely palpable when enlarged, such as the lateral retropharyngeal and accessory axillary.

Blood biochemistry and endocrinology: Blood was collected via venipuncture of the jugular or femoral veins and transported to the laboratory at ambient temperature. Serum was separated from whole blood within 8 hours of collection and stored at -60°C until analysis. Liver (alanine aminotransferase [ALT], alkaline phosphatase [ALP]) and kidney (blood urea nitrogen [BUN], creatinine [CRE]) biochemical markers, blood glucose (GLU), and total protein (TP) concentrations were assessed using the commercially available Prep II Profile (Abaxis Inc.; PN 500-7124) run on an Abaxis VS2 Vet Scan machine (Abaxis Inc., Union City, California, USA).

Leptin and ghrelin were evaluated as a measure of body condition (Kotler et al., 1984; Yamamoto et al., 1989; Roelke et al., 2009; Freeman et al., 2004). Leptin is secreted at times of high nutrient availability due to increased body fat, while ghrelin is secreted in response to food scarcity and resulting stomach emptiness (Shibata et al., 2003; Martin et al., 2010; Depauw et al., 2012). Serum for the ghrelin assay was collected from whole blood tubes, which were transported on ice until separation. Once separated, HCl was added to acidify the sample to prevent protein breakdown and all samples were stored at -60°C until use. Plasma for leptin was collected from heparin tubes and was also transported on ice until storage at -60°C. Both hormones were measured at Oregon State University using commercially available RIA kits and following manufacturer instructions (Millipore Inc., Billerica, Massachusetts, USA; PN XL-85K

and GHRA-88HK*).* The Leptin Multispecies RIA kit and Human Acylated Ghrelin RIA kit were used due to strong cross-reactivity with the corresponding feline hormones (Depauw et al., 2012; Martin et al., 2010).

Hematology and Immunity: Immune status of lions was examined using a combination of quantitative measures including clinical hematology, flow cytometry, erythrocyte sedimentation rate, and bactericidal killing capacity. White blood cell counts were obtained by electrical impedance counting via an automated hematology analyzer (Vet ABC, Scil Animal Care Company, Gurnee, Illinois, USA) from blood collected into EDTA tubes. Differential counts of lymphocytes and granulocytes were calculated via blood smears prepared using Hemacolor Diff-Quick Staining Solution (VWR International, Radnor, Pennsylvania, USA). Packed cell volume and total solids were measured from heparinized blood spun at 15000XG in a hematocrit centrifuge (Gemmy Industrial Corporation, Taipei, Taiwan; PN HKT-400) using microcapillary tubes and a refractometer (Optika, Ponteranica, Italy; PN HR-160).

Immunolabeling and flow cytometry were used to count populations of lymphocytes including CD4+ T-helper cells, CD8+ cytotoxic T cells, and CD21+ B cells, which are known targets of FIV infection (Roelke et al., 2006). Cells were stored as whole blood in Cyto-Chex® BCT cell preservation tubes (VWR International, Radnor, Pennsylvania, USA; PN 87005-196) for no longer than two weeks at 4°C. For quantification, cells were labeled with feline-specific antibodies against CD4, CD8, or CD21 cell surface markers and allowed to interact for 15 minutes. Following incubation, red cells were lysed to prevent sample contamination using lysis buffer (500 mls of deionized water with 4.13g NH_4Cl, 0.5g $KHCO_3$ [Sigma Aldrich, St. Louis, Missouri, USA; PN A0171, 60339, respectively], and 0.185g Na_4EDTA [Fisher Scientific, Waltham, Maryland, USA; PN S657]). Antibodies were then conjugated to horse anti-mouse IgG-FITC over a second 15-minute incubation period (Vector Labs, Burlingame, California, USA; PN FI-2000). Control tubes with unlabeled cells were also provided for proper gating of cell populations. Felid-specific antibodies and protocols for their use were provided by Peter Moore at UC Davis (Leukocyte Antigen Biology Laboratory, University of California, Davis, CA, USA) and final counts for each subset of lymphoid cells were performed by faculty at Onderstepoort Veterinary Institute's Flow Cytometry Laboratory in Pretoria, South Africa.

Erythrocyte sedimentation rate (ESR), which has been used as a gauge of systemic inflammation in late stage AIDS patients, was assessed following protocols validated in humans

(Ndakotsu et al., 2009). Samples were collected into citrate ESR tubes and processed immediately upon return to the lab (Greiner Bio-One, Lasec Laboratory and Scientific, Cape Town, South Africa; Vacuette ESR Systems). ESR was measured at 60 and 120 minutes post initiation of the assay.

A bactericidal assay, which measures the ability of whole blood (cellular and protein constituents) or plasma (protein constituents) to kill bacteria, was also conducted as a measure of innate immune function (Beechler et al., 2012). Samples for this assay were collected into sodium heparin tubes and stored at ambient temperature until analysis. All bactericidal assays were conducted within 8 hours of sample collection to avoid cell death. Published protocols for wildlife species (Tielman et al., 2005; Matson et al., 2006; Beechler et al., 2012) were modified for use in lions by combining 20ul of either whole blood or plasma with 120ul sterile phosphate buffered saline (PBS) and 15ul of serially diluted *Escherichia coli (*Fisher Scientific, Waltham, Maryland, USA; PN 23-021-087*).* The final concentration for the mixture contained approximately 1500 colony forming units (CFUs). Bacteria for this assay were obtained from actively growing colonies plated on Criterion Tryptic Soy Agar (TSA; Hardy Diagnostics, Santa Maria, California, USA; PN C7122), which were created using an *Escherichia coli* Kwik-Stik (Fisher Scientific, Waltham, Maryland, USA; PN 23-021-087). Consistent numbers of CFUs for the original stock concentration were collected via the Kirby-Bauer technique using a BD BBL Prompt Inoculation System (Fisher Scientific, Waltham, Maryland, USA; PN B26306). After addition of bacteria, samples were vortexed to ensure an equal suspension. The blood-bacteria mixture was then allowed to incubate for 30 minutes at 37°C. After 30 minutes, 15ul of the mixture was plated onto sterile TSA plates in triplicate and allowed to incubate for 16 hours at 37°C. To determine bactericidal ability, the average number of CFUs growing on each experimental plate was compared to the average number of CFUs growing on three control plates containing only bacteria and PBS using the following equation:

$$BKA = \frac{Mean\ \#\ CFUs\ on\ Control\ Plates - Mean\ \#\ CFUs\ on\ Experimental\ plates}{Mean\ \#\ CFUs\ on\ Control\ Plates}$$

Behavior: Aggressive encounters may speed the transmission of both FIV and other pathogens (Hawley et al., 2011; McCaig et al., 2009; Fromont et al., 2003). AIDS in other species is often associated with an increase in sex steroids that promote these behaviors (Tejerizo et al., 2012). As such, wounds and testosterone levels were quantified as proxies for aggression

in lions. Wounds from bites and scratches were identified visually and noted when severe enough to penetrate the skin or if there was evidence of suppurative inflammation. Testosterone was measured using a commercially available EIA kit (Cayman Chemical, Ann Arbor, Michigan, USA; PN 582701) from serum samples stored at -60°C.

Coinfections

Virology: Diagnostic virology was run on serum samples using in-house ELISAs based on methods developed at Onderstepoort Veterinary Institute. Exposure to canine distemper, feline parvovirus, feline enteric coronavirus, and feline calicivirus was measured qualitatively using antibody-based ELISAs. Feline immunodeficiency virus was also detected using antibody-based methods, but since FIV is a life-long infection, presence of circulating antibodies was presumed indicative of active infection.

Gastrointestinal parasitology: Fecal parasites were detected through the use of standardized fecal sugar flotation and centrifugation techniques (Foreyt, 2001). An approximate number of worm eggs for each species of parasite was identified visually through microscopy and quantified as:

$$Parasite\ eggs\ per\ gram = \frac{\#eggs\ counted}{\#grams\ feces}$$

Parasite families identified were solely those detected using flotation methods and included nematodes, cestodes, and protozoa (for a complete list of those identified reference Table 2.1**)**.

Blood parasitology: Hemoparasite DNA to be used for infection diagnostics was extracted from stored EDTA whole blood using a Qiagen DNeasy kit (Qiagen, Hilden, Germany; PN 69506) and run using a polymerase chain reaction (PCR) based reverse line blot. This method was chosen due to its ability to detect multiple parasites at once, as well as its capability for catch-all detection of unidentified species. The PCR membranes used for this study contained existing markers for parasites previously identified in cheetahs and lions and included *Theileria, Hepatozoon, Babesia,* and *Ehrlichia* species (for a complete list reference Table 2.1). All PCR diagnostics were run by M. Oosthuizen (Molecular Diagnostic Services, Department of Veterinary Tropical Diseases, Onderstepoort Veterinary Institute, Pretoria, South Africa) according to established protocols (Bosman et al., 2010; Kelly et al., 2014).

Data Analysis

Statistical analysis: All data for inclusion in our analyses were investigated for normality, constant variance, linearity, and leverage points using histograms, Cook's Distance, and plots of

residuals versus fit values in R (*plot* function; R Core Team, 2016). Data that appeared normal were further investigated for normality using Shapiro-Wilk tests (stats package; R Core Team, 2016). All independent variables of interest were modeled using either linear mixed models (LMMs) or generalized linear mixed models (GLMMs) as indicated by the data (nlme and lme4 packages; Pinhiero et al., 2015; Bates et al., 2015, respectively), with the exception of flow cytometric cell counts. Full models included fixed terms for age, sex, body condition score, FIV, and two-way interactions between age and FIV and sex and FIV. For immune models, an additional variable was included for suppurative wounds, as wounds are a strong predictor of large fluctuations in certain inflammatory markers and cell numbers (Thrall, 2004; Levinson, 2012). In addition, all full models included a random effect for pride to account for the fact that animals from the same pride are not fully independent (Bolker et al., 2008) due to commonalities in disease exposures, prey availability, and relatedness.

For model selection, data that were either normal or log transformable to normality as confirmed by the Shapiro-Wilk Test were modeled as LMMs in R using the nlme package (Pinhiero et al., 2015). The final model was selected via stepwise selection using AIC values provided by the stepAIC function (MASS package; Venables & Ripley, 2002) and modeled using a restricted maximum likelihood (REML) estimation. Contrastingly, data that were continuous, but were not transformable to normality, were modeled as GLMMs using a Laplace Gaussian distribution with a log link and stepwise comparison using a combination of manual likelihood ratio tests, chi-square values, and AIC values provided by the dredge function in the MuMIn package (Barton, 2016*)*.

In addition to continuous parameters, several variables were discrete counts necessitating the use of Poisson distributions with a log-link. As overdispersion was a common problem, and means of Poisson modeled variables were not prohibitively small (>5), discrete count variables were modeled using penalized quasi-likelihood GLMMs in the MASS package (Venables & Ripley, 2002), as this method is more robust to overdispersion with minimal loss of accuracy. Final model selection for these variables relied on backwards selection via Wald T statistics (aod package; Lesnoff & Lancelot, 2012).

Some models included caveats as dictated by differences in data structure. For models where bactericidal activity was the dependent variable, pride was replaced by a different random effect for “batch”, as bacterial growth was similar for bacterial colonies plated on the same day,

but varied between days. In models for individual parasite abundance, samples with zero values were removed to avoid zero inflation and fecal egg/oocyte burden was compared only between positive samples for a given parasite species using a full model including fixed effects for age, sex, body condition, and FIV status. Interaction terms were excluded due to prohibitively small sample size once parasite groups were partitioned. To model parasite richness, the dependent variable was calculated as the total number of parasite and pathogen taxa diagnosed for each host:

$$Parasite\ richness = \frac{total\ no.\ parasite\ species}{individual\ host}$$

Information on the total number of parasite species contributing to this count was provided in terms of the prevalence of each of the parasite taxa in the lion population:

$$Prevalence\ (\%) = \frac{total\ no.\ animals\ with\ parasite}{total\ no.\ of\ animals\ captured}$$

Finally, for variables that were binary in nature, such as those included for parasite/pathogen diagnostics and clinical measures, statistical models employed Laplace GLMMs with a binomial distribution and a logit link (Bates et al., 2015). Final model selection was conducted using stepwise comparison of AIC values via the dredge function in the MuMIn package (Barton, 2016).

Due to small sample size of FIV-negative individuals for flow cytometric parameters (n=4), FIV status was evaluated separately for CD4+, CD8+, and CD21+ cell counts using t-tests (stats package; R Core Team, 2016). Cell counts were also modeled against demographic parameters for age and sex using generalized linear models with a Quasipoisson distribution to account for overdispersion as indicated by the dispersiontest function (AER package, Kleiber & Zeileis, 2008; R Core Team, 2016). Wound status, body condition score, and pride were not included as fixed effects in the full model to avoid further data partitioning. For quality control, animals with CD4+, CD8+, or CD21+ cell counts significantly greater than the gated T/B cell catch-all measure were also dropped from the final analysis. Model selection was conducted via manual backwards selection using AIC values and p-values via the drop1 function (stats package; R Core Team, 2016).

Path analysis: Structural Equation Models (SEM) include a diverse set of techniques that are very useful in establishing causal relationships in observational studies on natural processes (Serrano et al., 2014), including the impact of diseases (Fan et al., 2016). Several techniques

have been used to estimate model parameters in SEM, with Partial Least Square Path Models (PLS-PM) preeminent among them. Two types of relationships are described in PLS-PM. The first one involves relationships between the latent variables (e.g. a combination of physiological parameters defining an abstract concept such as "health status"), while the other considers the links between the latent variables (LVs) and its own block of manifest variables (MVs). These MVs are variables directly measured in the field or in the laboratory (e.g. body condition or leptin concentration). PLS-PM is an iterative algorithm that separately solves LVs (measurement model) and then in a second step estimates relationships between LVs by means of path coefficients (i.e. structural model). Here, we decided to use PLS-PM because it does not require strong assumptions with respect to the distributions of the sample size or the measurement scale (Wold, 1985).

For our purposes, we have defined five blocks of LVs in our path model: FIV infectious status (0 for FIV-free and 1 for FIV-infected lions), immune response (IMM), hemoparasite infections (Hemoparasites), helminth infections (Helminths), and health impairment (HealthIMP). Descriptive statistics for the set of manifest variables defining each block are shown in Table 2.2. Additional manifest variables describing immune response or health status that were initially included in our path model, but were finally excluded because of their low contribution to their own LVs, are also shown in Table 2.2. Other viral (e.g., FCV, FPV, CDV) infections, as well as *Toxoplasma gondii*, detected in our lions were initially considered for being included in independent LVs, but finally excluded for not contributing to the path model due to low co-occurrence and overall prevalence in the population. While noting that old lions were more prone to be infected with FIV (mean age of FIV-infected lions = 5.5 yrs. whereas age of FIV-free lions = 3.9 yrs; $t= -4.5; df= 131.8; p<0.01$) the age of animals was initially included as a LV. However, it was finally excluded due to lack of correlation with the rest of the LVs included in the final path model.

When specifying the conceptual model, it was assumed that FIV and IMM should have both a direct and indirect impact (through Helminths and Hemoparasites) on HealthIMP. For a scheme defining the structural model see Figure 2.4a of results. Each LV was considered as a linear combination of its own MVs. Thus, the vector of outer weights (Wj) associated at each block of MVs was obtained as the vector of the regression coefficients in the multiple regression of the inner estimate of the LVs on their associated MVs (Tenenhaus et al., 2005). Each outer

weight can be considered as a proxy for the importance of each MV in the construction of the LV. The path coefficients (β) were obtained by least squares regression of connected LV scores, and they were interpreted as standard regression coefficients (Esposito et al., 2010). Finally, once the full model including all possible relationships between LVs (FIV, IMM, Hemoparasites, Helminths and HealthIMP) was fitted, a model simplification was performed by removing those MVs uncorrelated with their own LVs and later those relationships between LVs with the lowest R2 values. The fit of the final model was measured by the goodness-of-fit index (Tenenhaus et al., 2004). Model parameters (the path coefficients and the weights of the MVs) and fit indices (the percentages of explained variance and the R2 values) were validated by bootstrapping. This statistical procedure was performed using the package 'plspm' version 0.4.736 (Sanchez et al., 2017) of the statistical software R version 3.3.3. (R Core Team, 2016).

2.3 RESULTS

FIV infection was common in KNP lions, affecting 142 of the total 195 sampled (72.8%). Prevalence of FIV infection was similar among male and female lions and between park regions, but increased with host age (n =195; β = 1.29; *p-value* = 0.00; for a complete demographic breakdown reference Fig. 2.1). Overall, results of this study showed negative effects of FIV infection on host nutrient balance, clinical health, and immunocompetence, as well as alterations to endocrine and behavioral parameters that may reflect increased aggression in FIV-positive hosts.

FIV infected lions are malnourished. Lions infected with FIV showed higher levels of serum ghrelin (n =194; β = 3.40; *p-value* = 0.00), indicating low frequency of feeding; lower levels of leptin (n = 194; β = 0.23; *p-value* = 0.00), indicating decreased fat reserves; and reduced blood urea nitrogen (n = 193; β = 0.88; *p-value* = 0.04), reflecting malnourishment/reduced protein intake. Together, these findings suggest impaired resource acquisition or retention in FIV-positive hosts (Fig. 2.2a; Tables 2.2 and 2.S1).

FIV infected lions show biochemical alterations and clinical signs suggestive of feline AIDS. FIV infection was associated with an increase in liver enzymes (ALP and ALT; n = 193; β = 1.89 and 1.65; *p-values* = 0.00 and 0.00, respectively; Table 2.2), suggesting damage to the liver such as inflammatory disease; an increased prevalence of ocular lesions (n = 184; β =10.19; *p-value* = 0.00); and increases in gingivitis with age (n = 181; β =1.63; *p-value* = 0.04).

Contrastingly, FIV infection was associated with a lower prevalence of oral papillomas (n = 180; β = 0.50; *p-value* = 0.04; Fig. 2.2a; Table 2.S1).

FIV alters inflammation and immunity. FIV-positive lions showed an increase in erythrocyte sedimentation rate (n = 100; β = 3.10; *p-value* = 0.02), indicating non-specific inflammation; and higher total solids (n = 94; β = 1.04; *p-value* = 0.05), suggesting elevated antibody titers. Infected animals also showed a reduction in total lymphocyte count (n = 90; β = 0.58; *p-value* = 0.04; Fig. 2.2a and 2.2b; Tables 2.2 and 2.S2), consistent with immunosuppression. While no difference was detected in flow cytometric counts for CD4+, CD8+, and CD21+ cells based solely on FIV status (n= 57*; t* = 1.69, 0.52, and -0.94; p = 0.17, 0.63, and 0.41, respectively; Table 2.2), CD4+ and CD8+ helper cell populations decreased with age (n = 57; β = 0.94 and 0.94; *p-values* = 0.00 and 0.02, respectively; Fig. 2.2c), suggesting progressive age-related impairment of adaptive immunity, including humoral and cell-mediated responses (Table 2.S3). Monocytes and neutrophils also showed progressive declines with age, with a more drastic decrease in FIV-infected lions, supporting a reduction in innate immunity alongside other immune impairment (n = 90; β = 0.85 and 0.94; *p-values* = 0.00 and 0.03, respectively; Table 2.S2).

FIV may increase aggressive behaviors or delay tissue healing. FIV-positive lions exhibited a higher risk for severe wounds when compared to FIV-negative individuals (n = 193; β = 3.76; *p-value* = 0.04), suggesting increased fighting, more severe outcomes of fights, or reduced ability to heal wounds. Male FIV-infected lions had higher testosterone levels (n = 73; β = 3.36; *p-value* = 0.03) than their FIV-negative counterparts, again pointing towards increased aggression; however, FIV-positive females had lower testosterone levels than healthy females (n =119; β = 0.44; *p-value* = 0.00; Fig. 2.2a; Tables 2.2 and 2.S4).

FIV increases the richness and abundance of coinfecting parasites. This study measured exposure to 21 different parasite and pathogen taxa known to infect lions. Overall, infections were common and diverse among KNP lions, with individuals typically hosting from one to twelve of the taxa we identified (*median =7; IQR* =3; Fig. 2.3a). Several of the taxa were detected only in FIV-infected lions: feline enteric coronavirus (4% prevalence), whipworms (*Trichuris spp.*; 9% prevalence), *Toxoplasma gondii* (12% prevalence), *Theileria annae (0.07%* prevalence*),* and *Theileria bicornis* (4% prevalence). Gastrointestinal parasite infections were strikingly common in FIV-positive lions (Fig. 2.3a). In addition to *T. gondii* and whipworms,

infections with tapeworms (*Taenia* and *Echinococcus* spp.; n = 114; β = 2.87; *p-value* = 0.03), hookworms (*Ancylostoma* spp.; n = 114; β = 8.51; *p-value* = 0.01), and coccidia (*Isospora* spp.*;* n = 114; β = 221.02; *p-value* = 0.02) were increased among FIV-positive lions (Table 2.S5). Total gastrointestinal parasite burden reflected this trend and was increased in FIV-positive hosts (n = 104; β = 4.73; *p-value* = 0.03), largely driven by a marginal increase in the intestinal burden of hookworm species (n= 48; β=4.67; *p-value*= 0.09; Table 2.S5). Finally, overall richness of gastrointestinal parasites was significantly higher in FIV-positive hosts (n = 114; β = 1.61; *p-value* = 0.02; Fig. 2.3b).

The prevalence of *Babesia microti*, a tick-transmitted hemoparasite, was also higher in FIV-positive lions (n = 190; β = 2.19; *p-value* = 0.05; Fig. 2.3a), as was hemoparasite richness (n = 190; β = 1.35; *p-value* = 0.04; Fig. 2.3c). Interestingly, despite these trends, overall parasite richness, including both gastrointestinal and hemoparasites, was not statistically different in FIV-positive and FIV-negative lions (n = 111; β = 1.17; *p-value* = 0.12; Table 2.S5), suggesting a trade-off in infection prevalence between groups.

FIV affects lion health directly and indirectly via immunity and coinfections. In our path analysis, we asked whether progressive wasting and organ damage associated with FIV are mediated directly due to FIV infection, or indirectly via increased coinfections. Our models reveal three major findings. First, FIV, hemoparasites, and gastrointestinal parasites have direct negative effects of similar magnitude on lion health (Fig. 2.4a and 2.4d), but they explain different aspects of ill health. All three contribute to emaciation based on body condition score, but FIV and hemoparasites are associated with liver pathology (ALT) while GI parasites are correlated with the most variation in leptin and ghrelin levels (Fig. 2.4b). Second, FIV-related immune impairment itself contributes strongly to ill health in lions in addition to the infections that we measured (Fig. 2.4a and 2.4c). In fact, the effects of immunity on health parameters are equal to, or stronger than, direct effects of FIV and coinfections (Fig. 2.4d). Negative health trends are associated primarily with low B cell populations and inflammation (ESR; Fig. 2.4b). Finally, FIV promotes hemoparasitic and gastrointestinal coinfections through its effects on host immunity (Fig. 2.4a and 2.4c). GI parasite coinfections show strong associations with low B cell populations and increased inflammation (ESR); whereas hemoparasites were associated with low populations of lymphocytes, as well as poor bactericidal activity of whole blood (Fig. 2.4b).

2.4 DISCUSSION

While previous studies have explored the negative health and immune consequences of FIV-ple infection in free-ranging lion hosts, this study is the first of its kind to characterize these associations against the broader context of parasite and pathogen communities known to co-occur within FIV-infected lion populations. As indicated by our results, FIV-infection with viral subtypes A, D, or E (the subtypes known to infect South African lions) is characterized by immune impairment and dysfunction leading to nutrient imbalance and progressive wasting, as well as clinical and biochemical changes commonly associated with feline AIDS (FAIDS; Roelke et al., 2009; Hartmann, 2011; Hartmann, 2012). Importantly, findings suggest that these health impairments may be driven in similar parts by FIV itself, and by gastrointestinal and hemoparasitic coinfections, which appear to be facilitated by FIV-related immune dysfunction. Overall, these findings support coinfections as an integral component of FIV disease progression, rather than merely a side effect.

Using quantitative metrics, immune dysfunction was common in FIV-positive hosts. Total lymphocyte counts were lower; CD4+ and CD8+ T cell populations decreased with age in infected animals, as did counts of innate effector cells including monocytes and neutrophils; and inflammation, as measured by erythrocyte sedimentation rate (ESR) and total solids, was elevated. Observed changes to immune cells were consistent with FIV's broader cell tropism compared to HIV and SIV, a difference that allows infection of B lymphocytes, T lymphocytes, regulatory T cells, and macrophages (Ackley et al., 1990; Reggeti et al., 2008). As a result, cellular destruction via viral replication and apoptosis results in lymphoid involution and anergy contributing to depletion of immune cells and cellular dysfunction (Brown et al., 1991; Yamamoto et al., 2007: Elder et al., 2008; Yamamoto et al., 2010; Murphy et al., 2013). These trends were further supported in FIV-positive lions by elevations in ESR and total solids. In humans, ESR has been shown to correlate well with a shift towards general immune overstimulation – a compensatory response to immune impairment due to CD4+ T-helper cell depletion (Ndakotsu et al., 2009). As such, ESR has been widely used as an indicator of disease progression and outcome in AIDS patients (Ndakotsu et al., 2009). Similarly, total solids have been used as a measure of systemic inflammation in HIV and FIV patients, and may be suggestive of hyperglobulinemia due to chronic immune overstimulation (Bendinelli et al., 1995; Roelke et al., 2009; Hartmann, 2011; Hartmann, 2012).

Coinfections were overwhelmingly common in the lion population. In FIV-positive lions, immune impairment appears to contribute to an increase in the richness and abundance of co-infecting parasites, which in-turn contributes to FIV disease progression. Positive associations with FIV were particularly striking for the gastrointestinal parasites we investigated, with prevalence of hookworms, tapeworms, and coccidia increased, and whipworms and *Toxoplasma gondii* exclusively found in FIV-positive lions. Overall intestinal parasite burdens were increased with FIV infection and were driven largely by increased egg shedding by hookworm species. These changes can most likely be attributed to impaired gastrointestinal immunity, as similar lentiviruses (HIV and FIV) have a strong affinity for CD4+ T cells exhibiting either CCR5 (HIV) or CXCR4 (FIV) chemokine coreceptors, which are commonly found in gut associated lymphoid tissue (GALT; Reggeti et al., 2008; Hartmann et al., 2011). As such, the correlations between FIV-infection and gastrointestinal parasite prevalence may reflect a breakdown in gut immunity as observed with HIV in humans and FIV in domestic cats (Willett et al., 2006; Howard & Burkhard, 2007; Costiniuk & Angel, 2012; Murphy et al., 2013). In humans with HIV, coinfection with GI parasites has been found to contribute to disease progression and severity via activation of the TH2 CD4+ T cell response, promoting cell populations for which the virus has a particular predilection (Bentwich et al., 1995; Kalinkovich et al., 1998; Bentwich et al., 1999; Bundy et al., 2000; Fincham et al., 2003; Walson et al., 2009; McSorely & Maizels, 2012). This mechanism is thought to facilitate HIV entry into and destruction of immune cells and release the virus from resource restriction until terminal phases of the disease (Bentwich et al., 1999). Further support for FIV-helminth CD4+ TH2 cell destruction comes from our path analysis, which revealed that GI parasite coinfections were also linked independently to decreased B cell populations. As TH2 cells are responsible for the secretion of interleukin-4, a cytokine responsible for promoting B cell growth (Levinson, 2012; McSorely & Maizels, 2012), suppression of the functional capacity of these cells may exacerbate immunosuppression across other cell lineages. Together, lymphoid involution and functional inhibition of immune cells may speed disease progression and predict an overall negative health outcome in FIV-positive lions.

Alongside gastrointestinal parasites, the richness of hemoparasite infections was increased in FIV-positive lions. This alteration was most apparent in the prevalence of *Babesia microti,* which was significantly higher in FIV-infected hosts. *B. microti* infection occurred

against a backdrop of other *Babesia*, *Ehrlichia*, *Theileria*, and *Hepatozoon* species that were also prevalent in combination, including one unknown *Hepatozoon* species that was visually identified in most of our blood smear samples despite a lack of affinity for our PCR markers. Of the species detected via PCR, hemoparasite coinfections responded positively to reductions in any of the immune cell populations, as well as bactericidal capacity of blood, likely reflecting the role of adaptive and innate immune defenses in limiting the invasion, growth, and persistence of these intracellular pathogens (Sher et al., 1992; Olivier et al., 2003; Levinson, 2012). Hemoparasites may thus benefit directly from a decrease in humoral and cell-mediated immunity brought on by clinical AIDS (Sher et al., 1992; Dean et al., 1998; Olivier et al. 2003).

Another hallmark of AIDs in humans and FAIDS in cats is progressive wasting and malnourishment. Here, wasting was also present and was characterized by high levels of ghrelin, the "hunger enzyme" of the gastrointestinal tract, and low levels of leptin, which is produced by adipose cells, together suggesting impaired nutrient balance (Appleton et al., 2000; Shibata et al., 2003; Ida et al., 2007; Martin et al., 2010; Depauw et al., 2012). Ghrelin, a gastric hormone secreted in response to nutrient deprivation and stomach shrinkage, plays an active role in promoting food intake and regulating energy homeostasis (Ida et al., 2007; Jensen et al., 2015). Adequate nutrient availability and stomach expansion following a meal suppresses gastric release of ghrelin while long periods of starvation upregulate its secretion (Klok et al., 2007; Martin et al., 2010). Leptin works in opposition to ghrelin and is an adipokine hormone that is secreted by adipocytes in response to high nutrient balance in order to down-regulate food consumption (Appleton et al., 2000; Klok et al., 2007; Witzel et al., 2015). Our findings on FAIDS-associated wasting were further supported by consistently low levels of blood urea nitrogen (BUN) in FIV-positive hosts, even in the face of dehydration. While BUN is more frequently used to evaluate renal health, low values of BUN may reflect decreased protein balance and subsequent muscle wasting due to insufficient alimentary intake or increased losses through the kidneys and intestines (Thrall, 2005; Stockham & Scott, 2008). Though either mechanism is possible, normal CRE levels in the face of elevated BUN suggest that findings here were not related to compromised renal health.

Of the parasites diagnosed, GI parasites contributed significantly to malnutrition in FIV-infected lions. In fact, they were more tightly associated with low levels of leptin and high levels of ghrelin than FIV itself or hemoparasites. Given that reduced immunity strongly and positively

affects the prevalence, richness, and abundance of GI parasites, our study thus provides evidence that FIV-GI parasite coinfection drives progressive weight loss in lions with FAIDS. According to the path analysis, FIV-GI parasite malnutrition is exacerbated by immune impairment, particularly with regards to alternate defenses as B cell populations wane. These findings suggest that lions with FAIDS may maintain condition while they are able to mount compensatory immune responses, whereas a breakdown of immune compensation once B and T cells decrease coincides with a loss of metabolic homeostasis.

In AIDS patients, systemic inflammation and antigen deposition along filtration barriers due to primary and secondary infections often leads to extensive immunopathology in the form of glomerulopathies and enteropathies (Kotler et al., 1984; Yamamoto et al., 1989; Freeman et al., 2004; Roelke et al., 2009). While markers for these pathologies were included in our biochemistry panels, BUN levels in FIV-positive lions typically remained within the normal range (Broughton et al., 2017), as did creatinine, suggesting that they do not suffer from overt renal pathology. Liver enzymes were increased with FIV-infection relative to uninfected lions, but again were within the normal range previously established for this species (Broughton et al., 2017). Recent studies on the pathological mechanisms of FIV in lions of Botswana found similar trends in liver enzymes, which were attributed to parasite visceral larval migrans as diagnosed via gross assessment and histopathology (Roelke et al., 2009). We worked with live animals only, and so were not in a position to evaluate liver histology; however, the tight association of FIV and GI parasite infections in our study is consistent with this explanation. However, increased richness and prevalence of hemoparasites contributed much more strongly to elevations in ALT, suggesting a dominant role for hemoparasites in driving liver pathology in lions with FAIDS. While levels were not consistently elevated outside of the normal range in infected animals, increases in ALT and wasting, despite normal nutrition based on parameters for leptin and ghrelin, support hepatocellular injury and muscle breakdown perhaps as the result of chronic hypovolemia with resultant hypoxia and nutrient restriction, rather than diminished resource intake (Reyers et al. 1998; Solano-Gallego et al. 2016).

Even though some changes in biochemistry and endocrine markers were subclinical, in that they did not fall outside reference intervals established for healthy wild lions, signs of immunopathology in the form of gingivitis and ocular lesions were present in FIV-positive lions, especially as they aged. Within the context of environmental variation to which wild animals are

exposed, particularly variation in food availability and parasitic exposures, the observed subtle, subclinical changes in organ function and minor clinical manifestations may thus be relevant to animal health.

While this was one of the most comprehensive predator disease studies to date, capture logistics within a free-ranging predator population did impose several limitations. First, we were unable to investigate the role of directly transmitted viral pathogens in FIV disease progression because our diagnostics for viruses were limited to detection of antibodies at a single time point. As such, we cannot be sure whether viral exposures were current or occurred at some point in the past and whether they occurred prior to, or after FIV infection. Directly transmitted pathogens would be of particular interest here due to the potential feedback between increased aggressive behaviors in FIV positive hosts and enhanced transmission – both of FIV itself, and of other directly transmitted infections. Indeed, we found evidence of increased aggression or delayed wound healing particularly in FIV-infected male lions, which showed a higher prevalence of combative wounds and elevated levels of testosterone. These results are consistent with findings in domestic cats with FIV and humans with HIV, which show upregulation of sex steroid levels and an increased propensity to engage in "risky" behaviors (increased fighting in cats and sexual encounters in humans; Fromont et al., 1997; Podell et al., 1999; Lloyd-Smith et al., 2005; Tejerizo et al., 2012). In the case of wild male lions, which compete for territory and access to females, alterations to aggressive behaviors may increase host contact rates and speed transmission of FIV and other directly transmitted infections (Fromont et al., 1997; Hartmann, 1998; Fromont et al., 2003; VandeWoude & Apetrei, 2006). In combination with immunosuppression, FIV may thus change the community and abundance of pathogens found in affected wild lion populations. Characterization of these associations in future studies may be facilitated through the use of novel methods, such as NextGen sequencing, which can detect active infection by a broad spectrum of pathogens while they are still in the host and compensate against the lack of longitudinal serological data.

In addition, our finding of strong direct effects of FIV-driven immunosuppression on host health (independent of coinfections) may also be related to diagnostic limitations of this study. It is possible that, in fact, immunosuppression causes wasting because it permits more co-infections, but that we simply did not detect some of the relevant parasites and pathogens. Alternatively, wasting in FIV-positive lions may result from increased costs for immune

maintenance and decreased immunocompetence for processes such as tissue healing – without the involvement of coinfections. Longitudinal data on infections and health in lions with and without FIV would be needed to assign causal pathways with more certainty.

Finally, while we attempted to avoid sampling bias by catching entire prides and conducting captures across all areas of KNP, our sampling methods were not truly random. Lions that were too weak to respond, or to compete with other lions at the carcass, were unlikely to be sampled. We may thus have missed the most emaciated individuals, as well nomadic sub adults, as these animals often do not associate with a pride (Schaller, 1972). On the other hand, lions that had recently eaten might not respond to a bait carcass, and as such we might also have missed especially well-fed individuals with higher fitness.

2.5 CONCLUSION

With a sample population of 195 wild lions out of 1700, which equates to 11.5% of the total population living in Kruger National Park, as well as diagnostic and health data on 22 different parasites and pathogens, ours is one of the largest and most comprehensive infectious disease studies of wild predators. Findings from this study significantly extend current knowledge of FIV's immunosuppressive effects in lions, as well as its potential role for structuring parasite and pathogen communities via these immune alterations. Both FIV's direct mechanisms, and indirect pathology mediated by the higher richness and abundance of other parasites in FIV-infected individuals, may in turn contribute to this pathogen serving as a key mediator of host pathology through organ injury, malnutrition, and further immune dysfunction. Most importantly, this is the first study to show strong supportive evidence that secondary infections play an essential part of FIV disease progression in lions, rather than just a symptom.

From a broader viewpoint, the above findings support that future wildlife, human, and domestic animal disease studies would benefit from examination of singular pathogens and parasites in the context of their parasite community. While randomized laboratory studies are useful in terms of establishing causal relationships between infectious agents and diseases of interest, the sterility of conditions under which they are conducted, as well as their necessary lack of confounding variables, impede these studies from determining health outcomes in the case of natural infection, where disease progression occurs under the influence of a multitude of natural biotic and abiotic factors. Of particular interest, chronic immunosuppressive pathogens,

such as that studied here, often occur in high prevalence in human and animal populations with poor access to health services and high exposure to neglected pathogens and parasites. Thus, extrapolating health outcomes from laboratory experiments to these populations is not only highly inaccurate, but may prove dangerous in terms of limiting negative health outcomes for entire populations. As such, studies examining the implications of parasitic and pathogenic associations for health within naturally occurring host populations are critical in terms of predicting future population responses to endemic and novel disease threats. The study presented here paves initial steps towards uncovering these associations in a threatened predator species, as well as serving as a model for other immunosuppressive pathogens occurring in host populations heavily impacted by neglected tropical diseases.

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2.6 TABLES AND FIGURES

Table 2.1 Coinfecting parasite species identified in this study.

COINFECTIONS		
Hemoparasites	***Gastrointestinal parasites***	***Viruses***
Ehrlichia/Anaplasma spp.	Ascarid spp.	Canine distemper virus
Babesia felis	Tapeworm spp.	Feline calicivirus
Babesia leo	Hookworm spp.	Feline enteric coronavirus
Babesia microti	Whipworm spp.	Feline parvovirus
Babesia lengua	Coccidia spp.	Feline immunodeficiency virus
Babesia rossi	*Toxoplasma gondii*	
Babesia vogeli		
Babesia canis		
Hepatozoon spp.	***Parasite abundance***	***Parasite richness***
Theileria annae	Worm eggs per gram of feces	Number of parasite species per host
Theileria bicornis		

* Viral measures are based on seroprevalence (exposure) and do not denote active infection.

Table 2.2 Description of latent and manifest variables used to fit a partial least squares path model in order to examine the causal relationships between FIV infection, immune status, coinfection with helminths or hemoparasites, and health impairment in 195 lions from Kruger National Park, South Africa.

Latent Variable	Manifest Variable (Units)	Descriptive statistics according to FIV infection status		Reference Range
		Free	Infected	
Feline Immunodeficiency Virus (**FIV**)	0 = FIV-free 1 = FIV-infected	27.20% n= 53	72.80% n = 142	-
Immune response (**IMM**)	Leukocytes (cells/ul)	17305.2 ± 1505.9 (5000 - 35050)	14200.8 ± 547.9 (5350 - 29000)	7139 - 31045
	Neutrophils (cells/ul)	10414.3 ± 970.4 (386.6-22256.3)	8654.7 ± 466.3 (583.3-28040.1)	3893 - 18545
	Lymphocytes (cells/ul)	2731.1± 308.4 (31.4-5390)	1726 ± 109.1 (22.7-4011)	761 - 7220
	Monocytes (cells/ul)	928.7 ± 139.4 (20.9-2430)	636 ± 62.5 (0-2804)	153 - 3606
	Eosinophils (cells/ul)	1709.4 ± 299.2 (73.2-7495)	1049.6 ± 98.4 (0-3985)	217 - 4015
	Basophils (cells/ul)*	37.8 ± 22.5 (0 - 350.5)	30.8 ± 9.49 (0 - 318)	0 - 1
	Packed cell volume (%)*	38 ± 0.9 (27.3 - 45)	38.2 ± 0.5 (20 - 51)	33.7 - 46.7
	Total solids (g/dl)*	8.4 ± 0.2 (8.0 - 9.5)	8.4 ± 0.1 (6.9 - 10.4)	7.5 - 9.1
	CD4 (cells/10000)	513.3 ± 17.9 (375 - 658)	412.5 ± 16.4 (77-978)	242 - 745
	CD21 (cells/10000)	434.7 ± 16.6 (223 - 542)	339.29 ± 16.7 (16 - 855)	119 - 1874
	CD8 (cells/10000)	277.7 ± 13.7 (100-401)	255.6 ± 10.6 (20-709)	51 - 1140
	ESR120 (units at 120 min)	49.3 ± 3.1 (8 - 104)	55.5 ± 2.3 (1 - 126)	7.8 - 107.6
	BKaWB (BKA)	35.7 ± 3.25 (2.5 – 59.2)	22.8 ± 1.5 (0 – 57.6)	-
	BKaPlasma (BKA)	15.2 ± 2.4 (0 – 53.9)	20.6 ± 1.2 (0 – 65.2)	-
Hemoparasite infections (**Hemoparasites**)	**Richness** (No. species /lion host)	2.4 ± 0.18 (0-4)	3.1 ± 0.10 (0-5)	-
Helminth infections (**Helminths**)	**Burden** (eggs/gram of feces)	4 ± 1.81 (0-81)	25 ± 6.6 (0-700)	-
	Richness (No. taxa /lion host)	0.34 ± 0.08 (0-2)	0.83 ± 0.08 (0-3)	-
Health impairment (**HealthIMP**)	**Emaciation** (- log BCS)	-1.14 ± 0.003 (-1.2 – -1.08)	-1.13 ± 0.002 (-1.2 – -1.04)	-
	ALT (U/L)	23.3 ± 1.5 (4 – 54)	39.2 ± 1.9 (4 – 133)	6.6 - 91.4
	ALP (U/L)	30.2 ± 3.6 (4 – 66)	32.1 ± 3.5 (4 – 129)	6.8 - 86.0
	GLU (mg/dl)	92.3 ± 5.9 (29-272)	96.2 ± 2.9 (9.8-244)	42.6 - 151.0
	BUN (md/dl)	48.1 ± 2.6 (19-96)	44.9 ± 1.42 (21-113)	8.3 - 78.5
	TP (g/dl)	10.1 ± 0.2 (7.5-14.9)	10.1 ± 0.11 (7.2-15.1)	-
	CRE (mg/dl)	1.5 ± 0.1 (0.1-3.2)	1.8 ± 0.14 (0.1-12.5)	0.8 - 2.6
	GHR (pg/ml)	21.3 ± 7.3 (10.5 – 35.2)	40.8 ± 37.3 (9.5 – 178.6)	11.4 - 37.5
	Lept (ng/ml)	3.1 ± 0.6 (0.07 – 27.1)	2.2 ± 0.3 (0.15 – 20.3)	0.3 - 4.8
Aggression*	Testosterone (ng/ml)*	12.1 ± 5.0 (0.6 - 91.8)	17.8 ± 3.9 (3.8 - 171.9)	0.06 - 10.5

Values in the table describe the sample mean ± standard error, range minimum, and range maximum for each manifest variable included in the full path model. Asterisked parameters were measured for the purposes of this study, but were not included as manifest variables in the path analysis. Manifest variables marked in bold represent those that made a statistically significant contribution towards predicting the outcome of their respective latent variable. Only those values were retained for the final path analysis. Reference intervals for wild lions are provided for comparison and were generated using clinically healthy animals from the same sample population (Broughton et al., 2017).

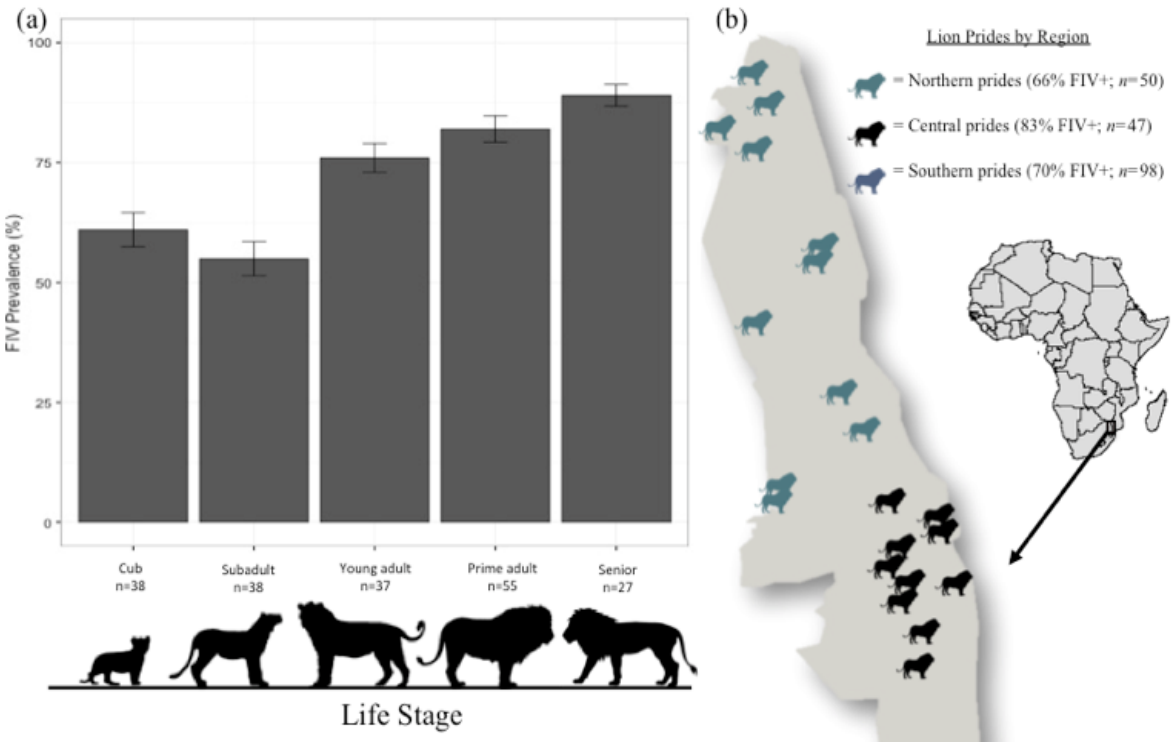


Fig. 2.1 FIV infection is common in lions of Kruger National Park. Within the sample population, 72.8% (142/195) of lions were infected with FIV. Prevalence of FIV infection was similar among male and female lions (75.6% versus 70.2%; n=74 and 121, respectively), but increased with host age (a). For ease of visualization, age has been broken up by life stage into cubs (0-2yrs), subadults (2.1-4yrs), young adults (4.1-6yrs), prime adults (6.1-8yrs), and seniors (>8yrs) based on previous age classifications (Schaller 1972). Regional prevalence of FIV within Kruger National Park was highest in the central region and lowest in the north (b). The map to the right shows locations where lion prides were sampled.

Box 2.2a: Independent variables for health evaluated in this study

CATEGORY	VARIABLE	CHANGE WITH FIV
Health	Alkaline Phosphatase (U/L) - ALT	↑
	Alanine Aminotransferase (U/L) - ALP	↑
	Blood urea nitrogen (mg/dl) -BUN	↓
	Creatinine (mg/dl) - CRE	Ø
	Glucose (mg/dl) - GLU	Ø
	Total protein (g/dl) -TP	Ø
	Gingivitis (% prevalence)	Ø
	Oral papillomas (% prevalence)	↓
	Ocular lesions (% prevalence)	↑
	Lymph node enlargement (% prevalence)	Ø
	Hygromas (% prevalence)	Ø
Nutrition	Dehydration (% prevalence)	↑
	Leptin (ng/ml) - GHR	↓
	Ghrelin (pg/ml) - Lept	↑
Immunity	Packed cell volume (%)	Ø
	Total solids (g/dl)	↑
	Leukocytes (cells/ul)	Ø
	Neutrophils (cells/ul)	Ø
	Lymphocytes (cells/ul)	↓
	Monocytes (cells/ul)	Ø
	Eosinophils (cells/ul)	Ø
	Basophils (cells/ul)	Ø
	CD4+ T lymphocytes (cells/10000)	Ø
	CD8+ cytotoxic T lymphocytes (cells/10000)	Ø
	CD21+ B lymphocytes (cells/10000)	Ø
	Erythrocyte sedimentation rate (units 120 min) -ESR120	↑
	Bactericidal activity whole blood (BKA) - BKaWB	Ø
	Bactericidal activity plasma (BKA) - BKaPlasma	Ø
Aggression	Testosterone in males (ng/ml)	↑
	Testosterone in females (ng/ml)	↓
	Severe wounds (% prevalence)	↑

↑Increase ↓Decrease Ø No change

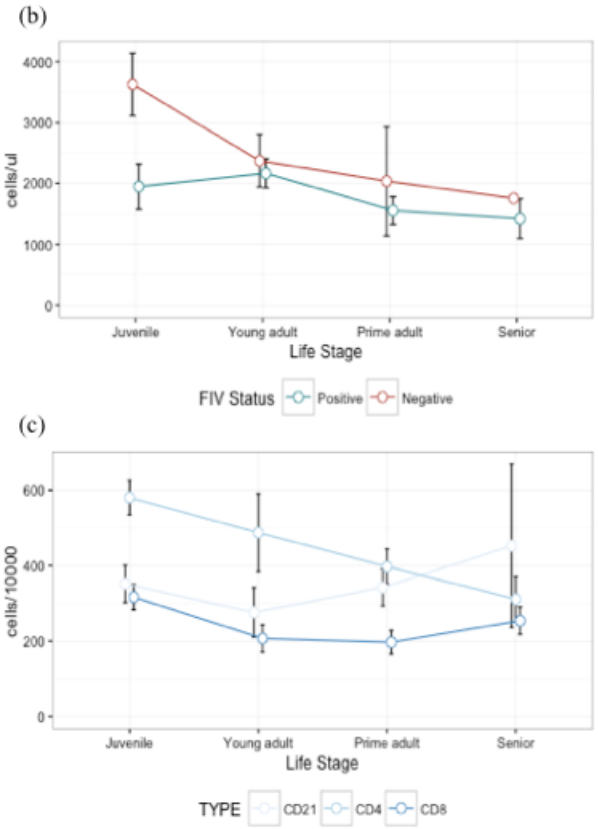


Fig. 2.2 FIV has broad effects on lion health and causes progressive immune impairment. Box (2.2a) above provides a complete list of the health metrics measured for the purposes of this study. Arrows to the right of each variable summarize the directionality of statistically significant changes with FIV infection. Descriptive statistics and reference values can be found in Table 2.2. Complete model output can be found in supplementary tables S1-S4 and S6. For ease of visualization, each parameter has been broken into categories of clinical relevance. To the right, age-related changes in lymphocyte profiles are shown for total lymphocyte counts in FIV-positive versus FIV-negative lions (b); as well as specific lymphocyte subsets in FIV-positive lions (c). Due to small sample size for lymphocyte subsets, subadults and cubs have been included together under the 'juvenile' category.

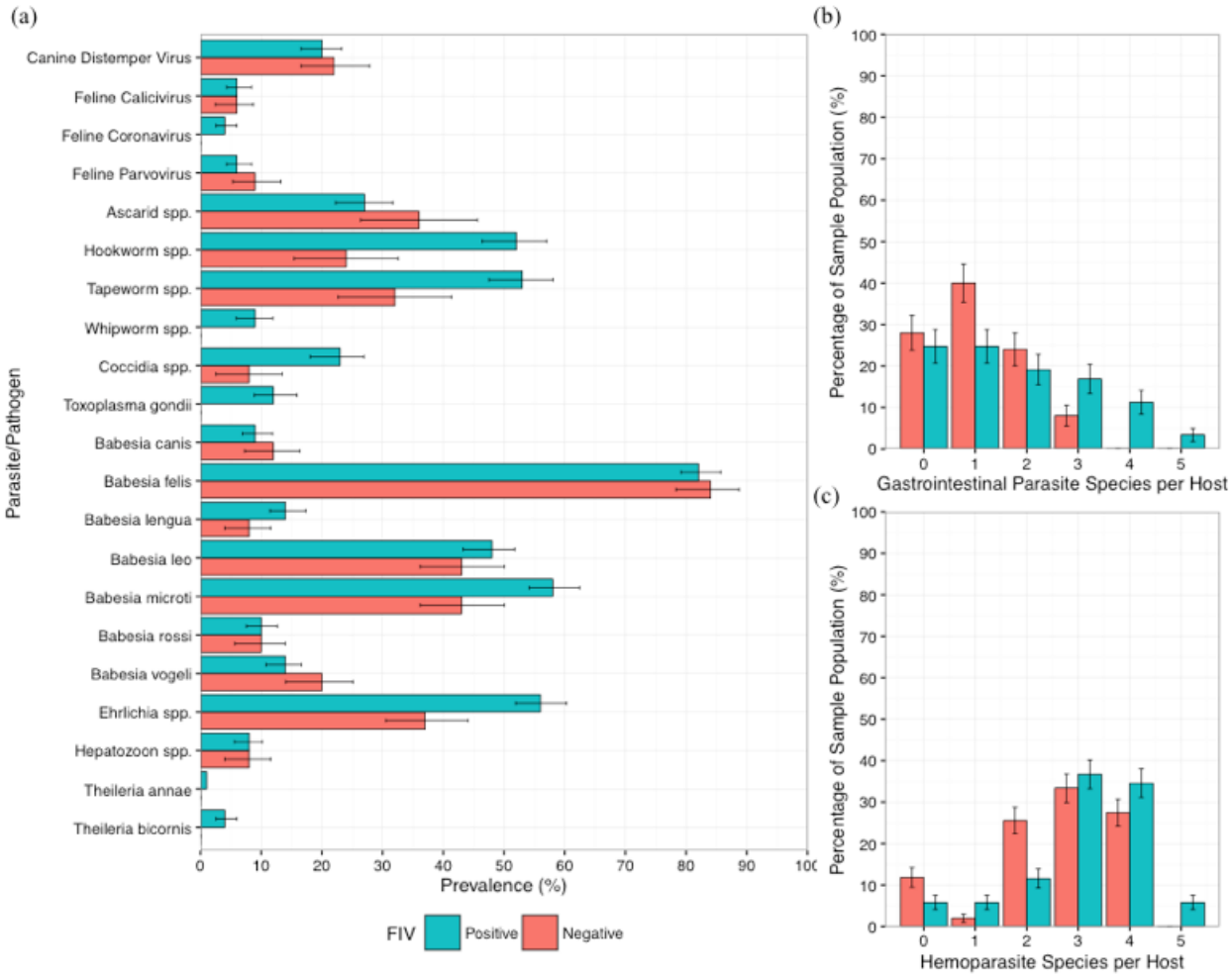
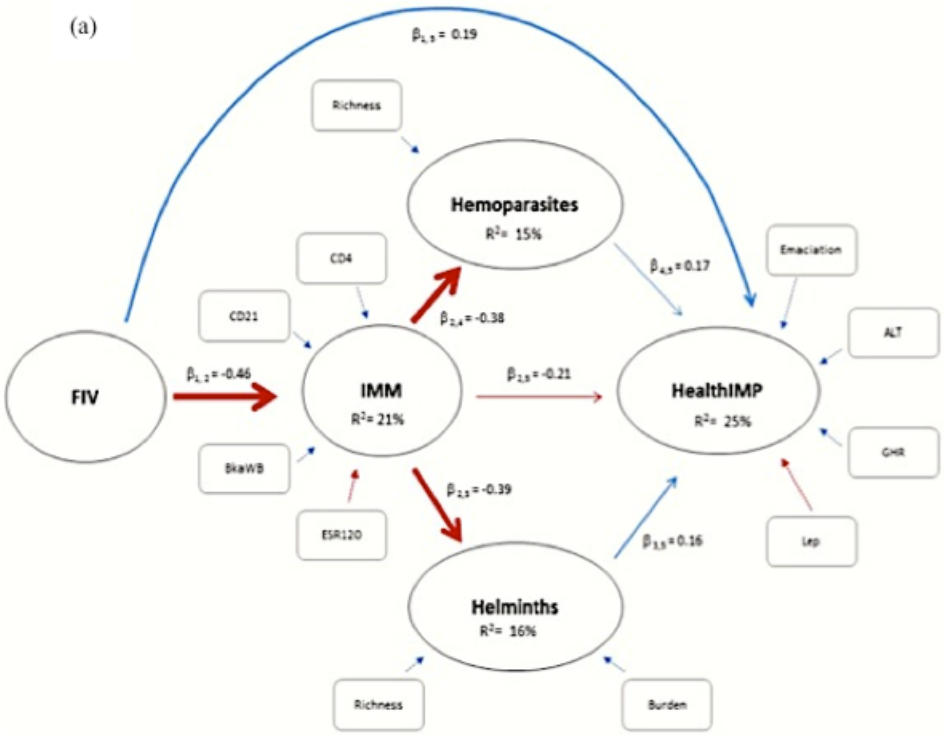


Fig. 2.3 FIV significantly increases the prevalence of select gastrointestinal and hemoparasitic coinfections, as well as overall parasite richness for both groups. Graph (a) shows the prevalence of coinfecting pathogens/parasites isolated in lions from this study. Coinfections are broken down by FIV status (positive versus negative). The two additional graphs illustrate the relationship between FIV status and gastrointestinal parasite richness (b) and hemoparasite richness (c).

Fig. 2.4 FIV has strong direct and indirect effects on overall health. The final path model (a) shows only statistically significant relationships between manifest variables (rectangles) and latent variables (circles) for FIV infection, immune response (IMM), co-infections with hemoparasites (Hemoparasites), co-infections with gastrointestinal parasites (Helminths), and health impairment (HealthIMP). Note that the parameter $\beta_{1,2}$ between each variable of interest represents the path coefficient obtained from least squares regression examining the relationship between one latent variable and the next (for example, FIV to IMM). Blue arrows represent positive relationships, whereas red arrows denote negative relationships. Small dashed arrows are used to represent significant relationships between manifest variables and their respective latent variable, and solid arrows are used for relationships between latent variables. Below, cross correlations generated by the partial least squares path model are shown for manifest and latent variables (Box 2.4b) and between latent variables (Box 2.4c). A summary of contribution (%) by each latent variable to global observed variability in health outcome is shown in (Box 2.4d). Overall, direct and indirect effects of FIV infection predicted 31% (R^2 = 31%) of health impairment.



Box 2.4b: Final cross correlations between significant manifest variables (MVs) and their respective latent variables (LVs).

VARIABLES	FIV	IMM	Hemoparasites	Helminths	HealthIMP
Immunity (IMM)	**-0.46**	**1**	**-0.38**	**-0.39**	**-0.43**
CD4	-0.38	0.62	-0.48	-0.05	-0.11
CD21	-0.29	0.88	-0.25	-0.46	-0.45
BKaWB	-0.39	0.57	-0.39	0.01	-0.16
ESR120	0.28	-0.56	0.03	0.41	0.23
Hemoparasites	**0.22**	**-0.39**	**1**	**-0.01**	**0.29**
Helminths	**0.2**	**-0.4**	**-0.01**	**1**	**0.28**
Burden	0.19	-0.29	0.03	0.86	0.29
Richness	0.19	-0.41	0.02	0.95	0.24
Health Impairment (HealthIMP)	**0.36**	**-0.43**	**0.29**	**0.28**	**1**
Emaciation	0.15	-0.15	0.18	0.11	0.44
ALT	0.34	-0.36	0.29	0.15	0.86
GHR	0.08	-0.19	-0.01	0.28	0.4
Lep	-0.04	0.14	0.01	-0.15	-0.23

*Relationships between latent variables are denoted by bold font.

Box 2.4c: Direct, indirect, and total effects of latent variables (LVs) on health impairment.

Paths	Direct Effects	Indirect Effects	Total	Mean Boot
FIV -> IMM	-0.46	0	-0.46	-0.46
FIV -> Hemoparasites	0	0.18	0.18	0.19
FIV -> Helminths	0	0.19	0.19	0.16
FIV ->HealthIMP	0.19	0.15	0.35	0.32
IMM ->Hemoparasites	-0.38	0	-0.38	-0.41
IMM ->Helminths	-0.39	0	-0.39	-0.33
IMM ->HealthIMP	-0.21	-0.13	-0.33	-0.27
Hemoparasites ->Helimnths	0	0	0	0
Hemoparasites ->HealthIMP	0.17	0	0.17	0.16
Helminths ->HealthIMP	0.16	0	0.16	0.15

Box 2.4d: Contribution (%) of each latent variable (LV) to global observed variability in negative health outcome.

LVs explaining health impairment	β	Correlation	Contribution to global R2 (%)
FIV	0.193	0.36	27.8
IMM	-0.204	-0.424	34.6
Helminths	0.16	0.28	17.9
Hemoparasites	0.17	0.29	19.7

CHAPTER 3: FELINE IMMUNODEFICIENCY VIRUS: THE ROLE OF COINFECTION FOR STRUCTURING PARASITE AND PATHOGEN COMMUNITIES IN AFRICAN LIONS (*PANTHERA LEO)*

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ABSTRACT

Within-host parasite and pathogen interactions can alter host susceptibility, pathogen invasion, persistence, and pathogenicity, with potential ramifications for both individual and population level disease dynamics, health, fitness, and mortality. Here, we use monopartite network analyses and a cluster model to evaluate population level parasite community composition as driven by the immunosuppressive pathogen, feline immunodeficiency virus (FIV), in a sample population of 195 free-ranging African lions (*Panthera leo).* To explore parasitic affiliations, we compare node and network statistics from our network models with findings from our cluster model to determine parasite community structure for FIV-positive and FIV-negative sample groups with known exposure to 21 other species of parasite or pathogen. We then discern whether interactions with FIV are direct or mediated via an alternate coinfection using generalized linear mixed effects models to account for differences with age, sex, body condition score, and pride membership while exploring associations between prevalence of single parasites and infection status for FIV and other coinfections known to increase with FIV infection. Results of the separate analyses show that, on average, FIV-positive parasite networks are more diverse; parasitic coinfections between species are more common; and parasitic affiliations across parasite groups are more homogenous than in FIV-negative networks. In addition, the cluster model suggests that parasites with tropism to the same body system tend to occur together. On an individual level, animals show direct associations between FIV infection and increased prevalence of some parasites (hookworms, coccidia, tapeworms, *Babesia microti*, whipworms, *Toxoplasma gondii,* and *Theileria* spp.); and indirect interactions with others (hookworm and *Babesia microti* drive associations with other parasites of their respective host system). Overall, the results of this study show that FIV may play a dominant role in structuring parasite communities at the population level, but that other coinfections may be of equal or greater importance when predicting host internal parasite community composition.

3.1 INTRODUCTION

Within-host environmental modification via simultaneous infection with parasites and pathogens, commonly known as co-infection, plays a large role in structuring host and population level parasite community assemblages – or infra- and component communities, respectively (Petney & Andrews, 1998; Pedersen & Fenton, 2007; Graham, 2008; Telfer et al., 2010; Ezenwa & Jolles, 2011). While the outcomes of these associations are widely variable, the predominant effects can be classified as either facilitative or inhibitory (Telfer et al., 2010). Facilitative interactions may increase the success of invasion and persistence by other coinfecting species through mechanisms such as the mechanical destruction of host barriers, cross-regulation of the host immune response, or direct immunosuppression (Petney & Andrews, 1998; Pedersen & Fenton, 2007; Ezenwa & Jolles, 2011). Conversely, interactions may limit host availability to other pathogens through cross-immunity or resource competition (Petney & Andrews, 1998; Graham, 2008; Telfer et al., 2010; Henrichs et al., 2016). Indeed, these dynamics play a large role in diverse host systems ranging from host-invasion by *Clostridium novyi* secondary to liver fluke (*Fasciola hepatica*) infestations in Black disease of sheep (Uzal et al., 2016); resource depletion by *Babesia microti* limiting susceptibility to *Bartonella taylori* in field voles (Telfer et al., 2010); and immune trade-offs driving persistence and outcome of coinfections between *Mycobacterium bovis* and parasitic helminths in African Cape buffalo (Ezenwa & Jolles et al., 2011). In addition, some interactions, such as the classically cross-protective effects of inoculation with certain pathogens for vaccination, are beneficial for the host and have been the subject of much research and manipulation.

Among the most recognized pathogens to alter host-parasite infracommunities are the lentiviruses, which have gained global attention for their ability to drive parasite community dynamics through wide scale immunosuppression, nutritional deficiency, and the destruction of host barriers. Infection with human immunodeficiency virus (HIV) in humans is recognized to increase susceptibility, morbidity, and mortality associated with a wide array of secondary parasites and pathogens including gastrointestinal helminths (Bentwich et al., 1999; Harms & Feldmeier, 2002; Fenton, 2013), malaria (Whitworth et al., 2000; Harms & Feldmeier, 2002; Fenton, 2013; Ivan et al., 2013), and tuberculosis (Selwyn et al., 1989; UNAIDS, 2012; Mor et al., 2013) among many others. Feline immunodeficiency virus, the feline analog of HIV, has

been shown to have similar effects in domestic cats (*Felis catus)*, leading to an AIDS-like syndrome characterized by immunosuppression, systemic immune dysregulation, nutritional wasting, neurologic and endocrine derangements, behavioral changes, and ultimately predisposition to secondary infections and neoplasia mediated via the aforementioned mechanisms (English et al., 1994; Pedersen et al., 2001; VandeWoude & Apetrei, 2006; Hartmann, 2011; Tejerizo et al., 2012). While the link between feline acquired immunodeficiency syndrome (FAIDS) and FIV infection has been clearly established in domestic cats, owing in large part to their use as a model species for HIV infection in humans (Bendinelli et al., 1995), behavior of other viral subtypes specific to wild species of felidae and hyenidae have historically been under-studied due to the inaccessibility of these species (Cleaveland et al., 2007).

Of particular interest, African lions (*Panthera leo*) show a high prevalence of FIV infection, with some populations ranging up to 100% (VandeWoude & Apetrei, 2006). Previous findings in this species have suggested that FIV is an innocuous, coevolved pathogen with few major clinical implications (Carpenter & O'Brien, 1995; Hofmann-Lehmann et al., 1997; Pecon-Slattery et al., 2008). However, recent studies on FIV-ple, the strain known to infect lion hosts, have shown broad immunosuppressive and pathologic effects including CD4+ depletion, panleukopenia secondary to myelosupression, lymphoid and thymic atrophy and involution, alterations to hepatic biochemical markers, changes to behavior and sex steroids that may drive aggression, nutritional wasting, and systemic immune dysregulation as indicated by elevations in inflammatory markers and hyperproteinemia (Bull et al., 2003; Troyer et al., 2004; Troyer et al., 2005; Brennan et al., 2006; Roelke et al., 2006; Troyer et al., 2008; Roelke et al., 2009). Despite the establishment of these FAIDs associated pathologies in infected lions, few studies have accounted for other coinfections when examining internal parasite community structure and the potential implications of internal parasite assemblages for lion health outcome.

While the compounding effects of coinfection dynamics are critical to the management of any wildlife system, understanding these impacts in African lions may be all the more pressing due to their critical role as an apex predator within several African ecosystems and their recent reclassification as threatened by the International Union for the Conservation of Nature (Bauer et al., 2016). Over the last 21 years, African lion numbers have declined by 43% (Bauer et al., 2016) due to a combination of overharvesting (Lindsey et al., 2012; Packer et al., 2009; Packer et

al., 2011; Riggio et al., 2013), human-wildlife conflict (Ikanda & Packer, 2008), population fragmentation (Dolrenry et al., 2014), prey source depletion (Ray et al., 2005; Becker et al., 2013), and localized epizootics (Roelke-Parker et al., 1996; Renwick et al., 2007), leading to reduction or extirpation over 80% of their historical range (Riggio et al., 2013). Commonly, sequela to these threats include reductions in heterozygosity, restriction to movement, physiologic stress, or malnutrition secondary to inaccessibility of resources, all of which may further facilitate local extinctions in the face of novel or compounding disease threats exacerbated by FIV (O'Brien et al., 1985; Ulrey, 1993; Lloyd, 1995; Murray et al., 1999). Because lions play a critical role in shaping the niche associations within their system, both as a top-down regulator via predation and an intra-guild competitor, critical reduction in lion population sizes may have knock-on effects on sympatric species across their range (Sergio et al., 2006; Valeix et al., 2009; Thaker et al., 2010; Ripple et al., 2014). Finally, lions share a wide array of infectious diseases with other vulnerable or endangered African carnivore species including bat-eared foxes, cheetahs, wild dogs, hyenas, and leopards among others (Hofmann-Lehmann et al., 1996; Osofsky et al., 1996; Murray et al., 1999; Cleaveland et al., 2007; Renwick et al., 2007; Alexander et al., 2008; Huang et al., 2014) leaving the potential for them to act as reservoir hosts for diseases that may be released from density dependence by jumping between alternate species (Begon & Bowers, 1995; McCallum & Dobson, 1995; Murray et al., 1999; Cleaveland et al., 2007). Thus, understanding the associations structuring parasite communities is paramount to the design of long term management strategies to mitigate impacts to host health, fecundity, and survival for both lions and at-risk sympatric carnivores for which lions may act as a reservoir.

To meet these end goals, this study utilized network analyses, a cluster analysis, and generalized linear mixed models to investigate parasite community structure in a population of 195 free-ranging African lions captured in Kruger National Park, South Africa. Network analysis provides a conceptual framework to describe the patterns of co-occurrence in ecological communities by comparing the units of interest (nodes) to the biological process of interest (edges; Poulin, 2010). Thus, use of network analyses on broad epidemiologic data can identify rules of ecosystem assembly (e.g. generalist vs. specialist species) or analyze the effect of environmental or ecological changes that may otherwise be missed by limited statistical models (Memmott et al., 2004; Memmott et al., 2007), while cluster analyses are useful in terms of

determining species groupings. Here we used these analytical tools to ask: 1) are coinfections more common in FIV-positive networks as compared to FIV-negative networks; 2) is the grouping of parasitic associations uniform across both FIV-positive and FIV-negative groups, or does one exhibit a higher degree of clustering; 3) on the individual level, are interactions with FIV direct or indirect via a secondary coinfection; and 4) which parasites tend to co-occur within lion hosts?

FIV-positive and negative networks were modeled separately to examine parasite and pathogen (nodes) co-occurrence within a shared host (edges) for both FIV-positive and FIV-negative networks. Parasites and pathogens were then compared across both groups using a cluster analysis to examine major parasite groupings (communities). Finally, statistical models accounting for variation due to demographics (age and sex), host health (body condition), and environmental exposure (location/pride) were used to explore direct versus indirect pathogenic and parasitic associations by host FIV status. Taken together, the use of these analyses in combination with the broad array of pathogens and parasites measured establish this study as one of the most comprehensive investigations examining the relationship between FIV and secondary infections in lions to date.

3.2 METHODS

Study System and Sample Collection

Collaboration with a larger parent study investigating lion behavior and demographics provided supportive infrastructure for the capture and sampling of 195 free-ranging African lions over a three-year period from March 2010 until September 2013. Captures took place over the dry season from March until November in Kruger National Park, South Africa, which supports an overall lion population of approximately 1700 individuals. Lions were attracted to the capture site using recordings of prey distress calls and a bait carcass. The lion pride was chemically immobilized en masse using combinations of either butorphanol (0.31 ± 0.034 mg/kg), medetomidine (0.052 ± 0.006 mg/kg), and midazolam (0.21 ± 0.024 mg/kg); or medetomidine (0.07 ± 0.01mg/kg) and tiletamine-zolazepam (1.8 ± 0.5mg/kg; Jacquier et al., 2006; Wenger et al., 2010). Tranquilizers were sourced from Kyron Laboratories (Pty) Ltd, South Africa and combined according to visual weight approximations. Drug cocktails were injected intramuscularly using a CO_2 powered Dan-inject dart rifle (Dan-Inject International, Skukuza,

South Africa; Jacquier et al., 2006; Wenger et al., 2010). Due to potential complications associated with α2 agonists, lions under six months of age were not immobilized and were thus not included in the datasets. All sedations were performed by SANPARKs veterinarians associated with the parent study (AUCC #FERSM5-767).

Heart rate and gum pallor were monitored regularly throughout immobilization and a full physical examination was performed to determine general health and identify any clinical signs that may be associated with infection status pertaining to the parasites/pathogens of interest. In addition, body condition score was measured on a five-point scale (1-5) using standardized techniques to score fat coverage over the sacrum, ribs, hips, and stomach (Ferreira and Funston, 2010). Animals with low fat coverage and readily palpable boney prominences received a score of one, while animals with a large degree of fat coverage and barely palpable prominences received a score of five. Blood was collected for use in ELISA and PCR based diagnostics for viruses and hemoparasites, while feces was collected for the identification of protozoal and helminth gastrointestinal parasites. After sampling, anesthesia was reversed using atipamezole (0.3 ± 0.1mg/kg; Wenger et al., 2010; Jacquier et al., 2006) to allow for a faster recovery and reduced risk from other animals while under sedation.

Demographics

While immobilized, animals were examined for age and sex, branded, and one from each pride was fitted with a GPS collar. Branding was performed using a branding iron with symbol combinations placed over the hip and shoulder to allow for individual identification. Pain control during branding was provided by butorphanol included in the dart cocktail or injected separately and the brand site was treated with topical antibiotic afterward to prevent any irritation or infection. GPS coordinates were recorded at the time of capture and used later to classify the regional distribution of lion prides and individuals into northern, central, and southern portions of the park, which are known to vary with regards to prey and water access, species density, and geologic substrate (Ferreira and Funston, 2010). Pride identification was provided through a combination of affiliation at the time of immobilization and observational data from long-term monitoring efforts.

General demographic parameters for age and sex were evaluated using standard techniques. Age was determined using tooth eruption patterns, breakage and wear to the canine teeth, pigmentation of the gums and nasal planum, which darken with age, and in males,

coloration and fullness of the mane (Smuts et al., 1978). The above demographic parameters were used to evaluate prevalence patterns for parasites/pathogens and were included in statistical models to account for observed trends both independently and in combination with body condition, FIV status, and other coinfections of interest.

Coinfections

Virology: Blood for use in viral diagnostics was collected via venipuncture of the jugular or femoral veins directly into redtop whole blood Vacutainer® tubes (Greiner Bio-One®) and stored at ambient temperature during transport from the capture site (< 8 hours). Once in the laboratory, serum was separated via centrifugation and stored at -60°C until use. Individual serum samples were transported frozen to Onderstepoort Veterinary Institute, where viral diagnostics were performed via antibody-based ELISA assays for canine distemper virus, feline parvovirus, feline calicivirus, feline enteric coronavirus, and feline immunodeficiency virus. While antibody ELISAs are considered indicative of exposure rather than active infection for most of the viral pathogens given the ability for recovery and development of long-term immunity, a positive ELISA for FIV was considered an active infection, as infection with FIV is lifelong after seroconversion.

Gastrointestinal parasitology: Feces for use in gastrointestinal parasite diagnostics was collected directly from the rectum into plastic flasks and refrigerated (short term) or frozen (long term) until use. Parasite eggs for gastrointestinal helminthes (ascarids, hookworms, tapeworms, and whipworms) and oocysts for protozoal parasites (coccidia spp. and *Toxoplasma gondii*) were detected using standardized fecal centrifugation and sugar flotation protocols (Foreyt, 2001).

Blood parasitology: DNA for a PCR-based reverse line blot hemoparasite panel was extracted from EDTA whole blood using a Qiagen DNeasy kit (Qiagen, Hilden, Germany; PN 69506). Blood for use in the assay was collected via jugular or femoral vein venipuncture directly into EDTA Vacutainer® tubes (Greiner Bio-One®). Samples were then aliquoted and stored at -60°C until extraction. The selected reverse line blot panel, which was custom made by Marinda Oosthuizen according to established protocols (Molecular Diagnostic Services Laboratory at Onderstepoort Veterinary Institute, Pretoria, South Africa; Bosman et al., 2010; Kelly et al., 2014), and was chosen for its ability to detect multiple parasites at once using markers for *Theileria*, *Babesia*, *Ehrlichia*/*Anaplasma*, and *Hepatozoon* species previously identified in lions and cheetahs. This, in combination with its inclusion of non-specific markers

for catch-all determination of major parasite groups, made it a sensitive and inclusive detection method for hemoparasite identification in a host where target parasite species have not been fully described. In addition, the panel's use of parasite DNA makes it a valid method for determining active infection status.

Statistical Analysis

Parasite prevalence and richness: Parasite prevalence was determined overall and for each demographic parameter using positive and negative status counts from each parasite and pathogen. For viruses, a positive ELISA was considered evidence of exposure rather than active infection, and thus exposure prevalence was determined for all viruses except FIV. Seroprevalence of FIV was considered active infection due to the lifelong course of the disease. For gastrointestinal and hemoparasites, a positive test was considered indicative of active infection. For all prevalence parameters, prevalence was determined using the following equation:

$$Prevalence\ (\%) = \frac{total\ no.\ animals\ with\ parasite}{total\ no.\ of\ animals\ in\ sample\ group}$$

Size of sample group was adjusted according to the demographic parameter of interest or, in the case of FIV, the number of FIV-positive or FIV-negative individuals. For ease of interpretation and visualization, age was subdivided into five age categories: cubs (0-2 years), sub adults (2-4 years), young adults (4 – 6 years), prime adults (6-8 years), and seniors (>8 years). For location, animals were divided by north, central, and south, as well as individual pride.

Parasite richness (i.e. the total number of parasite species in a given individual or group) was calculated both separately for viruses, gastrointestinal parasites, and hemoparasites, as well as combined for total parasite richness. While overall richness was selected to visualize the combined measure against FIV in statistical and population level network models, richness determination for individual parasite group was selected for mapping purposes due to differing sample size. This allowed all samples to be retained for final comparison (i.e. metrics for viruses and hemoparasites included far more samples than gastrointestinal parasites due to lack of availability of feces from each lion). Thus, overall richness includes only those samples for

which all metrics were available, while the mapped richness for each parasite category includes all samples from that particular parasite group.

Cluster and network analysis: Following methods used to examine viral community structure in rodents and bats, monopartite networks were constructed in this study to examine structural relationships between parasite and host communities in FIV-positive and negative subpopulations (Luis et al., 2015). All network and node statistics were calculated using the *igraph* network research package in R (Csardi & Nepusz, 2006). Two networks were constructed separately. The first, referred to as the parasite network, defined network nodes as a single parasite/pathogen species and edges as the shared lion host between two separate parasites. In this network, the weight of an edge represented the number of shared lion hosts between each pair of pathogens/parasites. The second network, referred to as the host network, compared shared parasites between hosts. Thus, hosts (lions) were defined as nodes in the second model, while shared parasite species became edges. Again, the weight of an edge reflected the number of shared parasite species between two lion hosts. For each of the two networks, node statistics were calculated for mean degree, diameter, transitivity, weighted assortivity, and betweenness in both FIV-positive and FIV-negative sample populations. Complete definitions of each of these node statistics can be found in Box 3.1.

In addition to node statistics for each monopartite network, network statistics were calculated for the combined host-parasite network and included parameters for connectance and degree distribution. Here, connectance is defined as the ratio of realized edges (link:node ratio of parasites sharing a single host) relative to the total possible number of edges (lion hosts) that could be shared between nodes (parasites). Degree distribution was defined as the distribution of contact numbers within the population – in this case parasite contacts via shared lion hosts (measured as parasite richness; Luis et al., 2015).

Next, to identify which groups of pathogens commonly co-occur, a cluster analysis was conducted using a community detection algorithm to identify communities in the overall parasite network. In this context, communities were defined as sets of nodes in the network with high levels of within-group connections and low levels of between-group connections (high modularity). Modularity, as used here, presents a metric for the amount of community structure – in this case the ratio of intra-group connections of parasites to intergroup connections (Luis et

al., 2015). We identified pathogen communities on the pathogen network by maximizing the modularity, Q, where:

$$Q = \frac{1}{2m}\sum_{i,j}[A_{i,j} - \frac{k_i k_j}{2m}]\ \delta(c_i, c_j).$$

In this equation, $A_{i,j}$ is the weight of the edge between *i* and *j*. If an edge exists, $k_i = \sum_j A_{i,j}$ is the sum of the edges attached to *i*, and c_i is the community to which *i* is assigned. The delta function is 1 if c_i=c_j and 0 otherwise. Modularity, calculated as $m = \frac{1}{2}\sum_{i,j} A_{i,j}$, ranges between -1 and 1. Higher values occur when the density of edges within communities is larger than the density of edges between communities.

We applied the community detection algorithm above, as described in Blondel et al., 2008, to detect communities in two stages and repeated iteratively until a maximum modularity was obtained. In the first stage of this method, each node is assigned to its own community. The algorithm sequentially evaluates the reassignment of each node to its neighboring communities and places the node in the community with the largest gain in modularity. This process is repeated for all nodes and until no reassignments improve the modularity. In the second stage, a new network is constructed with nodes representing the communities identified in the first stage and edges representing the between-community links. The first stage of the algorithm is applied to this new, community-level network, and community memberships of all nodes are updated. The two-stage process is then repeated through several iterations until no increases in modularity are achieved. Many alternative algorithms exist to determine communities in networks (Newman, 2010), but use of the algorithm described above (Blondel et al., 2008) performs well (Lancichinetti & Fortunato, 2009) in a range of contexts (Gorsich et al., 2016; Buhnerkempe et al., 2017).

Statistical models: Statistical models to explore parasite/pathogen associations with demographic parameters, FIV, and coinfections were conducted in four stages using R statistical software (stats package; R Core Team, 2014). Prior to modeling all data were explored for normality, linearity, constant spread, and leverage points using histograms, boxplots, Cook's Distance, matrixes, and plots of residuals vs fit values (*plot* function; R Core Team, 2014). Primary models to explore the effect of location were run as binomial generalized linear models with a logit link and included the parasite/pathogen of interest as a dependent binary variable with independent variables for age, sex, body condition, location, and FIV status (stats package;

R Core Team, 2014). All models were backwards selected using AIC and p-values provided by the *drop1* function in the AER statistical package (Kleiber & Zeileis, 2008; stats package; R Core Team, 2014).

In the aforementioned model, location showed consistent variation geographically for each individual parasite of interest, which was also evident at the pride level and lead to drastic reduction in residual degrees of freedom and statistical power. While location exhibited high variation for individual parasites, it is likely that the observed loss of residual degrees of freedom in the statistical models resulted from extreme data partitioning. As such, location was dropped from subsequent parasite models and raw prevalence values were provided for north, central, and south.

To avoid data partitioning and non-independence, subsequent models were run as Laplace generalized linear mixed models (GLMMs) with a binomial distribution, logit link, and a random effect for pride identification to account for pseudoreplication among locations and pride affiliation (lme4 packages; Bolker et al., 2008; Bates et al., 2015). Primary models to investigate parasite/pathogen associations with demographic parameters and FIV included fixed effects for age, sex, body condition score, and FIV, as well as interaction terms for age:FIV and sex:FIV modeled against the parasite/pathogen of interest. A separate model was run to examine demographic associations with FIV itself and contained fixed effects for age and sex, as well as the random effect for pride identification, but did not include body condition, as body condition was more likely an outcome of FIV status rather than a predictive factor.

Secondary and tertiary models to investigate whether parasite/pathogen associations were directly related to FIV, or indirectly related to FIV via an alternate coinfection, included fixed effects for age, sex, FIV, and coinfections highly associated with FIV (hookworms, coccidia, tapeworms, and *Babesia microti).* An interaction term between the aforementioned coinfections and FIV was added to the tertiary model to explore significant interactions between the two. Each coinfection was compared one at a time to increase statistical power and avoid parameters for which there was zero co-occurrence. Final model selection was conducted using stepwise comparison of AIC values via the dredge function in the MuMIn package (Barton, 2016). FIV was considered directly related to parasite/pathogen outcome if it was individually statistically significant in every model; both directly and indirectly related if it was statistically significant in every model and was significant in the FIV:coinfection interaction term; and indirectly related if

FIV was retained in the primary model, but dropped after accounting for another coinfection parameter that was statistically significant and more common in FIV infected hosts. For coinfections for which all animals were positive for a certain parasite/pathogen, that parasite was not included in the statistical analysis. Instead, prevalence was noted and coinfections were considered separately. Prevalence for dual infection was calculated separately to account for potential interactions between fixed effects.

3.3 RESULTS

FIV-positive host and parasite networks are larger and more connected than FIV-negative networks. Overall, node statistics for connectivity (mean degree, transitivity, diameter, and betweenness) were higher; node statistics for clustering (weighted assortivity) lower; and overall number of nodes higher in FIV-positive networks for both parasites and hosts (Table 3.1). In addition, network statistics for the combined networks showed a higher degree of connectance (link:node ratio of 21 versus 5; Fig. 3.1) in FIV-positive groups. Meanwhile, mean degree distribution was not markedly different between subpopulations (FIV-positive hosts supported an average of five parasite species in contrast to four species in FIV-negative hosts). Despite this finding, a right shift in the network distribution indicated that though average number of parasites was not markedly different, FIV-positive networks supported an overall greater parasite richness at the top end of their range (network variance of 4.9 in FIV-positive versus 2.1 in FIV-negative; Fig. 3.2) and increased species maximum in the right tail of their distribution.

Cluster analyses of parasite-host networks suggest that parasites co-occur within four primary communities. Using community detection algorithims to assign parasite groupings based on the frequency of shared hosts, parasites were divided into four main communities that included the following species and families: 1) hookworms, whipworms, tapeworms, coccidia, *Toxoplasma gondii*, *Theileria bicornis*, canine distemper virus, and feline enteric coronavirus; 2) feline immunodeficiency virus, feline parvovirus, *Hepatozoon* species, *B. felis*, and *B. lengua;* 3) *B. vogeli*, *B. rossi*, *B. canis*, and *T. annae;* and 4) feline calicivirus, ascarid spp., *B. microti*, *B. leo*, and *Ehrlichia spp*. (Fig. 3.3).

FIV is directly associated with increased prevalence of some gastrointestinal and hemoparasites, and may be indirectly associated with others via coinfection interactions. Further exploration of parasite interactions through statistical models suggests that FIV may be

directly and/or indirectly associated with various coinfections (Fig. 3.4**;** see supplementary Tables 3.S1 and 3.S2 for model output). Individually, FIV shows direct statistically significant interactions with increased prevalence of hookworm species (*β = 6.03; p-value = 0.00; n= 110*), tapeworm species (*β =3.21; p-value=0.02; n=110*), coccidia species (*β =221.02; p-value=0.02; n=110),* and *B. microti* (*β = 3.00; p-value=0.03; n=110).* Animals with whipworm species, coronavirus, *T. gondii*, and both species of *Theileria* also exhibited a 100% prevalence of FIV, as well as a frequent co-occurrence with the other FIV-linked parasites and pathogens (Fig. 3.4a).

Contrastingly, statistical association between FIV and *Ehrlichia* species approached significance (*β =2.62; p-value=0.06; n=110)* after a fixed effect for hookworms was added to the model. Hookworms, which occur in high prevalence associated with FIV infection, also appear to drive infections with canine distemper virus (*β =4.30; p-value=0.00; n=110)* and ascarid species (*β =2.32; p-value=0.06; n=110*), while decreasing coinfection with *B. lengua (β =0.04; p-value=0.00; n=110)* and *B. canis (β =0.24; p-value=0.08; n=110).* Similarly, coinfections with *B. microti* drive infection with *B. felis (β =4.43; p-value=0.00; n=110), B. leo (β =7.60; p-value=0.00; n=110),* and feline calicivirus (*β =6.79; p-value=0.08; n=110*), while lessening the prevalence of *B. lengua* (*β =0.36; p-value=0.05; n=110).* Interestingly, interactions between FIV and coccidia decrease after tapeworms are added as a fixed effect, indicating that this strong association may also be indirectly mediated (see Table 3.S2 for model output).

Prevalence of pathogens and parasites shows wide variation across location and pride membership. While exploratory models failed to show any statistically relevant association between overall parasite richness and geographic location or pride identity (*β = 1.12; p-value =0.15; n=110*), the prevalence of individual parasites and pathogens among prides and geographic locations showed wide statistical variation. As such, raw regional prevalence and overall pride parasite richness for each of the major pathogen categories are reported in Fig. 3.5 (and supplementary Tables 3.S3, 3.S4, 3.S5, and 3.S6). Overall, FIV and feline calicivirus seroprevalence was highest in the central region of the park, while exposure to other viral pathogens (feline parvovirus, feline coronavirus, and canine distemper virus) occurred disproportionately in the south. With the exception of ascarid species, which showed a higher prevalence in northern sections, infection with all other gastrointestinal parasites (hookworm spp., tapeworm spp., whipworm spp., coccidia spp., and *Toxoplasma gondii)* occurred most

frequently in the south. Contrastingly, all *Ehrlichia* species, *Hepatozoon* species, and major species of *Babesia* (*B. felis, B. leo,* and *B. lengua)* showed highest prevalence in the central region of the park with the exception of *Babesia microti,* which was highest in the south. Rare species of *Babesia* (*B. canis, B vogeli,* and *B. rossi)* showed highest prevalence in the north. Both *Theileria* species (*T. bicornis* and *T. annae)* were found in low enough prevalence that geographic trends were not readily discernable (see Fig. 3.5 for all observed trends and Tables 3.S1, 3.S2, 3.S3, and 3.S4 for raw data).

Prevalence of certain parasites correlated highly with age and body condition. Of the demographic parameters (age and sex), only age exhibited statistically significant associations with regards to the parasites and pathogens of interest after accounting for FIV infection status and body condition (Fig. 3.6; for all model outputs and demographics please reference Tables 3.S1 and 3.S7). FIV infection itself increased significantly with age (*β = 1.27; p-value = 0.001; n=195*). As for the four other viral pathogens, only feline calicivirus showed a statistically significant increase with age (*β = 1.28; p-value = 0.02; n = 195).* With regards to gastrointestinal parasites, infection with hookworm and tapeworm species decreased with age (*β = 0.80* and *0.89; p-value = 0.02* and *0.07; n = 114*, respectively), while prevalence of coccidia increased after accounting for FIV status and a significant interaction between the two parameters (*β = 2.64; p-value = 0.04; n=114*). Finally, *Ehrlichia* species, *Babesia felis, Babesia leo,* and *Babesia rossi* infection all increased with age (*β = 1.28, 1.15, 1.12,* and *1.20; p-values= 0.00, 0.07, 0.06,* and *0.08; n= 190,* respectively), though some findings only approached statistical significance. No other statistically significant associations were observed between age and the parasites of interest. Of all models considered, the control parameter for body condition showed marginal associations with increases in *Babesia lengua* and *Hepatozoon* species (*β = 2.61* and *2.36; p-values = 0.06* and *0.06; n=190,* respectively), and decreases with *Toxoplasma gondii* and *Ehrlichia* species (*β = 0.35* and *0.69; p-values= 0.02* and *0.09; n= 114* and *109,* respectively).

3.4 DISCUSSION

While major findings from this study support the overall importance of FIV for structuring parasite communities in lion populations, network and cluster analysis and statistical models suggest that the crux of this importance may be influenced more heavily by combinations

of parasite infracommunities rather than FIV infection alone. This finding is supported by the fact that, while FIV infection does correlate with increased infection from certain species of gastrointestinal and hemoparasite (hookworms, tapeworms, coccidia, and *B. microti),* these parasites showed greater statistical significance for determining infection risk with the vast majority of other parasites, which was reflected by shifts in parasite network and community affiliation.

Overall, parasite and host network analysis support a higher degree of connectivity, higher community membership, and lower clustering/modularity in FIV-positive networks versus FIV-negative networks, a trend that may easily be explained by observed immunosuppression and increased aggression/contact among FIV-positive hosts (Roelke et al., 2006, Roelke et al., 2009). Within this context, network findings suggest that FIV-positive lion populations are more likely to harbor a greater number of parasitic coinfections, and are more likely to share those coinfections with other hosts, than FIV-negative groups. While these findings are not surprising given known host-parasite interactions in both domestic cats and lions exhibiting feline AIDS (Fromont et al., 1997), the more perplexing result is that these mechanisms may be driven largely via the particular coinfection assemblage present rather than FIV infection alone.

Of the parasites that increase with FIV infection, hookworms and *Babesia microti* played a disproportionately large role in determining community structure with other parasites and pathogens. With regards to hookworms, mechanisms for these associations have been largely substantiated in the human HIV literature. Several studies, metanalyses, and theoretical models have shown that HIV-helminth coinfections may drastically alter co-morbidity, progression, and mortality from secondary infections in HIV-helminth coinfected patients (Bentwich et al., 2000; Harms & Feldmeier, 2002; Walson et al., 2009). Of particular interest, the immunomodulatory effects of chronic helminth infection, which favor an IL-4 rich cytokine environment and a shift towards Th2 CD4+ cell activation, have been shown to release HIV from density dependence, thus facilitating viral replication and AIDS progression (Kalinkovich et al., 1998; Shapiro-Nahor et al., 1998; Bentwich et al., 1999; Kallestrup et al., 2005; Kinter et al., 2007; Walson et al., 2009). In coinfected patients, elimination of helminth infections has been shown to drastically reduce viral load and boost overall CD4+ counts and immunocompetence (Kassu et al., 2003; Walson et al., 2009; Modjarrad et al., 2010, Sangaré et al., 2011). Contrastingly, certain helminth infections have also been shown to directly evade host immune attack and promote

immune tolerance by increasing the production of regulatory B cells and creating a cytokine environment dominated by interleukin-10 (IL-10), interleukin-35 (IL-35), and transforming growth factor β (TGF- β), which directly suppress pro-inflammatory lymphocytes (Rosser & Mauri, 2015; McSorley & Maizels, 2012). Thus, while FIV infection and hookworm infestations are likely to have marginal significance for host health and infection dynamics on their own, these effects may be exacerbated in areas with a high level of coinfection and exposure to novel disease threats.

As for observed trends with *Babesia microti,* FIV-*Babesia* coinfections are likely facilitated by FIV driven crashes in CD4+ T helper cell populations, which are critical for clearance of *Babesia* organisms (Ruebush & Hanson, 1980; Homer et al., 2000). Particularly, intracellular and free-floating merozoites of *Babesia microti* have been shown to trigger an immune response characterized by upregulation of Th1 cells (Ruebush et al.,1986). In the case of FIV-*Babesia* coinfections, upregulation of this immune branch may further release FIV from resource restriction and exacerbate FIV driven CD4+ Th1 cell crashes, leaving hosts vulnerable to other viruses and intracellular parasites normally controlled via Th1 mechanisms.

Contrastingly, high co-occurrence between FIV, hookworms, and *Babesia microti,* may lend further evidence to functional switching of the host immune response between Th1 and Th2 branches as a driver for observed trends independent of FIV infection. The same IL-4 dominated cytokine environment that favors Th2 activation and protection from macroparasites, such as the helminth species observed here, suppresses the Th1 immune response, which plays a critical role in protection from intracellular pathogens and parasites (Bentwich et al. 1995; Homer et al. 2000; Harms & Feldmeier 2002; Kassu et al. 2003; Sangare et al. 2011; Ivan et al. 2013). As such, while coinfection with the detected hemoparasite species is likely to be driven largely by mutual exposure through infected tick vectors, the fact that coinfection with these and other intracellular viral pathogens persists even after accounting for exposure potential (location/pride identification) suggests that these parasites may be largely mediated via an alternate means in FIV-infected and uninfected hosts. This notion is further supported by the large degree of interaction between parasite communities, specifically hookworms and *Babesia microti*, and certain common species of hemoparasite and virus independently of FIV infection. Hookworm infection is associated with an increased prevalence of *Ehrlichia* and canine distemper virus, and a decreased prevalence of *Babesia canis* and *Babesia lengua* independently of FIV infection.

Likewise, *Babesia microti* is associated with increased prevalence of *Babesia leo, Babesia felis,* and feline calicivirus and decreased prevalence of *Babesia leo* regardless of FIV status. As such, the fact that hookworm and *Babesia microti* infections seems to correlate highly with increased or decreased prevalence of other parasite species independently of FIV infection may suggest that immune responses resulting from infection by these parasites alone may drive infection dynamics with other species, with any associations with FIV resulting from secondary knock-on effects.

Parasite community structure, as investigated via cluster analysis, suggests that parasite affiliations may also occur largely due to shared environment within the host. Parasites detected in this study tended to affiliate within four primary communities largely dictated by tropism to a shared body system (in this case either the cardiovascular or gastrointestinal system). Gastrointestinal parasites and pathogens tended to occur together with a high level of interaction between parasites occupying the gut lumen (hookworms, whipworms, tapeworms, coccidia, and *Toxoplasma*; Bowman, 2003), and viruses targeting enterocytes and lymphoid tissue within the gastrointestinal tract (canine distemper virus and feline enteric coronavirus; Brown et al., 2009; Carvalho et al., 2012; Pedersen et al., 2012). Similarly, parasite and pathogen species that target cells of the hemolymphatic system (leukocytes and red blood cells) tend to occur together in two subgroups: one including the more common pathogens and parasites such as feline immunodeficiency virus, feline parvovirus, *Hepatozoon* species, *B. felis,* and *B. lengua (*Truyen & Parrish, 1992; Baneth & Weigler, 1997; Dean et al., 1999; Baneth et al., 2000; Bowman, 2003; Bosman, 2010)*;* and the second including the less common hemoparasites *B. vogeli*, *B. rossi, B. canis,* and *T. annae* (Bowman, 2003; Kelly et al., 2014). In contrast to these first three communities, the fourth community showed interactions mostly based on prevalence in the population rather than individual tropism and included feline calicivirus (Love & Wood, 1975), ascarid spp. (Bowman, 2003), and three species of highly prevalent hemoparasite (*B. microti, B. leo,* and *Ehrlichia* spp.; Bowman, 2003; Kelly et al., 2014). *T. bicornis* occurred in extremely low prevalence and tended to co-occur with the gastrointestinal parasites despite its tropism for the hemolymphatic system.

While the findings of this study are promising in terms of understanding parasite communities in FIV infected lion hosts and populations, it does have several major limitations. First, while methods to detect parasite species have been proven in clinical practice and domestic

species, unknown hypobiotic or pre-patent periods in wild species and single-sex gastrointestinal parasite infections may reduce sensitivity of parasite diagnostics for the detection of infection. Similarly, lack of PCR primers for previously unknown hemoparasite species may lead to artificially lower parasite richness count. Finally, cross-sectional rather than longitudinal data makes it impossible to determine disease incidence, as well as concurrent infection with directly transmitted viral pathogens, in order to nail down the mechanisms that may underlie these associations.

3.5 CONCLUSION

Despite these limitations, this study provides a sound starting block for the structure of future parasite community studies in this and other species and may help to inform wildlife management strategies aimed at mitigating the consequences of novel disease risks, such as that currently faced by wildlife populations impacted with *Mycobacterium bovis.* Future studies would benefit substantially from the use of longitudinal data collection and species-level parasite detection, though this may be logistically infeasible in large carnivore species due to low recapture rates and lack of previous data. For now, increased efforts should be dedicated to understanding the importance of these FIV-parasite associations in at-risk populations of lions and sympatric species for which they may act as a reservoir.

Acknowledgements: Sample collection and logistics for this project were part of an international collaborative effort including experts from several agencies and institutions throughout South Africa and the U.S. Funding for the project and capture infrastructure were provided by a larger parent study on lion behavior, disease, and demographics (PIs Paul Funston and Sam Ferreira). Sample and data collection/exchange were part of a team effort carried out by the wonderful capture teams at South African National Park Services (SANPARKs) - Veterinary Wildlife Services in Skukuza, Mpumalanga, South Africa. Scientific expertise, study design, and project approval were made possible by the expertise of the ecologists from SANPARKs Scientific Services also in Skukuza. Of particular mention, Drs. Sam Ferreira, Peter Buss, Markus Hofmeyr, Michelle Miller, and senior technician Jenny Joubert-Hofmeyr, as well as their associated teams and graduate students, headed up various directions of this multifaceted disease project to make it one of the most comprehensive predator disease projects to date. Additionally, lion behavioral monitoring and longitudinal regional and pride level data were

made possible by the diligent efforts of Darius Scholtz, Nkabeng Maruping, Trevor Valoyi, Travis Smit, and Katlego Makutulela.

On the feline immunodeficiency side of the project, undergraduate, graduate, and veterinary student teams made the collection of disease and immune data in the field not only possible, but one of the most memorable experiences of a lifetime. Special recognition is owed to these students, who included Tanya Kerr, Zaya McSky, Emily Magnuson, Kadie Anderson, Elena Alonso, Ignatius Viljoen, and Stephanie Caruana. Last but not least, funding for these study efforts was supplied both by the parent project (funded by Ajubatus) as well as independent fellowships and grants provided by the Biomedical Sciences Department at Oregon State University and Morris Animal Foundation (Grant # D13ZO-420). We would like to direct a special thanks to the many donors of the Morris Animal Foundation program, which have made this and many other wildlife studies possible and continue to allow Morris to be one of the leading agencies for animal welfare in the world.

3.6 TABLES AND FIGURES

Box 3.1 Definitions for node statistics calculated for monopartite host (lion) and parasite networks in FIV-positive and FIV-negative sample populations.

PARAMETER	Parasite Network	Host Network
Number of Nodes	Number of observed parasites in the network	Number of observed lions in the network
Mean Degree (Unweighted)	Average number of parasites each parasite is connected to via a shared lion host.	Average number of lions each lion is connected to via a shared parasite.
Diameter	Maximum number of steps in the shortest set of paths between all parasite pairs (connectedness).	Maximum number of steps in the shortest set of paths between all lion pairs (connectedness).
Transivity	If two parasites are connected via a shared host, the probablity that adjacent parasites are also connected.	If two hosts are connected via a shared parasite, the probability thay adjacent hosts are also connected.
Weighted Assortivity	Likelihood of highly connected parasites being connected to other highly connected parasites, and low to low.	Likelihood of highly connected hosts being connected to other highly connected hosts, and low to low.
Betweenness	Number of shortest paths through a focal parasite species (i.e. a metric of centrality).	Number of shortest paths through a focal lion (i.e. a metric of centrality).

*Network model design and node/network definitions created following protocols previously used in bats and rodents as outlined by Luis et al., 2015.

Table 3.1 Node statistics for monopartite host and parasite networks.

PARAMETER	Monopartite Parasite Network		Monopartite Host Network	
	FIV+ (avg ± SE)	FIV- (avg ± SE)	FIV+ (avg ± SE)	FIV- (avg ± SE)
Number of Nodes	21.00	21.00	88.00	23.00
Mean Degree (Weighted)	148.76 ± 30.29	23.71 ± 5.53	206 ± 9.66	40.87 ± 3.01
Mean Degree (Unweighted)	13.81 ± 0.88	5.81 ± 0.95	74.61 ± 1.63	18.87 ± 0.84
Diameter	0.10	0.20	0.02	0.07
Transivity	0.62 ± 0.01	0.42 ± 0.01	0.88 ± 0.002	0.75 ± 0.01
Weighted Assortivity	-0.66	-	-	-0.97
Betweenness (Weighted)	9.72 ± 2.99	4.94 ± 1.91	29.79 ± 4.92	5.37 ± 0.79

*Included values are network averages for each statistical measure based on values generated by each node (i.e. lions or parasites) present within the network. Note that FIV- networks had fewer lions and fewer parasites than FIV positive networks. To account for potential bias derived from variable sample size, repeated random subsamples of 23 FIV+ lions were generated to see if observed trends remained. Ultimately lion population sample size did not impact model outcome.

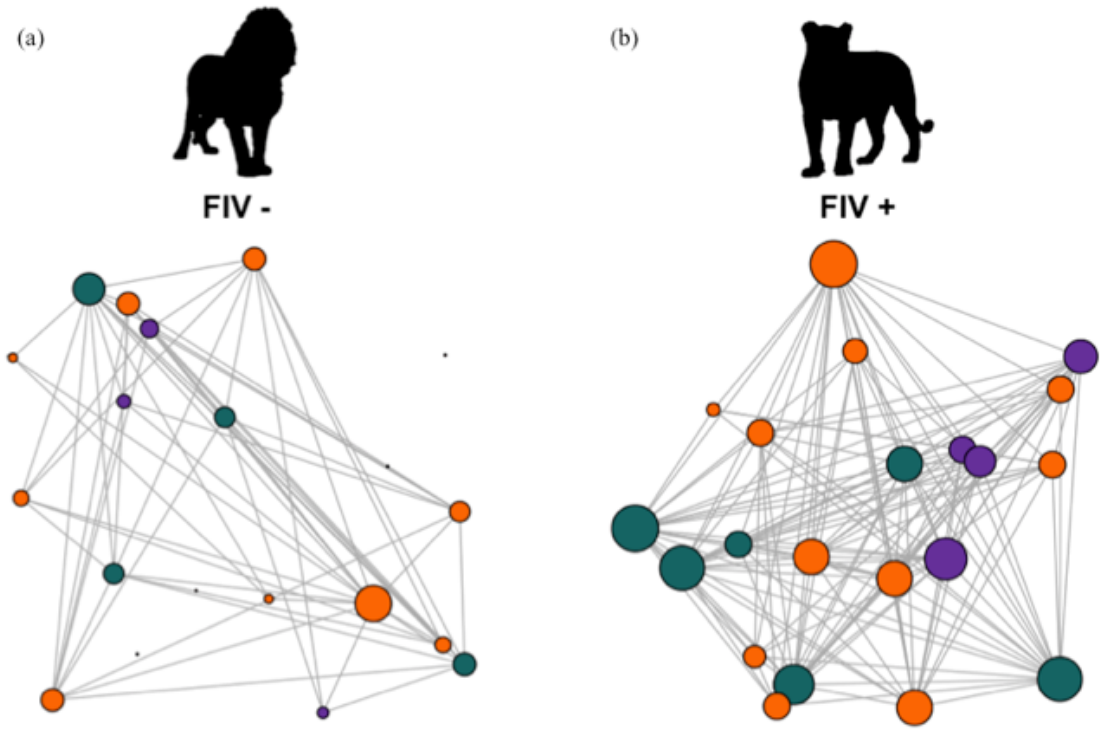

Fig. 3.1 FIV-positive networks are larger and more connected than FIV-negative networks. The figure above shows connectivity between FIV-negative (a) and FIV-positive (b) host-parasite networks based on calculated network statistics for connectance (scaled as the link:node ratio). Realized edges/links were defined as lions shared by two parasite species, while nodes represent each pathogen/parasite species detected in a given network. Each node is proportional to the number of times a given parasite/pathogen occurred within the network, with green nodes representing gastrointestinal parasites, orange nodes representing hemoparasites, and purple nodes representing viruses. In general, FIV-positive networks were larger, supporting 21 other parasite/pathogen species in total, with each pair of parasites/pathogens sharing an average of 21 lion hosts. Contrastingly, FIV-negative networks only supported 16 other parasites (nodes for coronavirus, *Toxoplasma gondii,* whipworms, *Theileria annae,* and *Theileria bicornis* did not occur) and parasites shared an average of 5 lion hosts.

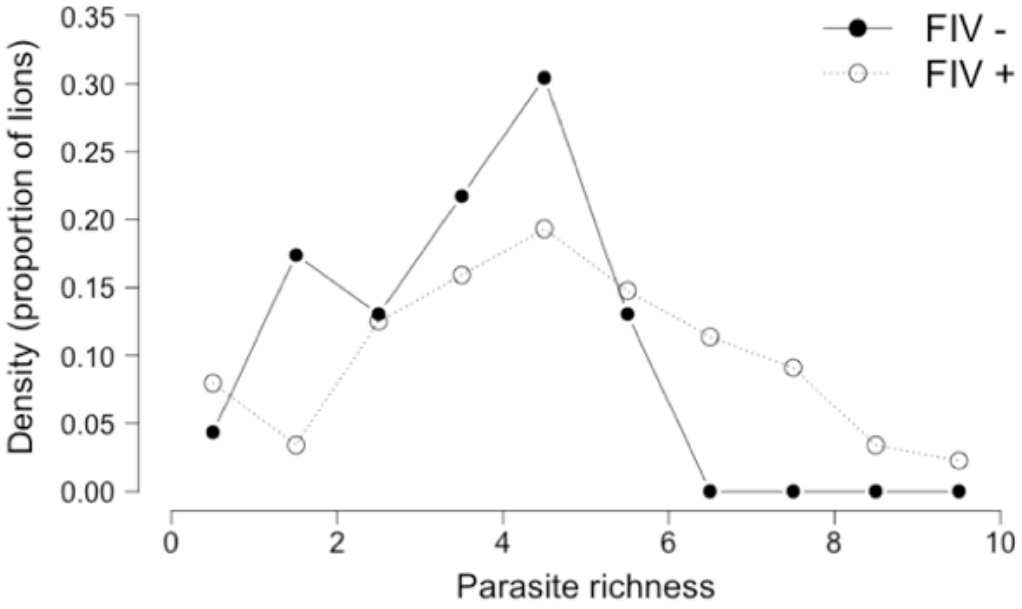

Fig. 3.2 Mean richness of parasite species does not vary between FIV-positive and negative networks, but total parasite richness is much higher in FIV-positive networks. The graph above provides a comparison of parasite network richness in FIV-positive versus FIV-negative subgroups as measured via degree distribution. While average parasite richness does not vary widely between FIV-positive and FIV-negative networks (an average of five parasite species compared to four parasite species, respectively), a right shift in the network distribution drives a higher variance in FIV-positive networks (4.9 as opposed to 2.1 in FIV-negative), with FIV-positive networks supporting a greater number of parasite species at the higher end of their range.

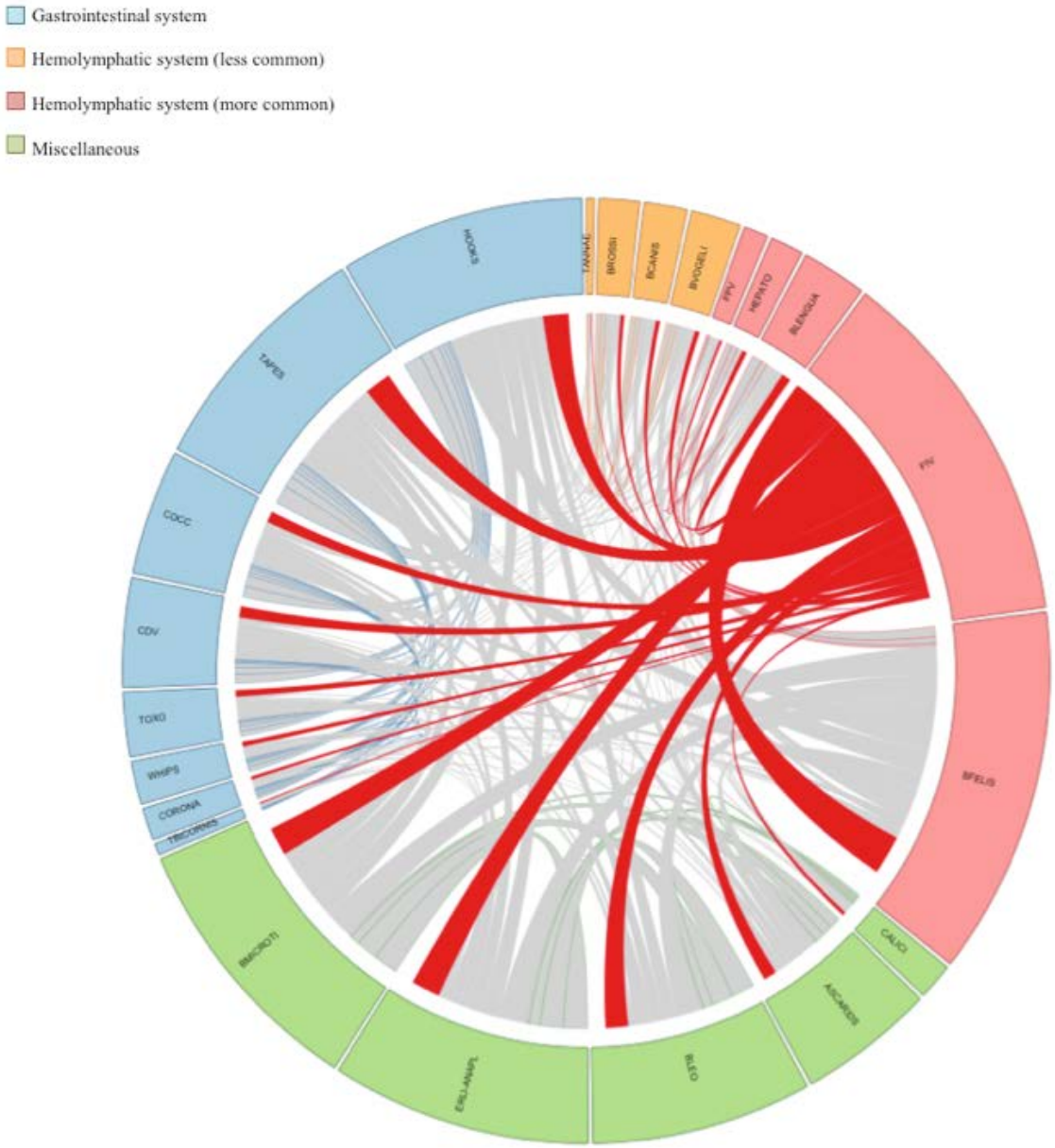

Fig. 3.3 Parasites of lion hosts exist within four main parasite communities. The diagram above shows the co-occurrence of various pathogens/parasites within the sample population. Each box represents a different pathogen/parasite, with the width of the box directly proportional to overall parasite/pathogen prevalence (i.e. a higher prevalence equates to a bigger box). Box color is coded to show community, defined in this context as a group of parasites that more commonly occur together within a single host. The flows between boxes represent co-occurrence, indicating that two pathogens/parasites share a single host. Larger flows indicate a higher degree of coinfection interaction within the population. For visualization, interactions with FIV are coded in red.

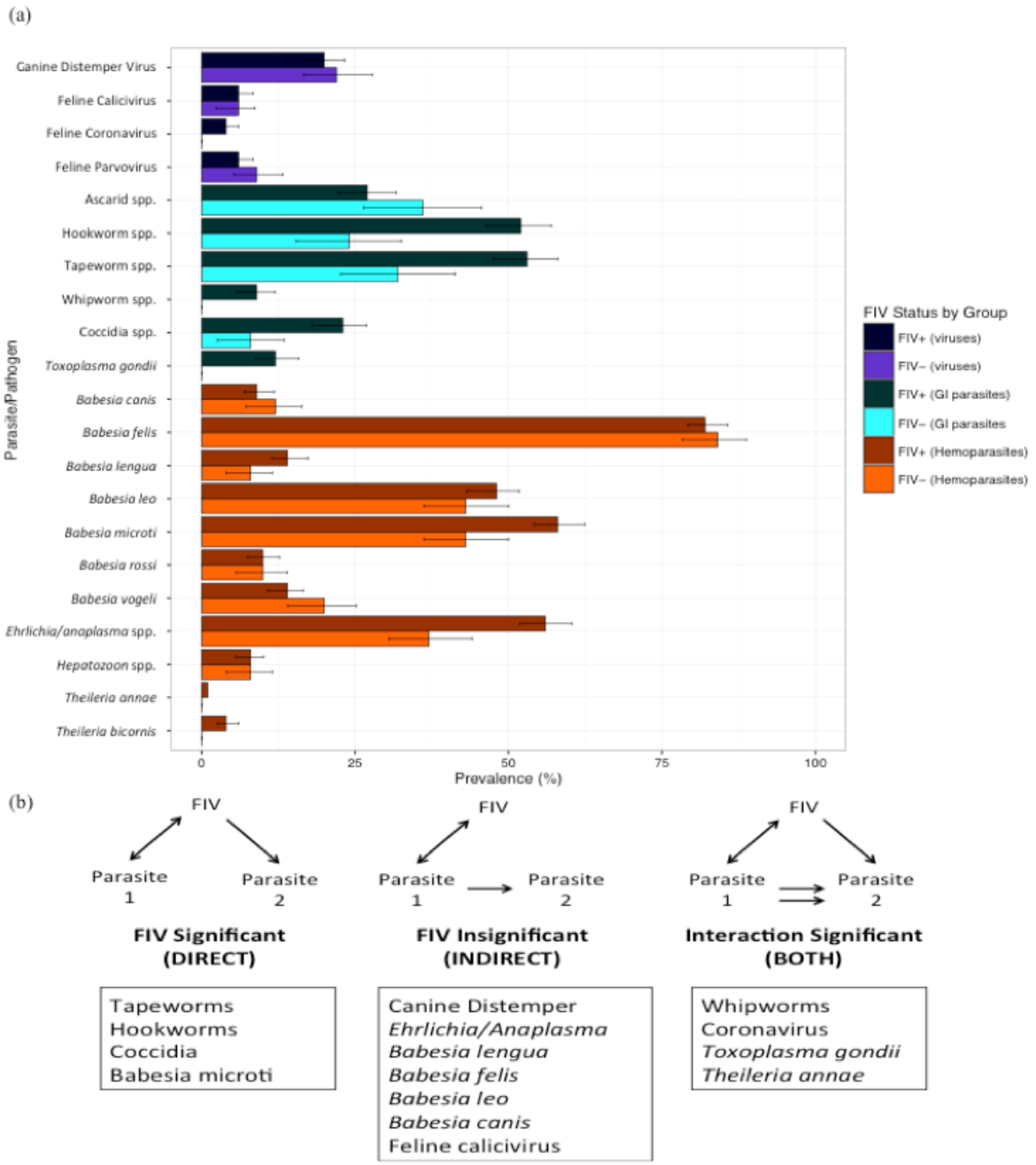

Fig. 3.4 FIV mediates parasite communities both directly and indirectly. Graph (a) shows overall pathogen/parasite prevalence by FIV status. On a host level, FIV positive status is both directly and indirectly (via intermediate coinfections) correlated with increases in certain key pathogens and parasites (b) as supported by generalized linear mixed effects models accounting for age, sex, body condition, and FIV status, as well as secondary coinfections and interactions between FIV and coinfections of interest. Correlations with FIV were direct when FIV was statistically significant in all models, both direct and indirect if it was both significant on its own and via the coinfection interaction term, and indirect if it was significant in the original model, but dropped from the final model after accounting for other coinfections found commonly in FIV-positive hosts.

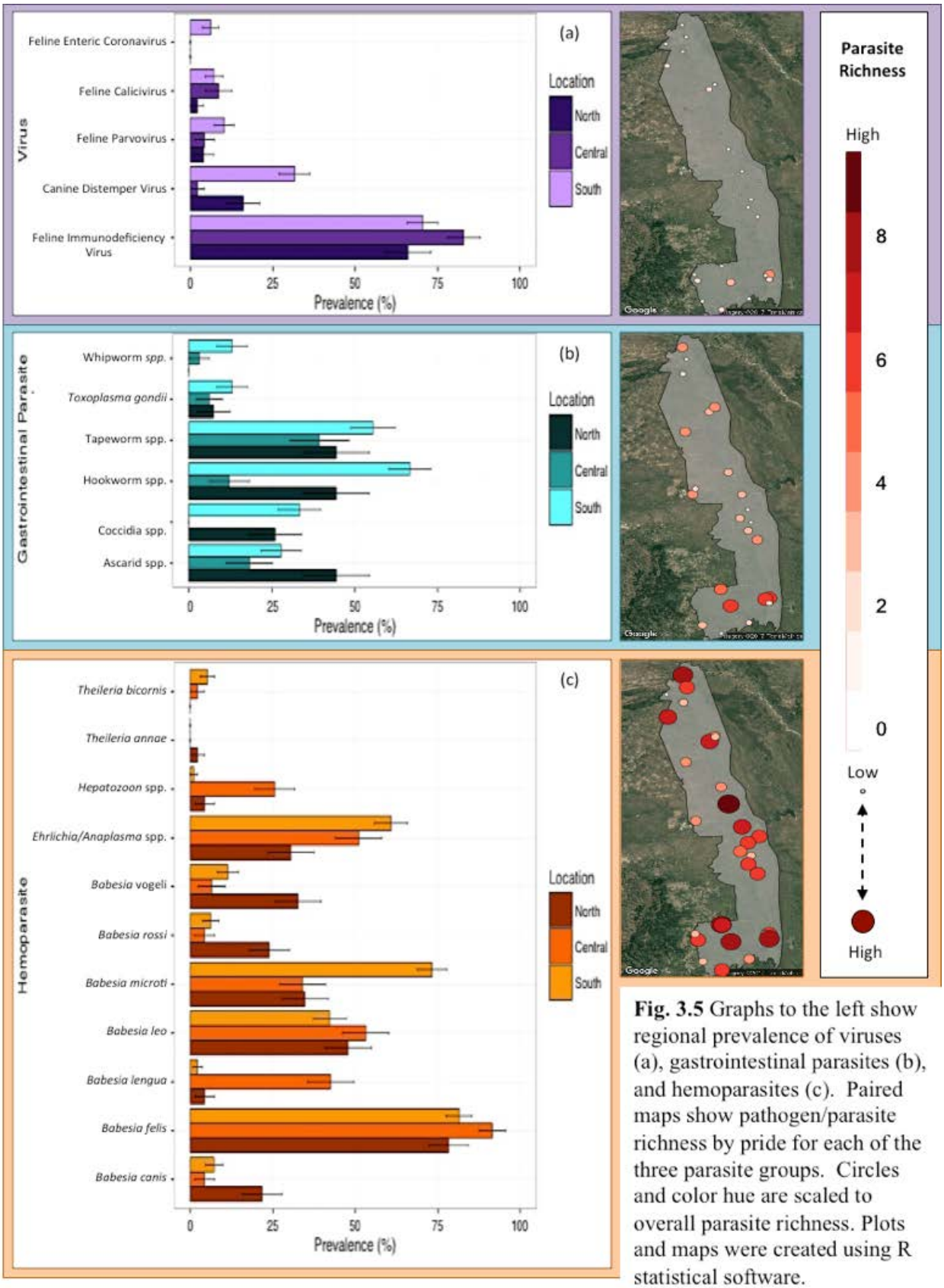

Fig. 3.5 Graphs to the left show regional prevalence of viruses (a), gastrointestinal parasites (b), and hemoparasites (c). Paired maps show pathogen/parasite richness by pride for each of the three parasite groups. Circles and color hue are scaled to overall parasite richness. Plots and maps were created using R statistical software.

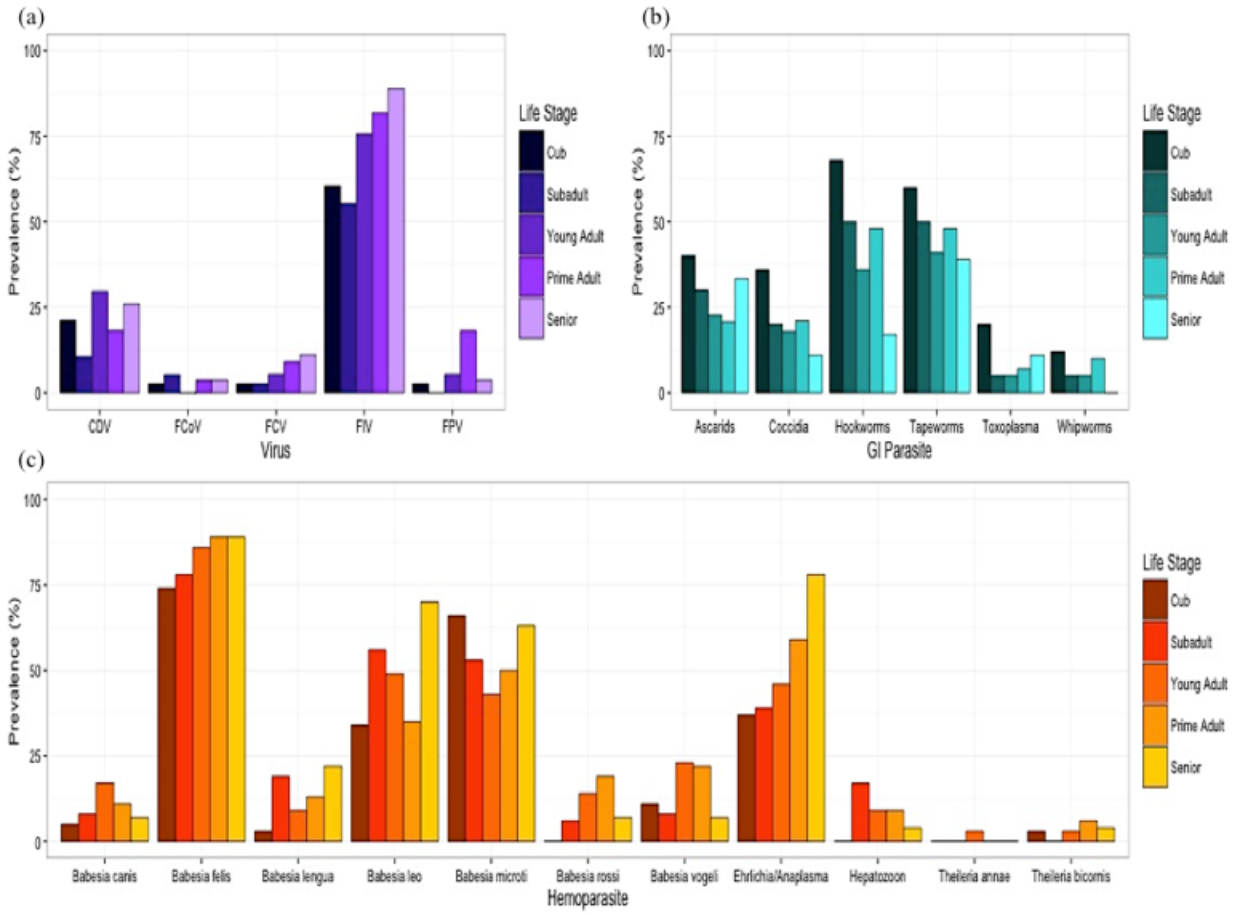

Fig. 3.6 The prevalence of certain parasites correlates highly with age. The figure above shows parasite/pathogen by age for viruses (a), gastrointestinal parasites (b), and hemoparasites (c). Age has been broken into life stages and includes cubs (0-2 years), sub adults (2-4 years), young adults (4-6 years), prime adults (6-8 years), and seniors (>8 years) for ease of data visualization. Overall, age was associated with statistically significant increases in prevalence of FIV, coccidia spp., *Ehrlichia* spp., *Babesia felis,* and *Babesia rossi,* as well as exposure to feline calicivirus. Age was associated with decreased prevalence of tapeworm and hookworm species (see **Tables S3.1 and S3.7** for model output and demographics).

CHAPTER 4: PREVALENCE OF PARASITES AND INFECTIOUS DISEASE IN URBAN FERAL CATS (FELIS CATUS): INFORMING PREVENTATIVE HEALTH STRATEGY

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ABSTRACT

Urbanization and the movement of rodent species into and around human dwellings, coupled with abundant feral cat populations, increases contact of feral cats with wildlife, humans, and domestic cats. As such, the need for disease surveillance and preventative medicine strategies for feral cats has grown. Feral cats play an important functional role for control of rodent populations and rodent borne-pathogens, but may serve as disease reservoirs for important feline and zoonotic pathogens given their lack of access to veterinary services. The objective of this study was to provide a snapshot of the prevalence and incidence of key veterinary and zoonotic diseases of feral cats living in an urbanized setting to better inform preventative health strategies aimed at the control of common infections of domestic and feral cats during trap-and-neuter programs. Disease prevalence and incidence were evaluated for 129 feral cats living in the greater Portland metro area for common feline viruses (Feline Herpesvirus-1 (FHV-1), Feline Immunodeficiency Virus (FIV), and Feline Leukemia Virus (FeLV)), the cat-scratch-fever causing bacterium *Bartonella henselae,* disease vectoring ectoparasites (fleas, ticks, and ear mites), and intestinal parasites, including the zoonotic species *Toxoplasma gondii, Toxacara cati,* and *Capillaria aerophilla*. FHV-1, FeLV, *Bartonella henselae,* fleas, ear mites, and intestinal parasites were common in the feral cat population, with prevalence between 24% and 85.18%. FIV was detected in only 4.05% of cats and ticks were not present. Incidence of infection in kittens and young cats was highest for intestinal parasites, followed closely by fleas, *Bartonella,* FHV-1, mites, and FeLV, respectively. Findings from this study suggest that targeted preventative health campaigns alongside spay-and neuter efforts in urban feral cat populations may not only promote feral cat health through direct medical treatment and indirect control of rodent populations, but may also promote health within surrounding communities by reducing the development of reservoir populations.

4.1 INTRODUCTION

Feral cats (*Felis catus*) are defined as cats that are not owned or confined to a household and often show various levels of human socialization, though many may not be distinguishable from pet cats in this regard (Levy & Crawford, 2004; Robertson, 2008). The total population of feral cats in the United States has been estimated between 60 and 73 million, with much of that population living in close proximity to human dwellings (Levy et al., 2003; Luria et al., 2004). Sympatric feral cat populations are often well tolerated and even encouraged by humans due to their important functional role as a biological control vector for rodent populations, as well as the general view of them as a pet species. Previous studies have shown that feral and domestic cats are the most effective species for limiting exponential growth of rodent populations, thus inhibiting invasion success of non-native species while driving down rodent-borne disease prevalence (Wodzicki, 1973).

While the importance of feral cats for the biological control of rodents is now well established, intraspecific competition and disease transmission among feral cat colonies due to overpopulation continues to present a hurdle for ensuring animal welfare (McCarthy et al., 2013). In addition, feral cat populations themselves may act as reservoirs for parasites and infectious diseases of humans, wildlife, and domestic pets due to decreased access to disease prevention and treatment (Bradshaw et al., 1999; Robertson, 2008).

Due to the importance of feral cats for controlling established rodent populations, as well as the concern for animal welfare with regards to feral cats and other sympatric species, this study investigated the prevalence and incidence of several pathogens for which feral cats may act as reservoirs. The study was accomplished using infrastructure already supported by the Feral Cat Coalition of Oregon (FCCO), which has established free spay and neuter clinics in the greater Portland metro area and surrounding communities. This project collaborated closely with the FCCO in order to determine the prevalence of key diseases in the feral cat population. Prevalence and incidence were determined for three chronic viral pathogens dangerous to cats, Feline Immunodeficiency Virus (FIV), Feline Herpes Virus-1 (FHV-1), and Feline Leukemia Virus (FeLV); a bacterial pathogen, *Bartonella henselae,* known to cause mild illness in cats and Cat-Scratch-Fever in humans and dogs; feline gastrointestinal parasites, including helminths (*Capillaria aerophilla, Taenia taeniaeformis*, and *Toxacara cati)* and protozoa (*Cystoisospora felis, Cystoisospora rivolta, Coccidia spp.,* and *Toxoplasma gondii)* responsible for disease in

humans and animals; and ectoparasites including fleas, ear mites, and ticks. Findings presented in this paper are meant to represent a snap shot of the diseases prevalent in the feral cat population and may serve as a guide to direct disease control and prevention efforts during spay and neuter campaigns.

4.2 METHODS

Study System and Sample Collection

All feral cats involved in this study were sampled from cat populations living in the upper Willamette Valley through collaboration with the FCCO, a Portland, Oregon-based non-profit organization. FCCO volunteers captured animals using humane live-trapping techniques between October and November 2012. Capture sites were located throughout the upper Willamette Valley and lower Washington, extending from Corvallis, OR to Longview, WA. Animals were presented to the clinics in metal live traps, with most cats having been captured within the previous 24 hours.

At the time of presentation, FCCO clinical staff sedated all cats to allow easy handling and safety. Sex was determined on visualization of external genitalia, or in the case of small kittens or cryptorchid males, during castration and from clinic records (Table 4.1). Aging of animals was based on the presence/absence and number of permanent versus deciduous teeth (Table 4.1). Kittens were classified as those individuals with all deciduous teeth (<6 months age), juveniles as individuals with the presence of mostly new adult teeth +/- deciduous teeth (approximately 6 months to one year), and adults as individuals with all adult teeth and evidence of some wear (Dyce et al., 2002). Tooth wear patterns were used to discern between animals in the adult (little to moderate wear) and senior age categories (significant wear +/- broken teeth).

Blood and fecal samples for the purpose of viral, bacterial, and intestinal parasite diagnostics were collected under sedation. Blood was collected into Vacuette EDTA (Greiner Bio-One®), sodium heparin (Greiner Bio-One®), and microbial isolator tubes (Wampole Isolator 1.5^{TM}) via venipuncture of the jugular or femoral veins. Feces was evacuated from the rectum using a fecal loop or collected from the cage when available. Sample sizes among diagnostic tests varied due to limited sample availability (i.e. no feces at time of presentation or inability to procure sufficient blood due to low blood pressure or small animal size).

Ectoparasite diagnostics

Flea infestation was diagnosed in animals where fleas were clearly present and/or in animals with evidence of significant flea dirt seen as small red dust in the patient's coat. Presence of ticks was evaluated visually, with special attention to common tick sites including periocular, perianal, inguinal, axillary, and interdigital locations. Ear mite diagnosis was presumptive and was based solely on clinical signs suggestive of mites, which included dark aural exudates, alopecia around the base of the ears, dermatitis, and excoriations suggestive of self trauma from repeated scratching (Farkas et al., 2007).

Viral Diagnostics

Heparinized serum and EDTA whole blood samples were used for all viral diagnostics, which were run by students and faculty at Oregon State University. Infection with FHV-1 was identified using serum neutralization, an antibody based diagnostic method with 70% test sensitivity (Dawson et al., 1998; Maggs et al., 1999). As herpes infection is chronic and incurable, this test was used to determine herpes exposure rather than active viremia. FIV and FeLV infection were determined in combination using a commercial two-way snap ELISA (IDEXX Laboratories Inc., One IDEXX Drive, Westbrook, Maine 04092). The FIV portion of the test relies on the presence of circulating antibodies to the virus, which is considered to be indicative of current infection due to the disease's chronic nature, and is approximately 93.5% sensitive and 100% specific. The FeLV portion of the snap test is based on circulating antigen consistent with presence of the virus itself and is 98.6% sensitive and 98.2% specific for detecting infection.

Bacterial Diagnostics

Blood for diagnosis of *Bartonella henselae* was obtained from EDTA and bacterial isolator tubes and used for both bacterial culture and polymerase chain reaction (PCR). We considered animals as positive for *Bartonella* if they showed a positive reaction to one or both tests. On culture, identification of *Bartonella* was confirmed by demonstrating that colonies were oxidase, urease, catalase, and nitrate reductase negative (Songer & Post, 2005). In humans, PCR as a diagnostic tool for the detection of *Bartonella henselae* is 81% sensitive and 100% specific, while bacterial culture shows low sensitivity (28%), but high specificity (100%) (Scola & Raoult, 1999; Hansmann et al., 2005).

Intestinal Parasite Diagnostics

Due to the short amount of time each cat spent in the cage, coupled with a mandatory fasting period and small animal size, feces was only available for 27 of the cats participating in the study. Intestinal parasite diagnostics were performed using a combination of standard fecal sugar flotation protocols and microscopy. While these methods allow direct visualization, specificity and sensitivity are variable and depend on the pre-patent/hypobiotic periods of many parasitic infestations or the species of parasite present (Foreyt, 2001; Liccioli et al., 2012).

Statistical Analysis

All statistical analyses were performed using the Microsoft Excel Package and R statistical software (R Core Team, 2014). Disease prevalence is the proportion of animals that are currently infected with a parasite or pathogen, representing the burden of infection in the population at the specified point in time:

$$Prevalence = \frac{\#\ of\ animals\ with\ disease}{total\ \#\ of\ animals\ in\ sample\ population}$$

Standard error for prevalence was calculated according to sample size and prevalence using the following equation:

$$SE = \sqrt{\frac{(1-f)p(1-p)}{n}}$$

Standard error is denoted by (*SE*). Sample prevalence is denoted by the symbol (p), sample size by (n), and fraction of the total population sampled by (f). Population fraction was ignored for our calculations due to its negligible value. 95% confidence intervals (*95%CI*) for prevalence were calculated using the calculated standard error in the following equation (Putt et al., 1988):

$$95\%CI = Estimated\ prevalence \pm 1.96\ x\ SE$$

The incidence rate is the number of new infections in a susceptible population over a period of time that the animals were at risk, representing the per-capita risk of becoming infected. Incidence was only calculated for the kitten and juvenile samples group, as all cats in these age categories could be considered initially susceptible, with infections occurring over a period of six months (kittens) or one-year (juveniles). The formula for incidence was modified to:

$$Incidence\ rate = \frac{\#\ of\ kittens\ and\ juveniles\ w/\ disease}{(total\ \#\ kittens * 0.5\ yr) + (total\ \#\ juveniles\ * 1\ yr)}$$

Age and sex risk factors for infection and disease prevalence were investigated using generalized linear models with binomial distribution and a logit link function in the R *stats package* (R Core Team, 2014).

4.3 RESULTS

Prevalence of Ectoparasites in the Feral Cat Population

Fleas and ear mites were common in the feral cat population, occurring at 48.8% (*SE*=0.044, *95%CI*=40.23-57.43%, *n*=129) and 24% (*SE*=0.038, *95%CI*=16.6-31.4%, *n* =129) prevalence, respectively. Fleas were significantly lower among the adult and juvenile age groups, holding all other variables constant (Table 4.2). Mites did not show associations with age or sex. Contrastingly, ticks were not found at the time of sample collection, though it cannot be determined whether this 0% (*SE*=0, *95%CI*=0, *n*=129) prevalence was an actual trend or simply an artifact of seasonal or environmental variation.

Prevalence of Viral Diseases in the Feral Cat Population

Viral diagnostics showed high prevalence of both FHV-1 and FeLV in the population, with over 29.59% (*SE*=0.046, *95%CI*=20.59-38.59%, *n*=98) and 22.97% (*SE*=0.049, *95%CI*=13.37-32.57%, *n*=74) of cats testing seropositive, respectively. The prevalence of FIV was fairly low, with only 3 cats (4.05%; *SE*= 0.023, *95%CI*=0-8.55%, *n*=74) determined to be seropositive (Fig. 4.1). We did not detect age or sex patterns in FHV-1 and FeLV infections (Table 4.2). The small sample size of cats testing positive for FIV limited our ability to detect age and sex patterns for this infection. The FIV-positive cats included two senior females and one adult male.

Prevalence of Bartonella henselae in Feral Cat Populations

Prevalence of *Bartonella henselae* was high in the feral cat population, showing 37.14% (*SE*=0.047, *95%CI*=27.94-46.34%, *n*=125) seroprevalence when PCR and culture results were combined (Fig. 4.1). Additionally, *Bartonella* was higher in males than females (Table 4.2) and feral cats showed a high prevalence (48.83%) of flea infestation, the vector for *Bartonella* infection. However, flea infestation did not correlate with *Bartonella* infection (X^2 = 1.3099, *df* = 1, *p-value* = 0.2524).

Prevalence of Intestinal Parasites in Feral Cat Populations

The majority of cats tested (85.18%; *SE*=0.068, *95%CI*=71.88-98.48%, *n*=27) were positive for intestinal parasites (Fig. 4.1), with several parasite species represented in the study population (Fig. 4.2). Nematodes included the feline ascarid *Toxacara cati* (63% prevalence; *SE*=0.092, *95%CI*=46-82%, *n*=27), and *Capillaria aerophilla,* the feline lungworm (4% prevalence; *SE*=0.038, *95%CI*=0-11.4%, *n*=27). Protozoans *Cystoisospora spp., Coccidia spp.,* and *Toxoplasma gondii* were identified in 30% (*SE*=0.088, *95%CI*=12.8-47.2%, *n*=27*)*, 4% (*SE=0.*0.038, *95%CI*=0-11.4%, *n*=27), and 7% (*SE*=0.049, *95%CI*=0-16.6%, *n*=27) of cats, respectively. *Taenia taeniaeformis* was present in 11% (*SE*=0.06, *95%CI*=0-22.8%, *n*=27) of cats sampled and was the only cestode detected. Prevalence of intestinal parasites was not associated with age or sex.

Incidence rate of Micro and Macro- Parasites in Feral Cats

Incidence rate of infections with viruses, bacteria, and parasites was high in the kitten and juvenile age groups (Fig. 4.3). Infection risk was highest for intestinal parasites at 67% (n=7) during the first year of life, followed closely by fleas at 59.1% (n=51), *Bartonella* at 49.4% (n=45), FHV-1 at 35.3% (n=39), and mites at 27.2% (n=51). Infection risk with FeLV was lower, at 22.7% (n=25) during the first year, while the incidence rate of FIV in the first year of life appears negligible (0%; n=25).

4.4 DISCUSSION

Results of this study suggest a high prevalence and incidence of several key parasites and pathogens of known veterinary importance and zoonotic potential within the feral cat population (Figs. 4.1 and 4.2). In the face of these findings, decreased health care among this population has the potential to exacerbate these effects. While domestic animals are likely to receive treatment for their infections and possible confinement, limiting the time period they remain a risk for disease transmission, feral cats are unlikely to receive treatment or be confined to prevent host-to-host contact (Gerhold & Jessup, 2012). This, coupled with findings that feral animals are more likely to have an active infection and potentially increased risk of pathogen shedding, may increase their potential to serve as a reservoir of infection for humans and animals (Chomel et al., 1995), while also carrying significant ramifications for the welfare of the afflicted felines.

Within our study population we found significant prevalence of fleas and ear mites, with kittens and seniors being represented disproportionately for the former. Ticks were absent in this

study. While true ectoparasite prevalence is difficult to determine due to seasonal and environmental variation, trends towards these age groups may be explained by differences among age groups in immunity, exposure, or grooming behaviors. Between the two ectoparasites represented, ear mites are unlikely to be pose a significant health threat and are associated primarily with secondary infections and self-trauma due to irritation (Akucewich et al., 2002). Contrastingly, the high burdens of fleas observed pose a significant danger for both human and animal health. Direct detriments to the host as the result of flea infestation can include a range of conditions from allergic dermatitis to severe life threatening anemia in high infestations (Slapeta et al., 2011). More insidiously, fleas serve as the primary vector for diseases including cat-scratch fever, plague, and rickettsiosis, diseases which can exhibit high case fatality in humans and animals (Case et al., 2006; Slapeta et al., 2011; Gerhold & Jessup, 2012).

In addition to the ectoparasites, two viral pathogens, FHV-1 and FeLV, were found at high prevalence in our study population, which was concerning for three reasons. First, these viruses cause a wide range of clinical signs in infected felids, ranging from mild gingivitis or ocular lesions to immunosuppression, secondary infections, and direct mortality (Stiles, 2003; Gould, 2011). FeLV in particular has been credited with a wider range of disease related mortality and clinical pathologies than any other infectious agent of felids (Cotter, 1991; Hartmann, 2011; Collado et al., 2012). FeLV may also contribute to significant disease and mortality through coinfections with other infectious agents in the population, some of which showed high prevalence in this study (Little et al., 2011; Bande et al., 2012). Second, the high incidence of these infections in kittens indicates a high force of infection and the need for early detection and intervention (Fig. 4.3). Third, both viruses can spread via saliva, nasal, and ocular secretions and can be detected on fomites and in other bodily fluids (Gould, 2011). Thus, these viruses may easily spread between domestic and feral cats through direct contact, allogrooming, fighting, or the use of shared food bowls and litter areas, as well as from mothers to kittens. Due to the chronic nature of both FHV-1 and FeLV, animals maintain infection throughout their lifetime and may shed virus during periods of acute viremia or viral recrudescence (Gaskell et al., 2007). Consequently, infected animals may act as carriers of the disease, passing new infections to susceptible cats while appearing clinically normal aside from brief relapses (Jarret, 1985; Gaskell et al., 2007; Thiry et al., 2009).

By contrast, the prevalence of FIV was low and incidence was undetectable in kittens and juvenile feral cats. However, these findings should be interpreted with caution. Prevalence and incidence for FIV in this study were based on a sample cohort composed mostly of young animals. FIV is spread primarily through saliva to blood contact via bite wounds and, as such, increases with age and is associated strongly with older male animals who frequently participate in aggressive encounters (Gibson et al., 2002). As a result, it could be expected that incidence and prevalence would be low in our young sample population. Contrastingly, prevalence and virulence of pathogens are often tightly linked, with increased case-fatality contributing to overall lower prevalence of the disease in a sample population (McCallum & Dobson, 1995). This may also explain our trends, as diseased animals would be less likely to be identified antemortem and it could be expected that animals that are at a disadvantage otherwise (i.e. kittens with poorly developed immunity), may die before detection. Due to the pathologic nature of this disease, which causes immunosuppression and AIDS like clinical signs similar to HIV in humans, it can be expected that relative thresholds for coping with both direct health effects of this pathogen, as well as health effects of secondary pathogens, would be fairly low to begin with (Bendinelli et al., 1995; Roelke et al., 2009).

Aside from the viral pathogens, *Bartonella henselae* infection was common among the feral cats we sampled, with nearly half of the cats positive for infection and a high incidence rate among kittens and juveniles. This finding implies that feral cats may serve as an important reservoir for *Bartonella* infection in nearby human and animal populations. Fleas, the insect vector for *Bartonella* transmission, were also abundant in our sample of feral cats. However, contrary to expectations, *Bartonella* infection was not associated with flea infestation. The timing of vector presence and *Bartonella* infection may not typically be synchronous, if there is an incubation period during which *Bartonella* is not yet detectable, or we may have detected flea species other than the primary vector for *B. henselae*, the cat flea *Ctenocephalides felis,* which might explain this trend (Cruz-Vasquez et al., 2001; McElroy et al., 2010). Contrastingly, males were shown to have a higher overall association with *Bartonella* infection, which may be due to underlying behavioral differences and increased aggression among this demographic group, as *B. henselae* has been shown to gain access to a new host in flea dirt introduced via cat scratch wounds.

Bartonella has been linked to a broad spectrum of feline upper airway issues and is the causative agent of Bartonellosis (commonly referred to as Cat Scratch Fever) in humans. Bartonellosis has been associated with a range of clinical signs in humans and dogs including prolonged fevers, meningitis, systemic bacillary angiomatosis associated organ damage, and endocarditis in dogs and immunocompromised humans (Songer & Post, 2005; Bitam et al., 2010). Thus, the high prevalence of *Bartonella* in feral cats is not only a concern with regards to feline health, but infected cats may serve as an endemic reservoir for infection to nearby human and animal populations.

Finally, our findings suggest that feral cats may serve as a reservoir to a broad spectrum of intestinal parasites, including several species of known pathogenic potential to humans, domestic cats, and wildlife. While the clinical signs of many of these parasites are considered mild, gastrointestinal parasites account for the greatest amount of feline and canine morbidity worldwide and can cause severe disease in accidental hosts including humans (Mircean et al., 2010). Tapeworms and ascarids most commonly affect host health by competing with their host for nutrients in the gut, leading to weight loss, stunted growth, and diarrhea (Foreyt, 2001; Bowman, 2003). However, migrating stages of ascarids in aberrant hosts, including humans and other animals, can cause ocular lesions and organ dysfunction through mechanical damage to organ tissues (conditions referred to as ocular and visceral larval migrans, respectively), and both ascarids and tapeworms can cause intestinal obstruction and death in high burdens (Foreyt, 2001; Bowman, 2003; Center for Disease Control and Prevention, 2013). Similarly, while lungworms such as *Capillaria aerophilla* rarely cause disease more than mild lower airway irritation on their own, secondary bacterial infections in parasite damaged pleural tissue can result in bronchopneumonia and aberrant migrations can cause overt, even fatal clinical disease in multiple wild and domestic species, as well as occasional human cases (Bowman, 2003; Riggio et al., 2013; Traversa, 2013).

Of additional concern with regards to public health and conservation was the high level of protozoal parasites in feral cat populations. The high prevalence of *Coccidia* and *Cystoisospora* species is primarily important with regards to domestic animals and some related wildlife species, generally being associated with mild to moderate gastrointestinal upset and wasting. The occurrence of *Toxoplasma gondii* in urban feral cats, however, is alarming from both public health and conservation standpoints (Montoya & Liensenfeld, 2004; Overgaauw et

al., 2009). Though *Toxoplasma* infection is typically associated with mild clinical disease in cats, which are its definitive host, it has been associated with a wide-range of neurologic and teratogenic effects in aberrant hosts, which include several species of conservation concern, as well as humans (Tenter et al., 2000; Montoya & Liensenfeld, 2004; Miller et al., 2008). Of note, encephalitic toxoplasmosis, a disease resulting from entrance of the parasite into the central nervous system, has been associated with fatal infections in humans and primates. Domestic cats have previously been implicated as reservoirs for *T. gondii* in investigations of population declines of environmentally important keystone species such as the California Sea Otter (Montoya & Liensenfeld, 2004; Conrad et al., 2005).

While prevalence for the diseases involved in this study are likely to vary geographically, and are by no-means an exhaustive set of the pathogens important to feline health, they serve as a starting point for understanding feline disease in feral cats and their implications for the surrounding community. However, it is important to note that several of the gastrointestinal parasites detected in this study population, as well as several other key pathogens and parasites that were not evaluated, may also be carried in naturally occurring rodent populations. As such, it is important to distinguish whether true reservoirs exist among feral cat versus rodent populations, and whether cats are primarily infected or only secondarily due to their prominent role as a biological control agent for rodent populations. Future studies would benefit from the collection of longitudinal data and higher sample size to better determine demographic and regional disease trends, as well as concurrent sampling of sympatric rodent populations to pinpoint true reservoirs.

4.5 CONCLUSION

The findings of this study underscore the importance of surveillance and disease treatment/prevention within feral cat populations, as well as the potential for trap-neuter-and-release programs to provide a critical service for public health and the prevention of disease in cats, humans, and sympatric wildlife species. Through collaboration between local and global public health entities, veterinary educational institutions, veterinary non-profit organizations, and community outreach, disease control measures, including screening, treatment, and prevention, may be implemented to target key pathogens harbored among feral populations. Through these collaborations, veterinary professionals can help to lessen the impact of infectious diseases in

feral and domestic cats and by extension the emotional and physical wellbeing of owners and caregivers, thus promoting animal and human welfare. Additionally, these strategies would decrease the economic impacts of healthcare for feline related injuries and illness, both in humans and domestic pets. Finally, disease prevention and control, alongside spay-and-neuter efforts, would support conservation measures of wild animals impacted by feline diseases while promoting healthy, socially stabile feral cat clowders.

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4.6 TABLES AND FIGURES

Table 4.1 Demographic breakdown of feral cat sample population.

Age Class	Sex		Total
	Female	*Male*	
Kitten	7	7	14
Juvenile	22	15	37
Adult	43	29	72
Senior	1	5	6
Total	73	56	**129**

Table 4.2 Age and sex risk factors associated with parasite infection.

Infectious Organism	Sex (Reference= female)			Age (Reference=kitten)									Sample size (n)
				Juvenile			*Adult*			*Senior*			
	Est.	*SE*	*p*	*Est*	*SE*	*p*	*Est*	*SE*	*p*	*Est*	*SE*	*p*	
Fleas	2	0.37	0.59	-1.17	0.68	.08*	-1.12	0.64	.08*	0.63	1.25	0.62	129
Ear mites	0.15	0.42	0.71	0.81	0.85	0.34	0.71	0.81	0.38	0.13	1.34	0.92	129
Bartonella henslea	0.64	0.38	.10*	-0.57	0.65	0.38	-0.84	0.6	0.16	-1.21	1.04	0.24	125
FHV-1	-0.52	0.47	0.3	-0.59	0.77	0.44	-0.55	0.72	0.44	-0.57	1.34	0.67	98
FeLV	0.13	0.57	0.82	0.26	1.24	0.83	0.53	1.15	0.64	-15.07	1385	0.99	74
FIV	-0.32	1.25	0.8	-	-	-	-	-	-	-	-	-	74
Intestinal Parasites	0.34	1.44	0.81	-19.02	4602	1	-15.63	4602	1	-0.18	7982	1	27

Asterisks indicate values of marginal statistical significance.

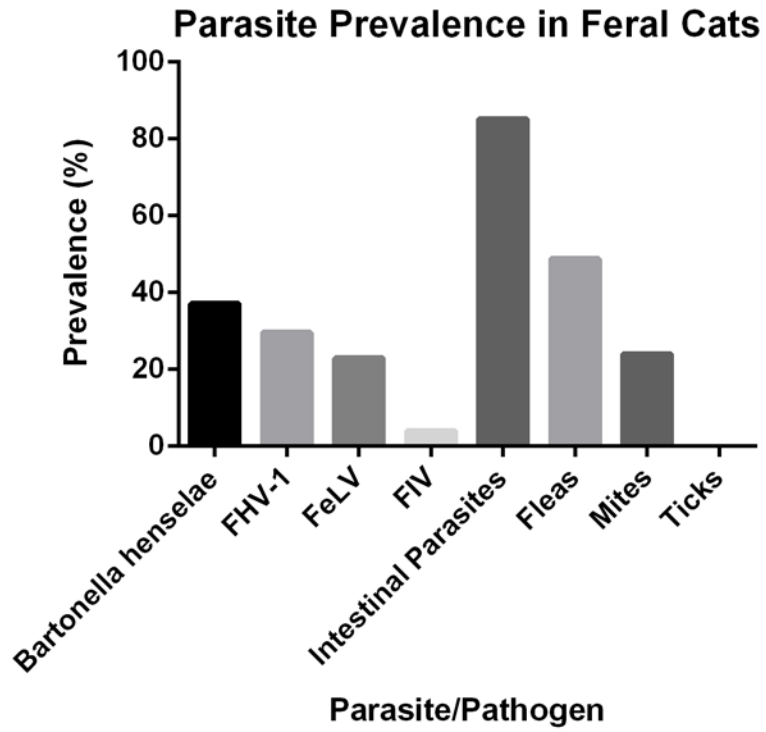

Fig. 4.1 Prevalence of Viral, Bacterial, and Endo-/Ecto- Parasites in Feral Cats.
Disease prevalence was determined as the number of feral cats with a positive test result (Serum Neutralization, ELISA, PCR, fecal flotation, or visual identification) over the total number of cats in the sample population. Prevalence was high for *Bartonella* (37.14%; *SE*=0.047, *95%CI*=27.94-46.34%, *n*=105), FHV-1 (29.59%; *SE*=0.046, *95%C1*=20.59-38.59%, *n*=98), FeLV (22.97%; *SE*=0.049, *95%CI*=13.37-32.57, *n*=74), intestinal parasites (85.18%; *SE*=0.068, *95%CI*=71.88-98.48%, *n*=27), fleas (48.83%; *SE*=0.044, *95%CI*=40.23-57.43%, *n*=129), and mites (24%; *SE*=0.038, *95%CI*=16.6-31.4%, *n*=129), while it was low for FIV (4.1%; *SE*=0.023, *95%CI*=0-8.55%, *n*=74) and non-existent for ticks (0%; *SE*=0, *95%CI*=0, *n*=129).

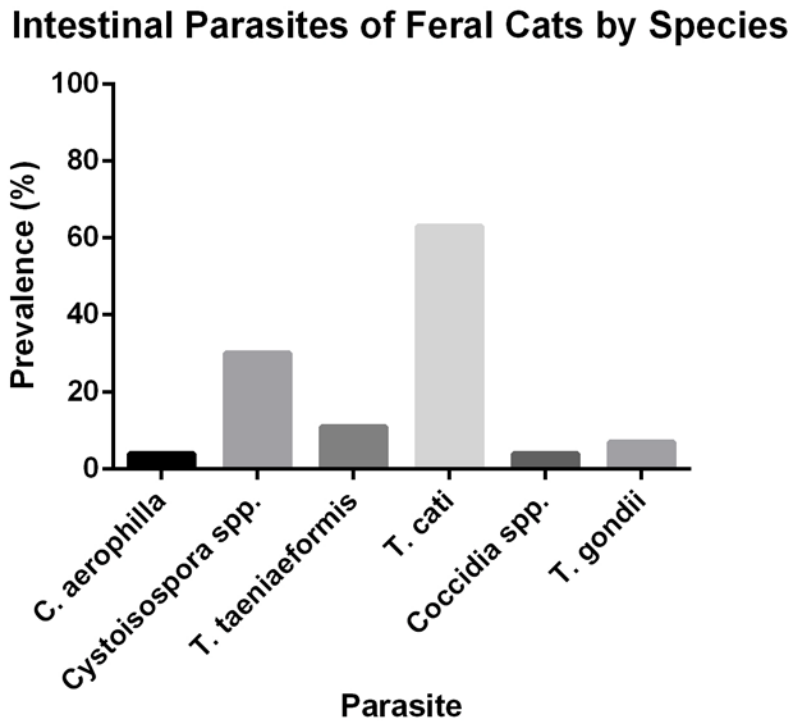

Fig. 4.2 Prevalence of Intestinal Parasites in Feral Cats by Parasite Species
The above chart shows the prevalence of gastrointestinal parasites whose eggs/sporulae were identified visually through microscopy. Data was not available from all animals and is based on a subset of the sample population (n=27). Overall, 4% (*SE*=0.038, *95%CI*=0-11.4%) of cats showed evidence of infection with the lungworm *Capillaria aerophilla*; 30% (*SE*=0.088, *95%CI*=12.8-47.2%) of cats had some species of *Cystoisospora (Cystoisospora felis or Cystoisospora rivolta),* a small intestinal protozoan of cats; 11% (*SE*=0.06, *95%CI*=0-22.8%) of cats had *Taenia taeniaeformis,* a tapeworm; 63% (*SE*=0.092, *95%CI*=46-82%) of cats exhibited infection with the ascarid worm *Toxacara cati*; 4% (*SE*=0.038, *95%CI*=0-11.4%) of cats had some species of the protozoan parasite *Coccidia*; and 7% (*SE*=0.049, *95%CI*=0-16.6%) of cats showed evidence of infection with *Toxoplasma gondii,* a protozoan parasite.

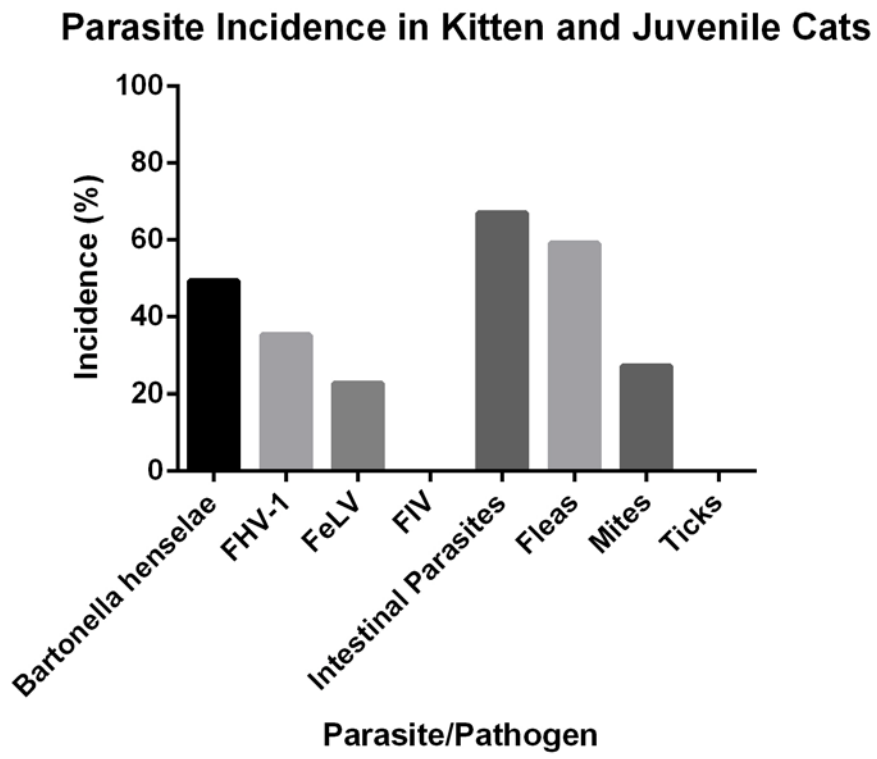

Fig. 4.3 Incidence of Viral, Bacterial, and Intestinal Parasites in Feral Cats.
Data on the exact time of infection was largely unavailable for our sample population. Thus, incidence was determined as the number of new infections over a given time period, using the kitten and juvenile age groups as proxies. Incidence was calculated as the total number of newly infected animals (infected kittens over six months and juveniles over one year) out of the total number of animals in the population (total number of kittens and juveniles). Incidence was highest for intestinal parasites (67%; n=7), followed by fleas (59.09%; n=51), *Bartonella* (49.4%; n=45), FHV-1 (35.3%; n=39), mites (27.2%; n=51), and FeLV (22.7%; n=25). Incidence for FIV was presumed to be low, as it was 0% (n=25) in the kitten and juvenile age groups. Ticks also showed 0% incidence (n=51).

CONCLUSION

In the face of drastically changing anthropogenic stressors, declines in healthy wildlife populations, as well as disease spillover from previously disparate animal and human communities, has made wildlife management an increasingly disease mediated task (Daszak et al., 2010). As these threats grow, the necessity for conservation entities to understand the implications of parasite-host dynamics, and which interactions may determine a species' ability to adapt to human derived changes such as disease emergence, is paramount (Daszak et al., 2000; Graham, 2008; Telfer et al. 2010; Fenton, 2013). In addition, as wild species do not exist in an ecological vacuum, an understanding of interspecific associations amongst host species, as well as each species' ability to serve as a reservoir of disease for the other, is essential. This dissertation has examined these associations through the lens of two separate host-parasite systems.

Using my first model system in African lions of Kruger National Park (KNP), South Africa, I worked to expand the available toolbox for wildlife health assessment by creating a range of normal biochemical, immune, and endocrine reference parameters against which future lion health studies may be compared (Chapter 1). Throughout that study, I demonstrated the potential pitfalls of using data from captive animals to determine normal clinical values in free-ranging species, as well as host level factors that should be taken into account with regards to clinical interpretation.

In Chapter 2, I used the tools established in Chapter 1 to investigate the direct health and immune consequences of FIV infection for lion hosts, as well as the role that various other coinfections of known significance within the KNP system may play in determining lion health outcome. Results yielded by that study show a strong association between FIV infection and wide scale immune dysregulation in lions through suppression of specific lymphocyte, monocyte, and neutrophil cell populations; as well as upregulation of hyperproteinemia and other non-specific systemic inflammatory responses. In turn, decreases in immune cell populations predict higher rates of coinfection with both gastrointestinal and hemoparasites. These trends are supported by an overall higher prevalence of hookworms, tapeworms, coccidia, and *Babesia microti* within FIV-infected hosts, as well as numerous infections seen exclusively in these animals including whipworms, *Toxoplasma gondii*, *Theileria annae*, *Theileria bicornis*, and feline coronavirus. Finally, while path analysis conducted as part of that study supports a strong

direct role for FIV in determining generalized health impairment within lion hosts, it also suggests that parasites (particularly gastrointestinal parasites) may play an equal or larger role in structuring host morbidity.

In Chapter 3, monopartite network and cluster models were used to evaluate host-parasite community structure in FIV-positive and negative networks, as well as to highlight common affiliations among parasites. Cluster analysis suggests that parasites tend to fall into four main communities dictated by cell or system tropism. In addition, FIV shows a strong association with increased host-parasite connectivity and decreased clustering, suggesting that parasitic in-host interactions may be more common in FIV-infected groups. However, despite the strength of these associations, parasite interactions mediated largely by coinfection with hookworms or *Babesia microti* point towards a more essential role of particular parasite combinations for determining host susceptibility to outside parasites. Together, findings presented in the first three chapters lend substantial support to FIV as an important direct and indirect mediator for host health in threatened lion populations, while showing that coinfection interactions may be of equal or greater importance in predicting host responses to novel threats.

In the fourth and final chapter I switched from a highly threatened, less adaptable species threatened by endemic and emerging disease, to a highly adaptable, common species that has benefitted from anthropogenic change. Using coprological and serological survey methods, I examined the potential implications of feral cats living in and around urban communities to act as reservoirs for disease within these areas. While scope of this last study was limited, results show high seroprevalence of viral, bacterial, and parasitic coinfections among this species, with several parasites of known zoonotic and conservation concern among those highly represented.

Ultimately, findings from both studies demonstrate the desperate need for more information on host-parasite community dynamics, and how they interact to shape health within heavily managed wildlife systems. In addition, results presented here highlight several points of intervention where dedicated health campaigns may be used to target disease threats in vulnerable populations in order to promote favorable health outcomes.

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APPENDIX I: CHAPTER 2 SUPPLEMENTARY FIGURES

Table 2.S1 Associations of FIV status with clinical health, blood biochemistry, and endocrine parameters.

PARAMETER	*(n)*	*df*	FIV			FIV : age			FIV : sex			SIGNIFICANT
			β	*CI*	*p*	*β*	*CI*	*p*	*β*	*CI*	*p*	FIXED TERMS
Biochemical												
Alkaline Phosphatase	193	187	1.89	1.47-2.43	**0.00**	0.89	0.82-0.96	**0.00**	-	-	-	age
Alanine Aminotransferase	193	189	1.65	1.64-1.65	**0.00**	-	-	-	-	-	-	none
Blood urea nitrogen	193	186	0.88	0.78-0.99	**0.04**	-	-	-	-	-	-	age, sex, BCS
Creatinine	193	186	1.08	0.95-1.23	0.22	-	-	-	0.82	0.67-1.01	**0.06**	age, sex
Glucose	193	189	-	-	-	-	-	-	-	-	-	age
Total protein	193	186	1.00	0.93-1.07	0.99	-	-	-	-	-	-	age, sex, BCS
Endocrine												
Leptin	194	156	0.23	0.12-0.43	**0.00**	1.24	1.10-1.41	**0.00**	-	-	-	age, sex
Ghrelin	194	185	3.40	1.99-5.79	**0.00**	0.92	0.83-1.01	**0.07**	-	-	-	age, sex
Clinical												
Gingivitis	181	176	0.28	0.03-2.70	0.27	1.63	1.01-2.65	**0.04**	-	-	-	age
Oral papillomas	180	177	0.50	0.26-0.97	**0.04**	-	-	-	-	-	-	none
Ocular lesions	184	179	10.19	2.25-46.18	**0.00**	-	-	-	0.05	0.01-0.40	**0.00**	sex
Lymph node enlargement	176	173	-	-	-	-	-	-	-	-	-	age
Dehydration	181	173	8000	1.2-5.2e+07	**0.04**	0.18	0.04-.81	**0.03**	74.00	0.51-11000	0.09	age, sex, BCS
Hygromas	184	181	-	-	-	-	-	-	-	-	-	age

*All estimates have been back-transformed for ease of interpretation. Associations between response variables of interest and FIV, as well as all two-way interactions with FIV, are reported above. Statistically significant associations with FIV, FIV:age, and FIV:sex are highlighted with bold-underline, while marginally significant associations are highlighted in bold. Missing values denote that the fixed effect was dropped from the final model. Other significant fixed effects, which were retained for the final model, are reported under "Significant Fixed Terms", but their estimates have not been reported. Pride membership, which was retained as a random effect in every model, has not been included due lack of interpretation.

Table 2.S2 Associations of FIV status with quantitative and functional hematologic and immune parameters.

PARAMETER	*(n)*	*df*	FIV			FIV : age			FIV : sex			SIGNIFICANT
			β	*CI*	*p*	*β*	*CI*	*p*	*β*	*CI*	*p*	FIXED TERMS
Quantitative												
Packed cell volume	135	130	-	-	-	-	-	-	-	-	-	sex, BCS
Total solids	94	73	1.04	1.01-1.09	**0.05**	-	-	-	0.89	0.81-0.89	**0.04**	sex, BCS
Leukocytes	121	89	0.90	0.85-0.96	0.19	-	-	-	0.77	0.58-1.03	0.08	sex, BCS, wounds
Neutrophils	90	61	1.16	0.84-1.59	0.37	0.94	0.89-0.99	**0.03**	-	-	-	age, BCS, wounds
Lymphocytes	90	63	0.58	0.35-0.96	**0.04**	1.07	0.98-1.18	0.15	-	-	-	age
Monocytes	90	62	1.88	0.98-3.58	0.06	0.85	0.76-0.94	**0.00**	-	-	-	age, sex
Eosinophils	90	65	0.79	0.56-1.13	0.20	-	-	-	-	-	-	none
Basophils	90	basophils were not modeled due to zero counts in all but 11 FIV+ and FIV- lions										
Functional												
Erythrocyte sedimentation rate	100	79	3.10	1.21-7.93	**0.02**	0.80	0.68-0.94	**0.01**	-	-	-	age
Bactericidal activity (Whole blood)	125	101	0.97	0.69-1.34	0.84	-	-	-	6.47	1.16-36.06	**0.04**	sex, BCS
Bactericidal activity (Plasma)	96	79	1.44	0.95-2.18	0.09	-	-	-	-	-	-	age

*All estimates have been back-transformed for ease of interpretation. Associations between response variables of interest and FIV, as well as all two-way interactions with FIV, are reported above. Statistically significant associations with FIV, FIV:age, and FIV:sex are highlighted with bold-underline, while marginally significant associations are highlighted in bold. Missing values denote that the fixed effect was dropped from the final model. Other significant fixed effects, which were retained for the final model, are reported under "Significant Fixed Terms", but their estimates have not been reported. Pride membership, which was retained as a random effect in every model except for bactericidal activity, has not been included due lack of interpretation. Models for bactericidal activity included a random effect for test batch in place of pride membership.

Table 2.S3 Associations of flow cytometric cell counts with age and sex in FIV-positive lions.

PARAMETER	*(n)*	*df*	age			sex		
			β	*CI*	*p*	*β*	*CI*	*p*
Quantitative								
CD4+ T helper cells	57	54	0.94	0.90-0.97	**0.00**	0.77	0.59-1.00	**0.06**
CD8+ cytotoxic T cells	57	54	0.94	0.91-0.99	**0.02**	0.74	0.53-1.02	**0.08**
CD21+ B cells	57	54	0.98	0.93-1.04	0.59	0.63	0.39-0.99	**0.06**

*All values have been back-transformed for ease of interpretation. Due to restricted sample size, which only included four FIV-negative individuals, cytometric cell counts were not modeled against FIV status. Instead cell counts were modeled against age and sex using a generalized linear model. FIV status was evaluated separately using a t-test. Statistically significant associations are highlighted in bold underline, while marginally significant associations are highlighted in bold.

Table 2.S4 Associations of FIV status with measures of aggression.

PARAMETER	*(n)*	*df*	FIV			FIV : age			FIV : sex			SIGNIFICANT
			β	*CI*	*p*	*β*	*CI*	*p*	*β*	*CI*	*p*	FIXED TERMS
Quantitative												
Testosterone (males)	73	42	3.36	1.12-10.09	**0.03**	0.79	0.63-0.98	**0.03**	N/A	N/A	N/A	age, BCS
Testosterone (females)	119	114	0.44	0.30-0.66	**0.00**	-	-	-	N/A	N/A	N/A	BCS
Severe wounds	193	189	3.76	1.08-13.15	**0.04**	-	-	-	-	-	-	BCS

*All estimates have been back-transformed for ease of interpretation. Associations between response variables of interest and FIV, as well as all two-way interactions with FIV, are reported above. Statistically significant associations with FIV, FIV:age, and FIV:sex are highlighted with bold-underline, while marginally significant associations are highlighted in bold. Missing values denote that the fixed effect was dropped from the final model. Other significant fixed effects, which were retained for the final model, are reported under "Significant Fixed Terms", but their estimates have not been reported. Pride membership, which was retained as a random effect in every model, has not been included due lack of interpretation. For testosterone, males and females were modeled separately due to the large variation in testosterone between sexes.

Table 2.S5 Associations of FIV status with coinfections, parasite richness, and parasite abundance.

PARAMETER	*(n)*	*df*	FIV			FIV : age			FIV : sex			SIGNIFICANT
			β	*CI*	*p*	*β*	*CI*	*p*	*β*	*CI*	*p*	FIXED TERMS
Viruses												
Canine distemper virus	195	192	-	-	-	-	-	-	-	-	-	age
Feline calicivirus	195	192	-	-	-	-	-	-	-	-	-	age
Feline enteric coronavirus	195	-	Could not compare for FIV status statistically, as all coronavirus-positive animals were also FIV-positive									
Feline parvovirus	195	192	-	-	-	-	-	-	-	-	-	age
Gastrointestinal Parasites												
Ascarid spp.	114	111	-	-	-	-	-	-	-	-	-	age
Tapeworm spp.	114	110	2.87	1.09-7.58	**0.03**	-	-	-	-	-	-	age
Hookworm spp.	114	110	8.51	1.75-41.35	**0.01**	-	-	-	-	-	-	age
Whipworm spp.	114	-	Could not compare for FIV status statistically, as all whipworm-positive animals were also FIV-positive									
Coccidia spp.	114	109	221.02	2.77-1.76E4	**0.02**	0.30	0.12-0.76	**0.01**	-	-	-	age
Toxoplasma gondii	114	-	Could not compare for FIV status statistically, as all toxoplasma-positive animals were also FIV-positive									
Hemoparasites												
Ehrlichia/Anaplasma spp.	190	186	-	-	-	-	-	-	-	-	-	age, BCS
Babesia felis	190	187	-	-	-	-	-	-	-	-	-	age
Babesia leo	190	187	-	-	-	-	-	-	-	-	-	age
Babesia microti	190	187	2.19	1.00-4.80	**0.05**	-	-	-	-	-	-	none
Babesia lengua	190	187	-	-	-	-	-	-	-	-	-	BCS
Babesia rossi	190	187	-	-	-	-	-	-	-	-	-	age
Babesia vogeli	190	187	0.56	0.21-1.50	0.25	-	-	-	-	-	-	none
Babesia canis	190	187	0.77	0.26-2.25	0.63	-	-	-	-	-	-	none
Hepatozoon spp.	190	187	-	-	-	-	-	-	-	-	-	BCS
Theileria annae	190	-	Could not compare for FIV status statistically, as all *T. annae*-positive animals were also FIV-positive									
Theileria bicornis	190	-	Could not compare for FIV status statistically, as all *T. bicornis*-positive animals were also FIV-positive									
Quantitaive Measures												
Parasite Richness (Overall)	111	84	1.17	0.96-1.45	0.12	-	-	-	-	-	-	none
Parasite Richness (GI Parasites)	114	85	1.61	1.07-2.41	**0.02**	-	-	-	-	-	-	age
Parasite Richness (Hemoparasites)	190	154	1.35	1.02-1.79	**0.04**	0.96	0.91-1.01	0.10	-	-	-	age
Parasite Abundance (Overall)	104	77	4.73	1.19-18.84	**0.03**	-	-	-	-	-	-	sex
Parasite Abundance (Ascarids)	28	14	-	-	-	Not included due to prohibitively small sample size						none
Parasite Abundance (Hookworms)	48	31	4.67	0.79-27.66	**0.09**	Not included due to prohibitively small sample size						age
Parasite Abundance (Tapeworms)	52	30	-	-	-	Not included due to prohibitively small sample size						none
Parasite Abundance (Whipworms)	8	-	Could not compare for FIV status statistically, as all whipworm-positive animals were also FIV-positive									
Parasite Abundance (Coccidia)	22	13	-	-	-	Not included due to prohibitively small sample size						age
Parasite Abundance (*T. gondii*)	11	-	Could not compare for FIV status statistically, as all toxoplasma-positive animals were also FIV-positive									

*All estimates have been back-transformed for ease of interpretation. Associations between response variables of interest and FIV, as well as all two-way interactions with FIV, are reported above. Statistically significant associations with FIV, FIV:age, and FIV:sex are highlighted with bold-underline, while marginally significant associations are highlighted in bold. Missing values denote that the fixed effect was dropped from the final model. Other significant fixed effects, which were retained for the final model, are reported under "Significant Fixed Terms", but their estimates have not been reported. Pride membership, which was retained as a random effect in every model, has not been included due lack of interpretation.

APPENDIX II: CHAPTER 3 SUPPLEMENTARY FIGURES

Table 3.S1 Associations of FIV status and demographics with coinfection prevalence.

PARAMETER	(n)	df	FIV			age			sex			BCS			SIGNIFICANT INTERACTION TERMS
			β	CI	p	β	CI	p	β	CI	p	β	CI	p	
Viruses															
Canine distemper virus	195	192	-	-	-	1.12	0.97-1.30	0.11	-	-	-	-	-	-	none
Feline calicivirus	195	192	-	-	-	1.28	1.04-1.56	**0.02**	-	-	-	-	-	-	none
Feline enteric coronavirus	195	193		All are FIV+		-	-	-	-	-	-	-	-	-	none
Feline parvovirus	195	192	-	-	-	1.20	0.92-1.56	0.18	-	-	-	-	-	-	none
Gastrointestinal Parasites															
Ascarid spp.	114	111	-	-	-	0.93	0.81-1.07	0.34	-	-	-	-	-	-	none
Tapeworm spp.	114	110	2.87	1.09-7.58	**0.03**	0.89	0.79-1.01	**0.07**	-	-	-	-	-	-	none
Hookworm spp.	114	110	8.51	1.75-41.35	**0.01**	0.80	0.66-0.97	**0.02**	-	-	-	-	-	-	none
Whipworm spp.	114	112		All are FIV+		-	-	-	-	-	-	-	-	-	none
Coccidia spp.	114	109	221.02	2.77-1.76E4	**0.02**	2.64	1.04-6.71	**0.04**	-	-	-	-	-	-	age:FIV
Toxoplasma gondii	114	111		All are FIV+		-	-	-	-	-	-	0.35	0.14-0.77	**0.02**	none
Hemoparasites															
Ehrlichia/Anaplasma spp.	190	186	-	-	-	1.28	1.13-1.45	**0.00**	-	-	-	0.69	0.46-1.05	**0.09**	none
Babesia felis	190	187	-	-	-	1.15	0.99-1.36	**0.07**	-	-	-	-	-	-	none
Babesia leo	190	187	-	-	-	1.12	0.99-1.25	**0.06**	-	-	-	-	-	-	none
Babesia microti	190	187	2.19	1.00-4.80	**0.05**	-	-	-	-	-	-	-	-	-	none
Babesia lengua	190	187	-	-	-	-	-	-	-	-	-	2.61	0.98-6.97	**0.06**	none
Babesia rossi	190	187	-	-	-	1.20	0.98-1.46	**0.08**	-	-	-	-	-	-	none
Babesia vogeli	190	187	0.56	0.21-1.50	0.25	-	-	-	-	-	-	-	-	-	none
Babesia canis	190	187	0.77	0.26-2.25	0.63	-	-	-	-	-	-	-	-	-	none
Hepatozoon spp.	190	187	-	-	-	-	-	-	-	-	-	2.36	0.95-5.84	**0.06**	none
Theileria annae	190	-	Too few positive animals to compare statistically. All animals with *T. annae* are FIV+.												
Theileria bicornis	190	-	Too few positive animals to compare statistically. All animals with *T. bicornis* are FIV+.												

All estimates have been back-transformed for ease of interpretation. Associations between response variables of interest and FIV, as well as all two-way interactions with FIV, are reported above. Statistically significant associations with FIV, age, sex, and body condition are highlighted with bold-underline, while marginally significant associations are highlighted in bold. Missing values denote that the fixed effect was dropped from the final model. Significant interactions retained in the final model are reported under "Significant Interaction Terms", but their estimates have not been reported. Pride membership, which was retained as a random effect in every model, has not been included due lack of interpretation.

Table 3.S2 Associations of coinfection prevalence with FIV and secondary pathogens/ parasites.

PARAMETER	(n)	FIV		Tapeworms		FIV:Tapeworms		Hookworm spp.		FIV:Hookworms		Coccidia		FIV:Coccidia		*Babesia microti*		FIV:*Babesia microti*	
		β	*p*	*β*	*p*	*β*	*p*	*β*	*p*	*β*	*p*	*β*	*p*	*β*	*p*	*β*	*p*	*β*	*p*
Viruses																			
Canine distemper virus	110	0.38	0.26	-	-	-	-	4.30	**0.00**	All coinfected FIV+		2.30	0.12	-	-	3.98	**0.01**	-	-
Feline calicivirus	110	-	-	-	-	-	-	-	-	-	-	100% coccidia+		-	-	6.78	**0.08**	-	-
Feline enteric coronavirus	110	100% FIV+		4.48	0.19	80% coinfected		100% hookworm+		100% coinfected		19.55	**0.00**	80% coinfected		-	-	80% coinfected	
Feline parvovirus	110	-	-	-	-	-	-	6.15	0.11	-	-	-	-	-	-	-	-	-	-
Gastrointestinal Parasites																			
Ascarid spp.	110	0.42	0.10	-	-	-	-	2.32	**0.06**	-	-	-	-	-	-	-	-	-	-
Tapeworm spp.	110	3.21	**0.02**	N/A	N/A	N/A	N/A	3.70	**0.00**	-	-	11.78	**0.00**	21.28	**0.07**	-	-	-	-
Hookworm spp.	110	6.03	**0.00**	2.89	**0.01**	-	-	N/A	N/A	N/A	N/A	11.88	**0.00**	0% coinfected		4.64	**0.00**	8.53	**0.07**
Whipworm spp.	110	100% FIV+		8.34	**0.05**	87.5% coinfected		100% hookworm+		100% coinfected		-	-	60% coinfected		7.13	**0.07**	87.5% coinfected	
Coccidia spp.	110	221.02	**0.02***	11.18	**0.00**	-	-	11.85	**0.00**	0% coinfected		N/A	N/A	N/A	N/A	**0.00**	0.99	All coinfected FIV+	
Toxoplasma gondii	110	100% FIV+		3.13	0.11	73% coinfected		6.48	**0.02**	82% coinfected		6.30	**0.00**	55% coinfected		-	-	63% coinfected	
Hemoparasites																			
Ehrlichia/Anaplasma spp.	110	2.62	**0.06***	-	-	-	-	4.10	**0.00**	-	-	2.27	0.12	-	-	19.24	**0.00**	-	-
Babesia felis	110	0.31	0.10	-	-	-	-	-	-	-	-	-	-	-	-	4.43	**0.00**	-	-
Babesia leo	110	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7.60	**0.00**	-	-
Babesia microti	110	3.00	**0.03**	-	-	-	-	5.42	**0.00**	-	-	0.00	0.99	All coinfected FIV+		N/A	N/A	N/A	N/A
Babesia lengua	110	2.73	0.15	-	-	-	-	0.04	**0.00**	-	-	0% coccidia+		-	-	0.36	**0.05**	-	-
Babesia rossi	110	1.16	0.89	-	-	-	-	0.32	0.17	-	-	3.73	**0.06**	0.05	0.10	0% B. microti+		-	-
Babesia vogeli	110	0.36	0.10	-	-	-	-	0.33	0.10	-	-	-	-	-	-	0% B. microti+		-	-
Babesia canis	110	-	-	-	-	-	-	0.24	**0.08**	-	-	-	-	-	-	0% B. microti+		-	-
Hepatozoon spp.	110	-	-	-	-	-	-	0% hookworm+		-	-	0% coccidia+		-	-	0% B. microti+		-	-
Theileria annae	110	100% FIV+		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Theileria bicornis	110	100% FIV+		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

All estimates have been back-transformed for ease of interpretation. Original models included fixed effects for age, sex, body condition, FIV, and a random effect for pride identification to account for non-independence. Coinfection models included these fixed effects as well as additional parameters for the coinfection of interest (one each of the original coinfections showing high co-association with FIV), as well as an interaction term for FIV and the coinfection parameter. Asterisks (*) denote parasite/pathogen infections for which statistical significance of FIV+ status changed after accounting for other coinfections (either gained or lost significance). N/A values indicate where a parameter was not compared against itself, while missing values marked by a dash indicate that the fixed effect was dropped from the final model. With some coinfection parameters, sample size was not sufficient to explore associations (all animals were positive/negative for one or more parameters of interest). For these values, prevalence for the individual infection or coinfection between the parameter of interest and FIV are indicated.

Table 3.S3 Regional parasite prevalence within Kruger National Park.

PATHOGEN/ PARASITE	LOCATION		
	Central	North	South
Viruses			
Feline Immunodeficiency Virus	83	66	70
Canine Distemper Virus	2	16	32
Feline Parvovirus	4	4	10
Feline Calicivirus	9	2	7
Feline Enteric Coronavirus	0	0	6
Gastrointestinal Parasites			
Ascarid spp.	18	44	28
Whipworm spp.	3	0	13
Tapeworm spp.	39	44	56
Hookworm spp.	12	44	67
Coccidia spp.	0	26	33
Toxoplasma gondii	6	7	13
Hemoparasites			
Ehrlichia/Anaplasma spp.	51	30	61
Babesia felis	91	78	81
Babesia leo	53	48	42
Babesia microti	34	35	73
Babesia lengua	43	4	2
Theileria bicornis	2	0	5
Hepatozoon spp.	26	4	1
Theileria annae	0	2	0
Babesia canis	4	22	7
Babesia rossi	4	24	6
Babesia vogeli	6	33	11

Regional parasite prevalence was calculated as the total number of animals exposed/infected with the pathogen/parasite of interest divided by the total number of animals sampled for that parasite/pathogen in the given region.

Table 3.S4 Viral seroprevalence by lion pride.

PRIDE	(n)	FIV (%) PREVALENCE	VIRAL PREVALENCE (%)			
			CDV	FPV	FCV	FCoV
Bangu	3	100	0	0	0	0
Berg-en-dal	5	100	0	20	20	0
Confluence	1	100	0	0	0	0
Crocodile Bridge	16	63	50	13	6	25
Crudzani	7	71	14	0	0	0
Dzudzini	2	100	0	50	0	0
Fayi loop	5	60	0	60	20	0
Jock	18	67	22	0	11	11
Kraglyn	15	67	40	0	0	0
Kumana	5	100	0	0	20	0
Lower Sabie	8	38	50	0	13	0
Lukimbi	4	100	75	0	0	0
Magamba	2	50	100	0	0	0
Mingerhout Dam	5	80	20	0	0	0
Mjejane	1	0	0	0	100	0
Monzo	1	100	0	100	0	0
Mpongholo	5	40	20	0	0	0
Ngotso	3	100	0	0	0	0
Nsemani	10	60	0	0	10	0
Nshawu	2	100	0	0	0	0
Nwaswitshaka	14	93	29	0	0	0
Nyamundwa	4	100	0	50	0	0
Olifants	14	86	0	0	7	0
Punda	2	100	50	0	0	0
Red Rocks	8	25	25	13	0	0
Robs Pride	2	50	0	50	0	0
Rodneys	2	50	0	0	0	0
Sable Dam	1	0	0	0	0	0
Shingwedzi	5	60	20	0	0	0
Shishangane	7	71	0	14	14	0
Skukuza	6	67	33	17	0	0
Sweni	6	100	0	0	17	0
Timbavati	5	100	0	0	0	0
Vlakteplaas	1	100	0	0	0	0

Prevalence was calculated as the total number of animals testing seropositive for the virus of interest over the total number of animals tested for that virus within each pride.

Table 3.S5 Gastrointestinal parasite prevalence by lion pride.

PRIDE	(n)	FIV (%) PREVALENCE	GASTROINTESTINAL PARASITE PREVALENCE (%)					
			Ascarids	Whipworms	Tapeworms	Hookworms	Coccidia	*Toxoplasma*
Bangu	1	100	0	0	0	0	0	0
Berg-en-dal	3	100	33	0	0	0	0	0
Confluence	0	100	-	-	-	-	-	-
Crocodile Bridge	10	63	40	20	80	100	70	30
Crudzani	2	71	50	0	50	0	0	0
Dzudzini	1	100	0	0	100	100	0	0
Fayi loop	0	60	-	-	-	-	-	-
Jock	14	67	36	7	50	50	43	7
Kraglyn	11	67	27	9	55	45	18	9
Kumana	3	100	0	0	67	0	0	0
Lower Sabie	1	38	100	0	0	100	0	0
Lukimbi	3	100	0	0	33	100	0	0
Magamba	1	50	100	0	100	100	100	0
Mingerhout Dam	4	80	50	0	25	75	0	0
Mjejane	0	0	-	-	-	-	-	-
Monzo	1	100	0	0	100	0	0	0
Mpongholo	2	40	0	0	50	0	0	0
Ngotso	1	100	0	0	100	0	0	0
Nsemani	10	60	10	0	30	30	0	0
Nshawu	0	100	-	-	-	-	-	-
Nwaswitshaka	11	93	0	27	73	82	18	18
Nyamundwa	0	100	-	-	-	-	-	-
Olifants	5	86	40	0	60	0	40	0
Punda	0	100	-	-	-	-	-	-
Red Rocks	6	25	50	0	17	33	0	0
Robs Pride	1	50	100	0	0	100	100	0
Rodneys	2	50	50	0	50	100	100	0
Sable Dam	1	0	0	0	100	0	100	0
Shingwedzi	4	60	75	0	25	50	0	25
Shishangane	5	71	0	0	0	0	0	20
Skukuza	0	67	-	-	-	-	-	-
Sweni	6	100	50	17	50	17	0	0
Timbavati	4	100	25	0	50	0	0	25
Vlakteplaas	1	100	0	0	100	100	100	100

Prevalence was calculated as the total number of animals testing positive for the parasite of interest over the total number of animals tested for that parasite within each pride.

Table 3.S6 Hemoparasite prevalence by lion pride.

PRIDE	(n)	FIV (%) PREVALENCE	HEMOPARASITE PREVALENCE (%)										
			Ehrlichia	*B. felis*	*B. leo*	*B. microti*	*B. lengua*	*T. bicornis*	*H. canis*	*T. annae*	*B. canis*	*B. rossi*	*B. vogeli*
Bangu	3	100	67	100	67	67	67	0	33	0	0	0	0
Berg-en-dal	5	100	80	100	60	80	0	0	0	0	20	0	20
Confluence	1	100	100	100	100	100	0	0	0	0	0	0	0
Crocodile Bridge	16	63	75	75	88	88	0	6	0	0	6	0	0
Crudzani	7	71	57	100	71	14	0	14	0	0	0	0	0
Dzudzini	2	100	0	100	50	100	0	0	0	0	0	0	0
Fayi loop	4	60	75	75	25	75	0	50	0	0	0	0	25
Jock	18	67	56	72	28	61	6	0	0	0	6	6	11
Kraglyn	15	67	27	47	27	53	0	0	0	0	0	0	0
Kumana	5	100	60	60	60	60	20	0	20	0	0	0	0
Lower Sabie	8	38	75	100	75	75	0	13	0	0	25	25	25
Lukimbi	4	100	100	100	50	100	0	0	0	0	0	0	0
Magamba	2	50	50	50	50	50	0	0	0	50	50	50	50
Mingerhout Dam	5	80	60	80	60	40	20	0	20	0	20	20	20
Mjejane	1	0	100	100	0	100	0	0	0	0	0	0	0
Monzo	1	100	0	100	0	0	100	0	100	0	0	0	0
Mpongholo	5	40	20	100	80	80	0	0	0	0	0	20	20
Ngotso	3	100	33	100	33	33	33	0	33	0	0	0	0
Nsemani	10	60	60	90	60	40	50	0	30	0	0	0	0
Nshawu	2	100	0	100	0	50	50	0	50	0	0	0	0
Nwaswitshaka	14	93	50	100	14	79	7	0	7	0	7	0	14
Nyamundwa	4	100	50	100	25	75	0	25	0	0	0	25	25
Olifants	13	86	31	85	31	31	0	0	0	0	31	31	54
Punda	1	100	0	0	100	0	0	0	0	0	0	0	0
Red Rocks	8	25	25	50	25	13	0	0	0	0	25	25	38
Robs Pride	2	50	100	100	0	100	0	0	0	0	0	0	0
Rodneys	0	50	-	-	-	-	-	-	-	-	-	-	-
Sable Dam	1	0	0	100	0	0	0	0	0	0	100	100	100
Shingwedzi	5	60	40	80	100	0	0	0	0	0	0	0	0
Shishangane	7	71	29	86	43	14	14	0	14	0	29	29	43
Skukuza	6	67	67	100	50	67	0	0	0	0	17	33	33
Sweni	6	100	67	100	50	67	100	0	17	0	0	0	0
Timbavati	5	100	40	100	40	0	60	0	60	0	0	0	0
Vlakteplaas	1	100	0	100	0	0	0	0	0	0	100	100	100

Prevalence was calculated as the total number of animals testing positive for the hemoparasite of interest over the total number of animals tested for that hemoparasite within each pride.

Table 3.S7 Parasite/pathogen prevalence by lion demographic group.

PATHOGEN/ PARASITE	LIFE STAGE					SEX	
	cub	subadult	young adult	prime adult	senior	female	male
	(n = 38)	*(n = 38)*	*(n = 37)*	*(n = 55)*	*(n = 27)*	*(n = 121)*	*(n = 74)*
FIV	61	55	76	82	89	70	76
CDV	21	11	30	18	26	22	19
FPV	3	0	5	18	4	8	5
Calicivirus	3	3	5	9	11	6	7
Coronavirus	3	5	0	4	4	3	3
	(n = 25)	*(n = 20)*	*(n = 22)*	*(n = 29)*	*(n = 18)*	*(n = 74)*	*(n = 40)*
Ascarid spp.	40	30	23	21	33	27	33
Whipworm spp.	12	5	5	10	0	7	8
Tapeworm spp.	60	50	41	48	39	50	45
Hookworm spp.	68	50	36	48	17	43	50
Coccidia spp.	36	20	18	21	11	19	28
Toxoplasma gondii	20	5	5	7	11	8	13
	(n = 38)	*(n = 36)*	*(n = 35)*	*(n = 54)*	*(n = 27)*	*(n = 117)*	*(n = 73)*
Ehrlichia/Anaplasma spp	37	39	46	59	78	53	48
Babesia felis	74	78	86	89	89	86	78
Babesia leo	34	56	49	35	70	45	48
Babesia microti	66	53	43	50	63	53	56
Babesia lengua	3	19	9	13	22	15	10
Theileria bicornis	3	0	3	6	4	4	1
Hepatozoon spp.	0	17	9	9	4	9	5
Theileria annae	0	0	3	0	0	0	1
Babesia canis	5	8	17	11	7	9	12
Babesia rossi	0	6	14	19	7	9	11
Babesia vogeli	11	8	23	22	7	15	16

Prevalence was calculated as the total number of animals testing positive for the parasite/pathogen of interest over the total number of animals tested within each demographic group.